# Root Genomics

Antonio Costa de Oliveira • Rajeev K. Varshney
Editors

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# Foreword

## Root Biology: An Inconvenient Truth

The truth is that roots usually are as extensively underground as the aerial portions are above the ground. Crop plants would not live without roots. Roots absorb water and nutrients and anchor the plant in the soil. So why do not we know more about roots? It is likely due to the inconvenience of phenotyping root characteristics – and many of today's phenotyping methods are destructive. While we recognize the essentiality of roots and their relation to plant performance, the scientific community has not placed a sufficiently high priority on their analysis to make the needed major advances. Many of the factors that affect root health can result in a 50% yield loss when deficient. Given that the predicted human population increase is 50% by 2050, the improvement of root health in crop plants could play a major role in meeting the world's need for increased food.

The study of root biology involves extensive plant–soil–water interactions that are complicated by the microorganisms and insects in the rhizosphere that can alter root development. Each of the possible interactions has feedback effects in the plant; many effects are long-range effects within the plant. The soil environment relates to nutrient availability and uptake, which reflects the condition of the soil including acidity. Even alternation of dry and flooded conditions changes various ion states, which can change with the duration of flooding. Many climate change scenarios predict water shortages, making the understanding of root biology even more important in the future.

Much of today's phenotyping of roots is based on root architecture, such as root length, root diameter, root proliferation, root biomass, root mass density at different soil depths, diameter, and distribution of meta-xylem vessels, and root-to-shoot ratios. Early maturity, early shoot-growth vigor, and depth and rapidity of water absorption also are often assessed among other factors. New nondestructive approaches need to be encouraged such as X-ray imaging, light transmission imaging, and time-lapse recordings of root growth.

This book clearly documents that many new genetic/genomic technologies are rapidly being applied to the study of roots, including high-throughput genome sequencing, TILLING, use of molecular markers such as SSRs, DArTs, and SNPs for introgression of favorable genes, QTL analyses, marker assisted breeding, gene discovery, comparative mapping, transcription factor identification, transcriptional profiling, posttranscriptional events regulating microRNAs, and proteome profiling with complete roots. Some genetic approaches are constrained – such as genome-wide selection and gene cloning – by the difficulty in phenotyping.

Plants coordinate root growth with the soil environment. Many factors can inhibit root growth. In this book, aluminum, iron, and salt toxicity are extensively reviewed, providing a great deal of useful information. The root system is the primary site of interaction with the soil environment, which includes exudates of organic compounds from the plants and the microbes. Some of these exudates are known to represent signals that regulate microbe behaviors and even germination of seeds.

As illustrated in this book, it is amazing what we know about roots and their importance, but equally amazing is what we do not know – and we know even less about the complicated interactions and feedback mechanisms. The work reviewed in this book also shows the value of using model species such as *Arabidopsis*; e.g., 22 genes have been reported in *Arabidopsis* on lateral root development, 19 genes on primary root development, and 8 genes on root-hair formation.

One of the goals of this book was to show how root research relates to sustainable crop productivity. The chapters taken together represent an extensive review of the topic focusing primarily on highly productive crops under rainfed conditions. Crops are mostly rainfed in the most populated areas of the world; this suggests that it is imperative that root biology be a major research emphasis in the coming years – but will that be the case? Will the "inconvenient truth" be recognized?

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# Preface

With the emerging recognition that agriculture needs to approach sustainability, the plant–soil–water interactions become of paramount importance in crop systems. In this scenario, roots arise from a minor to a major role in the understanding of plant growth and development. Novel technologies allow us to scan genomes in the fastest way ever, and there is not a day without further developments leading to cheaper and more precise genotyping techniques. However, the complexity of underground metabolism and the responses of root systems to a variety of stresses call for improvements in phenotyping as well as genotyping techniques.

The idea of organizing a book on Root Genomics dates as back as early 1990s in the graduate benches of Purdue University. The fascination with a system so important for the plant but yet so unknown served as both an incentive and a challenge to pursue this line of research. In 2002, an important opening for root biology occurred when the late Dr. Mike Gale, *FRS*, agreed to include a workshop in Root Genomics at the Plant and Animal Genome Meetings, held yearly at San Diego, CA. Since 2003, this workshop has generated fruitful discussions and created new paths for root research. Many speakers from different countries shared their experience in root genomics, regardless if they were working with model or crop species. One of the speakers, Rajeev Varshney, was very impressive in his enthusiasm and determination to target important aspects of drought stress. Sharing the same enthusiasm for studying roots and stress responses was crucial to put the idea of this book forward. Many of the authors have presented their work in the Root Genomics Workshop, but all were chosen by their significant contributions to agricultural and plant sciences and their common efforts for a better world. We are grateful to all the authors who not only provided a timely review of the published research work in their area of expertise but also shared their unpublished results to offer an updated view. We also appreciate their cooperation in meeting the deadlines, revising the manuscripts and in checking the galley-proofs.

We are thankful to Dr Jeff L. Bennetzen, who as a brilliant geneticist was a great role model and a friend (ACO) that has indirectly inspired this line of research. We thank Dr. Ronald Phillips, a major pioneer in the field of plant genetics and

genomics and the father of many ideas that influenced modern plant sciences, for writing the foreword.

Both of us also recognize that the editorial work for this book took away precious time that we should have spent with our respective families. ACO acknowledges the efforts of his parents, Glauco and Izabel, for providing an atmosphere of learning and investigative thought during his young years, his wife Carla for her continuous encouragement, patience, and friendship, and his children Victoria (Vickie) and Eduardo (Dudu). Similarly, RKV acknowledges the help and support of his wife Monika and his children Prakhar (Kutkut) and Preksha (Nanu) who allowed their time to be taken away to fulfill RKV's editorial responsibilities in addition to research, managerial, and other administrative duties at ICRISAT and Generation Challenge Programme (GCP).

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# Chapter 1
# Introduction to Root Genomics

**Antonio Costa de Oliveira and Rajeev K. Varshney**

**Contents**



## 1.1 Introduction

The twenty first century has been marked by climate awareness and an overall increase in conscience towards environmentally friendly agriculture. Despite the natural phenomena playing hard against most crops, we need to gather all the possible information on the plant–soil–water interactions in order to breed for this century. Abiotic and biotic stresses will be targeted as most of the frontiers for agriculture lie in nonoptimal areas, and genetic improvements through science will play a major role in this conquer.

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Root development, one of the major processes essential to the development of flowering plants, remains poorly understood. Roots are a hidden part of plants for many aspects and have not been the main subject of interest of researchers. Nevertheless, roots play a major role in the plant–soil interactions, regarding biological and physical aspects. The understanding of the physiological, molecular, and developmental processes that roots undergo may represent a giant step on the achievement of a more sustainable and energy-efficient agriculture. This book may serve as a reference book in this context. Some concepts about root genomics together with an overview on different chapters presented in this volume are given in this article.

## 1.2 Root Genomics: An Overview

Root genomics research can be divided in the following four areas of research: (1) root growth and development; (2) functional analyses of abiotic stress responses; (3) functional analyses of biotic stress responses; and (4) quantitative trait loci (QTL) analysis and molecular breeding. The understanding of basic mechanisms involving root development and the interactions of roots and soils under various abiotic and biotic stresses will pave the way for the next decades. Also, mutations obtained in model species through the use of high throughput techniques such as TILLING (targeted induced local lesions in genome) are turning root genomics an exciting subject in plant molecular biology. An attempt has been made to cover all the above-mentioned four areas of root genomics research.

### *1.2.1 Root Growth and Development*

The breakthrough depiction of root development has started with *Arabidopsis* roots (Dolan et al. 1993, 1994; Scheres et al. 1996). The events of division, enlargement, and differentiation of cells in the roots are spatially separated. At the root tip, there is a region of continuous cell division, the RAM (root apical meristem). The new cells formed enlarge by a factor of 100-fold through a process of cell elongation. After the cells reach a mature size, they differentiate into the various cell types of the root. Root growth is accompanied by the formation of a series of lateral roots, resulting in a branching pattern that covers higher volumes of soil space in every step of branching. A range of root systems can be found in different plants including from shallow patterns to very deep roots. Therefore, the identification of factors affecting the patterns of root development is the major point in decoding the genetic control of this organ.

In a paleontological context, the role of auxin in morphogenesis has allowed the identification of vascular patterns preserved in fossils as records of auxin gradients and growth dynamics (Boyce 2010). Roots evolved independently at least

in lycophytes and euphyllophytes (Gensel et al. 2001). Root traces have been found in early Devonian soil horizons, contemporaneous with attached roots in lycophyte related fossils. The presence of root hairs, root cap, and endogenous initiation shared by roots has been proposed to have highly divergent origins (Boyce 2010). Shared regulation by similar helix-loop-helix transcription factors (Menand et al. 2007) suggests a homology between rhizoids and root hairs. The origin of root caps, on the other hand, is suggested to be a response to the need of having a protective tissue to the root apical meristem, a fast-growing region constantly in contact with a solid surface, i.e., the soil. The appearance of adventitious roots may date the evolution of endogenous initiation combined with reversed auxin transport, since the first appears to have occurred repeatedly through times and is suggested to have been required for the establishment of vascular continuity (Boyce 2005). Anatomical homogeneity/heterogeneity is suggested as a reflection of stable/unstable environments faced by land plants and epiphytes/swamp plants, respectively. Despite the environmental differences, auxin transport mechanisms are thought to limit the anatomical variations in roots (Boyce 2005; Raven and Edwards 2001).

Studying root development requires model species with simple root architecture. *Arabidopsis* and rice are model species that have been fully sequenced and therefore can provide good models for monocot and dicotyledoneous root development. *Arabidopsis* root is composed of 15 distinct cell types arranged as concentric cylinders around the radial axis (Iyer-Pascuzzi et al. 2009). MicroRNA-mediated signaling has been reported to be involved in plant root development (Meng et al. 2010). Several of these miRNAs are interestingly shared by *Arabidopsis* and rice despite their differences in root patterns and architecture. However, only a few genes governing root development have been described in cereals, and differences between monocots and dicots are quite remarkable when one regards at the root system. Therefore, both models are necessary for the better understanding of the branching patterns and functional specificities of roots. Two crown rootless mutants, *crown-rootless4* (*crl4*) and *OsGnom1*, affect the gene orthologous to *GNOM1* in *Arabidopsis* (Kitomi et al. 2008; Liu et al. 2009). *GNOM1* is a membrane-associated guanine-nucleotide exchange factor of the ADP-ribosylation factor G protein (ARF_GEF) that regulates the traffic of PIN1 (PINFORMED 1) auxin efflux carrier proteins that regulates auxin transport. *GNOM1* is thought to be required for the formation of the lateral primordium in Arabidopsis, by acting on the asymmetrical division of pericycle cells (Coudert et al. 2010). Recently, a new notion on root system architecture (RSA) has been described (Dorlodot et al. 2007). Root architecture importance for plants lies in the fact that soil nutrients are not evenly distributed and the ability to spatially deploy roots can constitute an advantage.

Developmental models could be an alternative to improve phenotyping in this very plastic organ. Mapping the dynamics of roots per se or after inducing root development under different stresses could bring better understanding and establish genotype differences. Shoot-borne-root formation characterizes the difference between cereals and the dicot model plant Arabidopsis. Several mutants that are impaired in shoot-borne-root formation (4), lateral roots (4), primary root (6), and root hairs (4) have been described in maize and rice (Hochholdinger et al. 2004).

Some of these genes controlling root development have been recently cloned and will shed light on the influence of distinct root functions and architecture on grain yield and performance in water-limited conditions (Hochholdinger and Tuberosa 2009). However, the overall trend is that single mutant standard analysis is shifting to genome-wide approaches, leading to a speeding up of the process of generating information. Proteomics- and metabolomics-generated datasets will need integration with bioinformatics tools in order to translate the overwhelming amount of data into biological meaningful phenomena.

### *1.2.2 Biotic Stress Tolerance*

Biotic stress is caused by organism attacks to plants and can be caused by different pathogens (virus, bacteria, or fungi) or pests (insects). Pathogen infections trigger plant response mechanisms that are not restricted to the infection organ. The plant senses the pest attack and responds with a range of different expressions of genes regulating metabolites such as proteinase inhibitors, toxins, or volatiles that repel pests or attract natural enemies. Herbivores or pathogens can elicit different types of defense reaction. When vacuoles and trichomes are bursted as a consequence of a chewing herbivore attack, compounds such as organic isothiocyanates can be released (Bruce and Pickett 2007).

An interesting point of view is brought by on the cross-talk between shoot and root (Van Dam et al. 2004; Bezemer and van Dam 2005). Induced responses are complicated. The fact that hormone signaling pathways govern biotic and abiotic stress responses is characterized by the fact that ABA is involved in many abiotic responses and acts as a negative regulator of disease resistance (Fujita et al. 2006). Other phytohormones, such as Salycilic acid (SA), Jasmonic Acid (JA), and Ethylene (ET), play critical roles in biotic responses. Other responses are mediated by MAP-kinase cascades, which control many biotic and abiotic responses. Other evidence of this cross-talk is the presence of Reactive Oxygen Species (ROS) at converging points between biotic and abiotic response pathways. The integration of this network of responses is essential for the understanding of how roots participate in this process and the intricate process of cross-signaling that this may need.

### *1.2.3 Abiotic Stress Tolerance*

Roots are subjected to a wide range of stresses such as drought, flooding, salinity, as well as nutrient starvation and metal toxicity such as Al, Cd, Fe, As, and Hg. Cadmiun is a nonessential element for plants, its toxicity resulting in chlorosis and stunting. Chlorosis seems to be an indirect effect on the uptake, transport, and use of other elements such as Ca, Mg, Fe, Mn, Cu, Zn, P, and K. Cd also interferes with hormones and disturbs plant water status, causing reduction of root hydraulic

conductivity, decrease of transpiration, and increase of stomatal resistance (Prasad 1995; Das et al. 1997; Aina et al. 2007). A proteomics approach revealed the importance of two metabolic enzymes induced by 10 uM Cd that seems to play a key role in the response to several abiotic stresses: alanine aminotransferase (ALT) and Hexoquinase (HXK) suggest that these could be potential biomarkers for the study of Cd toxicity (Aina et al. 2007). The accumulation of NaCl at root peripheral regions limits growth by exerting osmotic and ionic stresses. Ionic stress is a consequence of $Na^+$ and $Cl^-$ accumulation, disturbing the $K^+/Na^+$ ratio in the plant cell (Hasegawa et al. 2000). Time-dependent effect of NaCl on the activities of tonoplast proton pumps, showing distinct profiles for vacuolar proton transporting ATPase and vacuolar proton transporting pyrophosphatase were reported. Activity alterations were found to be due to posttranslational changes (Kabata and Ktobus 2008). The effects of salinity on *Arabidopsis* cells have been recently investigated (Dinnenny et al. 2008). Transcriptional changes in response to salinity seem to be highly constrained by developmental parameters. Iron deprivation and salt stress data sets were compared. The largest set of coregulated genes displayed concerted down-regulation in the epidermis and encoded genes important for protein biosynthesis. Epidermis cells seem to present the least conserved patterns when different stresses are applied (13–15%). A range of 244 genes are cell-type-specific and whose expression pattern does not substantially change with stress. Chloroplast accumulation was found to be a novel feature of the cortex in light-grown roots. Interestingly, rice roots under excess iron stress seem to accumulate Rubisco peptides, as revealed by proteomic studies (Costa de Oliveira, unpublished).

The responses of roots to abiotic stresses are though amenable to environmental influences as well as cell-type. The high plasticity observed in the developmental patterns plus the range of abiotic factors affecting root growth through the development of plants picture a complex scenario composed of many players as well as interactions among them.

### *1.2.4 QTL Analysis and Molecular Breeding*

Root morphology is in most cases regulated by many genes with small effects and highly influenced by the environment. Therefore, the study of root system related genes will very often rely on QTLs analyses. A few examples on mapping and identification of QTLs explaining the variation for root traits have become available in some crop species (Price and Tomos 1997; Price et al. 2002; Giuliani et al. 2005). Adventitious rooting has been considered to improve phosphorus uptake and deep root growth to increase the ability to cope with drought (Ochoa et al. 2006; Macmillan et al. 2006; Steele et al. 2006). In some cases, QTLs associated with root traits have been cloned, e.g., root elongation in *Arabidopsis* (Sergeeva et al. 2006).

Although QTL analysis was developed to deal with environmental influence on target characters, the high degree of plasticity presented by roots can mislead studies and make it difficult to do a reliable phenotyping. However, at least in rice and

maize, QTL by environment interactions have been found to be weak, and marker-assisted selection studies have been successful (Macmillan et al. 2006; Kamoshita et al. 2002; Steele et al. 2006, 2007; Giuliani et al. 2005; Landi et al. 2005).

## 1.3 About the Book

This book covers all the four areas of research mentioned above. Some highlights of the chapters included in this book are given below.

During the past decades, a considerable number of genes and gene networks have been well described in the model species *Arabidopsis thaliana*. This knowledge can be adapted for more complex plant systems as barley, rice, or maize. Despite their agronomic importance, only a little is known about molecular basis of root formation in crop species, and only few mutants together with corresponding genes have been well characterized. In this context, Orman and colleagues from Silesian University, Poland, have described the EST (expressed-sequence tag)-based approach, in Chap. 2, to search for potential orthologous genes involved in root morphogenesis between Arabidopsis, rice, and barley. The comprehensive gene list, developed by authors, should provide strong platform for molecular studies and gene identification in barley and related species.

Roots are exposed to a range of microbe, and there are several studies, as mentioned above, which deal with discussions on root–microbe interactions as well as impact of biotic stresses on the root architecture. The Chap. 3, authored by Mathesius and van Noorden from Australian National University, Australia, present the updates on genomics of root–microbe interactions. Microbes influence roots by producing signals, toxins, altering nutrient cycling, and by invading roots as endosymbionts or endoparasites. Genomic tools have helped to elucidate the molecular changes induced in roots by microbes. This chapter highlights some of the recent advances gained by genomic and postgenomic studies to enhance knowledge in the area of root–microbe interactions. Similarly, Deshpande and colleagues from Purdue University (USA), University of Georgia (USA), Michigan Technological University (USA), and Instituto Nacional de Tecnología Agropecuaria (INTA, Argentina), in Chap. 4, discuss the advances in the plant genetics for study of the roles of root exudates and microbes in the soil. In order to dissect the relationships between soil microbes, plant exudates, and plant function, authors planned to use host genetics to identify exudate::microbe correlates that segregate with specific plant genes. Their studies indicated the great potential for future investigations of the plant-determined chemical and organismal diversity in the soil.

Abiotic stresses are the major stresses for limiting crop productivity in several crop species, especially in developing countries. In majority of such cases, roots are the first plant organs to be exposed as well as to respond. Some of these abiotic stresses in the context of root genomics have been discussed in a few chapters. For instance, in Chap. 5, Gruber and colleagues from Institut des Sciences du Végétal (ISV) and Université Paris Diderot Paris 7 from France discuss the impact of abiotic stresses

such as drought and salt on the action and number of root meristems to determine root architecture. In addition to Arabidopsis, authors have discussed recent results on model legumes able to interact symbiotically with soil rhizobia to form new meristems leading to the nitrogen-fixing nodule. Aluminum (Al) toxicity is another abiotic stress that limits agricultural productivity over much of the world's arable land by inhibiting root growth and development. Affected plants have difficulty in acquiring adequate water and nutrition from their soil environments and thus have stunted shoot development and diminished yield. Hoekenga from US Department of Agriculture (USDA) – Agricultural Research Station (ARS) (USA) and Magalhaes from EMBRAPA Maize and Sorghum (Brazil) discuss in Chap. 6 the Al-tolerance mechanisms. They propose and discuss the use of systems biology approaches to study the mechanisms of Al tolerance and apply this knowledge to crop improvement via marker-assisted breeding and translational genomics. Sousa and Costa de Oliveira from Eliseu Maciel School of Agronomy, Campus UFPel (Brazil) discuss, in Chap. 7, about root responses to other abiotic stresses such as soluble iron and short chain organic acids in flooded soils, especially in the context of rice. Authors review the progress on discovery of iron transporters as well as genetic variation present in rice genotypes for flooding tolerance.

A number of studies have described QTLs that provide access to valuable genetic diversity for the morphophysiological features that characterize root functionality. Although a number of major QTLs have been identified as mentioned above, none of these QTLs has been cloned so far in crop plants, mainly due to the difficulty to accurately phenotype the target traits in a sufficiently large number of plants. In this context, in Chap. 8, Tuberosa and colleagues present summary and discuss the strategies for QTL cloning, especially in the context of maize. QTL cloning should be facilitated by adoption of high-throughput phenomics platforms as well as by information made available through genome and the profiling of the transcriptome, proteome, and metabolome, all of which will contribute to the identification of plausible candidate genes. Sheshashayee and colleagues from University of Agricultural Sciences-Bangalore, India, in Chap. 9, have presented phenotyping methodology for root traits and biotechnological approaches to improve these roots traits with an objective of sustainable crop production. In Chap. 10, Varshney and colleagues from ICRISAT, India, and Hokkaido University, Japan, discuss the physiological and genomics approaches to dissect the root traits at genetic and molecular level in context of devising the strategies for breeding for root traits to enhance drought tolerance in chickpea. Authors have also discussed the use of next generation sequencing technologies towards gene discovery and marker development.

The last two chapters discuss the progress in the area of molecular breeding for root traits for crop improvement. For instance, Raman from Wagga Wagga Agricultural Institute, Australia, and Gustafson from University of Missouri, USA, in Chap. 11, review the progress made on various aspects of molecular breeding for Al resistance such as genetics, molecular mapping, comparative mapping, marker-assisted selection, candidate gene discovery and validation, and allele mining in key cereal crops including wheat, barley, rice, maize, oats, sorghum, and rye. Similarly, Ismail and

Thomson from International Rice Research Institute, Philippines, in Chap. 12, have summarized the progress made in unraveling molecular and physiological bases of tolerance of various abiotic stresses encountered in rice problem soils including salt stress and nutritional toxicities and deficiencies. Authors have also provided a brief account of the progress towards developing and using marker-assisted back crossing (MABC) for cultivar improvement in rice.

## 1.4 Concluding Remarks

The field of root genomics is an exciting and promising field of research. Some of these areas of research have been detailed in some chapters of the book. The technical advances in plant-*omics* are prone to generate enough data to push forward the science of root genomics. Candidate gene identification is a strategy that is getting stronger every year. The production of genomic sequences from many sequencing projects is making the availability of specific genes more frequent. Bioinformatic tools and reverse genetic approaches such as TILLING, gene knockout mutants, or RNAi are prone to increase the success in this strategy (Dorlodot et al. 2007). An ever neglected part of the plant, roots seem to hold the key for the next plant breeding revolution, leading to improved crop productivity in environmentally challenged situations.

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# Chapter 2
# EST-Based Approach for Dissecting Root Architecture in Barley Using Mutant Traits of Other Species

**Beata Orman, Aleksander Ligeza, Iwona Szarejko, and Miroslaw Maluszynski**

**Contents**



## 2.1 Introduction

There are increasing evidences that root architecture is a fundamental aspect of plant growth. The role of root system includes acquisition of water and nutrients, anchorage of the plant in the soil, synthesis of hormones, and also storage functions. It was generally considered that root characteristics could be important for breeding, to obtain genotypes of a higher adaptability to unstable soil and climatic conditions (Gorny 1992; De Dorlodot et al. 2007) and higher productivity (Lynch 1995). Despite their importance, little is known about genetic basis of root system formation and architecture in major crop species. A great progress in understanding the molecular processes underlying root development has been achieved only in *Arabidopsis thaliana* (Scheres et al. 2002; Casimiro et al. 2003; Casson and

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Lindsey 2003; Ueda et al. 2005; Zhang et al. 2007; Busov et al. 2008). This progress was accomplished through detailed analysis of root mutants with the use of advanced molecular, genomic, and bioinformatic tools available for *Arabidopsis*. Recently, several root mutants have been reported in three cereal species, rice (Ma et al. 2001; Zimmer et al. 2003; Liu et al. 2005; Inukai et al. 2005; Jiang et al. 2005; Li et al. 2006a; Kim et al. 2007), maize (Lim et al. 2005; Woll et al. 2005; Wen et al. 2005; Hochholdinger et al. 2008), and wheat (Wang et al. 2006). Some of them have become the subject of studies similar to *Arabidopsis* that have led to the identification of homologous and novel genes controlling root system formation in monocotyledons (Morita and Kyozuka 2007). There is, however, a lack of similar knowledge in barley. These differences in progress of knowledge between monocotyledonous and dicotyledonous species could be considered as a result of the more extensive size of adult cereal root systems and lack of such efficient screening strategies like those developed for *Arabidopsis*. Based on this, we will focus on root development in monocotyledons, especially in barley, which is the fourth most important crop in the world after maize, wheat, and rice. Recently, it is becoming a novel cereal model plant because of its true diploidy (Sreenivasulu et al. 2008).

Root system of monocotyledonous plants is generally composed of two fundamental parts: seminal root system, which develops from initials present in embryo, and nodal (often called adventitious or shoot-borne) root system, which originates from shoot (Hackett 1968). The dicotyledonous species develop a taproot system with one primary root and lateral branches, which remain active during the whole life cycle. However, dicotyledonous plants can also form roots called "adventitious" under unusual circumstances such as wounding or hormone application, etc., at uncharacteristic sites on a plant. Following Hochholdinger and coworkers (2004), we also suggest not calling monocotyledonous stem-derived crown and brace roots "adventitious" because they belong to the normal developmental program of cereals. Despite having to fulfill the same fundamental functions, the root systems of monocotyledons and dicotyledons differ both in morphology and anatomy. In monocotyledons, the secondary root growth do not occur, and root vessels are relatively uniform cylinders (in the absence of environmental stimuli) (Gorny 1992). The adult crop plant exhibits an extensive shoot-born root system, which plays a major role in the postembryonic root architecture (Hochholdinger et al. 2004; Hochholdinger and Zimmermann 2008). Nevertheless, it has been reported that maize seminal roots have relatively high water uptake capacity compared to other root types, which makes them important throughout whole plant life (Osmont et al. 2007).

## 2.2 Root Mutants of *Arabidopsis* Published in Pubmed

Both forward and reverse genetic approaches have been used to increase knowledge about root architecture. As there are many mutagenesis methods, the use of chemical mutagenesis mostly by EMS and insertional mutagenesis using T-DNA insertion, followed by mutant screening, apparently dominates. Using EMS, 147 gene alleles were obtained, 140 alleles by insertional mutagenesis (e.g., 19 by

transposable elements, 118 by T-DNA, 2 by promoter trap and 1 by activation tagging), whereas 22 alleles were obtained by physical approach (nine by fast neutrons, six by X-ray, seven by gamma rays). Reverse approach (e.g., RNAi, overexpression) were also commonly used to study influence of a gene of interest on root traits.

Using these strategies, it was possible to build the model pattern of root development in dicotyledons, based on data from reference *Arabidopsis*. Up to now, many genes have been shown to be involved in various aspects of *Arabidopsis* root development (Tables 2.1 and 2.2). Many of them have a pleiotropic effect not only on various stages of root development but also on whole plant per se. Nevertheless, we divided Arabidopisis genes controlling root system into formation of radial and longitudinal pattern, keeping in mind that assigning genes to only one chosen category could be misleading. The *Arabidopsis* radial pattern consists of a number of defined cell types organized in concentric layers, with the epidermis, ground tissue composed of cortex and endodermis, and the last main part called stele, which includes pericycle surrounding the central vascular cylinder (Scheres et al. 2002; Casson and Lindsey 2003). Based on this, we secondly divided genes responsible for root radial pattern into three groups, which assemble genes involved in epidermis, ground tissue, and stele development.

The first one (Table 2.1) includes genes involved in root hair development as a specific product of root epidermis. Both monocotyledonous and dicotyledonous root systems increase absorptive surface through the formation of root hairs. In *Arabidopsis*, root hairs always form on epidermal cells positioned over the radial cell wall between cortical cells (Dolan and Costa 2001). However, it is difficult to predict root hair-forming epidermal cells in cereals (Hochholdinger et al. 2004). In *Arabidopsis*, epidermis is composed of trichoblasts, which develop into root hair cells, and atrichoblasts, which remain hairless. The identity of these cells is regulated by positional information – hair-forming cells are located above two underlying cortical cells. The genetic analysis of root hair development has identified at least 39 genes that are required for the initiation and growth of the root hair. Some of them, such as *TRANSPARENT TESTA GLABRA1 (TTG1)*, *GLABRA3 (GL3)*, *ENHANCER OF GLABRA3 (EGL3)*, and *GLABRA2 (GL2)*, have been well described (Galway et al. 1994; Walker et al. 1999; Bernhardt et al. 2003). Both *TTG1* and *GL2* mutants have root hairs at nearly all root epidermal cells (Walker et al. 1999; Ohashi et al. 2003), whereas *GL3* and *EGL3* mutants have reduced numbers of atrichoblasts (Bernhardt et al. 2003). *TTG1* encodes a protein with WD40 repeats (Mendoza and Alvarez-Buylla 2000), which is localized in the nuclei of trichomes at all developmental stages (Zhao et al. 2008). It seems that *GL2* is a direct target of *GL3* and *EGL3*, whereas *TTG1* is directly regulated by *GL1* (Zhao et al. 2008).

The second group includes genes responsible for ground tissue pattering, composed of one cortex and one endodermis layer (Table 2.1), which originate from the common initial cell adjacent to the quiescent center (QC) (Scheres et al. 2002). Outside the endodermis, there are 4–6 layers in barley (Jackson 1922) and 8–15 in rice and corn (Hochholdinger et al. 2004) of bigger and thin-walled loosely packed

**Table 2.1** Mutated genes responsible for *Arabidopsis* root radial pattern

| Gene name (alias) | Accession number | Allele/mutation strategy/ reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| **Root hairs** | | | | |
| *TRANSPARENT TESTA GLABRA1 (TTG1)* | AT5G24520 | *ttg-1*/EMS | All cells with root hairs | Galway et al. (1994), Walker et al. (1999) |
| *GLABRA2 (GL2)* | AT1G79840 | *p777*/T-DNA insertion | All cells with root hairs | Ohashi et al. (2003) |
| *WEREWOLF (WER)* | AT5G14750 | *wer-1*/EMS | All cells with root hairs | Lee and Schiefelbein (1999) |
| *CAPRICE (CPC)* | AT2G46410 | *cpc-1*/T-DNA insertion | All cells without root hairs | Wada et al. (1997) |
| *GLABRA3 (GL3)* | AT5G41315 | *gl3-1*/EMS | Reduced number of atrichoblasts (much more root hairs) | Bernhardt et al. (2003) |
| *ENHANCER OF GLABRA3 (EGL3)* | AT1G63650 | *egl3-77439*/T-DNA insertion | Reduced number of atrichoblasts (much more root hairs) | Bernhardt et al. (2003) |
| *ENHANCER OF TRIPTICHON AND CAPRICE1 (ETC1)* | AT1G01380 | EMS | All cells without root hairs or root hairs are very sporadic | Kirk et al. (2004) |
| *ETROPIC ROOT HAIR 1 (ERH1)* | ? | *erh1*/fast neutrons | Reduced number of atrichoblasts (much more root hairs) | Hauser et al. (1995), Schneider et al. (1997) |
| *ETROPIC ROOT HAIR3 (ERH3)* | AT1G80350 | Gamma rays | Reduced number of atrichoblasts (much more root hairs) | Hauser et al. (1995), Schneider et al. (1997) |
| *TORNADO1 (TRN1)* | AT5G55540 | *trn1-1*/T-DNA insertion | Radial pattern is unsettled, pattern of root hairs is twisted like DNA helix | Dolan (2000) |
| *TORNADO2 (TRN2)* | AT5G46700 | *trn2-2*/EMS | Radial pattern is unsettled, pattern of root hairs is twisted like DNA helix | Dolan (2000) |
| *ROOT HAIRLESS 1 (RHL1)* | AT1G48380 | *rhl1-1*/T-DNA insertion *rhl1-2*/unknown | Root hairs very sporadic, pattern of trichoblasts and atrichoblasts unsettled | Schneider et al. (1997, 1998) |
| *ROOT HAIRLESS 2 (RHL2)* | AT5G02820 | T-DNA insertion | Root hairs very sporadic, pattern of trichoblasts and atrichoblasts unsettled | Schneider et al. (1997) |
| *ROOT HAIRLESS 3 (RHL3)* | AT3G20780 | *rhl3-1*/EMS | Root hairs very sporadic, pattern of trichoblasts and atrichoblasts unsettled | Schneider et al. (1998) |
| *CONSTITUTIVE TRIPLE RESPONSE (CTR1)* | AT1G01380 | ctrl-6/T-DNA insertion | Root hairs are formed on other place than usually | Kieber et al. (1993), Dolan et al. (1994) |
| | AT1G66340 | *etr1-1*/EMS | | Masucci and Schiefelbein (1996) |

| | | | | |
|---|---|---|---|---|
| *ETHYLENA RECEPTOR 1 (ETR1)* | | | Root hairs are formed near to the basal part of cell | |
| *ETHYLENE OVERPRODUCER 1 (ETO1)* | AT3G51770 | *eto1-1*/EMS | Root hairs are formed near to the apical part of cell | Masucci and Schiefelbein (1996), Yoshida et al. (2006) |
| *ROOT HAIR DEFECTIVE 6 (RHD6)* | ? | EMS | Root hairs are very sporadic and formed near to the basal part of cell, more than one root hair on one cell | Masucci and Schiefelbein (1994), Dolan (2001) |
| *SALT OVERLY SENSITIVE 4 (SOS4)* | AT5G37850 | *sos4-1*/EMS | Root hairs are very, very sporadic | Shi and Zhu (2002) |
| *ROOT HAIR DEFECTIVE 1( RHD1)* | AT1G64440 | *rhd1-2*/EMS<br>*rhd1-1*/EMS | Primordium is very big, root hairs with normal length | Schiefelbein and Somerville (1990) |
| *TIP GROWTH DEFECTIVE 1 (TIP1)* | AT5G20350 | *tip1-1*/EMS | Primordium is bigger, root hairs are shorter and often branched, sometimes there are 2–4 root hairs on one cell | Ryan et al. (1998) |
| *SUPERCENTIPEDE 1 (SCN1)* | ? | EMS | 1–5 primordiums on one cell | Grierson et al. (2001) |
| *TINY ROOT HAIR 1 (TRH1)* | AT4G23640 | *trh1*/EMS | Root hair growth stopped at primordium stage | Rigas et al. (2001), Vicente-Agullo et al. (2004) |
| *HAIR DEFECTIVE 2 (RHD2)* | AT5G51060 | *rhd2-1*/EMS | Root hair growth stopped at primordium stage | Schiefelbein and Somerville (1990) |
| *SHAVEN1,2,3 (SHV1,2,3)* | ? | EMS | Root hairs are shorter | Parker et al. (2000) |
| *KOJAK (KJK)* | AT3G03050 | *csld3-1*/T-DNA insertion | Root hairs rupture at their tip soon after initiation | Favery et al. (2001) |
| *MRH2* | AT3G54870 | *mrh2-1*/T-DNA insertion | Mutant exhibits wavy and branching root hair phenotype | Yang et al. (2007) |
| *LRR/EXTENSIN 1 (LRX1)* | AT1G12040 | lrx1/En-1 transposition | Root hairs are shorter, often branched | Baumberger et al. (2001) |
| *DEFORMED ROOT HAIRS 1 (DER1)* | ? | EMS | Root hairs are shorter, primordium is bigger, and sometimes there are 2 root hairs on one cell | Ringli et al. (2002) |
| | AT2G35630 | EMS | Root hairs are wavy and branched | Whittington et al. (2001) |

(*continued*)

**Table 2.1** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/ reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *MICROTUBULE ORGANIZATION 1 (MOR1)* | | | | |
| *INCOMPLETE ROOT HAIR ELONGATION (IRE)* | AT5G62310 | T-DNA insertion | Root hairs are shorter | Oyama et al. (2002) |
| *ROOT HAIR DEFECTIVE 3 (RHD3)* | AT3G13870 | *rhd3-1*/EMS | Root hairs are shorter and wavy | Schiefelbein and Somerville (1990), Galway et al. (1997), Zheng et al. (2004) |
| *ROOT HAIR DEFECTIVE 4 (RHD4)* | ? | EMS | Root hairs are shorter and wavy | Schiefelbein and Somerville (1990) |
| *CAN OF WORMS 1 (COW1)* | AT4G34580 | T-DNA insertion | Root hairs are shorter, wavy, and 1–3 on one cell | Grierson et al. (1997), Böhme et al. (2004) |
| *BRISTLED1 (BST1)* | AT5G65090 | Fast neutrons | Root hairs are shorter, wavy and branched | Parker et al. (2000) |
| *CENTIPEDE 1,2,3 (CEN1,2,3)* | ? | EMS | Root hairs are shorter, wavy and branched | Parker et al. (2000) |
| *ACTIN 2 (ACT2)* | AT3G18780 | *act2-3*/T-DNA insertion | Root hairs are shorter and branched | Ketlaar et al. (2003) |
| *SUPRESOR OF AUXIN RESISTANCEL (SAR1)* | AT2G33120 | EMS | Root hairs are longer and on almost all cells | Cernac et al. (1997) |
| *ROOT AND POLLEN ARFGAP (RPA)* | AT2G35210 | T-DNA insertion | Aberrant root hair phenotype, including bulged, branched and shorter root hairs | Song et al. (2006) |
| **Ground tissue pattern (cortex + endodermis)** | | | | |
| *POM-POM1 (POM1)* | AT1G05850 | *pom 1-1*/T-DNA insertion | Shorter root and significantly greater cell volume | Hauser et al. (1995), Scheres et al. (2002) |
| *POM-POM2 (POM2)* | ? | *pom2-1, 2-2*/fast neutron | Shorter root and significantly greater cell volume | Hauser et al. (1995), Scheres et al. (2002) |
| | ? | *qui-1*/X-ray *qui-2*/T-DNA insertion | Shorter root and significantly greater cell volume, decreased cell elongation, | Hauser et al. (1995), Scheres et al. (2002) |

| | | | | |
|---|---|---|---|---|
| *PROCUSTE1/QUILL/ ATCESA6 (PRC1/ QUI)* | | *qui-3*/EMS | specifically in roots and dark-grown hypocotyls | |
| *COBRA (COB)* | AT5G60920 | *cob1-4*/EMS<br>*cob-2*/X-ray<br>*cob-2*/T-DNA insertion | Abnormal root cell expansion, greatest in the epidermal cells | Benfey et al. (1993, Scheres et al. (2002), Roudiera et al. (2005) |
| *SHORT ROOT (SHR)* | AT4G37650 | *shr-1*/T-DNA insertion | Determinate root growth, very short root missing an internal cell layer, mutant layer has attributes of cortex only | Benfey et al. (1993), Scheres et al. (2002), Franco-Zorrilla et al. (2005) |
| *SCARECROW (SCR)* | AT3G54220 | *scr-4, scr-1*/T-DNA insertion | Defects in the division and/or specification of endodermis and cortex | Benfey et al. (1993), Scheres et al. (2002) |
| *KORRIGAN (KOR)* | AT5G49720 | *kor1-1*/EMS<br>*kor1-2*/*Agrobacterium* transformation | Radially expanded hypocotyl cells, impaired root expansive growth, formation of aberrant cell plates, incomplete cell walls, and multinucleated cells, leading to severely abnormal root morphology (cells divided randomly and often contained incomplete cell walls | Zuo et al. (2000), Scheres et al. (2002) |
| *LION'S TAIL* | ? | T-DNA insertion | Abnormal root cell expansion, greatest in the stele cells | Benfey et al. (1993), Hauser et al. (1995), Scheres et al. (2002) |
| *SABRE (SAB)* | AT1G58250 | EMS | Abnormal root-cell expansion, primarily in radial orientation. Expansion greatest in cortex cells | Benfey et al. (1993) |
| *FACKEL (FK)* | AT3G52940 | T-DNA insertion | Short roots and hypocotyl, defective cell shape, supernumerary cell layers and aberrant vascular patterning | Souter et al. (2002) |
| *HYDRA 1 (HYD1)* | AT1G20050 | *hyd1-2*/ T-DNA insertion | Short roots and hypocotyls, defective cell shape, supernumerary cell layers and aberrant vascular patterning. Root may cease cell division within 2 weeks after germination, or may continue to grow very slowly until the seedling dies | Souter et al. (2002) |

(*continued*)

**Table 2.1** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/ reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *CUDGEL-1* | ? | *cud-1*/X-ray | Shorter root and significantly greater cell volume | Hauser et al. (1995), Scheres et al. (2002) |
| *SCHIZORIZA (SCZ)* | ? | Ac/Ds | Subepidermal layer (ground tissue) develops root hairs – supernumerary layers in the ground tissue | Mylona et al. (2002) |
| *KNOPF (KNF)* | AT1G67490 | *knf-14*/EMS | Radially swollen root phenotype due to cellulose deficiency and isotropic embryo growth | Gillmor et al. (2002) |
| *RADIAL SWELLING 1 (RSW1)* | AT4G32410 | *rsw1-1, 1-2*/EMS | Radially swollen root phenotype due to cellulose deficiency and isotropic embryo growth | Gillmor et al. (2002) |
| *RADIAL SWELLING 3 (RSW3)* | AT5G63840 | *rsw3-1*/EMS | Temperature-sensitive, cellulose-deficient mutant with radially swollen roots | Burn et al. (2002) |
| *RADIAL SWELLING 4 (RSW4)* | ? | EMS | Radially swolling roots and temperature sensitive phenotype. Cortical microtubules and cellulose microfibrils are neither depleted nor disoriented | Wiedemeier et al. (2002) |
| *RADIAL SWELLING 7 (RSW7)* | ? | EMS | Radially swollen roots and temperature sensitive phenotype. Cortical microtubules and cellulose microfibrils are neither depleted nor disoriented | Wiedemeier et al. (2002) |
| *PLEYADE (PLE)* | AT5G51600 | *ple-1, -2*/EMS, *ple-3*/T-DNA | Shorter roots exhibit a wavy growth pattern and develop more lateral roots. Irregular cell expansion, multinucleated cells, cell wall stubs, epidermis, cortex and endodermis are radially enlarged; symmetry of the vascular tissues is disrupted and synchronized cell divisions in incompletely separated cells that are all characteristics of defective cytokinesis | Műller et al. (2002) |

| | | | | |
|---|---|---|---|---|
| *HYADE1 (HYA1)* | ? | *hya-1, -2, -3*/EMS | Shorter roots exhibit a wavy growth pattern and develop more lateral roots. Irregular cell expansion, multinucleated cells, cell wall stubs, epidermis, cortex and endodermis are radially enlarged; symmetry of the vascular tissues is disrupted and synchronized cell divisions in incompletely separated cells that are all characteristics of defective cytokinesis | Műller et al. (2002) |
| *BOTERO1 (BOT)* | ? | *bot1-1, 1-3, 1-4, 1-5*/EMS<br>*bot1-2*/retrotransposon Tnt1 bot1-7, 1-8/T-DNA insertion | Shorter and thicker root and hypocotyl. Affected in anisotropic growth, loosely organized microtubules | Bichet et al. (2001) |
| *CLUB* | ? | EMS | Lack of primary roo. Cell wall stubs, gapped walls and multinucleate cells. Incapable of growing long root hairs, likely to represent a tip growth defect. | Söllner et al. (2002) |
| *BUBLINA* | ? | EMS | Lack of primary root. Cell wall stubs, gapped walls and multinucleate cells. Long root hairs. | Söllner et al. (2002) |
| *BIMS* | ? | EMS | Lack of primary root. Cell wall stubs, gapped walls and multinucleate cells. Long root hairs. | Söllner et al. (2002) |
| *MASSUE* | ? | EMS | Stunted root, cell wall stubs, gapped walls and multinucleate cells | Söllner et al. (2002) |
| *BLOATED* | ? | EMS | Stunted root, cell wall stubs, gapped walls and multinucleate cells. Long root hairs | Söllner et al. (2002) |
| *ROD* | ? | EMS | Stunted root, cell wall stubs, gapped walls and multinucleate cells | Söllner et al. (2002) |
| *KEULE* | AT1G12360 | EMS | Lack of primary root. Cell wall stubs, gapped walls and multinucleate cells. Incapable | Söllner et al. (2002) |

(*continued*)

**Table 2.1** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/ reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| | | | of growing long root hairs, likely to represent a tip growth defect. | |
| *KNOLLE* | AT1G08560 | X-ray | Lack of primary root. Cell wall stubs, gapped walls and multinucleate cells. Cytokinesis defects are more severe than in *keule* mutants: severely perturbed epidermis and long root hairs | Söllner et al. (2002) |
| *HINKEL (HIK)* | AT1G18370 | EMS | Long root hairs, cell wall stubs, gapped walls and multinucleate cells | Söllner et al. (2002) |
| *SHORT BLUE ROOT (SBR)* | ? | EMS | Deformation of epidermal cells, larger meristematic cells, disorganized root vascular tissue. Reduced lateral root initiation, adventitious roots often form on hypocotyl | Subramanian et al. (2002) |
| **Stele pattern (pericycle + vasculature)** | | | | |
| *ALTERED PHLOEM DEVELOPMENT (APL)* | AT1G79430 | En-1 transposition | Defects in vascular tissue | Bonke et al. (2003) |
| *LONESOME HIGHWAY (LHW)* | ? | EMS | Lack of root bilateral symmetry: reduced the number of cells in the center of the root, single xylem and phloem poles | Ohashi-Ito and Bergmann (2007) |
| *WOODEN-LEG (WOL)* | AT2G01830 | *ahk4-1*/T-DNA insertion *wol-1*/EMS | Protoxylem is the only tissue in the vascular cylinder | Scheres et al. (2002), Sieberer et al. (2003), Franco-Zorrilla et al. (2005) |
| *KOBITO 1 (KOB1)* | AT3G08550 | *eld1-1*/gamma rays | Cellulose-deficient dwarf mutant. Randomized microfibrils occluded by a layer of pectic material | Pagant et al. (2002) |

| | | | | |
|---|---|---|---|---|
| *IRREGULAR XYLEM 1 (IRX1)* | AT4G18780 | *irx1-1*/EMS | Severe deficiency in the deposition of cellulose in secondary cell walls, which results in collapsed xylem cells | Taylor et al. (2000) |
| | | *irx1-5*/ T-DNA insertion | | |
| *IRREGULAR XYLEM 3 (IRX3)* | AT5G17420 | *irx3-1*/EMS | Severe deficiency in the deposition of cellulose in secondary cell walls, which results in collapsed xylem cells | Taylor et al. (2000) |
| *ECTOPIC LIGNIFICATION 1 (ELI-1)* | ? | EMS | Mutant exhibits altered patterns of lignification (ectopic lignification), stunted phenotype and disorganized xylem tissue | Caño-Delgado et al. (2000) |

cortical cells (Briggs 1978), whereas in *Arabidopsis*, root comprises only one endodermis and one cortical layer (Scheres et al. 2002). The one layer of endodermis is exceptionally thick-walled, just like that reported earlier in rice, maize, and onion (Jackson 1922) with a "Caspian strip" in the walls (Karas and McCully 1973). Many mutations that disrupt patterning of the ground tissue have been identified. For example, both the *SCARECROW (SCR)* and *SHORT ROOT (SHR)* mutants have a single layer instead of cortex and endodermis. These genes encode putative transcription factors of the GRAS family responsible for specifying QC and for controlling the periclinal cell division of the daughter cell of their common initial cell, which leads to two adjacent layers (Ueda et al. 2005). However, *SCR* mutant layer has differentiated attributes of both cortex and endodermis, whereas *SHR* layer attribute only to cortex (Scheres et al. 2002). *SCR* was previously shown to act downstream of *SHR* (Ueda et al. 2005), whereas Levesque and coworkers (2006) suggested that *SHR* not only directly regulates the transcription of *SCR* through binding to the chromatin upstream of the gene but also functions in development of the vascular tissue.

In the middle of the young barley root is a duct bordered by thin-walled cells, which becomes thickened during aging. The continuity of one layer of pericycle cells is broken by the xylem groups, which contain large vessels. The number of xylem groups in barley root is from 6 to 8 alternating with groups of phloem (Jackson 1922). Protoxylem elements abut directly to the single layer of endodermis, the walls of which thicken with age (Briggs 1978). Fully developed monocotyledonous root consists of much more thickened cell walls in stele, and sclerenchyma develops in the outer cortex (Briggs 1978). In contrast to monocotyledonous root radial pattern, the primary vascular pattern in *Arabidopsis* roots involves a xylem axis and two phloem poles, surrounded by one pericycle layer (Scheres et al. 2002). Only few *Arabidopsis* genes, which are responsible for stele pattern, have been described (Table 2.1). In the *WOODEN-LEG (WOL)* mutant, protoxylem is the only tissue in the vascular cylinder (Sieberer et al. 2003). It has been shown that this gene encodes a cytokinin receptor (Franco-Zorrilla et al. 2005), which is required for asymmetric cell divisions of phloem and procambium initial cells (Scheres et al. 2002). Defects in vascular tissue could be also observed in *ALTERED PHLOEM DEVELOPMENT (APL)* mutant. This gene, which encodes a MYB transcription factor, has a dual role both in promoting phloem differentiation and in repressing xylem differentiation during vascular development (Bonke et al. 2003).

Root meristem tissues are organized in longitudinal cell files. From the root tip to the plant base, three main regions could be distinguished: the division, elongation, and the differentiation zone (Table 2.2). During both monocotyledons and dicotyledons embryogenesis, first the primary or embryonic radicle and few seminal roots are formed, respectively, whereas lateral roots (LRs) originate from existing roots postembryonically. LRs originate from the group of pericycle cells in *Arabidopsis* (Malamy and Benfey 1997; Scheres et al. 2002), whereas in monocotyledons, endodermis is also involved (Hochholdinger et al. 2004; Karas and McCully 1975). In *Arabidopsis*, lateral roots emerge from the pericycle cells adjacent to

**Table 2.2** Mutated genes responsible for *Arabidopsis* root longitudinal pattern

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| **Meristematic zone** | | | | |
| *ROOT PRIMORDIUM DEFECTIVE 1 (RPD1)* | AT4G33495 | *rpd1-1*/EMS | Temperature-sensitive mutant with defects at the initial stage of root primordium development. Embryogenesis arrested at the globular stage | Konishi and Sugiyama (2006) |
| *HALTED ROOT (HLR)* | AT4G29040 | *hlr-1, hlr-2*/T-DNA insertion | In postembryonic meristems the cellular organization is disrupted, the activity of proteasomes is reduced | Ueda et al. (2004) |
| *RUB1 CONJUGATING ENZYME 1 (RCE1)* | AT4G36800 | T-DNA insertion | Dwarf phenotype. Reduced response to the change in the gravity vector deficient in auxin and jasmonate response, fewer lateral roots in response to auxin | Dharmasiri et al. (2003) |
| *PLETHORA 1 (PLT1)* | AT3G20840 | *plt1-1, 1-2, 1-3, 1-4, 1-5* /T-DNA insertion | Mutant shows an abnormal cellular organization of the hypophyseal derivatives | Aida et al. (2004) |
| *PLETHORA 2 (PLT2)* | AT1G51190 | *plt2-2*/ T-DNA insertion | Mutant shows an abnormal cellular organization of the hypophyseal derivatives | Aida et al. (2004) |
| *GNOM/EMB30 (GN)* | AT1G13980 | *emb30-1, emb30-2, gn*/EMS | Failure in maintenance of primary root meristem activity; reduced LR number | Shevell et al. (2000), Geldner et al. (2003) |
| *STEROL METHYLTRANSFERASE 1 (SMT1)* | AT5G13710 | EMS | Mutants displays several conspicuous cell polarity defects, primary and lateral roots are shorter | Willemsen et al. (2003) |
| *FASS (FS)* | AT5G18580 | EMS | Drastically changed the shape of the seedling without altering body pattern and affected cell elongation and orientation of cell walls | Torres-Ruiz and Jürgens (1994) |
| *HOBBIT (HBT)* | AT2G20000 | EMS | Postembryonic meristem activity is absent and the distal cell types (QC, columella- and lateral root cap) do not differentiate. The earliest defect found in mutants is disturbance of cell division planes in the hypophysis, the progenitor cell for the QC and columella | Blilou et al. (2002), Scheres et al. (2002) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *BODENLOS (BDL)* | AT1G04550 | EMS | Mutant failure to establish the hypophysis which caused severe primary root defects | Hamann et al. (1999, 2002), Scheres et al. (2002) |
| *MONOPTEROS (MP)* | AT1G19850 | EMS | Mutant failure to establish the hypophysis which caused severe primary root defects (loss-of-function) | Hamann et al. (2002) |
| *ACID-INDUCED PROTEIN 13 (IAA13)* | AT2G33310 | EMS | Lack of root caused by failure in the specification of the hypophysis and subsequent abnormal cell division patterns | Weijers et al. (2005) |
| *ROOT MERISTEMLESS 1 (RML1)* | AT4G23100 | EMS | Extremely short mature root (1–2 mm) composed of the same number of cells and cell files as the embryonic root, unable to establish and maintain an active, undifferentiated meristematic zone (mutation does not affect axial and radial patterns of root cell organization). Mutant produces lateral roots readily | Vernoux et al. (2000) |
| *ROOT MERISTEMLESS 2 (RML2)* | ? | EMS | Extremely short mature root (1–2 mm). Mutant produces nodule-like structures but not lateral roots. Limited number of cell divisions | Cheng et al. (1995) |
| *HISTIDINOL-PHOSPHATE AMINOTRANSFERASE (HPA)* | AT1G71920 | *emb-2196*/ T-DNA insertion *hpa1*/ EMS | Very short root system, unable to sustain primary root growth 2 days after germination | Mo et al. (2006) |
| *INCURVATA 4 (ICU4)* | AT1G52150 | *icu4-1, icu4-2*/En-2 transposition *icu4-3, icu4-4*/ T-DNA insertion | Longer root hairs, higher number of secondary roots, reduced root length and an aberrant cell pattern in the root apical meristem | Ochando et al. (2006) |
| *TEBICHI (TEB)* | AB192295 | *teb-1*/T-DNA insertion | Short root, split root tip and an aberrant pattern of cell division in postembryonic development | Inagaki et al. (2006) |
| *TONSOKU (TSK)* | AT3G18730 | T-DNA insertion | Short roots and altered responses to DNA damage | Inagaki et al. (2006) |

| | | | | |
|---|---|---|---|---|
| *RETINOBLASTOMA-RELATED (RBR)* | AT3G12280 | *rbr1-3*/ T-DNA insertion | Supernumerary stem cells | Wildwater et al. (2005) |
| *AUXIN INFLUX 1 (AUX1)* | AT2G38120 | *aux1110*, *aux12*, *aux1106*, *aux17*, *aux122*/EMS, *aux121*/X-ray | 50% reduction in number of LR primordia, reduced auxin-sensitive root elongation, perturbed in gravitropism | Casimiro et al. (2003) |
| *PIN-FORMED 1 (PIN1)* | AT1G73590 | En-1 transposition | Defects in auxin transport. Reduction of root length, meristem size and root elongation zone size | Friml et al. (2003) |
| *PIN-FORMED 2 (PIN2)* | AT5G57090 | En-1 transposition | Defects in auxin transport. Reduction of root length, meristem size and root elongation zone size | Muller et al. (1998) |
| *PIN-FORMED 3 (PIN3)* | AT1G70940 | *pin3-5*/ T-DNA insertion | Defects in auxin transport. Reduction of root length, meristem size and root elongation zone size. Subtle cell division defects in the QC and columella root cap | Friml et al. (2003) |
| *PIN-FORMED 4 (PIN4)* | AT2G01420 | *pin4-3*/transposon insertion | Defects in auxin transport. Reduction of root length, meristem size and root elongation zone size. Subtle cell division defects in the QC and columella root cap | Friml et al. (2003) |
| *PIN-FORMED 7 (PIN7)* | AT1G23080 | *pin7-1, pin7-3*/ transposon insertion *pin7-2*/T-DNA insertion | Defects in auxin transport. Reduction of root length, meristem size and root elongation zone size. Subtle cell division defects in the QC and columella root cap | Friml et al. (2003) |
| *PINOID (PID)* | AT2G34650 | *pid-1, pid-2*/EMS | Mutants do not display a root phenotype. Constitutive overexpression results in a consumption of the primary root meristem within a few days after germination: all cells at the root tip become elongated and root hairs cover the primary root tip | Shishkova et al. (2008) |
| *ISOPENTENYLTRANSFERASE 3 (IPT3)* | AT3G63110 | *atipt 3*/ T-DNA insertion | Triple cytokinin biosynthetic mutant with severely reduced cytokinin level. Enlarged RAM shows an increased number of meristematic cells | Dello-Ioio et al. (2007) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *ISOPENTENYLTRANSFERASE (IPT5)* | AT5G19040 | *atipt 5*/T-DNA insertion | Triple cytokinin biosynthetic mutant with severely reduced cytokinin level. Enlarged RAM shows an increased number of meristematic cells | Dello-Ioio et al. (2007) |
| *ISOPENTENYLTRANSFERASE (IPT7)* | AT3G23630 | *atipt 7*/T-DNA insertion | Triple cytokinin biosynthetic mutant with severely reduced cytokinin level. Enlarged RAM shows an increased number of meristematic cells | Dello-Ioio et al. (2007) |
| *ENDO-BETA-1,4-GLUCANASE (CEL5)* | ? | T-DNA insertion | Mutant forms the root cap and sheds root cap cells but sloughing is less efficient compared to wild type | Campillo et al. (2004) |
| *NO HYDROTROPIC RESPONSE (NHR)* | ? | EMS | No positive hydrotropic response. Abnormal root cap morphogenesis and reduced root growth sensitivity to abscisic acid (ABA) and the polar auxin transport inhibitor N-(1naphtyl) phtalamic acid (NPA). Homozygous condition results in a lethal phenotype | Eapen et al. (2003) |
| *MIZU-KUSSEI 1 (MIZ1)* | ? | EMS | Mutant impaired in hydrotropism but shows normal gravitropism and elongation growth | Kobayashi et al. (2007) |
| *SKU 5* | AT4G12420 | T-DNA insertion | Roots skewed and looped away from the normal downward direction of growth | Sedbrook et al. (2002) |
| *SPIRAL 1 (SPR1)* | AT2G03680 | *spr1-1*/EMS *spr1-5*/T-DNA insertion | Right-handed helical root growth | Nakajima et al. (2004) |
| *LEFTY 1* | ? | EMS | Left-handed helical growth: epidermal cell files of lefty roots begin to skew at the region where first root hair is emerging | Thitamadee et al. (2002) |
| *LEFTY 2* | ? | EMS | Left-handed helical growth: epidermal cell files of lefty roots begin to skew at the region where first root hair is emerging | Thitamadee et al. (2002) |

| | | | | |
|---|---|---|---|---|
| *WAVY GROWTH 2 (WAV2)* | AT5G20520 | *wav2-1, wav2-2*/ T-DNA insertion | Enhanced wavy root growth | Mochizuki et al. (2005) |
| *WAG1* | AT1G53700 | *wag1-1, wag1-2*/ T-DNA insertion | Wavy root phenotype | Santner and Watson (2006) |
| *WAG2* | AT3G14370 | *wag2-1*/ T-DNA insertion | Wavy root phenotype | Santner and Watson (2006) |
| *AUXIN RESPONSE FACTOR 10 (ARF10)* | AT2G28350 | *arf10-2*/ T-DNA insertion | *arf10 arf16* double mutant displays uncontrolled cell division and blocked cell differentiation in the root distal region and shows a tumor-like root apex and loss of gravity-sensing | Wang et al. (2006) |
| *AUXIN RESPONSE FACTOR 16 (ARF16)* | AT4G30080 | *arf16-2*/ T-DNA insertion | Uncontrolled cell division and blocked cell differentiation in the root distal region. Mutant shows a tumor-like root apex and loss of gravity-sensing | Wang et al. (2006) |
| *ADENYLATE KINASE 2 (AK2)* | ? | T-DNA insertion | Mutant exhibits significantly elevated root growth, cap morphogenesis defects, along with alterations in root sensitivity to gravistimulation and slower kinetics of root gravitropic curvature | Carrari et al. (2005), Young et al. (2006) |
| *ADENYLATE KINASE 3 (AK3)* | ? | T-DNA insertion | Mutant exhibits significantly elevated root growth, cap morphogenesis defects, along with alterations in root sensitivity to gravistimulation and slower kinetics of root gravitropic curvature | Carrari et al. (2005), Young et al. (2006) |
| **Elongation zone** | | | | |
| *QUASIMODO 1 (QUA)* | AT3G25140 | *qua1-1, qua1-2*/ T-DNA insertion | Reduced cell adhesion. Dwarf phenotype and rough aspect resulting from numerous cells protruding from their cotyledons, leaves, and hypocotyls | Bouton et al. (2002) |
| *PROPORZ 1 (PRZ1)* | AT4G16420 | *prz1-1*/T-DNA insertion | Mutant exhibits defects in cell and organ differentiation | Sieberer et al. (2003) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *DAWDLE (DDL)* | AT3G20550 | T-DNA insertion | Mutant plants exhibit shortened roots caused by lower number of cell divisions | Morris et al. (2006) |
| *CYTOKININ ROOT SYNDROM (CKR1)* | ? | *ckrl-7,-8,-12,-50,-09*/EMS | Mutant exhibits significantly elevated root growth with shorter root hairs and altered response to cytokinin | Su and Howell (1992) |
| *POLARIS (PLS)* | AT4G39403 | Promoter trap | Short-root phenotype, relatively short and radially expanded cells, altered response to exogenous auxins and cytokinins, enhanced ethylene-response phenotype, defective auxin transport and homeostasis, and altered microtubule sensitivity to inhibitors | Chilley et al. (2006) |
| *YADOKARI 1-D (YADK 1-D)* | ? | T-DNA insertion | Dwarf mutant: short hypocotyl and primary root, reduced apical dominance and reduced number of lateral roots | Takase et al. (2004) |
| *MURUS 1 (MUR1)* | AT3G51160 | *mur1-1, 1-2*/EMS | Root growth defects, altered cell walls which are more brittle | Freshour et al. (2003) |
| *BREVIS RADIX (BRX)* | AT1G31880 | T-DNA insertion | Roots composed of shorter as well as fewer cells. Reduction in mature cell size as well as cell proliferation causes slow primary root growth | Mouchel et al. (2004) |
| *PETIT 1 (PT1)* | ? | *pet1-1*/fast neutrons | Defective in aspects of root and hypocotyl elongation and presence of gaps in internal cortical and epidermal cell walls | Kurata and Yamamoto (1998) |
| *PROCUSTE 1 (PRC1)* | ? | *prc1-8, 1-10, 1-12, 1-19*/ T-DNA insertion | Decreased cell elongation in roots and dark-grown hypocotyls | Fagard et al. (2000) |
| *CONSTITUTIVE EXPRESSION OF VSP1 1 (CEV1)* | AT5G05170 | *ixr1-1, 1-2*/EMS | Stunted phenotype. Short hypocotyls in dark-grown seedlings. Roots have reduced cellulose content, increased production of jasmonate and ethylene | Ellis et al. (2002) |

| | | | | |
|---|---|---|---|---|
| *ARABIDOPSIS THALIANA ACT7 (ACT7)* | AT5G09810 | *act7-2, 7-3, 7-4*/ T-DNA insertion | Increased root twisting and waving, and retarded root growth. Root apical cells are not in straight files and contain oblique junctions between cells | Gilliland et al. (2003) |
| *WAVE-DAMPENED 2 (WVD2)* | AT5G28646 | *Overexpression wvd2-1*/Ac/Ds | Constitutive right-handed helical growth in both roots and etiolated hypocotyls and impaired anisotropic expansion. | Yuen et al. (2003) |
| *WVD2-LIKE 1 (WDL1)* | AT3G04630 | Overexpression/ Ac/Ds | Constitutive right-handed helical growth in both roots and etiolated hypocotyls and impaired anisotropic expansion | Yuen et al. (2003) |
| *PICKLE, SUPPRESSOR OF SLR 2 (PCL)* | AT2G25170 | EMS | Primary root differentiates improperly and expresses embryonic characteristics after germination | Li et al. (2005) |
| *STUNTED PLANT 1 (STP1)* | ? | EMS | Roots elongate more slowly than in the WT | Baskin et al. (1995), Beemster and Baskin (2000) |
| *XYLOGLUCAN ENDOTRANGLUCOSYLASE/ HYDROLASE 21 (XTH21)* | AT2G18800 | T-DNA insertion | Stunted phenotype with shorter root hairs and perturbation in epidermis cell formation | Liu et al. (2007) |
| *ZINC FINGER OF ARABIDOPSIS THALIANA 6 (ZAT6)* | ? | RNAi/ overexpression | RNAi mediated silencing results in lethality. Overexpression affects root development and retards seedling growth as a result of decreased Pi acquisition | Devaiah et al. (2007) |
| *MULTIDRUG RESISTANCE P –GLICOPROTEIN (PGP4)* | AT2G47000 | *pgp4-1, 4-3, 4-4*/ T-DNA insertion | Reduced root gravitropic bending and elongation as well as lateral root formation | Terasaka et al. (2005) |
| *RESISTANT TO IBA (RIB1)* | ? | Ac/Ds | Shorter primary root, increased number of lateral roots and elongation defects in root gravitropism. Less sensitive to growth inhibition by IBA and less sensitive to IBA in stimulation of lateral root formation | Poupart and Waddell (2000), Poupart et al. (2005) |
| *XIPOTL 1* | ? | T-DNA insertion | Short primary root, a high number of lateral roots and short epidermal cells with aberrant morphology and few root hairs | Cruz-Ramírez et al. (2004) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *PHOSPHOLIPASE DS 1 (PLDζ1)* | ? | T-DNA insertion | Slower elongation of primary root and longer lateral roots in low phosphate conditions | Li et al. (2006a, b) |
| *PHOSPHOLIPASE DS 2 (PLDζ2)* | ? | T-DNA insertion | Slower elongation of primary root and longer lateral roots in low phosphate conditions | Li et al. (2006a, b) |
| *WEAK ETHYLENE INSENSITIVE 2 (WEI2)* | AT5G05730 | *wei2-1, 2-3*/EMS | Root-specific ethylene insensitivity. Upregulation of WEI2/ASA1 and WEI7/ASB1 by ethylene results in the accumulation of auxin in the tip of primary root, whereas loss-of-function mutations in these genes prevent the ethylene-mediated auxin increase | Stepanova et al. (2005) |
| *WEAK ETHYLENE INSENSITIVE 7 (WEI7)* | AT1G25220 | *wei7-1, 7-2*/Ac/Ds | Root-specific ethylene insensitivity. Upregulation of *WEI2*/*ASA1* and *WEI7*/*ASB1* by ethylene results in the accumulation of auxin in the tip of primary root, whereas loss-of-function mutations in these genes prevent the ethylene-mediated auxin increase | Stepanova et al. (2005) |
| *HISTONE MONOUBIQUITINATION 1 (HUB1)* | AT2G44950 | *hub1-1*/EMS *hub1-2, hub1-3*/T-DNA insertion | Slow primary root growth | Fleury et al. (2007) |
| *SCARFACE (SFC)* | AT5G13300 | sfc-9/T-DNA insertion | Shorter roots | Sieburth et al. (2006) |
| **Differentiation zone: lateral roots (LR)** | | | | |
| *ARABIDILLO-1* | AT2G44900 | T-DNA insertion | Fewer lateral roots | Coates et al. (2006) |
| *ARABIDILLO-2* | AT3G60350 | T-DNA insertion | Fewer lateral roots | Coates et al. (2006) |
| *SUPERROOT 1 (SUR1)* | AT2G20610 | *sur1-2, 1-3, 1-4, 1-5, 1-6*/EMS | Increased LR number and formation of additional adventitious root | Celenza et al. (1995) |

| | | | | |
|---|---|---|---|---|
| *SUPERROOT 2 (SUR2)* | AT4G31500 | En-1 transposition | Numerous adventitious roots begin to grow from the hypocotyl, lateral root primordial develop at high frequency, root hairs appear at higher density and root elongation is reduced | Casimiro et al. (2003), Casson and Lindsey (2003) |
| *ABERRANT LATERAL ROOT FORMATION 4 (ALF4)* | AT5G11030 | *alf4-1*/gamma rays | Unable to produce lateral roots and does not respond to exogenous auxins | Celenza et al. (1995), Casimiro et al. |
| *CEGENDUO (CEG)* | ? | T-DNA insertion | Increased lateral root production | Dong et al. (2006) |
| *KIP-RELATED PROTEIN 2 (KRP2)* | AT3G50630 | Overexpression | Mutations do not give any remarkable morphological phenotypes what indicates the presence of redundant functions. The number of lateral roots in overexpression line was reduced by 60% compared with that in the wild type | Himanen et al. (2002) |
| *KANADI (KAN)* | AT5G16560 | *kan1-2* /EMS | Reduced primary root length and reduced lateral root (LR) number | Hawker and Bowman (2004) |
| *KANADI 2 (KAN2)* | AT1G32240 | *kan2-1*/EMS | Reduced primary root length and LR number | Hawker and Bowman (2004) |
| *KANADI 3 (KAN3)* | AT4G17695 | *kan3-1*/EMS | Reduced primary root length and fewer lateral roots | Hawker and Bowman (2004) |
| *PHABULOSA 6 (PHB6)* | AT2G34710 | EMS | Reduced LR number | Hawker and Bowman (2004) |
| *PHAVOLUTA 5 (PHV5)* | AT1G30490 | T-DNA insertion | Reduced LR number | Hawker and Bowman (2004) |
| *REVOLUTA 10 (REV10)* | AT5G60690 | T-DNA insertion | Reduced LR number | Hawker and Bowman (2004) |
| *AUXIN RESPONSE FACTOR 8 (ARF8)* | AT5G37020 | *arf8-1*/ T-DNA insertion | Long-hypocotyl phenotype in light conditions and increased formation of LR | Tian et al. (2004) |
| *AUXIN RESPONSE FACTOR 10 (ARF19)* | AT1G19220 | *arf19-1*/ T-DNA insertion | Mutant with reduced LR development | Okushima et al. (2007) |
| *TRANSPORT INHIBITOR RESPONSE 1 (TIR1)* | AT3G62980 | *tir1-9*/T-DNA insertion | Reduced LR number. Reduced auxin-transport-inhibitor-sensitive root elongation | Xie et al. (2000), Casimiro et al. (2003) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *ENHANCER OF TIR1-1 AUXIN RESISTANCE (ETA3)* | AT1G19220 | EMS | Auxin-resistant root growth in seedlings and reduced LR development | Gray et al. (2003) |
| *ARABIDOPSIS SERINE/THREONINE KINASE 1 (ASK1)* | AT1G10940 | *ask1-1*/Ac/Ds | Decreased number of LR | Fukaki et al. (2005) |
| *ASK2* | AT3G61160 | *ask2-1*/ T-DNA insertion | Decreased number of LR | Fukaki et al. (2005) |
| *CULLIN-ASSOCIATED AND NEDDYLATION DISSOCIATED, HEMIVENATA (CAND1)* | AT2G02560 | EMS | Decreased number of LR | Fukaki et al. (2005) |
| *TRANSPORT INHIBITOR RESPONSE 3 (TIR3)* | AT3G02260 | *tir3-1*//EMS and gamma rays | Reduced LR number. Reduced auxin-transport-inhibitor-sensitive root elongation | Ruegger et al. (1997), Lopez-Bucio et al. (2005) |
| *ANR1* | AT2G14210 | *ANR1*-KO/dSpm transposon insertion | Does not show the nitrate-induced stimulatory effect (down-regulated expression) | Montiel et al. (2004) |
| *ARABIDOPSIS DUAL-AFFINITY NITRATE TRANSPORTER GENE AtNRT1.1 (NRT1.1)* | ? | T-DNA insertion | Does not show the nitrate-induced stimulatory effect | Zhang et al. (2007) |
| *LATERAL ROOT INITIATION (LIN1)* | AT1G08090 | EMS | LR development insensitive to high-sucrose, low-nitrogen medium | Cerezo et al. (2001), Casimiro et al. (2003), Zhang et al. (2007) |
| *IAA-ALANINE RESISTANT 2 (IAA28/ IAR2)* | AT5G25890 | *iaa28-1*/EMS | Defective in LR formation, reduced LR number. Defects in root hair development, resistance to the stimulatory effects of low P on root hair and LR formation | Lopez-Bucio et al. (2002) |
| *MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 5 (MRP5)* | AT1G04120 | *mrp5-1*/T-DNA insertion | Increased LR number, decreased root length | Gaedeke et al. (2001), Casimiro et al. (2003) |

| | | | | |
|---|---|---|---|---|
| *DWARF IN LIGHT 1 (DFL1)* | AT5G54510 | Activation-tagging plasmid | Altered hypocotyl length in light and reduced LR number but the primary root length is almost the same as in the WT. Auxin insensitive | Nakazawa et al. (2001), Casimiro et al. (2003) |
| *AUXIN RESISTANT 1 (AXR1)* | AT1G05180 | *axr1-3*/EMS | Agravitropic root, reduced LR number. Reduced auxin-sensitive root elongation | Lopez-Bucio et al. (2002), Casimiro et al. (2003) |
| *AUXIN RESISTANT 2 (AXR2)* | AT3G23050 | *axr2-5*/T-DNA insertion | Agravitropic root, reduced LR number. Short hypocotyls in dark conditions. | Lopez-Bucio et al. (2002) |
| *AUXIN RESISTANT 3 (AXR3)* | AT1G04250 | *axr3-3*/EMS | Reduced root elongation and increased lateral root number | Lopez-Bucio et al. (2002) |
| *AUXIN RESISTANT 4 (AXR4)* | AT1G54990 | *axr4-2*/gamma rays | Reduced LR number. Reduced auxin-sensitive root elongation | Lopez-Bucio et al. (2002), Casimiro et al. (2003) |
| *AUXIN RESISTANT 5 (AXR5)* | AT4G14560 | *axr5-1*/unknown | Gain-of-function mutation; mutants are resistant to auxin and display a variety of auxin-related growth defects including defects in root and shoot tropisms and reduced LR number on auxin | Yang et al. (2004), De Smet et al. (2006) |
| *AUXIN RESISTANT 6 (AXR6)* | ? | *atcul1-5*/ T-DNA insertion | Reduced lateral root number. Reduced auxin-sensitive root elongation | Lopez-Bucio et al. (2002), Casimiro et al. (2003) |
| *NO APICAL MERISTEM CUP-SHAPED COTYLEDON (NAC1)* | AT1G56010 | RNAi/ overexpression | RNAi lines have reduced LR number; overexpressing lines have increased LR number. | Montiel et al. (2004), Scheres et al. (2002), Xie et al. (2000) |
| *PEROXISOMAL ABC TRANSPORTER 1 (PXA1)* | AT4G39850 | EMS | Reduced IBA-sensitive root elongation. Reduced LR number | Zolman et al. (2001), Casimiro et al. (2003) |
| *SEVEN IN ABSENTIA HOMOLOG 5 (SINAT5)* | AT5G53360 | Overexpression | Overexpression leads to reduced LR formation | Casimiro et al. (2003) |
| *PASTICCINO 1 (PAS1)* | AT3G54010 | *pas1-1*/T-DNA insertion | Reduced LR number and short primary root | Faure et al. (1998), Casimiro et al. (2003) |
| *PASTICCINO 2 (PAS2)* | AT5G10480 | EMS | Increased LR number and short primary root | Faure et al. (1998) |
| *PASTICCINO 3 (PAS3)* | ? | | Reduced LR number and short primary root | Faure et al. (1998) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| | | *pas3-1*, *3-2*, *3-3*, 3-4/ EMS | | |
| *SOLITARY ROOT1/ IAA14 (SLR1/ IAA14)* | AT4G14550 | *iaa14-1*/ T-DNA insertion | Absence of LR development, reduced number of root hairs | Scheres et al. (2002), Casimiro et al. (2003), Montiel et al. (2004), Fukaki et al. (2006) |
| *LATERAL ROOT PRIMORDIUM 1 (LRP1)* | AT5G12330 | Gene trap transposon | Delayed lateral root initiation | Smith and Fedoroff (1995) |
| *NON-PHOTOTROPHIC HYPOCOTYL (NPH4)* | AT5G20730 | *nph4-1, 4-2, 4-3, 4-4*/ fast neutrons | Decrease in lateral and adventitious root formation | Stowe-Evans et al. (1998), Casimiro et al. (2003) |
| *SHORT HYPOCOTYL (SHY2)* | AT1G04240 | *shy2-2*/EMS | Significantly shorter roots of *shy2-2* mutants with very few lateral roots, whereas the *shy2-22* and *shy2-24* mutants form more and much longer LR than the WT | Tian and Reed (1999) |
| *KNOTTED-LIKE (KNAT6)* | AT1G23380 | T-DNA insertion | Down-regulation of *KNAT6* expression by RNA interference was associated with an increased total number of lateral roots | Dean et al. (2004), Montiel et al. (2004) |
| *MASSUGU2/IAA19 (MSG/IAA19)* | AT3G15540 | EMS | Defective in lateral root formation and root gravitropism | Tatematsu et al. (2004) |
| *PUCHI* | ? | *puchi-1*/T-DNA insertion | Disturbed cell division patterns in the lateral root primordium, resulting in swelling of the proximal region of lateral roots | Hirota et al. (2007) |
| *CYTOPLASMIC-INVERTASE 1 (AtCYT-INV1)* | ? | EMS | Insensitivity to osmotic stress-induced inhibition of lateral root growth. Short primary root, smaller size of leaves and siliques | Qi et al. (2006) |

| | | | | |
|---|---|---|---|---|
| *LATERAL ORGAN BOUNDARIES-DOMAIN16 (LBD16)* | AT2G42430 | Overexpression | Overexpression induces lateral root formation | Okushima et al. (2007) |
| *LATERAL ORGAN BOUNDARIES-DOMAIN29 (LBD29)* | AT3G58190 | Overexpression | Overexpression induces lateral root formation | Okushima et al. (2007) |
| *XB3 ORTHOLOG 2 IN ARABIDOPSIS THALIANA 32 (XBAT32)* | AT5G57740 | T-DNA insertion | Poor root system and severe defects in lateral root production. Defective in cell divisions that are required for lateral root initiation | Nodzon et al. (2004) |
| *IAA-LEUCINE RESISTANT 2 (ILR2)* | AT3G18485 | *ill2-1*/T-DNA insertion | Defective in lateral root formation and primary root elongation | Magidin et al. (2003) |
| *IAA-LEUCINE RESISTANT 1 (ILR1)* | AT3G02875 | *ilr1-1*/EMS | Shorter hypocotyl and fewer lateral roots on unsupplemented medium | Rampey et al. (2004) |
| *IAA-LEUCINE-RESISTANT (ILR)-LIKE GENE 2 (ILL2)* | AT5G56660 | *ill2-1*/T-DNA insertion | Shorter hypocotyl and fewer lateral roots on unsupplemented medium | Rampey et al. (2004) |
| *IAA-ALANINE RESISTANT 3 (IAR3)* | AT1G51760 | *iar3-1*/EMS | Shorter hypocotyl and fewer lateral roots on unsupplemented medium | Rampey et al. (2004) |
| *HOMEOBOX-LEUCINE ZIPPER PROTEIN HAT4 (ATHB-2/HAT4)* | AT4G16780 | Overexpression | Elevated *ATHB-2* levels inhibit specific cell proliferation such as secondary growth of the vascular system and lateral root formation. Reduced LR number. | Steindler et al. (1999) |
| *ROOTS CURL IN NP (RCN1)* | AT1G25490 | T-DNA insertion | Lateral roots exhibit reduced NPA sensitivity, gravitropic response and increased auxin transport. | |
| *RHO-RELATED PROTEIN FROM PLANTS 2 (ROP2)* | AT1G20090 | Overexpression | Constitutively active GTP-bound rop2 (CA-rop2): increased LR number; and dominant negative GDP-bound rop2 (DN-rop2) reduced LR number | Li et al. (2001) |
| *SEUSS (SEU)* | AT1G43850 | *seu-3*/EMS | Pleiotropic phenotype that includes reductions in several classic auxin responses such as apical dominance, lateral root initiation, sensitivity to exogenous auxin | Pfluger and Zambryski (2004) |
| *ARABIDOPSIS RESPONSE REGULATOR 3 (ARR3)* | AT1G59940 | T-DNA insertion | Lateral root formation is more sensitive to cytokinin inhibition | To et al. (2004) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *ARABIDOPSIS RESPONSE REGULATOR 4 (ARR4)* | AT1G10470 | T-DNA insertion | Lateral root formation is more sensitive to cytokinin inhibition | To et al. (2004) |
| *ARABIDOPSIS RESPONSE REGULATOR 5 (ARR5)* | AT3G48100 | T-DNA insertion | Lateral root formation is more sensitive to cytokinin inhibition | To et al. (2004) |
| *ARABIDOPSIS RESPONSE REGULATOR 6 (ARR6)* | AT5G62920 | T-DNA insertion | Lateral root formation is more sensitive to cytokinin inhibition | To et al. (2004) |
| *ARABIDOPSIS RESPONSE REGULATOR 8 (ARR8)* | ? | T-DNA insertion | Lateral root formation is more sensitive to cytokinin inhibition | To et al. (2004) |
| *ARABIDOPSIS RESPONSE REGULATOR 9 (ARR9)* | AT3G57040 | T-DNA insertion | Lateral root formation is more sensitive to cytokinin inhibition | To et al. (2004) |
| *CYTOKININ OXIDASE/ DEHYDROGENASE 1 (CKX1)* | AT2G41510 | Overexpression | Increased growth of the primary root and increased number of lateral roots | Werner et al. (2003) |
| *CYTOKININ OXIDASE/ DEHYDROGENASE 3 (CKX3)* | AT5G56970 | Overexpression | Increased growth of the primary root and increased number of lateral roots | Werner et al. (2003) |
| *E1-CONJUGATING ENZYME-RELATED1-1 (ECR1)* | AT5G19180 | *ecr1-1*/EMS | Resistant to the auxin-like compound indole-3-propionic acid, produces fewer lateral roots than wild type, displays reduced adult height | Woodward et al. (2007) |
| *RING-BOX 1 (RBX1)* | AT5G20570 | Overexpression | Transgenic plants (*35S::RBX1)* had smaller cotyledons and produced fewer lateral roots than WT plants | Gray et al. (2002) |
| *BUSHY AND DWARF (BUD1)* | AT1G18350 | Sense/antisense RNA expression system | Significantly fewer lateral roots, loss of apical dominance, shorter hypocotyl at high temperature (29°C) under light. Deficiency in polar auxin transportt | Dai et al. (2006) |
| *LONG HYPOCOTYL 5 (HY5)* | ? | T-DNA insertion | Altered hypocotyl length in light and increased LR number. Emergence of LR occurs earlier than in WT, resulting in overall enhanced root system growth. The gravitropism of *hy5* roots is reduced | Casimiro et al. (2003), Sibout et al. (2006) |

| | | | | |
|---|---|---|---|---|
| *WALL-ASSOCIATED SER/THR KINASE (WAK4)* | AT1G21210 | Antisense gene | Impaired cell elongation and blocked LR formation | Lally et al. (2001) |
| *LIKE AUX1 (LAX3)* | AT1G77690 | T-DNA insertion | Nearly 40% reduction in numbers of emerged lateral roots | Swarup et al. (2008) |
| *ABA DEFICIENT 1 (ABA1)* | AT5G67030 | aba1-1/EMS | ABA sensitive, reduced ABA inhibitory effect on LR length. Shorter primary root | Signora et al. (2001) |
| *ABA DEFICIENT 2 (ABA2)* | AT1G52340 | aba2-1, 2-3, 2-4/ EMS | ABA sensitive, reduced ABA inhibitory effect on LR length. Shorter primary root | Signora et al. (2001) |
| *ABA DEFICIENT 3 (ABA3)* | AT1G16540 | aba3-1/EMS 3-2/gamma rays | ABA sensitive, reduced ABA inhibitory effect on LR length | Signora et al. (2001) |
| *ABA DEFICIENT 4 (ABA4)* | ? | *aba*4-1/T-DNA insertion | ABA insensitive, reduced ABA inhibitory effect on LR length | Signora et al. (2001) |
| *ABA DEFICIENT 5 (ABA5)* | ? | unknown | ABA insensitive, reduced ABA inhibitory effect on LR length | Signora et al. (2001) |
| *ABSCISIC ACID INTENSIVE 1 (ABI1)* | AT5G57050 | EMS | Reduced ABA inhibitory effect on LR length | De Smet et al. (2003) |
| *ABSCISIC ACID INTENSIVE 1 (ABI2)* | AT3G24650 | EMS | Reduced ABA inhibition on LR length | De Smet et al. (2003) |
| *ABSCISIC ACID INTENSIVE 1 (ABI3)* | AT3G24650 | EMS | Reduced ABA inhibitory effect on LR length | De Smet et al. (2003) |
| *ABSCISIC ACID INTENSIVE 1 (ABI4)* | AT2G40220 | Gamma rays | Reduced ABA inhibitory effect on LR length | De Smet et al. (2003) |
| *ABSCISIC ACID INTENSIVE 1 (ABI5)* | AT2G36270 | abi5-1/T-DNA insertion | Reduced ABA inhibitory effect on LR length | De Smet et al. (2003) |
| *ENHANCED RESPONSE TO ABA 1 (ERA1)* | AT5G40280 | unknown | Increased number of lateral roots | Brady et al. (2003) |
| *LATERAL ROOT ABA-INSENSITIVE (LABI)* | ? | EMS | Shorter primary root phenotype and ability to produce visible LRs in the presence of ABA. Mutants are less sensitive to the high-nitrate induced inhibition on LRs | Zhang et al. (2007) |
| *FLOWERING TIME CONTROL PROTEIN ALPHA (FCA)* | AT4G16280 | EMS | Reduced sensitivity to the inhibitory effect of ABA on LRs | Zhang et al. (2007) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *HOMEODOMAIN-LEICINE ZIPPER PROTEIN HAT2 (HAT2)* | AT5G47370 | Overexpression | Mutations of the *HAT2* gene did not produce any remarkable morphological phenotypes (mutants responded to gravity, exogenous auxin, and auxin transport inhibitor in similar ways to wild-type plants) indicating the presence of redundant functions among the HD-Zip II subfamily genes. 35S::HAT2 plants showed reduced lateral root elongation, and reduced auxin sensitivity compared to wild-type plants | Sawa et al. (2002) |
| *PI DEFICIENCY RESPONSE 2 (PDR2)* | ? | EMS | Disrupted Pi sensing | Ticconi et al. (2004) |
| *UBIQUITIN-LIKE MODIFIER (SUMO) E3 LIGASE (AtSIZ1)* | AT5G60410 | *siz1-1, 1-2, 1-3*/ T-DNA insertion | Cessation of primary root growth, extensive lateral root and root hair development | Miura et al. (2005) |
| *WRKY75* | AT5G13080 | RNAi | Suppression of *WRKY75* expression through RNAi silencing results in significantly increased LR length and number, as well as root hair number | Devaiah et al. (2007) |
| *ABERRANT LATERAL ROOT FORMATION 3 (ALF3)* | ? | *alf3-1*/EMS | Mutant is able to initiate lateral root primordium formation but then arrests at the emergence stage | Celenza et al. (1995), Casimiro et al. (2003) |
| *RELATED TO ABI3/VP1 1 (RAV1)* | AT1G13260 | Overexpression | Overexpression causes retarded LR development | Hu et al. (2004) |
| *INDOLE-3-BUTYRIC ACID-RESPONSE (IBR5)* | AT2G04550 | *ibr5-1*/EMS | Light-grown seedlings have longer primary roots with slightly fewer lateral roots. Lateral roots in *ibr5-1* elongate less than in the WT; hypocotyl and roots elongate normally in the dark | Monroe-Augustus et al. (2003) |
| *LRD2* | ? | EMS | Mutant has an altered response to exogenous ABA | Deak and Malamy (2005) |

**Adventitious roots**

| | | | | |
|---|---|---|---|---|
| *ARGONAUTE 1 (AGO1)* | AT1G48410 | EMS | Reduced formation of adventitious roots in response to auxin. Defect of hypocotyl elongation in response to auxin | Sorin et al. (2005) |
| *AUXIN RESPONSE FACTOR 17 (ARF17)* | AT1G77850 | T-DNA/ overexpression | Overexpression line produces fewer adventitious roots than the WT | Sorin et al. (2005) |
| *HASTY (HST)* | AT3G05040 | *hst-6*,/EMS *hst-7*/X-ray | Mutants have reduced size of roots and form adventitious roots from the base of the hypocotyl | Bollman et al. (2003) |
| *ROOT INITIATION DEFECTIVE 1 (RID1)* | ? | EMS | Temperature-sensitive, defective in the initial or the pre-morphogenic stage of adventitious root formation | Konishi and Sugiyama (2003) |
| *ROOT INITIATION DEFECTIVE 2 (RID2)* | ? | EMS | Temperature-sensitive, defective in the initial or the pre-morphogenic stage of adventitious root formation | Konishi and Sugiyama (2003) |
| *ROOT INITIATION DEFECTIVE 3 (RID3)* | ? | EMS | Temperature-sensitive, defective in the initial or the pre-morphogenic stage of adventitious root formation | Konishi and Sugiyama (2003) |
| *ROOT INITIATION DEFECTIVE 4 (RID4)* | ? | EMS | Temperature-sensitive, defective in the initial or the pre-morphogenic stage of adventitious root formation | Konishi and Sugiyama (2003) |
| *ROOT INITIATION DEFECTIVE 5 (RID5)* | ? | EMS | Temperature-sensitive, reduced frequency of root initiation at 28°C without affecting the later stages of root formation. The rate of adventitious rooting generally depends on the concentration of exogenous auxin | Konishi and Sugiyama (2003) |
| *ROOT PRIMORDIUM DEFECTIVE 1 (RRD1)* | ? | EMS | Temperature-sensitive, mutant can establish adventitious roots but fails to maintain their growth. Strongly inhibited subsequent growth of adventitious roots at 28°C | Konishi and Sugiyama (2003) |

(*continued*)

**Table 2.2** (continued)

| Gene name (alias) | Accession number | Allele/mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *ROOT PRIMORDIUM DEFECTIVE 2 (RRD2)* | ? | EMS | Temperature-sensitive, mutant can establish adventitious roots but fails to maintain their growth. Strongly inhibited subsequent growth of adventitious roots at 28°C | Konishi and Sugiyama (2003) |
| *ROOT PRIMORDIUM DEFECTIVE 4 (RRD4)* | ? | EMS | Temperature-sensitive, mutant can establish adventitious roots but fails to maintain their growth. Strongly inhibited subsequent growth of adventitious roots at 28°C | Konishi and Sugiyama (2003) |

*LR* lateral root, *RAM* root apical meristem, *QC* quiescent centre, *WT* wild type

the xylem poles (Benjamins and Scheres 2008), whereas in barley from pericycle and endodermis adjacent to phloem (Briggs 1978) just like in rice and corn (Hochholdinger and Zimmermann 2008). The general structure of barley lateral roots seems to be the same as the seminal and nodal roots, despite their different origins. The transverse section exhibit typical thick-walled endodermis and single large axile duct surrounded by much more thicker tissue (Gorny 1992). For LR initiation, auxin plays a crucial role in both monocotyledonous (Chhun et al. 2007) and dicotyledonous (Tian and Reed 1999; Casimiro et al. 2003) species.

More than 170 genes have been described as important for longitudinal pattern in *Arabidopsis*. Alterations in these genes cause often severe phenotype, such as in the case of *GNOM* (*GN*). Mutants of this gene display a range of phenotypes, but all of them lack a root (Shevell et al. 2000). This gene encodes an ARF GDP/GTP exchange factor involved in embryonic axis formation and polar localization of PIN1 (Geldner et al. 2004). It was shown that mutations in this gene disrupt the polarity of auxin transport and thereby cause defects not only in gravitropism (Geldner et al. 2003) but also hydrotropism (Miyazawa et al. 2009). Lack of a primary root is characteristic for *BODENLOS (BDL)* and *MONOPTEROS* (*MP*) mutants. The *MP* gene encodes a transcription factor ARF5 (AUXIN RESPONSE FACTOR 5) that activates auxin-responsive target genes, whereas *BDL* encodes INDOLACETIC ACID-INDUCED PROTEIN 12 (IAA12) (Shevell et al. 2000). Hamann and coworkers (2002) suggested inhibitory effect *BDL* on *MP*, but exact mechanism of their action is unknown (Weijers et al. 2006). Alterations in root length could be an output of decreased number of cell divisions such as in the case of *DAWDLE (DDL)*, cell elongation – *PHOSPHOLIPASE DS 1,2 (PLDζ1)* or cell-wall formation – *MURUS 1* (*MUR1*). *DDL* mutant plants exhibit shortened roots. This gene seems to influence transcription activation by recruiting proteins to transcription complexes; however, its precise function is still unknown (Morris et al. 2006). Slower elongation of primary roots and faster of lateral roots in low phosphate conditions are characteristic for *PLDζ1* and *PLDζ2* mutants. These genes are involved in root elongation during phosphate limitation – they promote primary root growth but inhibit lateral root elongation (Li et al. 2006b). *MUR1* mutants exhibit root grow defects, where more brittle altered cell walls are observed. This gene is necessary to form essential pectin cross-links within the cell wall and proper composition of cell wall polysaccharides (Freshour et al. 2003).

Up to now, many genes have been described as involved in lateral root formation in the differentiation zone. Lateral roots are formed from the pericycle "founder cells," which undergo a series of periclinal and anticlinal divisions to generate a new meristem (Casson and Lindsey 2003). One of the earliest genes involved in lateral root formation is *ALF4 (ABERRANT LATERAL ROOT FORMATION 4)*. The *ALF4* mutant is unable to produce lateral roots or adventitious roots and does not respond to exogenous auxins (Casimiro et al. 2003). It was suggested by DiDonato and coworkers (2004) that *ALF4* functions in maintaining the pericycle in the mitotically competent state needed for lateral root formation. There are only few mutants described as involved in lateral root emergence. *LAX3*, which has been described recently by Swarup et al. (2008), encodes an auxin influx carrier that

facilitates emergence of new primordia. Mutants exhibit nearly 40% reduction in numbers of emerged lateral roots. Many genes involved in lateral meristem activation are related to ABA, such as *ABA DEFICIENT 1 (ABA1)*. This mutant has shorter primary root, is ABA-sensitive, and exhibit reduced ABA inhibition of LRs length (Signora et al. 2001). As auxin is involved in all steps of lateral root formation, genes involved in ABA metabolism determine auxin-independent checkpoint for lateral root development. The product of the *ABA1* gene – zeaxanthin epoxidase – generates the epoxycarotenoid precursor of the ABA biosynthetic pathway (Barrero et al. 2005).

Little is known about adventitious root formation in *Arabidopsis*. Among those genes, *ARGONAUTE 1 (AGO1)* has been well described. Mutants are barely able to form adventitious roots in response to auxin and exhibit defect of hypocotyl elongation in response to auxin. Sorin et al. (2005) suggested that *AGO1* regulates genes required for adventitious root development through its action on the regulation of *ARF17* expression. Mutation in *AGO1* results in the higher levels of *ARF17* expression in hypocotyl, which in turn leads to fewer adventitious roots. *ARF17*-overexpressing line also forms fewer adventitious roots than the wild type (Sorin et al. 2005).

## 2.3 Root Mutants in Monocotyledonous Species Published in Pubmed

The deepest monocotyledonous root system is usually of seminal origin, whereas the upper layers of the soil are penetrated by the nodal roots (Gorny 1992). In addition to their white color, nodal roots are much thicker and less branched than seminals and maintain larger number of root hairs. The anatomy of nodal roots differs from seminal roots. Young ones have all thin-walled stele cells. There are several (four to six) large ducts in the center surrounded by parenchymatous cells. Moreover, the xylem and phloem are undetectable. Eight to nine layers of parenchymatous cells form the cortex separated from the stele by the endodermis. The fully developed roots exhibit four large ducts separated by the more thick-wall cells. Each of twelve to sixteen xylem groups contains one large vessel. The groups are separated from each other by parenchyma cells and phloem poles hard to distinguish. Outside the endodermis, there are six to eight layers of large parenchymatous cortical cells (Jackson 1922).

Up to now, little is known about genes involved in root architecture in monocotyledons. The main information came from three species: rice, maize, and wheat (Table 2.3). Similar to dicotyledons, also forward and reverse approaches were used to study root traits. At least six mutants were obtained trough Mu transposition, four by γ-irradiation, three by $NaN_3$, and one by each: MNU, Tos17, and tissue culture. Reverse approach (e.g., RNAi, overexpression) were also used to study influence of a gene of interest on root traits. Several mutants have been described, which are responsible for monocotyledonous root traits. Lim and coworkers (2005) described

**Table 2.3** Mutated genes responsible for monocotyledonous plants root architecture

| Gene name (alias) | Accession number | Mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| **Root traits** | | | | |
| *RAN-RELATED GTP-BINDING PROTEIN (TaRAN1)* | AF488730 | Overexpression | Increased primordial tissue, reduced number of lateral roots and stimulated hypersensitivity to exogenous auxin | Wang et al. (2006) |
| *SCARECROW (ZmSCR)* | AF263457 | EST-based isolation | Not reported | Lim et al. (2005) |
| *OsASR1* | ? | ? | Defective seminal roots | Ge et al. (2004) |
| *CROWN ROOTLESS 1 (ZmCRLL1)* | BG873644 | ? | Not reported | Inukai et al. (2005) |
| *CROWN ROOTLESS 2 (ZmCRLL2)* | BE050765 | ? | Not reported | Inukai et al. (2005) |
| *CROWN ROOTLESS 1 (ZmCRL1)* | AY736375/ AB200234 | MNU | Impaired initiation of nodal root primordia | Liu et al. (2005), Inukai et al. (2005) |
| *CASEIN KINASE 1 (OsCKL1)* | AJ487966 | Antisense | Reduced primary root length and fewer lateral and nodal roots | Liu et al. (2003) |
| *CROWN ROOTLESS 1 (OsCRLL1)* | AB200235 | ? | Not reported | Inukai et al. (2005) |
| *CROWN ROOTLESS 2 (OsCRLL2)* | AB200236 | ? | Not reported | Inukai et al. (2005) |
| *CROWN ROOTLESS 3 (CRLL3)* | AB200237 | ? | Not reported | Inukai et al. (2005) |
| *CROWN ROOTLESS 4 (CRLL4)* | AB200238 | ? | Not reported | Inukai et al. (2005) |
| *GLUTAMATE RECEPTOR LIKE CHANNEL 3.1 (GLR3.1)* | DQ305408 | T-DNA insertion | Short root phenotype | Li et al. (2006a, b) |
| *GLUCOSAMINE-6-PHOSPHATE ACETYLTRANSFERASE (GNA1)* | AY772189 | T-DNA insertion | Short root phenotype | Jiang et al. (2005) |
| *ROOT ARCHITECTURE ASSOCIATED 1 (OsRAA1)* | AY659938 | Overexpression | More nodal roots, shorter primary and lateral roots compare to WT | Ge et al. (2004) |
| *ROOTHAIR DEFECTIVE 3 (TaRHD3)* | AY557340 | mRNA differential display | Not reported | Shan et al. (2005) |
| *OsCRL2* | ? | Gamma rays | Impaired initiation and growth of nodal root primordia, longer primary root than WT | Inukai et al. (2001) |

(*continued*)

**Table 2.3** (continued)

| Gene name (alias) | Accession number | Mutation strategy/reverse approach | Mutant phenotype | References |
|---|---|---|---|---|
| *ROOTLESS WITH UNDETECTABLE MERISTEMS 1 (RUM1)* | ? | Mu insertion | Deficient in the initiation of the embryonic seminal roots and the postembryonic lateral roots on the primary root | Woll et al. (2005) |
| *ALTERED LATERAL ROOT FORMATION (OsALF)* | ? | Retrotransposon Tos17 | Significantly shorter lateral roots as compared with the wild type | Rani Debi et al. (2003) |
| *REDUCED ROOT LENGTH 1 (OsRRL1)* | ? | Gamma rays | Reduced root length | Inukai et al. (2001) |
| *REDUCED ROOT LENGTH 2 (OsRRL2)* | ? | Gamma rays | Reduced root length | Inukai et al. (2001) |
| *SHORT LATERAL ROOTS 1 (ZmSLR1)* | ? | Mu insertion | Short lateral roots as a result of impaired root cell elongation | Hochholdinger et al. (2001) |
| *SHORT LATERAL ROOTS 2 (ZmSLR2)* | ? | Mu insertion | Short lateral roots as a result of impaired root cell elongation | Hochholdinger et al. (2001) |
| *SHORT LATERAL ROOTS 5 (OsSRT5)* | ? | $NaN_3$ | Reduced root length | Yao et al. (2003) |
| *SHORT LATERAL ROOTS 6 (OsSRT6)* | ? | $NaN_3$ | Reduced primary root length and diameter, the mutant at the seedling stage also shows inhibited lateral root elongation and altered root hair formation | Yao et al. (2003) |
| *PIN-FORMED 1 (PIN1)* | NM_001063762 | RNAi | Significantly inhibited nodal root emergence and development | Xu et al. (2005) |
| *PINOID (PID)* | NM_001073800 | Overexpression | Delay of nodal root development, curled growth of shoots and agravitropic roots | Morita and Kyozuka (2007) |
| *SLENDER (SLR1-1)* | XM_469478 | Gamma rays | Reduced number and root length compared with the wild-type plant | Ikeda et al. (2001) |
| *ADP-RIBOSYLATION FACTOR (ARF) GTPASE-ACTIVATING PROTEIN (GAP) (AGAP)* | NM_001062427 | Overexpression | Reduced apical dominance, shorter primary roots, increased number of longer nodal roots. | Zhuang et al. (2005) |
| *AUXIN EFFLUX MUTANT (AEM1)* | ? | ? | Short lateral roots, reduced development of root hairs, agravitropic root | Rani Debi et al. (2005) |
| *ADVENTITIOUS ROOTLESS 1 (OsARL1)* | ? | Tissue culture | Defective in nodal root formation | Liu et al. (2005) |

**Root hairs**

| | | | | |
|---|---|---|---|---|
| *CELLULOSE SYNTHASE-LIKE D1 (OsCSLD1)* | BK000089 | Ds gene trap | Root hair development is initiated normally, the hairs elongate less than the wild-type hairs and they have kinks and swellings along their length | Kim et al. (2007) |
| *ROOTHAIRLESS 3 (ZmRTH3)* | ? | Mu insertion | Impaired in root hair elongation | Wen and Schnable (1994) |
| *ROOT HAIRS (OsRH2)* | ? | $NaN_3$ | Defective in the formation of root hairs | Ma et al. (2001) |
| *ROOTHAIRLESS 1 (ZmRTH1)* | AY265854 | Mu insertion | Impaired in root hair elongation | Wen and Schnable (1994), Wen et al. (2005) |
| *ROOTHAIRLESS 3 (ZmRTH3)* | AY265855 | Mu insertion | Impaired in root hair elongation | Wen and Schnable (1994), Hochholdinger et al. (2008) |

*$NaN_3$* sodium azide, *WT* wild type

maize *ZmSCR* gene. They suggested that this gene is *Arabidopsis SCR* ortholog based on sequence and expression pattern similarity to the members of the GRAS family. It was then confirmed due to the ability to complement the *Arabidopsis SCR* mutant phenotype, which suggests conservation of function. Although the main knowledge about lateral root development came from *Arabidopsis*, rice mutant *ALF1* (*ALTERED LATERAL ROOT FORMATION*) has been isolated by Rani Debi and coworkers (2003). This mutant displayed not only significantly shorter lateral roots as compared with wild type but also reduction in both the number and length of root hairs. In maize, *SHORT LATERAL ROOTS1 (SLR1)* and *SLR2* mutants have been reported with defective lateral root elongation (Hochholdinger et al. 2001). The defects in both mutants act specifically during early postembryonic root development, and crown roots at all the stages produced normal lateral roots similar to the wild type. In contrast, the *ALF1* mutant displays shorter lateral roots in both embryonic seminal and postembryonic crown roots up to later growth stages (Rani Debi and coworkers, 2003). Rice mutants that lack *CELLULOSE SYNTHASE-LIKE D1 (OsCSLD1)* function develop abnormal root hairs that elongate less. It appears that *OsCSLD1* may be the functional ortholog of *Arabidopsis KOJAK*, which is involved in root hair elongation (Kim et al. 2007). The similar phenotype is observed in maize *roothairless 3 (ZmRTH3)*, which encodes a COBRA-like protein (Hochholdinger et al. 2008).

## 2.4 Strategy for EST Data-Mining

The goal of this work was to find an optimal, short, and efficient procedure in a search for potential orthologs between *Arabidopsis* and barley using rice for confirmation and between already reported genes in other monocotyledons and barley. The first step was to review the literature in searching for genes that are described as involved in root development. Out of 259 *Arabidopsis* and 35 monocotyledonous genes found in this search, it was possible to analyze a total number of 192 *Arabidopsis* and 21 monocotyledonous genes, whose nucleotide and protein sequences were available in GenBank database. Potential orthologs between *Arabidopsis* and barley and between other monocotyledons and barley were analyzed separately.

### 2.4.1 *Searching for Potential Orthologs Between* Arabidopsis *and Barley*

The strategy included two pipelines (Fig. 2.1). First, a search in the GeneBank for rice potential orthologs using BLASTn and BLASTp based on *Arabidopsis* nucleotide and protein sequences, respectively, was done. To minimize false positive results, more restrictive criteria (E value $10^{-5}$ or less) were chosen than suggested

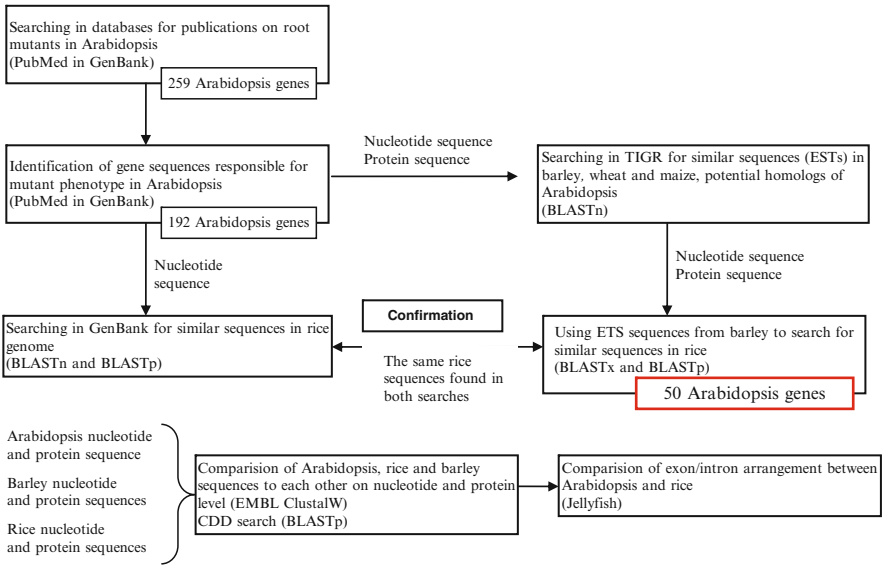


**Fig. 2.1** Strategy for selection of potential barley orthologs to *Arabidopsis* genes. E value (GenBank)/Expect (TIGR) $10^{-5}$ or less

by Pevsner (2003). However, it should be noticed that BLAST is a heuristic version of Smith-Waterman algorithm, so it generates an output that is a list of sequences based on Score value obtained for each corresponding fragment (Koonin and Galperin 2004). In other words, the more points the alignment gets, the higher on the output list will the sequence be. Moreover, change of the parameters of BLAST searching modifies the Score value for each alignment and may automatically have an influence on the order of sequences in the result list. That is the main reason for the need to manually verify the results from BLAST searches using multiple alignment tool ClustalW.

Parallel to this, the search for barley ESTs in TIGR and GenBank databases was performed to select *Arabidopsis* genes, which have good EST coverage. To minimize false positive results, more restrictive criteria were chosen (Expect $10^{-5}$ or less) just like in the previous searches. The barley EST sequences were then used as a query in TIGR database in search for rice ESTs. Rice ESTs obtained through this searching were then aligned with rice nucleotide and protein sequences obtained through GenBank searching.

Using this approach, 22 genes involved in LR formation, 19 genes controlling root development, and 8 genes involved in root hair formation in *Arabidopsis* (which lead to total number of 49 genes) were identified (Table 2.4). To determine the level of similarity between *Arabidopsis*, barley, and rice, the sequences were compared on nucleotide and protein level. Nevertheless, the success of this approach depends heavily on the quality of EST sequences, which cannot be guaranteed. This is mostly due to the existence in EST artifacts during cDNA library construction and inherent errors caused by DNA sequencing procedures

**Table 2.4** *Arabidopsis* genes which have potential orthologs in barley and rice genome

| *Arabidopsis* | | Rice | | Barley | Similarity [%] | | | | References |
|---|---|---|---|---|---|---|---|---|---|
| Alias | Gene acc. no. | Alias | Gene acc. no. | EST(s) acc. no. | At-rice | | At-barley | | |
| | | | | | N | P | N | P | |
| Lateral root development | | | | | | | | | |
| *ABA1* | At5g67030 | ? | Os04g0448900 | TC159565 | 60.6 | 57.1 | 69.6 | 59.8 | Signora et al. (2001) |
| | | | | TC189509 | | | 70.2 | 61.5 | |
| *AUX1* | AT2G38120 | *LAX2* | **Os01g0856500** | TC177829 | 51.6 | 79.9 | 70.5 | 86.4 | Fukaki et al. (2005) |
| | | | Os05g0417200 | | 32.7 | 8.84 | | | |
| *AXR1* | At1g05180 | ? | Os03g0820100 | TC168107 | 60 | 66.1 | 66.3 | 66.4 | Lopez-Bucio et al. (2002) |
| *AXR2* | At3g23050 | *IAA30* | **Os12g0601300** | TC183661 | 49.4 | 55.8 | 62.6 | 83.1 | Lopez-Bucio et al. (2002) |
| | | *IAA13* | Os03g0742900 | TC182343 | 59.6 | 53.1 | | | |
| *CKX1* | AT2G41510 | ? | **Os01g0940000** | TC153934 | 53.6 | 55 | 63 | 64.7 | Werner et al. (2003) |
| | | | Os05g0374000 | | 11.6 | 4.69 | | | |
| *CKX3* | At5g56970 | ? | **Os05g0374200** | TC153934 | 51.6 | 40.1 | 56.5 | 46.3 | Werner et al. (2003) |
| | | | Os01g0197700 | | 39.9 | 27.1 | | | |
| | | | Os01g0187600 | | 28.7 | 17.7 | | | |
| | | | Os01g0775400 | | 53.1 | 45.1 | | | |
| *LIN1* | At1g08090 | ? | Os02g0112600 | TC163374 | 60.5 | 69.9 | 68.3 | 72 | Casimiro et al. (2003), Zhang et al. (2007) |
| *RCN1* | AT1G25490 | ? | Os09g0249700 | TC163839 | 66.5 | 83.5 | 75.0 | 81.8 | Rashotte et al. (2001) |
| | | | | TC174461 | | | 74.7 | 82.6 | |
| *SINAT5* | AT5G53360 | *SINAT5* | Os05g0238200 | TC156156 | 53.8 | 53.9 | 45 | 82.9 | Xie et al. (2002), Casimiro et al. (2003) |
| | | | Os02g0293400 | | 48.2 | 56.5 | | | |
| *SLR1* | AT4G14550 | *IAA14* | **Os12g0601300** | TC182343 | 56.7 | 53.0 | 75.5 | 79.5 | Casimiro et al. (2003) |
| | | | Os03g0742900 | | 58.2 | 53.1 | | | |
| *TIR1* | AT3G62980 | *TIR1* | Os05g0150500 | TC168970 | 48.9 | 57.7 | 64.8 | 64.0 | Casimiro et al. (2003) |
| *WRKY75* | AT3G01970 | *WRKY75* | Os11g0490900 | TC185610 | 46.1 | 37.1 | 74.5 | 78.4 | Devaiah et al. (2007) |
| *PHV* | AT1G30490 | *ATHB14* | **Os03g0640800** | TC176589 | 62.0 | 72.8 | 69.5 | 71.4 | Hawker and Bowman (2004) |
| | | *HOX33* | Os12g0612700 | | 67.5 | 69.6 | | | |
| *CAND1* | AT2G02560 | *TIP120* | Os02g0167700 | TC192976 | 65.4 | 76.0 | 69.7 | 72.6 | Cheng et al. (2004) |
| *PAS2* | AT5G10480 | ? | **Os01g0150200** | TC166551 | 56.8 | 75.5 | 70.4 | 71.6 | Faure et al. (1998) |
| *ILL2* | AT5G56660 | *ILL1* | Os01g0706900 | TC173699 | 51.4 | 51.6 | 67.6 | 62.5 | Rampey et al. (2004) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *IAR3* | AT1G51760 | *ILL1* | Os01g0560000 | TC173699 | 57.1 | 63.3 | 67.0 | 68.0 | Magidin et al. (2003) |
| | | | | TC158327 | | | 63.8 | 63.5 | |
| *ECR1* | AT5G19180 | ? | Os01g0271500 | TC165467 | 60.5 | 67.8 | 71.1 | 78.7 | Woodward et al. (2007) |
| | | | | TC172176 | | | 68.7 | 69.8 | |
| *RBX1* | AT5G20570 | *RBX1a* | Os01g0106800 | TC161421 | 59.1 | 80.1 | 77.0 | 85.0 | Gray et al. (2002) |
| *REV* | AT5G60690 | *HOX9* | **Os10g0480200** | TC177627 | 68.6 | 70.4 | 63.3 | 62.2 | Hawker and Bowman (2004) |
| | | *HOX10* | Os03g0109400 | TC176589 | 68 | 69 | 70.6 | 70.7 | |
| | | | | TC182801 | | | 72.7 | 80.9 | |
| *AGL21* | AT4G37940 | *MAD21* | Os02g0579600 | TC187224 | 65.3 | 60.1 | 56.2 | 39.4 | Zhang et al. (2007) |
| *SUR1* | AT2G20610 | ? | Os11g0552000 | TC164962 | 53.4 | 46 | 59.2 | 50.7 | Casimiro et al. (2003) |
| Root hair development | | | | | | | | | |
| ERH3 | AT1G80350 | *KATANIN p60* | Os01g0683100 | TC160566 | 63.3 | 81.0 | 71.3 | 76.6 | Schneider et al. (1997) |
| *RHL2* | AT5G02820 | *RHL2* | Os03g0284800 | TC174707 | 56.8 | 61.4 | 67.8 | 63.4 | Schneider et al. (1997) |
| *RHD1* | AT1G64440 | *GEPI48* | Os05g0595100 | TC192755 | 60.8 | 74.2 | 61.4 | 57.4 | Schiefelbein and Somerville (1990) |
| | | *GEPI48* | Os08g0374800 | | 60.6 | 62 | | | |
| *TIP1* | AT5G20350 | *TIP1* | **Os02g0184000** | BU996187 | 58.7 | 63.8 | 52.1 | 57.3 | Hemsley et al. (2005) |
| | | *TIP1* | Os06g0644500 | EH091151 | 64.5 | 64.3 | 44.5 | 40.2 | |
| | | | | TC179087 | | | 42.9 | 46.1 | |
| *RHD2* | AT5G51060 | *RBOHD* | **Os11g33120** | TC153922 | 42.2 | 47.7 | 56.0 | 63.8 | Schiefelbein and Somerville (1990) |
| | | | Os12g0541300 | | 55.7 | 49.5 | 58.4 | 54.7 | |
| | | | | | | | 67.5 | 66.4 | |
| *KJK* | AT3G03050 | *CSLD2* | **Os06g0111800** | TC164787 | 64.4 | 79.8 | 73.7 | 85.8 | Favery et al. (2001) |
| | | *CSLD1* | Os10g0578200 | TC187976 | 59 | 75.5 | 64.9 | 64.3 | |
| | | | | | | | 65.5 | 60.7 | |
| | | | | | | | 59.2 | 54.4 | |
| *RHD3* | AT3G13870 | *RHD3* | Os01g0575000 | TC168229 | 64.0 | 62.4 | 69.0 | 70.1 | Schiefelbein and Somerville (1990) |
| | | | | TC181503 | | | 73.8 | 82.9 | |
| | | | | TC169425 | | | 66.8 | 66.5 | |
| | | | | TC177006 | | | 65.2 | 63.7 | |
| *ROP2* | AT1G20090 | *RAC6* | Os02g0120800 | TC179704 | 56.4 | 88.3 | 78.3 | 89.3 | Li et al. (2001) |

(*continued*)

**Table 2.4** (continued)

| *Arabidopsis* | | Rice | | Barley | Similarity [%] | | | | References |
|---|---|---|---|---|---|---|---|---|---|
| Alias | Gene acc. no. | Alias | Gene acc. no. | EST(s) acc. no. | At-rice | | At-barley | | |
| | | | | | N | P | N | P | |
| Primary root architecture | | | | | | | | | |
| *SMT1* | AT5G13710 | *SMT1* | **Os07g0206700** | TC155984 | 63.4 | 77.3 | 71.5 | 77.0 | Willemsen et al. (2003) |
| | | | Os03g59290 | | 66.1 | 65.2 | | | |
| | | | Os11g19140 | | 45.7 | 32. | | | |
| *RML1*/ *GSH1* | AT4G23100 | *GSH1B* | **Os05g0129000** | TC162201 | 57.6 | 65.3 | 72.4 | 78.8 | Vernoux et al. (2000) |
| | | | Os07g0462000 | TC194971 | 62.4 | 61.2 | 74.3 | 80.2 | |
| *HPA* | AT1G71920 | *HPA* | Os02g0709200 | TC155290 | 60.7 | 68.9 | 69.2 | 76.8 | Mo et al. (2006) |
| *DDL1* | AT3G20550 | *SNIP1* | **Os05g0545600** | TC161796 | 36.3 | 23.7 | 70.3 | 69.7 | Morris et al. (2006) |
| | | | | TC158236 | | | 62.4 | 58.2 | |
| *CEV1*/ *CESA3* | AT5G05170 | *CESA8* | **Os07g0208500** | TC187976 | 66.2 | 79.5 | 76.0 | 84.9 | Ellis et al. (2002) |
| | | *CESA2* | Os03g0808100 | | 72.7 | 69.8 | 61.9 | 50.9 | |
| | | | | | | | 59.9 | 39.8 | |
| *AtXTH21* | AT2G18800 | ? | Os06g0697000 | TC166673 | 46.5 | 53.4 | 63.4 | 59.1 | Liu et al. (2007) |
| *PAS1* | AT3G54010 | *PAS1A* | Os03g0367000 | TC162239 | 63.4 | 64.2 | 72.8 | 71.7 | Casimiro et al. (2003) |
| | | | | | | | 70.6 | 64.0 | |
| | | | | TC158273 | | | 70.6 | 64.0 | |
| *COB* | AT5G60920 | *COBL3* | **Os05g0386800** | TC170213 | 48.1 | 69.9 | 67.9 | 66.1 | Scheres et al. (2002) |
| | | *COBL4* | Os03g0754500 | TC165216 | 45.5 | 44.5 | 66.7 | 37.5 | |
| | | *COBL1* | Os10g0497700 | m | 59.9 | 54.2 | | | |
| | | *COBL2* | Os07g0604300 | | 62.8 | 64.5 | | | |
| | | | Os03g0416300 | | 64.9 | 62.6 | | | |
| *KEULE* | AT1G12360 | *SEC1A* | **Os04g0252400** | TC190473 | 36.6 | 56.6 | 61.2 | 58.9 | Söllner et al. (2002) |
| | | | | | | 29.1 | 68.3 | 65.7 | |
| | | | | | | | 70.1 | 70.8 | |
| | | | | | | | 74.6 | 80.3 | |
| | | | Os02g0452500 | TC161457 | 31.8 | 29.1 | 68.3 | 65.7 | |
| | | | | TC184788 | | | 70.1 | 70.8 | |
| | | | | TC174383 | | | 74.6 | 80.3 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *KNOLLE* | AT1G08560 | ? | **Os03g0736500** | TC179685 | 58.1 | 56.8 | 56.8 | 45.8 | Söllner et al. (2002) |
| | | | Os12g08980 | | 44.6 | 25.9 | | | |
| *IRX1* | AT4G18780 | *CESA4* | Os01g0750300 | TC160053 | 60.6 | 73.4 | 70.3 | 61.7 | Taylor et al. (2000) |
| | | | | | | | 75.2 | 82.6 | |
| | | | | | | | 72.7 | 77.7 | |
| *IRX3* | AT5G17420 | *CESA9* | Os09g0422500 | TC154161 | 66.7 | 80.6 | 70.0 | 76.2 | Taylor et al. (2000) |
| | | | | | | | 66.6 | 72.1 | |
| | | | | | | | 64.8 | 67.0 | |
| *HLR* | At4g29040 | ? | Os03g0298400 | TC156203 | 68.9 | 95.1 | 76.9 | 93.6 | Ueda et al. (2004) |
| *SKU 5* | AT4G12420 | ? | **Os10g0508000** | TC158012 | 57.5 | 62.4 | 65.6 | 53.5 | Sedbrook et al. (2002) |
| | | | Os06g0104300 | BG300903 | 64.3 | 67.5 | 61.4 | 66.4 | |
| *WAV2* | AT5G20520 | *MAD18* | Os07g0608300 | TC166812 | 57.3 | 69.3 | 69.9 | 74.1 | Mochizuki et al. (2005) |
| *KOR1* | AT5G49720 | *GUN9* | **Os03g0329500** | TC154162 | 68.4 | 74.6 | 67.4 | 72.1 | Zuo et al. (2000) |
| | | *GUN10* | Os03g0736300 | | 56.4 | 59 | | | |
| *RCE1* | AT4G36800 | ? | **Os08g0374100** | TC161238 | 43.9 | 30.4 | 69.3 | 48.2 | Larsen and Cancel (2004) |
| | | | Os09g0321900 | | 41.4 | 28.2 | | | |
| | | | Os10g0190000 | | 43.6 | 20.1 | | | |
| *FASS* | AT5G18580 | *TONNEAU2* | Os05g0149800 | TC172751 | 73.9 | 85.1 | 76.8 | 88.5 | Torres-Ruiz and Jürgens (1994) |
| *MUR1* | AT3G51160 | *GMD2* | Os06g0137700 | TC162999 | 62.3 | 70.2 | 70 | 79.3 | Freshour et al. (2003) |

*Acc. no* accession number, *At Arabidopsis thaliana, N* nucleotide, *P* protein
Bold indicates the most probable ortholog

(Liang et al. 2007), because ESTs are single pass reads. This leads to comparison of only corresponding fragments of sequences to determine similarity. Moreover, ESTs may often provide information on only a partial segment of an entire cDNA, whereas random sampling of clones leads to redundancy in EST datasets, as mention by Parkinson et al. (2002). To minimize false negative results in generation of barley consensus sequences, the CAP3 program was used, which has an ability to clip 5′ and 3′ low quality regions of reads (Huang and Madan 1999). To prevent "domain hits" (e.g., similarities that are caused by the conservation of fragments within families), only these *Arabidopsis*/monocotyledons sequences were chosen, which have extended barley EST coverage beyond the domain zone. Each time, the domain area on a nucleotide sequence, based on CDD search using Jellyfish, was established manually. As previously suggested by McGinnis and Madden (2004), the fastest way to compare the function of a protein is to perform a CDD search, which uses a database of motifs to characterize "conserved-domains" in a protein sequence. Following this idea, each selected sequence, which led to the confirmation of the existence of the same conserved domain in all cases (data not shown), was submitted into such analysis.

### 2.4.2 Arabidopsis *and Rice Genes Comparisons*

The definition of gene homology implies the existence of a common ancestor gene, which existed before speciation (in the case of orthologs) or before duplication (in relation to paralogs) (Alexeyenko et al. 2006). This implies the conservation in exon/intron arrangement between homologous genes, which led to the comparison of exon/intron organization in selected *Arabidopsis* and rice genes. In most cases, the arrangement was highly conserved between putative homologs, whereas some of them exhibited deletions or insertions (Fig. 2.2). Nevertheless, these changes have not disturbed an overall order in exon/intron arrangement.

### 2.4.3 *Searching for Potential Orthologs Between Other Monocotyledons and Barley*

Due to the lack of genomic sequences for most of monocotyledonous genes, it was not possible to check the level of conservation of exon/intron arrangement. Just like in the previous case, the first step was to search for barley ESTs in TIGR and GenBank databases (Fig. 2.3). This allowed selection of monocotyledonous genes, which have good EST coverage in barley genome, following the rules described above. Parallel to this, searching was done for the rice (in case of maize and wheat genes) and *Arabidopsis* sequences in GenBank. The barley ESTs were then used in a search for rice ESTs, which were compared with rice sequences from GenBank. As mentioned above, this step was performed to establish whether these sequences

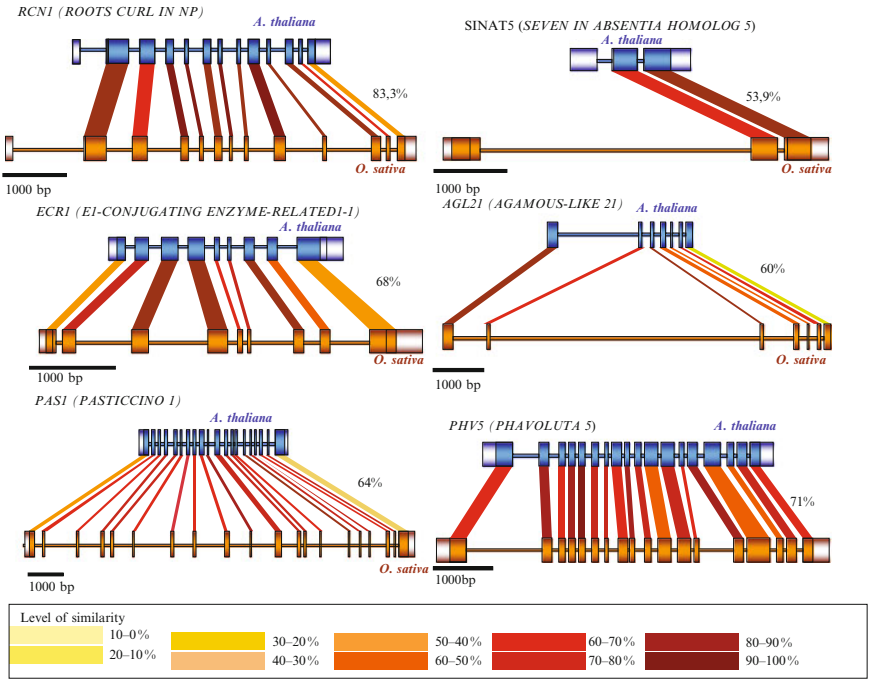


**Fig. 2.2** Examples of exon/intron arrangement in othologous *Arabidopsis* and rice genes. corresponding fragments are shaded using appropriate color in response to similarity between these fragments on protein level; *black line = scale bar*

are the same to confirm that the "hit" did not occur only by chance. This analysis led to the total number of ten genes, including six rice, two maize, and two wheat genes, which have potential orthologs in barley genome (Tables 2.5 and 2.6). ClustalW was also used for determining the similarity between other monocotyledons and barley sequences on nucleotide and protein level, respectively. To establish potential domains of barley proteins, CDD search was performed and confirmed in all cases the existence of the same conserved domains as in monocotyledonous proteins (data not shown).

### *2.4.4 Phylogenetic Analysis*

Even if the pairwise approach was theoretically the most powerful one-to-one methodology to predict true orthologs, many phylogenetic methods have been well described up to now (Chiu et al. 2006; Hulsen et al. 2006; Conte et al. 2008). In order to confirm the output from manually created BLAST-based approach and to establish the relationships between each of *Arabidopsis* and rice genes, it was decided to use GreenPhyl pipeline, which has been described as the

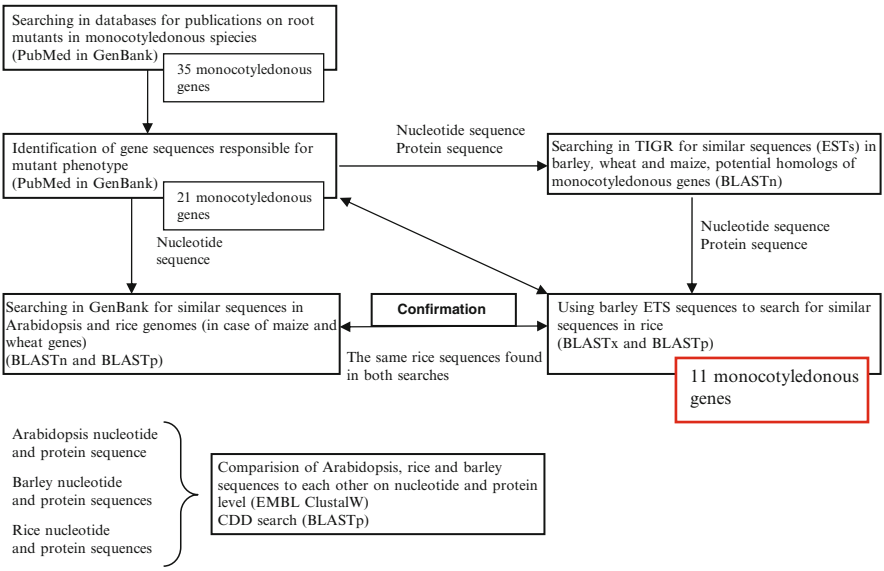


**Fig. 2.3** Strategy for selection of potential barley orthologs to monocotyledonous genes. E value (GenBank)/Expect (TIGR) $10^{-5}$ or less

most efficient phylogenetic method (Conte et al. 2008). In many cases, a large number of proteins showing high sequence similarity to *Arabidopsis* were encoded in the rice genome (data not shown). This is likely to be the result of multiple rounds of gene and genome duplications, followed by differential gene loss (Adams and Wendel 2005; Sterck et al. 2007). Following Conte et al. (2008), only ortholog associations in which a bootstrap value was 50% and more were taken into account as statistically significant. The total number of 50 *Arabidopsis* and 11 monocotyledonous genes were analyzed using this approach. From this number, 26 *Arabidopsis* genes (13 genes involved in LR formation, 3 genes involved in root hair formation, and 13 genes involved in root development) were confirmed as potential orthologs with a bootstrap value 50% or more (Fig. 2.4). Only in case of three *Arabidopsis* and one monocotyledonous genes, the orthologs detected by GreenPhyl were different from these selected on the basis of BLAST searching. Although genes selected as potential orthologs using BLAST approach were on the phylogenetic tree, they had lower bootstrap value. For genes typed by phylogenetic approach, the GreenPhyl bootstrap values were higher than values for genes selected using BLAST and were above 50%.

### *2.4.5 Synteny Detection in* Arabidopsis *and Rice Genomes*

To establish whether gene orders remained conserved between *Arabidopsis* and rice putative orthologs, the "Cinteny" pipeline was used (Sinha and Meller 2007). From 50 *Arabidopsis* sequences selected as having potential orthologs in rice

**Table 2.5** Monocotyledonous genes which have potential orthologs in barley and *Arabidopsis* genomes

| Monocotyledonous species | | Rice | | *Arabidopsis* | | Barley | Similarity [%] | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alias | Gene acc. no. | Alias | Gene acc. no. | Alias | Gene acc. no. | EST(s) acc. no. | Mc-Os | | Mc-Hv | | At-Os | | |
| | | | | | | | N | P | N | P | N | P | |
| *Ta RAN1* | AF488730 | *RAN1B* | Os05g0574500 | *RAN3* | AT5G55190 | TC176612 | 75.9 | 98.1 | 94.3 | 100 | 81.3 | 94.5 | Wang et al. (2006) |
| *Zm RTH1* | AY265854* | *RTH1* | Os03g0625700 | – | AT1G47550 | TC160970 | 56.5 | 65.6 | 86.8 | 92.6 | 24.9 | 25.8 | Wen et al. (2005) |
| | | | | | At1g47560 | TC187549 | | | | | 24.7 | 25.3 | |
| *Zm RTH3* | AY265855 | *COBL7* | Os03g0301200 | *COBL7* | AT4G16120 | TC157935 | 61.9 | 86.7 | 82.2 | 58.5 | 57.7 | 50 | Hochholdinger et al. (2008) |
| | | | | *COBL8* | At3g16860 | | | | | | 57.6 | 48.2 | |
| | | | | *COBL9* | At5g49270 | | | | | | 56 | 49.2 | |
| *Ta RHD3* | AY557340 | *RHD3* | Os01g0575000 | *RHD3* | AT3G13870 | TC168229 | 79.6 | 90.1 | 97.2 | 97.4 | 68.9 | 69.7 | Shan et al. (2005) |
| | | | | | AT1G72960 | TC177006 | | | 95.4 | 96.8 | 64.1 | 63.8 | |

**Table 2.6** Rice genes which have potential orthologs in barley and *Arabidopsis* genomes

| Rice | | *Arabidopsis* | | Barley | Similarity | | | | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Os-At | | Os-Hv | | |
| Alias | Gene acc. no. | Alias | Gene acc. no. | EST(s) acc. no. | N | P | N | P | |
| *Os CRL1* | Os03g05510 | *JLO* | AT4G00220 | NP9937331 | 46.6 | 36.6 | 64 | 41.8 | Inukai et al. (2005) |
| *Os CKL1* | Os02g0622100 | *CKI1* | **AT4G14340** | TC176778 | 49.4 | 66.8 | 86.5 | 92 | Liu et al. (2003) |
| | | *CKL10* | At3g23340 | | 67 | 66.6 | | | |
| *Os CSLD1* | Os10g0578200 | *KJK* | AT3G03050 | TC164787 | 59 | 75.5 | 72.4 | 78.7 | Kim et al. (2007) |
| | | | | TC157331 | | | 80.7 | – | |
| *Os RAA1* | Os01g0257300 | *FLP1* | **At4g31380** | BM816685 | 66.5 | 58.9 | 75.0 | 78.1 | Ge et al. (2004) |
| | | *FPF1* | AT5G24860 | | 62.8 | 58.9 | | | |
| | | | At5g10625 | | 41.5 | 39.7 | | | |
| *Os PIN1* | Os02g0743400 | *PIN1* | At1g73590 | TC188592 | 57.1 | 68.3 | 86.5 | 53.4 | Xu et al. (2005) |
| | | | | TC164300 | | | 82.9 | 82.1 | |
| *Os SLR1-1* | XM_469478 | *GAI* | **At1g14920** | TC156386 | 44.6 | 53.9 | 84.8 | 85.0 | Ikeda et al. (2001) |
| | | *RGA1* | At2g01570 | | 45.7 | 53.1 | | | |
| | | *RGL1* | At1g66350 | | 46.5 | 51.4 | | | |
| | | *RGL3* | At5g17490 | | 48.2 | 49.4 | | | |
| | | *RGL2* | At3g03450 | | 44.5 | 53.1 | | | |
| *Os AGAP* | Os05g0489600 | *ATARFA1B* | **AT5G14670** | TC166447 | 51.7 | 60.6 | 83.3 | 75.7 | Zhuang et al. (2005) |
| | | | AT1G70490 | | 61.7 | 59.5 | | | |
| | | | At1g10630 | | 60.6 | 59.5 | | | |
| | | | AT3g62290 | | 61.6 | 29.5 | | | |
| | | | At2g47170 | | 59.5 | 59.5 | | | |

*Acc. no.*accession number, *At Arabidopsis thaliana, Os Oryza sativa, Hv Hordeum vulgare, N* nucleotide, *P* protein
Bold indicates the most probable ortholog

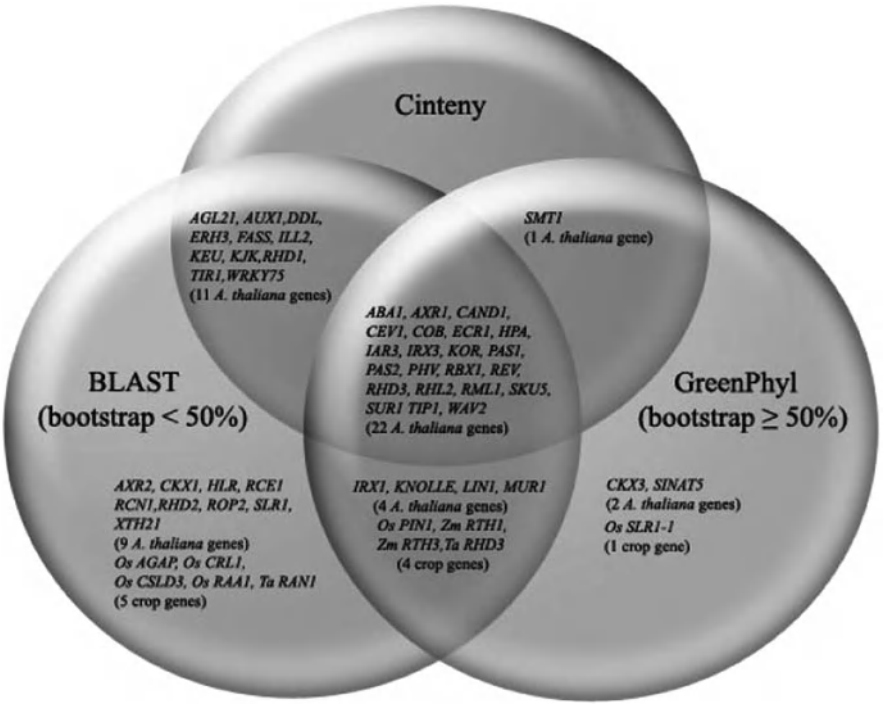


**Fig. 2.4** The bird's eye on in silico analysis: best candidates for molecular cloning. Using GreenPhyl, potential ortholog associations in barley genome were considered to be significant if the supporting bootstrap value was 50% and more. Similarity searching was proceeded using E value (GenBank)/Expect (TIGR) $10^{-5}$ or less. Genes that are situated in the middle (belonging to all three wheels) represent genes that have been selected by smart "best hit" strategy using BLAST searching and obtained a phylogenetical confirmation using GreenPhyl (bootstrap value 50% and more), and the conservation of gene order has been confirmed by Cinteny. Genes that are listed in the BLAST wheel were selected based on "best hit" strategy and have a GreenPhyl bootstrap value lower than 50%. GreenPhyl wheel corresponds to those genes that have candidates with bootstrap value higher than 50%, while "best hit" approach selected other candidate genes that have lower bootstrap values. Those genes, which belong to Cinteny wheel, preserved conservation in gene order. Genes that belong to BLAST and GreenPhyl wheels were selected by "best hit" approach and have bootstrap value 50% and more, but Cinteny did not display synteny blocks and/or orthologs in *Arabidopsis* or rice genome. Genes that belong to both GreenPhyl and Cinteny and separately to BLAST and Cinteny exhibit conservation of gene order for genes that belong to GreenPhyl and BLAST wheels, respectively

genome, 34 exhibited conservation in gene order (15 genes involved in LR formation, 6 genes involved in root hair formation, and 12 genes involved in root development). For the rest of 16 *Arabidopsis* genes, orthologs were not detected in rice genome using synteny-based approach. Nevertheless, it has been shown previously that, where microcolinearity is broken, it is possible to find "missing" gene in nonorthologous locus (Xu et al. 2002; Ware and Stein 2003). That is the reason why the lack of synteny does not imply the absence of homology. On the

other hand, the conservation of gene order during evolution could be treated as a valuable confirmation.

## 2.5 In Silico vs. Laboratory Approach to Gene Identification

Information from model species could be used in gene identification in two general ways. The first one is based on laboratory approach, where designing of degenerate starters (Ma et al. 1990; Finnegan and Dennis 1993) or probes for screening libraries (Schmidt et al. 1993; Nomura et al. 2003) have been commonly used. The second one is a bioinformatic approach, which in most cases is based on sequence similarity search using BLAST, phylogenetical analysis (Conte et al. 2008), as well as on the existence of synteny, as suggested by Fritz-Laylin and coworkers (2005). In general, the combined strategy is commonly used, which is based on bioinformatic analysis followed by molecular verification, like suggested in this paper.

In spite of their obvious successes in the past, laboratory strategies alone are inappropriate for large-scale analysis. The main disadvantage is their pure sequence-based nature, which can generate false-positive results, especially in correspondence to evolutionary divergence, where the level of similarity based on sequence comparison could be very low.

The improvements in sequencing technology led to hundreds of complete genome sequences, though most come from microorganisms. Till the end of 2008, only the genomes of three dicotyledonous species (*A. thaliana, Populus trichocharpa* and *Vitis vinifera)*, one monocotyledonous species (*O. sativa*), and a moss (*Physcometrilla patens*) have been fully sequenced. Recently also, complete draft assembly of the soybean (*Glycine max*) and maize (*Zea mays*) were released. Although, new sequencing technologies are now available, the assembly of large and complex genomes is still hampered by a significant content of repetitive DNA and, in allopolyploids, by the presence of homoeologous genomes. Most of economically important crops, specifically bread (16,979 Mbp) and durum (12,030 Mbp) wheat, barley (5,100 Mbp), oat (12,961 Mbp), rye (7,933 Mbp), and maize (2,793 Mbp), have large genomes (Doležel et al. 2007). For most of them, deep collections of full-length cDNA sequences are not available. In silico methods that are based on phylogenomic analysis suffer because of the lack of universal and efficient method for generating phylogenetic trees (Fu and Jiang 2007). Even the full genomic sequence does not guarantee the propriety of such analysis. It has to be taken into account that this could straightly lead to mistakes because of wrongly generated phylogenetic tree, as suggested by Dutilh et al. (2007). However, before the start of the genome sequencing projects, large-scale EST-sequencing projects were undertaken in several cereal species, and a large number of ESTs have become available for most of them. In spite of their importance (Varshney et al. 2006; Liang et al. 2007), EST projects yielded mostly partial

cDNA sequences, which are not adequate for direct comparison and assembly of entire genes. Nevertheless, the increasing amount of ESTs unlocks the gene contents of many species and automatically creates a need to elaborate new strategies to use this knowledge. They could be analyzed using only sequnce-based approach, like BLAST or FASTA, but such strategy can generate mistakes (Koonin and Galperin 2004).

Here is proposed the EST-based combined procedure for selecting potential orthologs, which is based on BLAST analysis combined with phylogenetic- and synteny-based approaches. The strategy includes a simple searching procedure used as a confirmation, which can avoid most common pitfalls during BLAST exploitation. Moreover, manual verification of the position of the evolutionary conserved fragments of proteins in domain zones using CDD search and Jellyfish program minimizes the risk of the so-called "domain hits," especially when the protein family is large. Although it should be noticed that lack of synteny does not imply absence of homology, such searching can be very handful during selection of genes. It was demonstrated in the presented paper that bioinformatic analysis is a powerful tool, which gives the possibility to find potentially homologous sequences between two species. The procedure that combines three most commonly used in silico approaches allowed to shortlist the number of potential orthologs as good candidates for molecular cloning.

## 2.6 Methods

### *2.6.1 Rice and* Arabidopsis *Searches*

Searches for rice and *Arabidopsis* genes were carried out in publicly available genome databases. *Arabidopsis* sequences were obtained from The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/). *O. sativa* sequences being potential homologs of *A. thaliana* genes were chosen using mRNA and protein sequences of *A. thaliana* genes searched against the GenBank database using BLASTn and BLASTp with default parameters, respectively (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Among a large number of output sequences obtained from the search, we selected the potential orthologs based on carefully selected criteria. First, E value was very restrictive and lower than $10^{-5}$ (Pevsner 2003). Each of the searches has been done in both directions to avoid hits obtained just "by chance." These sequences were also identified as potential orthologs through phylogenetic analysis using GreenPhyl (http://greenphyl.cines.fr/cgi-bin/greenphyl.cgi) or OrthologID; alternatively (http://nypg.bio.nyu.edu/orthologid/) synteny detection was proven using Cinteny (http://cinteny.cchmc.org/).

### 2.6.2 Sequence Analysis

The next stage of bioinformatic analysis was to check the degree of similarity on protein level between *A. thaliana* and *O. sativa*. The putative *O. sativa* and *A. thaliana* orthologous genomic sequences retrieved were then aligned with mRNA sequences for intron/exon junction positions, respectively, using Jellyfish program (http://jellyfish.labvelocity.com). This application was also used to align exon(s) of *A. thaliana* to the corresponding ones in *O. sativa* on protein level. Alignments of protein sequences were performed at The European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/ ) using the CLUSTALW program (Chenna et al. 2003) with default parameters.

### 2.6.3 ESTs

Searches for ESTs used in the presented publication were performed in publicly available EST libraries in The TIGR Gene Indices (Quackenbush et al. 2001) using the BLASTn and tBLASTx program with default parameters (http://www.tigr.org/db.shtml). This includes: barley sequences release 10.0 (June 3, 2008), wheat release 11.0 (July 13, 2008), maize release 18.0 (July 18, 2008), and rice release 17.0 (June 20, 2006). Searches for barley EST sequences corresponding to chosen monocotyledonous and *Arabidopsis* genes were also made in the GenBank EST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) using the tblastn program and default parameters.

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# Chapter 3
# Genomics of Root–Microbe Interactions

**Ulrike Mathesius and Giel E. van Noorden**

**Contents**



## 3.1 Introduction

All plants coinhabit their environment with a multitude of microorganisms, including bacteria, viruses, fungi, nematodes, and protozoans. In many cases, plants interact with specific microbes, leading to symbiotic relationships, where both partners are intimately associated and can either mutually benefit, or one partner can live at the other's expense. Roots are in close contact with the soil and an array of microorganisms that inhabit the rhizosphere. Easily available carbon is usually in short supply in soils, and microorganisms can benefit from root exudates and dead root material as a food source. Sometimes they specifically invade living root tissues to access nutrients from the plant. In the case of pathogenic interactions, this may lead to damage or death of the plant tissue. Common root-pathogen relationships

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include interaction of roots with pathogenic root knot (*Meloidogyne* sp.) or cyst (*Heterodera* and *Globodera* sp.) nematodes, infection of roots by pathogenic fungi, oomycetes, or bacteria. In contrast, plants and microbes have also evolved important mutualistic symbioses, most notably the interaction of plants with nitrogen-fixing bacteria and with mycorrhizal fungi. In both cases, the invading microbial partner provides nutrients in the form of ammonia (nitrogen-fixing bacteria) or phosphorus (mycorrhizal fungi), in exchange for carbon sources from the plant.

Because of the economic importance of the latter two mutualistic interactions, a major research effort has focused on unraveling the molecular basis of these symbioses. One of the best studied interactions is that between legumes and nitrogen-fixing soil bacteria called rhizobia. Rhizobia invade the roots of specific legume partners through root hairs or via crack entry, largely avoiding plant defense responses. Rhizobia produce species-specific lipochitin oligosaccharides (Nod factors) which are perceived by plant LysM-like receptors and activate a signal transduction pathway required for the invasion process and the subsequent development of a new root organ, the nodule (Geurts et al. 2005; Riely et al. 2004). Rhizobia remain outside the plant cytoplasm and are engulfed in a symbiosome membrane, which functions to regulate nutrient exchange between the partners. Nodules arise from redifferentiating root pericycle and cortical cells and are later invaded by rhizobia (Hirsch 1992). After further growth and differentiation of the nodule, the rhizobia start converting nitrogen from the air into ammonia, which is exported to the plant as amino acids. In exchange, rhizobia import carbon from the plant. This nutrient exchange requires coordination of transport processes by both partners (Prell and Poole 2006). The *Rhizobium*-legume (hereafter abbreviated RL) symbiosis also requires feedback mechanisms, so that symbiosis can be limited at times of sufficient nitrogen supply of the plant (Caetano-Anollès and Bauer 1988).

In contrast to the limited host range of rhizobia on legumes, most land plants form a mutualistic symbiosis with mycorrhizal fungi. Fungal hyphae show increased hyphal branching in the vicinity of host roots and invade root tissues, forming either arbuscular structures inside root cortical cells (arbuscular mycorrhizae or AM) or extracellular hyphal structures (ectomycorrhizae or EM). In AM symbioses, which are the most widespread associations and have existed for the last 450 M years, fungal hyphae first colonize the root surface where they form appressoria, invade roots intercellularly through clefts formed by the plant partner between epidermal cells, followed by intracellular invasion of root cortical cells and the formation of arbuscules in the inner root cortex (Harrison 2005). Similar to rhizobia, the fungal partner remains separated from the plant cytoplasm by a perifungal membrane. There is intensive nutrient exchange across membrane interfaces between the fungus and the plant. The most important nutrient provided by the AM fungal partner is phosphorus, while the plant provides carbon and lipid sources for the fungal symbionts (Harrison 1999). Again, feedback regulation functions to limit the carbon supply of the plant to the symbiont, which has been estimated to reach 30% of the total plant assimilated carbon (Nehls et al. 2007).

Root endoparasitic nematodes can cause enormous losses to crop plant production and have thus been extensively studied. The most common of these nematodes

include root knot and cyst nematodes, obligate sedentary endoparasites that complete their life cycle within the roots of host plants. Both invade roots and form feeding structures into which they divert large amounts of plant nutrients, leading to plant deformation or death (Bird and Koltai 2000; Williamson and Gleason 2003). The mechanism of gall or cyst formation is not well understood, but most likely a result of injections from nematode glands. Root knot nematodes induce giant cells, resulting from acytokinetic mitosis (mitosis without cell division) and endoreduplication of xylem parenchyma cells, which is accompanied by cell proliferation in cortical and pericycle cells, leading to root gall formation (Goverse et al. 2000a). Cyst nematodes induce the formation of syncytia, multinucleate cells resulting from fusions of cell contents of multiple root cells as well as endoreduplication of those cells (Goverse et al. 2000a). Both feeding structures alter transfer of nutrients from the xylem into the feeding site in a one-way relationship, in contrast to mutualistic symbionts.

Studying root–microbe interactions has provided insight into a number of biological processes, for example, recognition and communication of partner organisms (Cooper 2007), elicitation and suppression of defense responses (Samac and Graham 2007), formation and maintenance of endosymbiotic structures (Kistner and Parniske 2002), remodeling of plant development and meristem activity by the microbial partner (Ferguson and Mathesius 2003), nutrient exchange (Benedito et al. 2006), and long distance signaling in the plant (Beveridge et al. 2007). We will focus our review on aspects of these processes after discussing some of the major model organisms and genomic tools available for studies into root– microbe interactions.

## 3.2 Genomics Resources for Studying Root–Microbe Interactions

### *3.2.1 Legume Resources*

As neither the RL nor AM symbioses are formed in *Arabidopsis*, model legumes have been in the forefront of genomics research into root–microbe interactions. The selection of *Medicago truncatula* and *Lotus japonicus* as model plants for the study of RL and AM symbioses by a large community of researchers greatly contributed to the amount resources that are available for genomic approaches (Cook 1999; Udvardi et al. 2005). Both legumes have small diploid genomes of 470–550 Mb in size, have short regeneration times, are self-fertile, and are relatively easy to transform and regenerate. Both *M. truncatula* and *L. japonicus* are currently targets of genome sequencing projects, which have helped significantly in the map-based cloning of genes required for root–microbe interactions. As of January 2007, 176 Mb of nonredundant sequences of the *L. japonicus* and 189 Mb of nonredundant sequences of the *M. truncatula* genomes have been released. These correspond to approximately 40% of the entire genome of both legumes and cover 69 and 58% of public expressed sequence tags (ESTs) of *L. japonicus* and *M. truncatula*, respectively (Sato et al. 2007). The crop legume soybean (*Glycine max*) has been proposed as a third model

legume and sequencing is well underway (Jackson et al. 2006; Stacey et al. 2004). Soybean is a model legume for other bean species with more complex genomes and has been extensively studied for its interactions with rhizobia and cyst nematodes.

In addition to the genome sequencing projects, large EST databases are available for legumes that have been useful for transcript analyses as a basis for protein identification in proteomics studies and for the development of transcript profiling arrays (Journet et al. 2002). EST frequency analyses (in silico Northers) have also been used for transcript profiling (Tesfaye et al. 2006). For *M. truncatula*, around 200,000 ESTs are available (MtDB2 http://www.medicago.org/MtDB/; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago). ESTs from *L. japonicus* are available from Kazusa at http://est.kazusa.or.jp/en/plant/lotus/EST/ and from Harvard University at http://compbio.dfci.harvard.edu/tgi/cgibin/tgi/gimain.pl? gudb=l_japonicus. EST sequences from soybean are numerous (330,436) and can be found at http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=soybean.

For *M. truncatula*, both a 16k microarray and The Affymetrix GeneChip® *Medicago* Genome Array are available. The 16K microarray (*Medicago truncatula* Mt16kOLI1 70mer oligonucleotide-based microarray) is based on all tentative consensus sequences (TCs) from the DFCI *Medicago* gene index release 5.0 (Hohnjec et al. 2005). The Affymetrix GeneChip® *Medicago* Genome contains about 48,000 transcripts of *M. truncatula*, 1,850 transcripts of *M. sativa* (alfalfa), and all the genes of *Sinorhizobium meliloti*, the symbiont of *M. truncatula* and *M. sativa*. An Affymetrix chip is also available for soybean and includes over 37,500 soybean transcripts as well as 15,800 transcripts for *Phytophthora sojae* (an oomycete pathogen of soybean) as well as 7,500 transcripts from the soybean cyst nematode (*Heterodera glycines*). Genomics resources for *L. japonicus* include cDNA arrays and Serial Analysis of Gene Expression (SAGE) (Sato et al. 2007). In addition, suppressive subtractive hybridization (SSH) has been used in a number of studies to identify transcripts differentially displayed in specific cDNA libraries.

Proteomics is another postgenomic tool that has gained steadily in popularity and has been used in several root–microbe studies (Bestel-Corre et al. 2004). For both *M. truncatula* and *L. japonicus*, protocols for proteomic analysis are available in protocol handbooks (see below), although much development is needed for detection of low abundance proteins, phosphoproteins, and other posttranslational modifications.

Metabolic profiling is a third postgenomic tool and is the most complex in scope and so far limited in its use. To measure metabolites on a genomics scale requires specialized equipment such as high-performance liquid chromatography, capillary electrophoresis, and gas chromatography in combination with mass spectrometry. In addition, the metabolite profiling data are highly complex, which presents challenges for identification and quantification of the metabolites. Metabolomics was used to study metabolite profiles in mature nodules in *L. japonicus* (Desbrosses et al. 2005) and *M. truncatula* (Barsch et al. 2006), as well as in mycorrhizal roots (Schliemann et al. 2008). Carbon, nitrogen, and phenylpropanoid metabolism have been the major focus of published metabolomic studies. In addition, a metabolic

pathway database has been established (Urbanczyk-Wochniak and Sumner 2007). A major current limitation is the availability of chemical reference databases for identification of a larger number of metabolites.

Research into the biology of root symbiosis in these model legumes is supported by a range of postgenomic resources (Ané et al. 2008; Colebatch et al. 2002b, c) including reverse genetic approaches as gene silencing by RNA interference (RNAi), virus-induced gene silencing, T-DNA and transposon tagging, fast neutron and EMS (ethyl methanesulfonate) mutagenesis (Tadege et al. 2005), and bioinformatics resources (Cannon et al. 2005; Küster et al. 2007a; Lamblin et al. 2003). A TILLING (Targeting Induced Local Lesions IN Genomes) service has been set up for both legumes at the John Innes Centre, Norwich, UK. Further descriptions of these resources and detailed protocols for the study of root–microbe interactions in these species can be found in the handbooks for *M. truncatula* (http://www.noble.orgMedicagoHandbook/) and *L. japonicus* (Marquez et al. 2005).

### 3.2.2 Microorganism Resources

In recent years, several *Rhizobium* strains have been sequenced (MacLean et al. 2007) including *Sinorhizobium meliloti*, the symbiont of *M. truncatula* (Galibert et al. 2001), *Mesorhizobium loti*, the symbiont of *L. japonicus* (Kaneko et al. 2000), and *Bradyrhizobium japonicum*, the symbiont of soybean (Kaneko et al. 2002). The complete sequenced genomes of these rhizobia allowed many genomic studies including profiles of transcript (Perret et al. 1999) and protein expression (Djordjevic et al. 2003; Djordjevic 2004). The AM fungus *Glomus intraradices* and the EM fungus *Laccaria bicolour* are two symbiotic fungal genomes being sequenced (http://darwin.nmsu.edu/~fungi/index.php). Sequencing projects for several root knot and cyst nematodes (http://www.nematode.net) and for root pathogenic fungi and oomycetes (http://www.broad.mit.edu/annotation/fgi/, http://genome.jgi-psf.org/) are underway. Recent reviews give an update on genomics of fungal partners (Soanes et al. 2002, 2007), discuss studies on transcript profiling during host–pathogen interactions, and give an excellent overview on design of these experiments (Wise et al. 2007). We will therefore not cover these areas in our chapter in detail.

## 3.3 Insights into Root–Microbe Interactions Using Genomics

### 3.3.1 Initial Communication Between Roots and Microbes

The first step in root microbial interactions is mutual recognition and subsequent attraction of the microbe to the root surface. Following signal molecule recognition, signal transduction is necessary to initiate defense reactions, morphological changes, or physiological adaptations of the root and whole plant.

Both plants and microbes release chemical signals into the rhizosphere that aid in mutual recognition and attraction. Legumes have long been known to release species-specific mixtures of (iso) flavonoids into the soil, which are recognized by a number of organisms. Rhizobia perceive flavonoids of their host legumes by binding of the flavonoid to a protein called NodD, which then activates a suite of nodulation genes inside the bacteria (Redmond et al. 1986). This gene induction by flavonoids appears specific to nodulation-related genes: a proteome analysis of *Rhizobium leguminosarum* in response to flavonoids revealed only four altered proteins (Guerreiro et al. 1997), and a transcriptome study of *S. meliloti* showed only nine altered gene transcripts (Capela et al. 2005). The requirement for root flavonoids for the successful induction of *Nod* genes and subsequent nodulation has recently been shown in soybean, where silencing of the isoflavonoid pathway by RNAi led to an inhibition of nodulation, which could be overcome by inoculating plants with a flavonoid hypersensitive *Bradyrhizobium* strain or purified Nod factors (Subramanian et al. 2006). Flavonoids of certain structures are also active as stimulators for mycorrhizal fungi and can trigger hyphal growth and branching that can be observed before AM fungi infect the root (Steinkellner et al. 2007). However, the successful infection of plants defective in flavonoid synthesis has cast doubt on a strict requirement for flavonoids for the AM symbiosis (Becard et al. 1995). Strigolactones are a class of sesquiterpenoid compounds that are released from roots of mycorrhizal host, but not from nonhost plants, and have been the first identified compounds with activity as stimulators of hyphal branching in AM fungi (Akiyama et al. 2005).

Microorganisms in turn produce a range of signaling molecules that mediate root–microbe interactions and have extensive effects on the host. The best studied of these signals are the Nod factors synthesized by rhizobia. Nod factors are necessary for nodulation and sufficient for the early signaling events in the root. Nod factors not only induce specific nodulation-related responses but also have effects on root growth and lateral root formation (Olah et al. 2005). A large-scale SSH approach identified many new regulatory genes activated in roots following the first 48 h after Nod factor treatment (Godiard et al. 2007).

Most bacteria release so-called "quorum sensing" signals (QSS) that are used in communication between bacteria and the regulation of a range of bacterial behaviors that require coordination between bacterial cells, including pathogenic behaviors of rhizosphere bacteria (von Bodman et al. 2003). While several studies have shown the extent of gene expression changes in the bacteria in response to QSS (Arevalo-Ferro et al. 2003; Chen et al. 2003; Gao et al. 2007; Schuster et al. 2003), it has become apparent that plant hosts can also detect and broadly respond to QSS. A proteome analysis of *M. truncatula* roots showed over 100 protein changes in response to QSS, and these were specific for the QSS structure and concentration (Mathesius et al. 2003). In addition, treatment of roots with QSS led to changes in the expression of disease-related genes in the shoots of tomato plants, indicating that QSSs have systemic effects in the plant that alter plant defense (Schuhegger et al. 2006). Therefore, it is likely that eukaryotes have evolved detection systems for signals

that could alert plants to the presence and density of bacterial symbionts of pathogens in the rhizosphere (Bauer and Mathesius 2004).

The existence of a mycorrhizal factor ("Myc factor") has long been suggested. Firm evidence for a diffusible factor from AM fungi comes from experiments, where expression of a plant reporter gene, *ENOD11::gusA*, was activated in response to AM fungi that were physically separated from the root by a membrane (Kosuta et al. 2003). Interestingly, the diffusible factor still stimulated *ENOD11* gene expression in a *dmi* (does not make infections) mutant that is unable to form either nodules or arbuscules, suggesting that the "Myc factor" triggers early signal transduction pathways outside this essential signal cascade. So far the "Myc factor" has not been structurally identified.

A recent study has suggested the existence of a "Nem factor," a signaling molecule released by parasitic root knot nematodes (Weerasinghe et al. 2005). This signal is likely to act on the same signal transduction pathway as Nod factors, as the nematode signal was unable to initiate root responses in mutant host plants lacking a functional Nod factor receptor (Weerasinghe et al. 2005). Identification of the "Myc" and "Nem" factors would be an important advance, together with characterization of genes involved in their synthesis and regulation, which is expected to progress with the sequencing of fungal and nematode genomes (Bird et al. 2005; McCarter et al. 2005).

### *3.3.2 Signal Transduction*

Unraveling of the signal transduction pathways required for successful microbial invasion and symbiosis has been accelerated in recent years through the positional and mapped-based cloning of key genes of the signal transduction pathways, especially in RL and AM symbioses. Importantly, Nod factor receptor candidates, as well as a calcium signaling cascade and several crucial transcription factors, were identified. An interesting finding of those studies was that there is a group of early signal transduction genes in legumes that are required for both RL and AM symbioses. Several detailed recent reviews have covered the identification and characterization of these genes (Cook 2004; Gianinazzi-Pearson and Brechenmacher 2004; Harrison 2005; Kinkema et al. 2006; Oldroyd and Downie 2006; Parniske 2004; Stacey et al. 2006), and therefore these studies will not be discussed in detail here. The identification of one of the signal transduction genes, the calcium-calmodulin dependent kinase, *DMI3*, has been one of the first examples of transcript-based cloning (Mitra et al. 2004a), whereby a transcript profiling comparison of the mutant and wild type was used to identify a few candidate genes with changed expression, including the mutant gene.

The nodulation mutants are now being used increasingly as tools in postgenomic analyses to study the downstream effects these mutations have on root–microbe interactions. For example, a transcriptome analysis found that gene expression changes induced in wild-type roots in response to rhizobia were not activated in

six early nodulation-deficient (nod$^-$) mutants and only partially induced in a later nod$^-$ mutant (*hcl, hair curling*). In addition, it was shown that the responses of 46 selected genes were specifically due to Nod factor synthesis by the rhizobia (Mitra et al. 2004b). Similar results were found in a micro- and macroarray analysis of *M. truncatula* that identified more than 750 gene differentially displayed during the first 10 days of nodule development (El Yahyaoui et al. 2004). Expression changes can be detected within 1 h of inoculation with rhizobia and showed stage-specific patterns (Lohar et al. 2006).

In the AM symbiosis, the *dmi3* mutant, which does not form AM or RL symbioses, fails to regulate several genes altered by AM in the wild type, including a receptor kinase, transcription factors, an ABC transporter, and an auxin response gene (Sanchez et al. 2005). Similarly, several other genes were only induced by AM in wild type but not the *dmi3* mutant, and interestingly, these genes could be induced even in absence of physical contact between fungi, suggesting that a diffusible "Myc" factor triggers the responses (Weidmann et al. 2004). In addition, an extensin and a Nod-like gene with similarity to membrane proteins showed reduced induction in the *dmi3* mutant at the appressorium stage and this might be linked to cell wall modifications necessary for the infection structure (Siciliano et al. 2007). A study of gene expression changes in response to AM fungi in seven early signal transduction mutants in *L. japonicus* that are affected in AM-colonization identified several gene expression changes dependent on the mutations (Kistner et al. 2005).

Additional components of signal transduction pathways that are shared and specific to the RL and AM symbiosis were identified in gene expression profiles, including a large number of transcription factors and kinases, but their roles remain to be investigated (Deguchi et al. 2007; Frenzel et al. 2005; Hohnjec et al. 2005, 2006; Liu et al. 2003; Manthey et al. 2004). A combination of in silico and transcript profiling has highlighted (AM and RL)-symbiosis-specific genes and promoter elements in *M. truncatula*, as reviewed by Küster et al. (2007b). Since the finding that most of the early signal transduction genes are required for both AM and RL symbioses, it has been interesting to search for genes specific for each symbiosis. Of interest are a group of lectin-like genes that are specifically induced during AM and RL symbioses and could play a role in binding cell wall carbohydrates of the microsymbiont and recognition of the partners (Frenzel et al. 2005; Mitra and Long 2004). A large (>300) group of short proteins with a signaling peptide and a cysteine motif has been identified to be specific for nodules in indeterminate legumes (Mergaert et al. 2003). This was confirmed and extended by in silico studies searching for nodule-specific genes (Fedorova et al. 2002; Tesfaye et al. 2006). Comparative transcript profiling of AM- and RL-infected roots showed AM-specific expression of two putative transcription factors that could be involved in gibberellic acid (GA) signaling (Manthey et al. 2004). Interestingly, comparisons of AM-induced genes with those induced in interaction of roots with the pathogenic fungi *Magnaporthe grisea* and *Fusarium moniliforme* (Guimil et al. 2005) and with the growth promoting bacterium *Pseudomonas fluorescens* (Sanchez et al. 2005) showed large overlaps in the root's response to these very different microbes, suggesting similarities in their perception.

### 3.3.3 Root Endosymbiosis, Endoparasitism, and the Regulation of Defense Responses

The successful invasion of microbes into plant roots requires physical changes in the root, formation of infection structures, and the regulation of defense responses, so that the invading microbe is tolerated by the root and restricted to certain tissues. In many legumes, rhizobia infect roots through infection threads (ITs) that form in infected root hairs. Other legumes are infected at so-called crack-entry sites at lateral root bases and these differences might reflect evolutionary stages in nodulation (Sprent 2007). The aquatic legume *Sesbania rostrata* can be infected in both ways, depending on growth conditions. When flooded, ethylene build-up inhibits IT entry and rhizobia invade by crack entry. A transcriptome comparison of *S. rostrata* roots infected via IT and crack entry identified multiple transcripts specific for each process (Capoen et al. 2007). A calcium-dependent protein kinase (CDPK1) was shown to be necessary for effective infection by rhizobia (and AM fungi) and transcript analysis of roots in which CDPK1 was silenced showed altered expression of cell wall and defense-related proteins (Ivashuta et al. 2005).

It has been suggested that rhizobia inhibit plant defense responses for successful invasion (Mithöfer 2002). In recent years, transcriptomics and proteomics studies found evidence for large-scale changes in root defense responses. Transcript profiling of early stages of nodulation showed that the majority of defense-related transcripts was induced early (from 1 h) after inoculation but was repressed during later stages, especially during IT development (Lohar et al. 2006). In nodules, there is evidence for enhanced expression of defense-related genes, and this might reflect the ongoing control of the bacterial partner by the plant (Colebatch et al. 2002a, 2004; El Yahyaoui et al. 2004; Tesfaye et al. 2006). Ethylene is one of the hormones mediating defense responses. The notion that nodulation is restricted by abortion of infection events by the plant was supported by the hyperinfection and hypernodulation of an ethylene insensitive mutant (*sickle*) (Penmetsa and Cook 1997). This mutant shows an altered expression of putative defense-related proteins, for example Kunitz proteinase inhibitor, trypsin inhibitor, and a pathogen-related protein (Prayitno et al. 2006a). Salicylic acid (SA) and jasmonic acid (JA) also play a role in regulating defense responses, and there is evidence that Nod factors downregulate defense responses mediated by SA (Martinez-Abarca et al. 1998) and that JA biosynthesis is enhanced during the early stages of infection (Kouchi et al. 2004).

In AM roots, fungal hyphae are restricted to cortical cell layers, and defense responses are likely to limit hyphal spread. Several studies have used transcriptomics and proteomics to identify candidates that play a role in defense and disease resistance. Successful infection by AM fungi appears to be related to a week early but transient expression of defense-related genes (or often just a downregulation without induction), followed by later induction of defense gene expression in arbuscule-containing cells, similar to the RL symbiosis (Deguchi et al. 2007;

Garcia-Garrido and Ocampo 2002; Gianinazzi-Pearson and Brechenmacher 2004; Liu et al. 2003).

The AM- and RL-deficient *dmi3* mutant of *M. truncatula* showed induction of a disease resistance gene during early appressorium formation in the AM symbiosis, suggesting that DMI3 might be involved in early downregulation of defense responses as part of successful invasion (Siciliano et al. 2007). Amiour et al. (2006) showed in a proteomic study that several glutathione-S-transferases (GST) are downregulated in appressorium-forming roots, which could play a role in defense. In contrast, SSH studies have shown increased abundance of GSTs in AM-infected roots and suggested that in addition to defense, this gene might be involved in arbuscule senescence (Brechenmacher et al. 2004; Wulf et al. 2003). In addition to local gene expression changes, mycorrhizal fungi were also shown to induce systemic changes in the shoot that led to increased pathogen resistance in *M. truncatula*, accompanied by expression changes of defense- and stress-related genes (Liu et al. 2007). The latter study also showed that most of the induced genes are common between roots inoculated with three different species of mycorrhizal fungi (*G. intraradices*, *G. versiforme*, and *Gigaspora gigantea*). Similar findings were made in *M. truncatula* inoculated with a range of different AM fungi that induced largely similar responses (Massoumou et al. 2007). Defense-related changes to gene expression found in many studies by genomic techniques could explain well known observations that AM-infected roots are more resistant to pathogen attack (Cordier et al. 1998; Liu et al. 2007) and might become important targets in improving plant health.

The extent of defense responses appears to be affected by the combination of host and fungal partner. A study by Feddermann et al. (2008) differentiated responses between *G. intraradices*, *G. mosseae*, and *Scutellospora casanea* and found that in addition to a common set of AM-related genes, there were significant differences in host responses to the different fungal species, although this correlated with different infection types. Similarly, Gao and colleagues reported that induction or repression of defense-related genes correlated with the infecting fungi and their ability to penetrate the root (Gao et al. 2004). AM fungi can form two developmental patterns, the *Arum*-type and the *Paris*-type, the former penetrating with one hyphae into one arbuscule-containing cell, whereas in the latter, hyphae can grow from cell to cell and thus penetrate many more cell walls. Increased defense gene expression was observed mainly in interaction with high fungal penetration rates in the Paris-type interactions, although analysis of a tomato mutant with reduced infection suggested that induction of plant defense genes does not necessarily restrict infection by AM fungi (Gao et al. 2004).

Proteomic analyses of *M. truncatula* roots in response to the oomycte pathogen *Aphanomyces euteiches* have shown a correlation between expression levels of PR10 (pathogenesis-related) proteins and pathogen infection levels in plant lines with various levels of resistance (Colditz et al. 2004). This study also showed the preinfection of roots with mycrorrhizal fungi protects from subsequent pathogen infection, and that this was accompanied with induction of proteins of the phenylpropanoid pathway and proteolytic proteins which could be involved in protection

from pathogens. Subsequent RNAi studies have confirmed that silencing of certain PR10 genes increased plant resistance to *A. euteiches*, concomitant with the induction of a different class of PR proteins in the silenced roots (Colditz et al. 2007).

Gene expression studies of roots responding to infection with endoparasitic nematodes have demonstrated downregulation of many defense-related genes (Jammes et al. 2005; Puthoff et al. 2003) including JA biosynthesis genes (Ithal et al. 2007b). This suggests that nematodes, which move through host roots either intercellularly (root knot nematodes) or intracellularly though vascular tissue (cyst nematodes), actively inhibit host defense responses. Thioredoxin peroxidase, a nematode secreted protein, could mediate reduced defense responses by repressing formation of reactive oxygen species (Robertson et al. 2000). However, other studies have reported increased expression of defense- and stress-related genes (e.g., Alkharouf et al. 2006; Gheysen and Fenoll 2002; Ithal et al. 2007b). Comparative analyses of gene expression changes in susceptible and resistant plants have identified several candidates for resistance to nematodes, including a glycosyltransferase in tomato (Schaff et al. 2007), a range of syncytial-specific genes including a WRKY transcription factor and a receptor-like kinase in soybean (Klink et al. 2007a). In addition, responses of the same soybean species to compatible and incompatible cyst nematodes have also shown extensive differences in gene expression in the roots within 12 h, again involving defense-related WRKY transcription factors (Klink et al. 2007b). A parallel study of gene expression changes in soybean roots and infecting cyst nematodes has highlighted the extent to which the genomes of both partners adapt during the interaction, with 429 of 35,611 (1.2%) plant genes and 1,850 of 7,431 (24%) nematode genes showing altered expression levels during different stages of infection (Ithal et al. 2007a).

### *3.3.4 Alteration of Root Development by Microbes*

Many rhizoshere microbes can alter the development of roots. Some bacteria synthesize hormones which can alter root growth, lateral root formation, and cell division activity. Most of the other microbial signals that alter root development, or their mechanism of action, remain unknown.

Rhizobia induce new cell divisions inside roots of host plants, which differentiate in an organized fashion to develop into a mature nodule. Purified Nod factors are sufficient to induce cortical and pericycle cell divisions, and their action has been linked to the reactivation of key cell-cycle regulators in legumes (Foucher and Kondorosi 2000). One explanation for their action on cell cycle is their potential to alter auxin and cytokinin signaling in the root. Both auxin and cytokinin levels and ratios are crucial for activation of the plant cell cycle (Foucher and Kondorosi 2000). Nod factors alter auxin transport at the site of nodule initiation in indeterminate legumes and this might cause an accumulation of auxin where cell division occurs (Mathesius et al. 1998b). The alteration of auxin transport is most likely mediated by an induction of root flavonoids (Mathesius et al. 1998a), and silencing the flavonoid pathway in *M. truncatula* by RNAi was shown to abolish the ability of

rhizobia to initiate nodules and to regulate auxin transport (Wasson et al. 2006). Auxin transport is also altered in the ethylene-insensitive *M. truncatula sickle* mutant, and this is linked to hypernodulation (Prayitno et al. 2006b). The involvement of cytokinin in nodulation has been demonstrated in two *L. japonicus* mutants. Whereas a mutant defective in cytokinin perception is unable to form nodules (Murray et al. 2007), a gain-of-function mutant conferring constitutive cytokinin signaling in the root forms nodules spontaneously (Tirichine et al. 2007). In *M. truncatula*, silencing of the cytokinin receptor CRE1 resulted in reduced nodulation (Gonzalez-Rizzo et al. 2006). If Nod factors alter hormone signaling in the root, they could be expected to alter other aspects of root development affected by these hormones, and this has been observed in several studies. The *cre1* mutant has significantly increased numbers of lateral roots (Gonzalez-Rizzo et al. 2006), and similarly in *L. japonicus*, overexpression of a cytokinin oxidase (which reduces cytokinin response) increased lateral root but decreased nodule formation (Lohar et al. 2004), suggesting a negative role for cytokinin in lateral root and a positive role in nodule formation. Nod factors and a signal from mycorrhizal fungi also stimulate lateral root formation and this was shown to require early nodulation signal transduction genes (Olah et al. 2005). Transcriptome and proteome studies have identified multiple genes that could be involved in developmental changes induced by rhizobia, including hormone response genes, transcription factors, and cell division-related genes, although their function remains unstudied (El Yahyaoui et al. 2004; Kouchi et al. 2004; Lohar et al. 2006; van Noorden et al. 2007).

Root endoparasitic nematodes cause major developmental changes in host roots as a result of creating feeding structures (Williamson and Gleason 2003). The mechanisms of feeding site induction are largely unknown, but results from injection of nematode secretions into plant cells. Some of the secreted proteins have been analyzed using a proteomic approach (Jaubert et al. 2002) and at least one secreted peptide belongs to the plant encoded CLE peptide family that includes CLAVATA3, a peptide regulating shoot meristem activity in plants (Wang et al. 2005). CLE peptides have recently also been observed in other cyst nematodes and it has been suggested that they mimic plant ligands for receptors involved in cell differentiation (Mitchum et al. 2007). Of particular interest in these root–nematode interactions have been genes involved in the induction of cell division and differentiation in the feeding structures. Microarray, subtractive cDNA cloning, and SAGE have begun to characterize the extensive changes occurring in host roots in response to cyst and root knot nematodes (Alkharouf et al. 2006; Bar-Or et al. 2005; Bird 1996; Fuller et al. 2007; Ithal et al. 2007a, b; Jammes et al. 2005; Khan et al. 2004; Klink et al. 2007a; McCarter et al. 2003; Puthoff et al. 2003, 2007; Uehara et al. 2007). The induction of cell cycle and auxin and cytokinin response genes indicates that nematodes activate the plant cell cycle by alteration of hormone levels (Bird and Koltai 2000; Gheysen and Fenoll 2002; Goverse et al. 2000b). Interestingly, there are several overlaps in gene expression and hormone changes between galls and *Rhizobium*-induced nodules (Favery et al. 2002; Hutangura et al. 1999; Koltai et al. 2001). Concomitant changes in cell wall modifying enzymes and cytoskeletal proteins are likely also involved in the activation of cell cycle and cell

expansion during giant cell and syncytium formation (Jammes et al. 2005). In *Arabidopsis*, a comparative analysis of gene expression in response to root knot and cyst nematodes revealed similar expression of certain cytoskeletal and organ development genes which might have a role in formation of both types of feeding structures, whereas lipid transfer proteins, hypothesized to be involved in cell expansion and/or organ development, were differentially expressed between the two interactions (Fuller et al. 2007). Studies on the global responses of hosts to nematodes (and other microbes) have been limited by the difficulty of collecting sufficient plant material of infection structures, and the use of laser capture microdissection to collect individual infected cells (Klink et al. 2007a; Ramsay et al. 2004) is a step toward obtaining more localized expression data.

In general, it has been difficult to distinguish responses related to invasion from those related to development. In future studies, it would be useful to analyze mutants defective either in invasion or in developmental changes to separate these effects.

### *3.3.5 Nutrient Exchange*

Endosymbioses with mutualistic bacteria and fungi are formed preferentially under conditions of nutrient deficiency, in particular of nitrogen and phosphorus, respectively. Both partners of these symbioses play an active part in regulating nutrient exchange across membranes in the infection structures. Rhizobia invade dividing cortical cells but remain separated from the plant cytoplasm by the peribacteroid or symbiosome membrane (derived from the plant plasma membrane). Often several bacteroids are housed together in a symbiosome, where nitrogen fixation by nitrogenase takes place. Leghemoglobin is an abundant protein inside nodules protecting nitrogenase from oxygen (which inhibits nitrogenase) at the same time as delivering oxygen to the electron transport chain. Bacteroids are differentiated rhizobia that show significantly altered gene and protein expression patterns compared with free-living bacteria (Ampe et al. 2003; Becker et al. 2004; Djordjevic 2004; Djordjevic et al. 2003; Pessi et al. 2007). Fixed nitrogen is exported to the plant cytoplasm as amino acids, and carbon, mainly in the form of tricarboxylic acids, are taken up by the bacteroids (Lodwig et al. 2003; Prell and Poole 2006). Transcript analyses for functioning root nodules demonstrated a high activity of sucrose breakdown, glycolysis, and carboxylic and amino acid assimilation (Colebatch et al. 2002a, 2004; Tesfaye et al. 2006). Nodule tissues of plant origin express a large number of nutrient transporters (carbon, nitrogen sulfate, and potassium), metal-binding proteins, aquaporins, ATPases related to nutrient uptake, and osmoregulaton inside the nodule (Benedito et al. 2006; El Yahyaoui et al. 2004; Kouchi et al. 2004; Küster et al. 2004; Manthey et al. 2004). Interestingly, these studies also found a large number of regulatory proteins that could be important in the ongoing regulation of enzyme and transport activity inside nodules (Colebatch et al. 2004). Proteomics studies of the peribacteroid membrane have identified about 100 proteins, including many transporters, aquaporins, especially of the nodulin 26 family, ATP-ases, signaling

and defense proteins, and endomembrane proteins, which could be a result of the endocytotic origin of the peribacteroid membrane (Panter et al. 2000; Wienkoop and Saalbach 2003). Metabolomic approaches confirmed elevated levels of amino acids, organic acids, and certain sugars in nodules (Barsch et al. 2006; Colebatch et al. 2004; Desbrosses et al. 2005). Because significant amounts of photoassimilates can be diverted to nodules for nitrogen fixation, it could be expected that plants limit carbon supply to ineffective (fix$^-$) nodules. Metabolome analysis of fix$^-$ nodules showed that carbon restriction to nodules occurs as a limitation of carboxylic acid synthesis in nodules, rather than photoassimilate transport to the nodule (Barsch et al. 2006). Sucrose synthase, which acts in unloading and cleavage of sucrose in the nodule, appears as another important metabolic control point and its repression led to major transcriptome and metabolome changes in nodules, particularly repressing amino acid synthesis (Baier et al. 2007). Senescing nodules can become a nutrient source for the plant, and this often coincides with pod filling. Transcriptome analysis of aging nodules identified many regulatory genes that could be involved in controlling the senescence process and revealed a role for ethylene, JA, and GA in nodule senescence (Van de Velde et al. 2006).

Mycorrhizal fungi depend on carbon allocation from their host and create a carbon sink in the infected roots. This is accompanied by increased expression of hexose transporters, activation of fungal glycolysis, and subsequent carbohydrate storage in ectomycorrhizal associations (Nehls et al. 2007). Induction of specific phosphate transporters is localized to arbuscules and is crucial for provision of phosphorus to the plant partner and some of these transporters have recently been cloned (Harrison et al. 2002; Paszkowski et al. 2002). A plethora of other nutrient and water transporters and enzymes of primary metabolism have been detected in AM-infected roots using transcript profiling (Hohnjec et al. 2005; Liu et al. 2003, 2007). Combined transcriptome and metabolome approaches have highlighted the role of metabolites from plastids and mitochondria in AM-infected roots. Amino acid, fatty acid, and carotenoid metabolism were activated in AM roots both at the transcript and metabolite level, and phosphate levels were increased (Lohse et al. 2005; Schliemann et al. 2008). A detailed review of genome-wide gene expression changes relating to nutrient exchange and concomitant cell wall modifications has recently been published (Balestrini and Lanfranco 2006).

Similar to the symbiotic structures, nematode feeding sites develop into massive nutrient sinks, although the plant appears to fail to regulate this process. Nematode feeding site development is accompanied by increases in expression of sucrose transporters and enzymes of carbohydrate metabolism and water channels and other transport proteins (Gheysen and Fenoll 2002; Hammes et al. 2005; Jammes et al. 2005; Uehara et al. 2007).

### *3.3.6 Feedback Mechanisms*

The acquisition of nutrients by roots is intimately linked with the available carbon supply from photosynthesis in the shoot. Therefore, long distance communication is

necessary to balance the extent of symbiosis in the root with carbon supply from the shoot. Both RL and AM symbioses are limited by a feedback mechanism called autoregulation (Caetano-Anollès and Bauer 1988). The number of nodules and arbuscules in the root is regulated by a gene that acts in the shoot and has been identified as a leucin-rich receptor like kinase (LRR-RLK) from soybean (Gm*NARK*), *L. japonicus* (Lj*HAR1*), and *M. truncatula* (Mt*SUNN*), as reviewed by Kinkema et al. (2006). Interestingly, this LRR-RLK has high similarity to the Clavata 1 (*CLV1*) gene from *Arabidopsis* that regulates shoot meristem activity (Gresshoff 2003). Mutation of NARK leads to supernodulation or super-mycorrhization of the root and overall plant growth is often stunted. Grafting studies have shown that autoregulation is a result of a signal initiated in the root upon infection with rhizobia or mycorrhizal fungi, which is received by NARK in the shoot, and a second signal is generated that travels back to the root and inhibits further symbiosis (Delves et al. 1986; Gresshoff 2003). So far it is unknown why both symbioses are affected by the action of NARK, or what the autoregulation signal is. Metabolite analyses of alfalfa found that flavonoid synthesis is limited in both RL and AM symbioses by the autoregulation signal, possibly limiting availability of symbioses-enhancing flavonoids (Catford et al. 2006). Metabolome analysis also suggested that the accumulation of isoflavonoids inhibitory to fungal germination in AM-infected roots could be part of the autoregulation system (Cordier et al. 1998; Schliemann et al. 2008). In the *M. truncatula sunn* (super numeric nodules) mutant, it was shown that inoculation of roots with rhizobia causes an inhibition of auxin translocation from the shoot to the root and that the supernodulation mutant does not show this long-distance auxin transport inhibition (van Noorden et al. 2006). In addition, *sunn* had higher levels of auxin in the inoculation zone of the root, suggesting that auxin is a positive regulator and long-distance signal in autoregulation (van Noorden et al. 2006). Proteome analysis of wild type and *sunn* roots supported this, showing that the large majority of proteins induced by rhizobia are also auxin-inducible. The study also identified proteins differentially expressed between wild type and *sunn,* including PR10 proteins, a protein involved in JA synthesis, a glutathione-dependent peroxidase, and a trypsin inhibitor (van Noorden et al. 2007). A transcriptome study also found several defense-related genes differing in expression between *sunn* and wild type suggesting a reduced defense response in supernodulating plants (El Yahyaoui et al. 2004). Proteome analysis of mycorrhizal fungi-infected wild type and *sunn* roots showed protein expression changes of two annexins, a narbonin, a quinine reductase, and a Kunitz proteinase inhibitor (Amiour et al. 2006). Liu et al. (2007) showed differential expression of several defense related and other genes including an aquaporin in the uninoculated and inoculated part of roots of a split-root system infected with mycorrhizal fungi, but also several genes similarly regulated by mycorrhizal fungi in both split root parts, confirming that mycorrhizal fungi have long-distance effects on uninfected parts of the plant. Comparison of gene expression changes in leaves of inoculated soybean wild type and a supernodulation mutant identified over 100 differentially amplified cDNA fragments of which most changed in wild type but not in the mutant (Lestari et al. 2006). Of particular interest in this study was differential expression of several receptor kinases and transcription factors that

might be involved in autoregulation. These studies have highlighted the complex changes occurring in shoot and root in response to rhizobia and mycorrhizal fungi and how they are affected by the autoregulation signal, yet the signal itself remains elusive. Proteome analysis of xylem sap of soybean wild type and NARK mutants identified some proteins that could potentially travel long distances in the xylem, including a lipid-binding protein and Kunitz proteinase inhibitor, although none of these differed between wild type and mutant (Djordjevic et al. 2007). In future, phosphoproteomics might reveal some of the early targets of the receptor-like kinases that control autoregulation.

## 3.4 Conclusions and Future Directions

One of the most interesting findings of recent years has been the overlap in the signaling pathways utilized by rhizobia and mycorrhizal fungi to invade legume roots, leading to the hypothesis that the more ancient mycorrhizal symbiosis was the precursor for the more recent interaction of legumes with rhizobia (Kistner and Parniske 2002; Sprent and James 2007). Furthermore, genomic tools have revealed evidence that root parasitic nematodes also share signal transduction pathways, genes and maybe signaling molecules with RL and AM symbioses (Bird and Koltai 2000; Favery et al. 2002; Gheysen and Fenoll 2002; Koltai et al. 2001; Weerasinghe et al. 2005). Interestingly, genome sequencing projects have revealed aspects of the evolution of genes involved in root–microbe interactions. Several nematode genes, in particular cell wall-degrading enzymes, appear to have higher similarity to bacterial genes than to eukaryotic genes, suggesting horizontal gene transfer between root-infecting bacteria and nematodes (Scholl et al. 2003). Future challenges remain to determine which parts of the microbial genomes are necessary for their symbiotic or pathogenic behavior, and these questions might become clearer with comparative genomic studies of a growing number of sequenced organisms. Likewise, it will be interesting to reveal the whole extent to which similar plant genes are required for infection, signaling, and developmental changes in response to soil microbes. The current wealth of genes and proteins identified in genomics studies will need to be tested in functional, e.g., reverse genetic, studies to explain how they are involved in root–microbe interactions. It would be particularly interesting to test the effect of specific mutations on the interaction of plants with a range of microbes to highlight commonalities and differences. For parasitic interactions, it will be of interest to identify nematode- and infection structure-specific genes that could be targeted in strategies to increase nematode resistance in crop plants.

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# Chapter 4
# Plant Genetics for Study of the Roles of Root Exudates and Microbes in the Soil

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## 4.1 Introduction

Although plants can be grown in sterile soil in aseptic growth chambers, their natural lives involve an intense and intimate interaction with a vast number of microbes, especially those found in soils. The number of different bacterial species in a single gram of soil has been estimated to be anywhere from a few thousand to many millions, depending on the soil source and the method of analysis (Foster 1988; Schloss and Handelsman 2006; Aislabie et al. 2008), with still-undescribed species making up a large share of the total. In addition to eubacteria and archaebacteria, many species of fungi, protists, and algae are also found in the soil, often in association with plant roots. The great majority of these soil microbes have not been studied to any significant degree, partly because conditions for their axenic culture have not been developed. For instance, only 26 of the approximately 52 identified major lineages, or phyla, within the domain Bacteria have cultured representatives. In fact, it is estimated that less than 1% of the bacterial species in the soil could be grown in culture with current approaches (Leadbetter 2003; Handelsman 2004; Leveau 2007), and this number is certain to be much lower if one considers that most rare microbial components of the soil are completely unknown.

Plants actively secrete very large quantities, and a great diversity, of organic compounds into the soil. Exudation of anywhere from 5 to 60% of total photoassimilate has been reported and found to be highly variable across environmental conditions (e.g., soil type, time of day, soil moisture, temperature) and plant genotype or growth stage (Bekkara et al. 1998; Groleau-Renaud et al. 1998; Hughes et al. 1999; Iijima et al. 2000; Aulakh et al. 2001; Garcia et al. 2001; Prosser et al. 2006). The roles of only a few of these compounds are known or guessed at (Merbach et al. 1999). Citrate is secreted, sometimes in very large quantities, to help acidify the soil and thereby promote root growth (Jones and Darrah 1994; Hinsinger et al. 2006), and this compound also helps bind aluminum in the soil, thereby decreasing its phytotoxic effects (Hoekenga et al. 2003). Some plants have been shown to exude phenolic compounds that exhibit allelopathic effects like the sorghum exudate sorgoleone that is an inhibitor of broadleaf and grass weeds at concentrations as low as 10 μM in hydroponic assays (Nimbal et al. 1996). Many other compounds, such as amino acids and sugars, are believed to be secreted by plant roots in order to promote rhizosphere microbial growth (Brimecombe et al. 2001), although the value to the plant of $\ll$1% of the rhizosphere microbes are not known in any system. Specific secreted phenolic compounds have been shown to be signal molecules that attract root colonization by useful microbes, nitrogen-fixing bacteria such as *Rhizobium*, and mycorrhizal fungi (reviewed in Bais et al. 2006).

The question remains, what do most of these soil microbes do? The active secretion of so much of the fixed carbon produced by a plant suggests that these microbes are very important to the plants, but this idea is challenged by the observation that plants can grow efficiently in sterile soil. Of course, plants that are grown with fertilizers in a controlled environment do not need symbiotic relationships that yield limiting growth substances, like the fixed nitrogen provided

by rhizobia or the phosphate access provided by mycorrhizae. Perhaps, a more frequent value of rhizosphere microbial associations to a plant is exemplified in the "take-all" disease, where the *Gaeumannomyces graminis* var. *tritici* fungus that infects wheat roots is overcome in the soil by a beneficial bacterial competitor, a specific isolate of *Pseudomonas fluorescens* (Thomashow and Weller 1988; Capper and Higgins 2007). Unlike sterile soil, potential microbial pathogens in field soil may exist in staggering numbers and variety, and only attraction of beneficial or neutral microbial competitors of these pathogens to the rhizosphere would provide comprehensive protection to host plants.

In the absence of the ability to grow most soil microbes in pure culture, it is difficult to test their possible contributions to plant growth or plant disease. One cannot simply inoculate the soil with a single microbe and see its effects on a potential host plant if one cannot first grow that microbe. However, we have postulated that we can use our control over host plant genetics to accomplish the same goals of understanding the roles of microbes in the soil (Deshpande 2006). If one can find mutations in plants, or segregating natural variation, which determines the presence/absence or abundance of specific rhizosphere microbes, then this demonstrates a specific relationship between the product of the mutated or varying plant gene(s) and the biology of the affected microbe. For instance, if one finds a natural variation for a low level of sorgoleone production, and sees that this causes the root to no longer be colonized by mycorrhizae, then this indicates that sorgoleone is involved in mycorrhizal colonization (Akiyama et al. 2005).

We have been pursuing this approach to use plant host genetics to dissect plant–microbe interactions in the soil for the last 10 years. This research has proceeded very slowly because of the need to establish a foundation for the experiments, a very limited tool set, a challenging level of environmental variation in the experiments, a surprisingly low level of plant genetic variation for rhizosphere exudates (at least in *Arabidopsis thaliana*, see below), and the lack of funding for such research in the absence of compelling preliminary results. However, recent advances in DNA sequencing technology have offered the possibility that studies of plant genetic control of microbial interactions in the rhizosphere and root can be analyzed comprehensively. This chapter describes our initial results with the genetic and metagenomic analysis of these interactions.

## 4.2 Natural Variation and Mutagenesis in *Arabidopsis* to Identify Alterations in Root Exudate

We used a model dicot angiosperm, *Arabidopsis thaliana*, as a target for our initial studies of plant host genetic effects on rhizosphere microbial populations. Because high pressure liquid chromatography (HPLC) is such a powerful technique to separate and display low molecular weight organic compounds like phenolics, we decided to determine reproducible conditions for exudate production by the roots of *Arabidopsis* seedlings by scoring the production from seedlings grown under sterile

conditions. Seeds were first surface-sterilized by gently agitating them in a solution containing two volumes of 0.1% Triton X-100 and one volume bleach. Seedlings were grown on filter paper set atop moist glass beads for 15 days in Gamborg's B5 medium at a temperature of 24°C and an artificial light intensity of 100 μE $m^{-2}$ $s^{-1}$. On day 15, fresh Gamborg's B5 medium diluted to 5% of its original concentration was added to the roots, and the medium (now with root exudates) was collected after 2 days of additional growth. Pools of ~100 seedlings were grown together in single vials for this analysis because smaller numbers of seedlings did not yield sufficient quantities of exudates for HPLC analysis. The liquid samples were frozen and dried in a Beckman lyophilizer and then resuspended in 98% methanol for reverse phase HPLC analysis. Under these conditions, a broad array of peaks representing different compounds were observed, and these were not produced by dead seeds or the growth media in the absence of growing seedlings (Fig. 4.1). Many of these peaks were found not to be reproducible from experiment to experiment, however, so a smaller range of peaks was chosen for specific focus. These six peaks gave qualitatively consistent profiles detected at 360 nm (Fig. 4.2). These peaks were both consistent across experiments and had the general properties of phenolics and related compounds that were good candidates as signal molecules.

Having established a reproducible assay system, we then looked at *A. thaliana* ecotypes Columbia, Landsberg *erecta*, Kashmir-1, Wassilewskeja, and Cape Verde Islands (CVI) for their root exudation of related compounds. Surprisingly, we saw no dependable variation for the compounds represented by these six peaks on the HPLC chromatogram. The ecotype CVI was included in this study because, at the level of DNA markers, it was the most different of any *Arabidopsis* ecotype available at that time. Hence, it was not possible to map genes responsible for variation in these compounds in any of the various mapping populations developed in *Arabidopsis* from crosses between these ecotypes.

Having failed to detect useful natural variation for exudate production, we next investigated the production of the compounds represented by these six peaks in EMS mutagenized Columbia and Landsberg *erecta* backgrounds, with M3 seed provided by Lehle Seeds. Most surprisingly, out of 2,000 M3 populations analyzed, not a single reproducible variation in any of these peaks was identified. Given the mutation rate in these EMS populations, we expected that 2,000 M3 would have provided an average of 1–2 homozygous and 3–4 segregating knockout mutations per gene for every gene in the *Arabidopsis* genome. Hence, for the first time in the history of genetics, we apparently identified a series of biological processes to produce numerous compounds that are not affected by mutational inactivation of any single gene. This astounding result remains unexplained.

Because it was expected that many of the compounds in the studied six peaks were phenolics, we also looked at known mutations in phenolic pathways, including knockout mutations in *fad1-2*, *fae2-1*, *gsm1-1*, *gsm1-2*, *hy5-1*, *mur2-1*, *mur4-1*, *mur5-1*, and *rhd1-1*, plus the double mutants *fah1-7*/*tt3-1*, *tt3-1*/ *tt7-1*, and *tt4-1*/*tt5-1* (Koornneef 1990; Lemieux et al. 1990; Haughn et al. 1991; Miquel and Browse 1992, Reiter et al. 1997) obtained from the ABRC. In addition, a line exhibiting transgenic *F5H* overexpression, generously provided by the laboratory of Dr. Clint

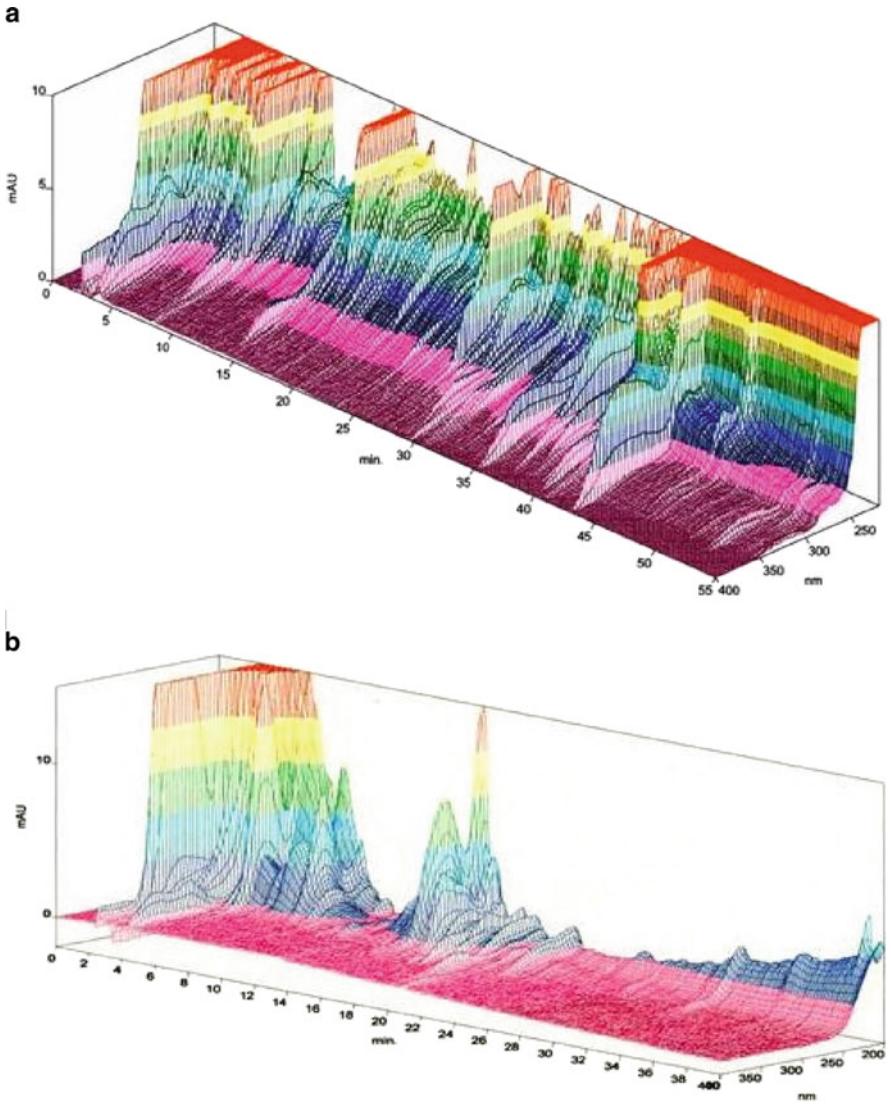


**Fig. 4.1** A three-dimensional metabolite profile of root exudates showing the retention time (*X*-axis), peak intensity (*Y*-axis), and the UV range of 200–400 nm (*Z*-axis). (**a**) Root exudates of wild-type *Arabidopsis thaliana* plants, ecotype Columbia; (**b**) negative control (growth media processed as exudate)

Chapple (Purdue University), was also investigated. *Arabidopsis* lines that were mutant in these genes were found to not exhibit any qualitative changes in the six putative phenolic peaks that we focused on throughout this project.

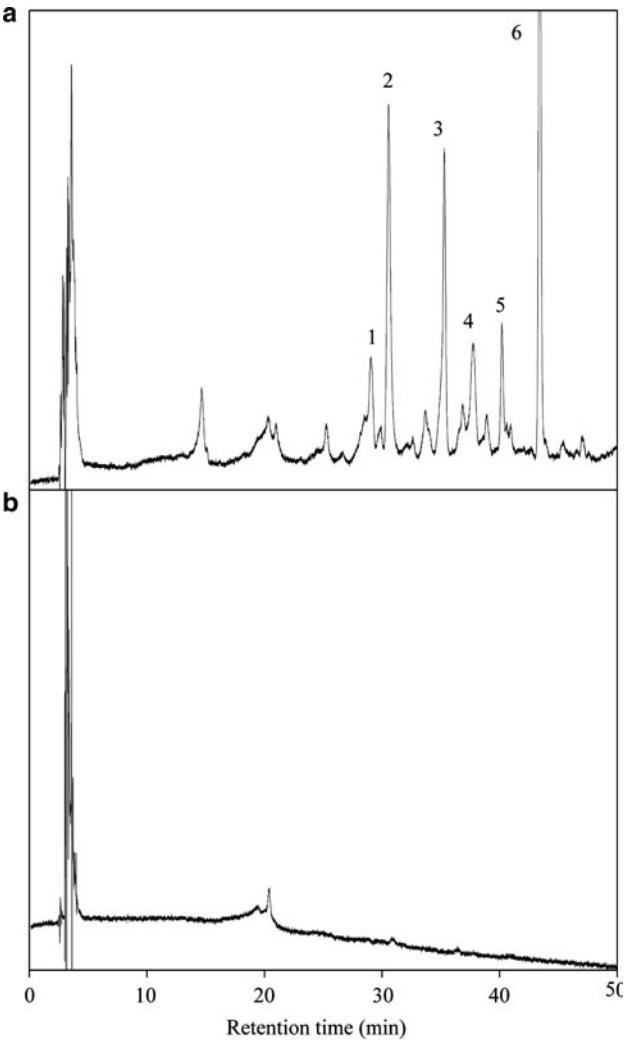


**Fig. 4.2** Chromatograms of wild-type *Arabidopsis thaliana* root exudates showing the six major peaks detected at 360 nm. (**a**) Ecotype Columbia; (**b**) negative control

The rationale of the *Arabidopsis* studies had been to identify genetically determined exudate variation and then to follow this up with the characterization of both the exudate compound(s) affected and the degree to which this variation altered soil microbial populations associated with the *Arabidopsis* root. In the absence of identified genetic variation, such follow-up studies were not performed.

## 4.3 Plant Genetic Determination of Natural Variation in Rhizosphere and Root-Associated Microbes in the Grasses

After arriving at the University of Georgia (UGA) in 2003, our lab decided to look at several grass species as targets for the study of root–microbe interactions. These studies have not yet involved exudates analysis but went directly to a metagenomic analysis of soil microbes. The soil used was from different UGA fields, but each experiment involved mixing one field soil source with a uniform potting mixture (to make roots easier to subsequently extract) and then placing equal amounts of this mixture in each large pot used in the experimental study. Seeds for host plants were germinated in these soils, and seedlings were then grown in the greenhouse under the same conditions for each duplicated or triplicated plant in the experiment. The assay system has been to sequence either total DNA or 16s ribosomal DNA amplicons prepared from the soil that clings to an extracted root ("rhizosphere" or Rh), the microbes firmly attached to a root washed with water ("root-external microbes" or REM), and the microbes remaining after the root is treated with chitinase, lysozyme, and various levels of hydrogen peroxide ("root-internal microbes" or RIM). Of course, the sample termed REM contains both root-internal and root-external microbes, while the Rh sample is certainly contaminated by broken root fragments that would yield some root-internal and root-external microbes.

In order to guarantee that the DNA analyzed would provide a comprehensive description of the microbes that were present, a vigorous DNA extraction protocol (http://fgp.bio.psu.edu/methods/ctab.html) was followed. Hence, the DNA extraction procedure for Rh, REM, and RIM samples yields not just the microbial DNA but also DNA from any other organisms or tissue fragments that were present in the sample. Especially in the case of the REM and RIM samples, this meant that there was a tremendous amount of host plant DNA present. Hence, random shotgun sequencing of all root-associated samples was mostly an exercise in sequencing the host plant genome, with yields of 10–20% of cloned DNA (Table 4.1) that was verified as nonplant. At the time of these analyses, neither the sorghum nor maize genomes had been fully sequenced, so many of the sequences labeled unknown could be screened for homology to these genomes once the ongoing sequencing projects are completed. Regardless, it was clear that this was an expensive route to pursue for metagenomic discovery.

Because the majority of maize nuclear DNA is methylated at the cytosines in 5′-CG-3′ and 5′-CNG-3′ sequences, we decided in one experiment to transform all of our soil DNA into DH5-α because cytosine methylated DNA such as that seen in maize and other grasses is often destroyed by this *Escherichia coli* strain (Palmer et al. 2003). Sequences of the resulting clones provided a significant decrease in maize DNA, and a significant increase in the percent of bacterial sequences recovered (Table 4.1) but decreased the amount of mycorrhizal DNA that was observed (data not shown). Hence, this potential metagenomic enrichment technology

**Table 4.1** Shotgun sequence analysis of DNA from the plant–soil interface

| Host plant species | Microbial fraction targeted[a] | Seq, type[b] | Number of sequences | Percent of sequences with highest homology to DNA from the listed organisms | | | | | | | | Unknown |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Host plant sequences | Moss | Eubact. | Archaea | Fungi | Protist | Diatom | Animal | Phage | |
| *Zea mays* | Rh+REM+RIM | RS | 732 | 88.3 | – | 3.7 | – | 2.2 | – | 0.1 | 0.4 | – | 5.3 |
| | | MF | 218 | 30.2 | – | 48.6 | 0.5 | 6.9 | – | 0.5 | – | – | 13.3 |
| | REM+RIM | RS | 259 | 89.2 | – | 1.9 | – | 4.6 | – | – | 0.8 | – | 3.5 |
| | | MF | 249 | 34.1 | – | 44.6 | – | 6.0 | – | – | 0.8 | – | 14.5 |
| | RIM | RS | 382 | 89.3 | – | 1.1 | – | 1.3 | 0.3 | – | – | – | 8.1 |
| | | MF | 176 | 43.7 | – | 46.0 | – | 2.8 | – | 0.6 | 1.7 | 0.6 | 4.6 |
| *Sorghum bicolor* | Rh+REM+RIM | RS | 366 | 88.3 | – | 4.6 | – | 0.5 | – | – | – | – | 6.6 |
| | | MF | 95 | 48.4 | 2.1 | 36.8 | – | 4.2 | – | – | – | – | 8.4 |
| | REM+RIM | MF | 84 | 76.2 | – | 13.1 | – | 3.6 | – | – | – | – | 7.1 |
| | RIM | MF | 83 | 44.5 | – | 30.1 | – | 1.2 | – | – | 1.2 | – | 22.9 |
| *Sorghum propinquum* | Rh+REM+RIM | RS | 352 | 80.7 | – | 5.1 | – | 1.2 | – | – | – | – | 13.1 |
| | | MF | 89 | 58.4 | – | 12.4 | – | 3.4 | – | – | – | – | 25.9 |
| | REM+RIM | MF | 83 | 60.2 | 1.2 | 24.1 | – | – | – | – | – | – | 14.5 |
| | RIM | MF | 85 | 51.8 | – | 21.2 | – | 1.2 | – | – | – | – | 25.9 |

[a]*Rh* + *REM* + *RIM* rhizospheric + root-external + root-internal microbes, *REM* + *RIM* root-external + root-internal microbes, *RIM* root-internal microbes
[b]*RS* random shotgun, *MF* methyl-filtered

was abandoned because it was not likely to yield a representative description of the microbes present in the soil, rhizosphere, or root samples. We also abandoned the hydrogen peroxide treatment in our RIM purification process because the level of treatment that we employed (2 min in 3% $H_2O_2$) appeared to lead to degradation of some DNA inside roots (data not shown). Moreover, although hydrogen peroxide treatment greatly lowered the number of sequences that were recovered from the extracted DNA, it did not show any obvious effect upon the relative abundances of classes of eubacteria that were recovered (data not shown). Hence, further investigation of hydrogen peroxide treatment, to identify an appropriate level of exposure for removing external microbes without damaging root-internal DNA, is warranted but may not be necessary.

Our first experiments were on the plant species *Zea mays* (maize), *Sorghum bicolor* (sorghum), and *S. propinquum* (a wild and interfertile relative of sorghum). The results with random shotgun sequencing of Rh, REM, and RIM microbes (Table 4.2) indicated that sequences representing many different kingdoms and phyla of microbes (archaebacteria, eubacteria, fungi, protists), small animals (e.g., nematodes and insects), mosses, and even a bacteriophage were present in the data, although most of the sequences were either from the host plant or of unknown origin. Interestingly, the organisms in the RIM sample (presumed root-internal microbes) included protists like *Cercozoa* (a flagellate protozoan that consumes bacteria) and the diatom *Thalassiosira*. These DNA sequences were annotated in early 2009, when internal funding for this project was exhausted, so reannotation at this date would be much more informative because additional plant sequences could be identified, and more of the unknown sequences would be attributed to many of the additional microbes that have been sequenced since that time.

For reasons of cost effectiveness, we decided to primarily switch to the standard process for amplification of rRNA genes (Weisburg et al. 1991; Tringe and Hugenholtz 2008) for microbe identification. This has the disadvantage of a potential for differential degrees of amplification of different sequences (thus providing a skewed quantitative description of the microbes present) and the possible lack of amplification of highly diverged microbes. For cost reasons with the maize and sorghum samples, only a few eubacterial rRNA sequences were investigated, providing between 173 and 191 eubacterial reads per duplicated data set. Even with this limited amount of data, certain patterns were clear. The most abundant eubacteria both outside and inside roots were from the class betaproteobacteria, although the deltaproteobacteria were about equally abundant in the REM (root external) samples for both *S. bicolor* and *S. propinquum* (data not shown).

**Table 4.2** Analysis of soil and root-associated organisms with 16s, 17s, and 18s rRNA sequences in switchgrass cultivar "Alamo"

| Species | Treatment | Eubact. phylotypes | Archaea phylotypes | Fungal phylotypes | Protist phylotypes | Animal phylotypes |
|---|---|---|---|---|---|---|
| Switchgrass | Rh | 668 | 13 | 37 | 19 | 46 |
| Switchgrass | REM | 409 | 3 | 53 | 6 | 5 |
| Switchgrass | RIM | 284 | 2 | 50 | 8 | 8 |

The alphaproteobacteria and gammaproteobacteria were also relatively abundant in both REM and RIM samples. Such species as the acidobacteria, bacilli, chloroflexi, clostridia, and deinococci were found both in REM and RIM samples but at low abundances. The sphingobacteria were of moderate abundance in the REM samples, but much rarer in the root-internal samples (RIM). Most dramatic, the *Sorghum* samples (especially *S. propinquum*) had a >2X lower percentage of eubacteria from known classes compared to maize, suggesting that a greater number/variety of exotic microbes associate with the roots of plants in the genus *Sorghum* than with maize.

Recently, we have begun studies of the microbial populations associated with the candidate biofuel crop called switchgrass (*Panicum virgatum*). In our first experiments, we have observed that the Rh, REM, and RIM populations for switchgrass are quite distinct (Table 4.2). For instance, archaebacteria were very abundant in the soil sample employed and frequent in the Rh populations but were very rare in the REM and RIM samples. As seen with maize and sorghum previously, mycorrhizal DNA was greatly enriched within the roots (the RIM samples). In general, bacterial, archaebacterial, protist, and animal diversity dropped off dramatically on and inside the roots compared to the rhizosphere, but detected fungi were actually more diverse both on and inside roots compared to the rhizosphere (Table 4.2). Preliminary results indicate that different switchgrass cultivars yield very different abundances for some microbial species (data not shown), suggesting that host genetics might be used to characterize the factors that determine the specific host–microbe associations involved.

## 4.4 Implications and Perspectives

The relationship between plant growth and soil microbes remains one of the great mysteries in the life sciences. Other than nitrogen fixation by root-internal or root-associated bacteria (Elbeltagy et al. 2001), only a few cases are known where a soil microbe provides some benefit to an associated plant (Thomashow and Weller 1988; Bais et al. 2006; Capper and Higgins 2007; Javot et al. 2007; Evelin et al. 2009). However, the tremendous contribution of photosynthate and a great variety of apparent signaling compounds that are actively released into the soil by roots indicate that most rhizosphere microbes are intentionally attracted by the plant. The simplest model for the role(s) of these microbes is protection from disease caused by that subset of microbes or animals in the soil that can pathogenize or parasitize plants via their roots. It is striking that the species diversity of microbes in the soil is orders of magnitude greater than that available to the aerial parts of the plants, yet soil-vectored/root-targeted pathogens of plants are relatively rare compared to those that infect above the ground. In one very preliminary experiment, we observed that greenhouse-grown maize, sorghum, and sunflower were slightly less vigorous if grown on field-derived soil than they were on sterilized field soil. Least healthy of all were plants grown on the same field soil that had been treated

with erythromycin, a broad-spectrum antibiotic that should have killed many of the eubacteria, suggesting that these bacteria provide some nutrients or protection from other microbes in the soil.

The most surprising results in this study were that no *Arabidopsis* mutants were identified for exudate production. There exists the very trivial explanation that the stocks that we obtained were not actually mutagenized. It is also possible (however unlikely) that every one of these exudates compounds is synthesized by enzymes and regulated by proteins that are encoded by redundant genetic pathways. The lack of natural variation in exudate production by *Arabidopsis* was also a surprise, and it reinforces the idea that these compounds are so important that their composition and approximate levels are fixed within the species. However, a recent study has found that two *Arabidopsis* ecotypes in our study (CVI and Landsberg *erecta*) were quite different in their exudates profile, and that this strongly affected rhizosphere microbial composition (Micallef et al. 2009). We have no explanation for the dramatic difference in conclusions about exudate variability between our results and those of Micallef and coworkers, other than the differences in the exudate assay systems employed. It has also been recently observed that some ATP-binding cassette (ABC) transporter mutants of *Arabidopsis* lead to altered root secretion of phytochemicals and significantly altered fungal and bacterial communities in the rhizosphere (Badri et al. 2009). It is puzzling that such mutations were not detected in our experiments.

The much-greater diversity of microbes outside the root compared to on the root (REM) and inside the root (RIM) suggests that there is a much greater diversity of environments and niches to fill in the soil than within a plant. The absence of archaebacteria from inside the roots makes sense, given the facts that the great majority of archaebacteria are extremophiles and that plants (like all other organisms) attempt to maintain a consistently moderate internal environment that is necessary for the physiology associated with efficient growth and development.

The most promising results to date are the differences observed in microbial populations associated with different cultivars of switchgrass. The tetraploidy and near-obligate outcrossing nature of this grass species makes it ideally unsuited for genetic dissection of any trait, including plant determination of soil microbial populations. Nonetheless, a perennial plant like switchgrass is particularly dependent on a durable and very efficient root system, so studies in the switchgrass rhizosphere are important. However, if funding were available, such studies would probably move much more rapidly if performed in diploid grasses with excellent genetics, such as maize, rice, or the close switchgrass relative called foxtail millet (*Setaria italica*) (Doust et al. 2009).

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# Chapter 5
# Impact of the Environment on Root Architecture in Dicotyledoneous Plants

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**Contents**



## 5.1 Introduction

The pattern of lateral root formation is a complex developmental process that is tightly regulated to achieve efficient nutrient and moisture acquisition from the soil in all land plants (Osmont et al. 2007). In addition to lateral roots, legume roots are capable to develop post-embryonically another organ resulting from the symbiotic interaction with soil rhizobia, the so-called symbiotic nitrogen-fixing nodules. Efficient use of symbiotic nitrogen fixation in legumes is an important agricultural trait (Stacey et al. 2006). Common mechanisms affecting lateral roots and *Rhizobium*–legume interactions seem to exist to regulate the action of meristems in root tissues to optimize root growth with a particular soil environment.

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Abiotic stresses impact severely plant development and productivity. To cope with these environmental stresses, plants have evolved complex cell signaling pathways in response to environmental stimuli and have acquired plasticity in metabolic functions and developmental switches to gain a new equilibrium between growth, development, and survival. In plants, the root system is the primary site of perception of the soil environment and diverse stresses, including salinity and drought (Osmont et al. 2007; Nibau et al. 2008).

In this chapter, we describe first the different meristems arising from the root system in two model plants, *Arabidopsis* and *Medicago*, and then discuss common mechanisms controlling lateral root development and the formation of the symbiotic root nodules. In contrast, we will not describe mycorrhizal interactions here, even though they have an important impact on root nutrition and architecture, because these interactions do not involve formation of new meristems. We referred to other nice reviews for this topic (Bending et al. 2006; Osmont et al. 2007; Reinhardt 2007; Parniske 2008). Additionally, we focus on the role of plant hormones in lateral root development in *Arabidopsis* and nodule organ formation in legumes.

## 5.2 Root System Development

### *5.2.1 Lateral Root Development*

In most eudicot plants, only primary roots are formed during embryogenesis and emerge during seed germination. The branching process in roots depends on the formation of new meristems starting from a limited number of pericycle lateral root (LR) founder cells (Fukaki et al. 2007). After germination, pericycle cells in the root, which constitute a cylindrical layer of cells surrounding the central vascular tissue, become competent to undergo a characteristic program of cell divisions and expansions to form lateral root primordia (LRP) post-embryonically. The primordium emerges from the primary root by cell expansion particularly apparent in cells near the base of the primordium. Then, the new LR meristem begins to elongate, cell numbers increase at the root tip, and the LR emerges from the parental primary root (Malamy and Benfey 1997; Malamy 2005; Dubrovsky et al. 2006; Osmont et al. 2007).

In *Arabidopsis* and most other dicots, LRs are formed only from pericycle cells overlying the protoxylem poles of the parent root (Barlow et al. 2004). After stimulation and dedifferentiation of the pericycle founder cells, cell re-entry and asymmetric cell divisions of pericycle derivatives produce a dome-shaped primordium with a radial organization similar to that of the mature root tip (Dubrovsky et al. 2000, 2001; Beeckman et al. 2001; Casimiro et al. 2001, reviewed in De Smet et al. 2006; Osmont et al. 2007). Pericycle founder cells acquire a different developmental fate during the first stages of lateral root initiation. In dicots, lateral root founder cells are recruited from the pericycle cells adjacent to the xylem pole

and formed from a minimum of three or six founder cells depending on longitudinal unicellular or bicellular initiation (Dubrovsky et al. 2001). The subset of cells associated with the xylem is strongly competent to initiate cell division contrary to those associated to phloem, which remain quiescent. Indeed, xylem pole pericycle cells, from which founder cells are recruited, carry cytological meristematic features such as large nuclei, dense cytoplasm, and small vacuoles (Himanen et al. 2004; Parizot et al. 2008).

To gain insight into the specification process, de Smet et al. (2008) performed live imaging on longitudinal pericycle cell files during lateral root initiation in *Arabidopsis*. Time-lapse recordings revealed a repeated cell division pattern composed of two successive rounds of asymmetric cell divisions, generating a central core of four small cells and two larger flanking cells. To achieve this, the original pericycle lateral root founder cells undergo an initial asymmetric division to generate a smaller daughter cell and a larger flanking cell. The latter will undergo another asymmetric division, resulting in a central core of small cells. Hereafter, the process of anticlinal asymmetric cell divisions stops, and the two central cells change their axis of division by 90° and divide periclinally. The flanking and the adjacent undivided pericycle cells undergo few or no anticlinal divisions and will only contribute modestly to the flanks of the primordium (Fukaki et al. 2007).

Potential factors involved in regulating asymmetric cell division pattern were identified using transcript profiling on sorted pericycle cells undergoing lateral root initiation (de Smet et al. 2008). Among them, the receptor-like kinase *Arabidopsis CRINKLY4* (*ACR4*) appears as a key factor both in promoting formative cell divisions in the pericycle and in constraining the number of these divisions once organogenesis has been started. *ACR4* is transcribed specifically in the small daughter cells after the first asymmetric pericycle cell division. *ACR4* represses supernumerary formative divisions of root cells, both in pericycle cells during lateral root initiation and in the columella in the root apex. *ACR4* signaling is therefore a critical homeostatic mechanism in mediating formative divisions in pluripotent root tissue during organogenesis and might act both cell- and non-cell autonomously. Cell autonomously, *ACR4* might be required for correct specification of lateral root primordia cells whereas non-cell autonomously, ACR4 signaling might prevent neighboring pericycle cells from becoming triggered for LR initiation. Although specification of founder cells is a key event in postembryonic organ formation, the mechanisms underlying this process are largely elusive. The restriction of formative cell division to a few pericycle cells and the specification of stem cell identity in the branching process in roots are not yet well understood.

Auxin promotes organ formation (Reinhardt et al. 2000, 2003; Tanaka et al. 2006), and locally increased levels of auxin response have been reported to mark positions of organ initiation and distal tips of developing organ primordia (Benkova et al. 2003; Heisler et al. 2005; Laskowski et al. 2006). LR initiation has since long been considered to occur after re-entry of pericycle cells into the cell cycle from an arrested G2 phase through the action of auxin (Blakely and Evans 1979; Laskowski et al. 1995; Malamy and Benfey 1997). However, experimental evidence argues against this dedifferentiation concept. Studies in young apical region of the

*Arabidopsis* root, just above the elongation zone, emphasize the mitotic competency of the pericycle and counter the G2 re-entry hypothesis as most of the pericycle remains in the G1 phase, with only the xylem pole pericycle cells progressing from G1 to G2 phase (Beeckman et al. 2001). Correspondingly, xylem pole pericycle cells continue to cycle without interruption after leaving the root apical meristem (Dubrovsky et al. 2000). Taken together, these data question the differentiated nature of pericycle cells and argue for the concept of a monolayered extended meristem. Nevertheless, new LRs can also initiate in more mature parts of the root, between earlier ones, which necessitate therefore a dedifferentiation and cell cycle re-entry for pericycle cells (Casimiro et al. 2003).

In *Arabidopsis*, the pericycle has been shown to have competence to divide due to the constitutive expression of at least two core cell cycle genes, the cyclin-dependent kinase *CDKA;1* and the cyclin *CYCA2;1* (Beeckman et al. 2001; Roudier et al. 2003). Furthermore, pericycle cells continue to divide at the xylem pole, but most of the divisions do not result in LR initiation and are purely proliferative. Accordingly, based on the genetic and phenotypic characterization of the *Arabidopsis alf4-1* mutant (Celenza et al. 1995), which is not capable to initiate any LR. DiDonato et al. (2004) have shown that *ALF4* is required to maintain the pericycle in a mitosis-competent state needed for LR formation. The competent state appears to be a prerequisite for the very first asymmetric divisions, because no such divisions and no mitotic cyclin *CYCB1;1* expression are observed in the mutant. Moreover, Himanen et al. (2002) reported that the *KRP2* gene, encoding the CDK inhibitor of the G1- to S-phase transition, is strongly expressed in non-dividing protoxylem pericycle cells. Overexpression of *KRP2* decreases the number of LRs regulating negatively the cell cycle progression during pericycle reactivation. The G1- to S- phase is therefore one of the targets for auxin-mediated LR initiation (Himanen et al. 2002, 2004 , Vanneste et al. 2005).

Despite the importance of the cell cycle in LR initiation, increasing the mitotic index in roots or forcing excessive cell divisions in the pericycle does not stimulate LR initiation or morphogenesis (Vanneste et al. 2005; Wang et al. 2006). Lateral root initiation takes place only when cell cycle activation is accompanied by cell fate re-specification of pericycle cells triggered by auxin-induced degradation of the SLR/IAA14 protein. Lateral root founder cell specification and patterned cell division in the pericycle can be separated both temporally and genetically indicating that the primary event during LR primordium initiation may not be exclusively an auxin-induced activation of the cell cycle (De Smet et al. 2006). Dubrovsky et al. (2008) have shown that an increase in auxin levels and signaling in individual pericycle cells always accompanies lateral root organogenesis, and that such increases are sufficient for the acquisition of lateral root founder cell identity. This process is not directly coupled to subsequent division of the founder cells, as the specification event can be genetically separated from the patterned division during primordium morphogenesis. The local accumulation of auxin in individual xylem pericycle cells could result from either directed transport or local synthesis and serves as a local instructive signal for cell fate reprogramming. This mechanism of local auxin maxima can thus select given pericycle cells and convert them into

founder cells, thereby determining a spatial pattern of lateral root formation. A model whereby auxin serves as a morphogenetic trigger for LR initiation was proposed (Dubrovsky et al. 2008). Interestingly, using the DR5::GUS auxin reporter line, De Smet et al. (2007) have reported that an oscillating auxin response maximum in the basal region of the meristem seems responsible for priming pericycle cells for lateral root initiation suggesting that early events in the life of a pericycle cell might affect its future competence for lateral root formation.

The polar auxin transport is required to form lateral roots as demonstrated with mutants defective in polar auxin transport failing to produce lateral roots (Benkova et al. 2003; Geldner et al. 2004). Roots use a transcellular auxin signaling network designed to synchronize lateral root development and emergence processes. The AUX/IAA-dependent repositioning of auxin efflux carriers toward the tip of the newly formed LR is linked to an important change in the direction of auxin flow favoring a LR growth perpendicular to the primary root (Benkova et al. 2003; Sauer et al. 2006). The PIN and AUX/LAX (such as AUX1) classes of auxin transport proteins have key roles in transmitting or localizing the inductive IAA signal, respectively (Kramer 2004). Although PIN class of auxin efflux carrier expressed by the lateral root facilitates the transmission of the inductive IAA signal, the ability to localize the auxin response to cells directly overlaying lateral root primordia is dependent on the auxin influx carrier LAX3 (Swarup et al. 2008). Auxin induces the expression of *LAX3* in cortical and epidermal cells directly overlaying new LR primordia creating a positive-feedback loop. *LAX3*-expressing cells will become more efficient sinks for auxin. *LAX3* therefore functions to amplify the signal emitted by the lateral root primordium tip while limiting its action to a few cells in close proximity with this auxin source.

Hirota et al. (2007) reported that *PUCHI* acts downstream of auxin signaling and contributes to lateral root morphogenesis through affecting the pattern of cell divisions during the early stages of primordium development. Indeed, the expression of *PUCHI* is regulated by auxin through ARF transcription factors (TFs) during the early stages of LRP development, in particular *ARF7* and *ARF19*, which are key regulators of LR initiation, whose activities are negatively regulated by the IAA protein SLR/IAA14 (Fukaki et al. 2005; Okushima et al. 2005). Moreover, ectopic expression of a stabilized mutant IAA14 protein in early LRPs results in the formation of disorganized primordia, suggesting that the normal auxin response mediated by Aux/IAA signaling is required for proper patterning of LRP (Fukaki et al. 2005). Microarray analyses have indicated that the induction of *PUCHI* expression by auxin does not occur in the *slr-1* or *arf7 arf19* mutant background (Okushima et al. 2005; Vanneste et al. 2005). Although it is not known whether the ARF7 and ARF19 proteins are involved not only in LR initiation but also in subsequent morphogenesis of the LRP, it is possible that *PUCHI* expression may be directly regulated by these ARF proteins. Alternatively, expression of *PUCHI* may be regulated by other unknown ARF proteins that are activated by auxin during early LRP development.

In addition to auxin, other hormone signals are important for LR emergence as recently reviewed by Fukaki and Tasaka (2009). Lateral root growth is regulated

antagonistically by auxin and cytokinin. Cytokinin is a negative regulator of LR formation in many plant species, including *Arabidopsis* and *Medicago* (Werner et al. 2003; Gonzalez-Rizzo et al. 2006; Li et al. 2006; Laplaze et al. 2007). Cytokinin signaling is repressed in xylem-pole pericycle cells (Mahonen et al. 2006). Transactivation of the *Arabidopsis* cytokinin-degrading enzyme cytokinin oxidase 1 in lateral root founder cells results in increased lateral root formation. Laplaze et al. (2007) observed that cytokinins perturb the expression of *PIN* genes in lateral root founder cells and prevent the formation of an auxin gradient that is required to pattern lateral root primordia.

Ethylene has a stimulatory effect on adventitious root formation in many plant species (Clark et al. 1999). Negi et al. (2008) reported that ethylene negatively regulates *Arabidopsis* LR formation by altering auxin transport. This ethylene-enhanced IAA transport depends on AUX1, an IAA influx carrier, because the *aux1-7* mutant is insensitive to ethylene as an enhancer of acropetal and basipetal IAA transport, and thus for the inhibition of LR formation.

Abscisic acid (ABA) can reversibly block meristem activation post-emergence by inhibiting the cell cycle gene expression necessary for meristem activity, leading to LR growth arrest (De Smet et al. 2006). Interestingly, ABA appears to have the opposite effect on LR emergence in legumes, stimulating LR formation in *Medicago* (Liang and Harris 2005). The *Medicago latd* mutant has a reduced root surface area with short primary roots, arrested LRPs, and disorganized meristems (Bright et al. 2005). However, exogenous application of ABA rescued at least partly the latd phenotype, and latd mutants seem to be impaired in ABA perception or signaling (Liang et al. 2007).

Lateral root formation is modified to optimize the growth of the root system in a particular soil environment. LRP initiation and emergence are separable processes providing therefore greater plasticity to the root system (Dubrovsky et al. 2006). Cells in the parent root overlaying new lateral root primordia actively participate in organ emergence. In several plant species, cells from root tissues overlaying new primordia are recruited to form a temporary root cap that assists organ emergence (Casimiro et al. 2003; Dubrovsky and Rost 2003; Ivanchenko et al. 2006). However, the principles that govern the longitudinal positioning and spacing of lateral root primordia are not yet understood (Malamy 2005).

### 5.2.2 Symbiotic Interactions and Legume Root Architecture

Legume roots are capable to interact symbiotically with nitrogen-fixing soil bacteria known as rhizobia, to form the so-called root nitrogen-fixing nodules. In this symbiosis, compatible rhizobia and plant partners recognize each other through the exchange of chemical signals (Limpens and Bisseling 2003). Host plants produce compounds acting as inducers of the bacterial *nod* genes, whose products are involved in the synthesis and secretion of a specific rhizobial lipochitooligosaccharide signal named the Nod factor. The Nod factor signal triggers a series of host

responses, culminating in the development of the root nodule, in which rhizobia convert atmospheric nitrogen to nitrogen-containing compounds (Oldroyd and Downie 2008). The signal perception by the host initiates epidermal infection and stimulates the cortical cell divisions that give rise to the first cells of the new root-derived organ. In *Medicago truncatula* and other temperate legumes, inner cortical cells dedifferentiate and proliferate, whereas in *Lotus japonicus* and other tropical legumes outer cortical cells are recruited (Stacey et al. 2006). Other bacterial surface components, such as exopolysaccharides or lipopolysaccharides, are also required for the elongation of infection threads and further stages of nodulation (Jones et al. 2007).

Nodule initiation involves two primary processes: root infection and nodule primordium induction. These processes occur predominantly in the developmentally receptive zone-of-elongation in legume roots. Rhizobia gain entry into the root tissues via plant-derived infection threads which route the bacteria toward the developing primordium (Limpens and Bisseling 2003; Fournier et al. 2008). In tropical-type nodules, the meristematic activity of the nodule occurs only at early stages of organogenesis, leading to round-shaped nodules with determinate growth. The meristem is transient, and all the primordia cells differentiate into mature nitrogen-fixing nodule cells. In temperate legumes exhibiting indeterminate nodules, *Rhizobium*-derived Nod factors stimulate pericycle, endodermal, and inner cortical cells at proximal xylem poles to enter the cell cycle, divide, generate new symplasmic connections with phloem cells in the stele, and form a primordium containing pluripotent stem cells (Timmers et al. 1999; Complainville et al. 2003). In this section, we will only discuss common mechanisms affecting LRs and *Rhizobium*–legume interactions. Indeed, nodules and roots share many aspects of their development consistent with the theory that nodulation may have evolved from pre-existing mechanisms dealing with lateral root formation (Hirsch and LaRue 1997; Mathesius et al. 2000; Mathesius 2003; Ferguson et al. 2005).

Symbiotic nodules and LRs form adjacent to xylem poles, develop meristems, and emerge through various cell layers from the primary root. However, unlike LRs, legume nodules lack a root cap and have a peripheral stem-like vasculature, rather than the central vasculature of roots. In addition, nodule and LR primordia are formed primarily from different tissues: the nodule from cortex and LRs from pericycle (Oldroyd and Downie 2008). Nevertheless, pericycle cells are activated and divide during nodule formation in *M. truncatula* (Timmers et al. 1999; Complainville et al. 2003), suggesting that the pericycle is at the origin of both organs. A further similarity between nodule and lateral root development in legumes is the involvement of cortical cells in the formation of lateral roots (Oldroyd and Downie 2008). Cortical cells activated during lateral root formation can be induced to form nodule primordia in mature regions of the root in white clover (Mathesius et al. 2000). Thus, the same root tissue layers are involved in nodule and lateral root development in legumes, but to different degrees. Differences in the ontogeny of lateral roots and indeterminate nodules may be more quantitative than qualitative, with divisions of the inner cortex providing the bulk of the nodule primordium, whereas predominantly pericycle-derived cells compose the LR primordium. Indeed, roots treated with auxin

transport inhibitors lead to the formation of nodule-like structures with some histological traits typical of lateral roots in alfalfa and pea (Hirsch et al. 1989; Scheres et al. 1992).

Insight in the cellular origin of nodule and LR has been obtained through genetic approaches. In the homeotic mutant *cochleata* of *Pisum sativum*, hybrid structures between nodules and roots are formed. The organs start as nodule, but once the meristem is formed (characteristic of indeterminate nodules), this meristem turns into a lateral root. These *cochleata* nodules appeared functional (able to fix nitrogen) and contained a root cap, a LR-like meristem, with the peripheral vasculature leading to a central vasculature and root hairs, similar to a LR (Ferguson and Reid 2005). These pea mutants are also deficient in gibberellins (GAs) and the reduction in lateral root and nodule formation could be complemented by exogenous application of GAs, suggesting therefore a role for GAs in both type of legume root-derived organogeneses (Ferguson et al. 2005). The existence of intermediate lateral organs known as root nodule hybrids in certain legumes or following inoculation with specific *Rhizobium* strains further supports the fact that nodule formation evolved from developmental pathways activated during lateral root formation (Ferraioli et al. 2004). However, these root nodule hybrids differed morphologically from those typically detected in the *cochleata* mutant, as the nodule zonation pattern and multiple root, nodule and callus structures characteristic of *cochleata* hybrids were not observed. In bean, ectopic roots from abortive nodule primordia develop after infection with different *Rhizobium etli* mutants called "root inducer" (RIND) affected in different anabolic pathways (Ferraioli et al. 2004). These mutants induced a wild-type early sequence of morphogenetics events, including root hair deformation and nodule primordia development. Later on, however, from the resulting root outgrowths, instead of nodules, one or more ectopic roots (spaced closely related and agravitropic) emerged.

The identification of common genes involved in both types of root-derived organogenesis revealed common regulatory pathways. One example reported by Wopereis et al. (2000) is the *HAR1* (for hypernodulation aberrant root formation) gene of *Lotus japonicus* which is involved in the regulation of lateral root and nodulation numbers and is a shoot-derived trait. This gene codes for a Clavata receptor-like kinase involved in the regulation of meristem number and nitrate regulation (Oldroyd and Downie 2008). In addition, analysis of the *latd* (for lateral root organ defective) mutant revealed that the *LATD* gene is required for both lateral root and nodule development, as well as for maintenance of the primary root meristem. The *latd* mutant plants initiate LRs and nodule formation but do not complete their development resulting in immature, non-nitrogen-fixing nodules and short bump-like LRs. *LATD* provides therefore a strong evidence for shared genetic components between nodule and LRs (Bright et al. 2005). Exogenous ABA rescues not only meristem organization of *latd* primary and lateral roots but also meristem function, restoring cell division and local inhibition of differentiation (Liang et al. 2007). This suggests a direct role for ABA in meristem function and organization in legume roots as well as in a later step of nodule formation. Secondary root organogenesis has also been shown to be controlled by a cytokinin receptor homolog,

MtCRE1 (Gonzalez-Rizzo et al. 2006). Down-regulation of MtCRE1 leads to cytokinin-insensitive roots, which show an increased number of LRs and a strong reduction in nodulation, supporting interactions between lateral root and nodulation pathways. This suggests a cross-talk between cytokinin signaling pathways and development of root lateral organs in legumes (Frugier et al. 2008). Further evidences for cross-talk between symbiotic nodule and LR developmental pathways have been obtained through the identification of genes, such as the *AUX1*-like genes *MtLAX1*, *MtLAX2* (de Billy et al. 2001), *MtANN1* (De Carvalho-Niebel et al. 2002), *Medicago sativa* cyclinA2;2 (Roudier et al. 2003), and the early nodulin (enod) genes *ENOD11*, *ENOD12*, and *ENOD40*, that are highly expressed in both developing nodules and LRs (Stacey et al. 2006). For example, during LR and nodule development, the *MtLAX* genes are expressed in the primordia, particularly in cells that are probably derived from the pericycle. At slightly later stages, these genes are expressed in the regions of the developing organs where the vasculature arises consistent with the involvement of *MtLAX* genes in local auxin transport. Auxin seems required at two common stages of lateral root and nodule development: formation of the primordia and differentiation of the vasculature (de Billy et al 2001; Mathesius 2008). Finally, the discovery of microRNAs (miRNAs) as post-transcriptional regulators of many developmental processes, including the formation of vascular tissues (Voinnet 2009), suggested a possible involvement in legume root architecture. Recently, overexpression of *MtMIR166* in *M. truncatula* was shown to perturb the organization of root vascular bundles and increased the number of xylem and phloem poles in roots. This consequently reduced the number of symbiotic nodules and lateral roots generated from these roots (Boualem et al. 2008) and was linked to *MtMIR166*-mediated post-transcriptional regulation of several *HD-ZIP III* genes in roots and nodules.

Both symbiotic interactions and soil environmental stresses can profoundly affect the growth and development of the root and influence the final architecture of the root system.

## 5.3 Plasticity: How the Action of the Environment on the Regulation of Gene Expression Affects Root Growth and Development

Plants are exposed to a plethora of stress conditions throughout their life cycle. The two major environmental constraints that currently reduce plant productivity are drought and salinity. More than 10% of arable land is affected with desertification and salinization rapidly increasing on a global scale the decline of average yields for most major crop plants (Bray et al. 2000; Botella et al. 2005). Exposure to both stresses triggers many common reactions in plants including cellular dehydration and removal of water from the cytoplasm into the extracellular space resulting in a reduction of the cytosolic and vacuolar volumes (Verslues et al. 2006). Plants have evolved complex cell signaling pathways activating metabolic functions and developmental switches to permit their adaptation to these conditions (Shao et al. 2006; Umezawa et al. 2006; Yamaguchi-Shinozaki and Shinozaki 2006; Sreenivasulu

et al. 2007). The regulatory circuits include stress sensors, signaling pathways comprising a network of protein–protein interactions, TFs and promoters, and finally the output proteins or metabolites. A critical step controlling stress responses involves thus transcriptional regulation, generally mediated by TFs that may govern and coordinate the expression of large groups of genes. Plant genomes dedicate a large number of their coding sequences to TFs reaching about 5.9% (>1,500 TF genes) in the fully sequenced *Arabidopsis* genome (Riechmann et al. 2000). In legumes, extensive sequencing highlighted around 2,000 TFs per genome, less than 1% of them genetically characterized (Udvardi et al. 2007).

Roots are in direct contact with the soil and hence are primary sites for perception of the soil environment. Abiotic stresses have the ability to elicit morphological, structural, and physiological responses to an unfavorable environment in root growth in order to maximize the acquisition of resources, a property linked to the so-called root developmental plasticity (Lynch and Ho 2005). TF networks are known to control root cell identity during development and adaptation to abiotic stresses also in roots (Montiel et al. 2004; Nibau et al. 2008). The development of genomic resources and information for model species as *Arabidopsis thaliana*, *Medicago truncatula*, and *Lotus japonicus* increased considerably the analysis of TF gene expression on a global genome-wide scale based on their regulation in response to abiotic stresses (Chen and Zhu 2004; Maggio et al. 2006; Tuteja 2007), including available publicly databases (e.g., Genevestigator, Ma et al. 2006). In fact, microarray studies revealed large-scale changes in the transcriptome in response to specific abiotic stresses (Kreps et al. 2002; Seki et al. 2002; Jiang and Deyholos 2006; Dinneny et al. 2008). In the model legume *M. truncatula*, microarrays covering 16,000 genes revealed more than hundred TF genes responding to early salt stress in root apexes (Gruber et al. 2009). In chickpea, the application of SuperSAGE (Serial Analysis of Gene Expression) technology to profile transcripts of drought- and salt-stressed roots from chickpea identified TF genes exclusively expressed under both stresses, but not in non-stressed controls (Molina et al. 2008).

Several reports have shown a role of TFs as major modulators of stress responses as salt and drought, in roots. Although the WRKY-type TFs are involved in multiple abiotic stress responses, the expression of GmWRKY13 in transgenic plants showed a higher sensitivity to salt and mannitol stress as well as an increase in LRs when compared to wild-type plants (Zhou et al. 2008). In contrast, *GmWRKY54* confers salt and drought tolerances possibly through the regulation of TFs like *DREB 2A* (drought-responsive element binding factor 2A) and *STZ*/*Zat10* (Yamaguchi-Shinozaki and Shinozaki 2005). Hence, *GmWRKY* genes play differential roles in abiotic stress tolerance, and *GmWRKY13* may function in both lateral root development and abiotic stress responses. In addition, a subclass of APETALA2 (AP2) – and ethylene-responsive element-binding protein – type TFs, such as *DREB2A,* expressed in all root cell layers under salt stress conditions controls semi-ubiquitous responses to abiotic stresses (Sakuma et al. 2006). Potential direct targets of DREB2A up-regulated by salt have been identified. Another *AP2*/*ERF*-like (APETALA 2/Ethylene Responsive Factor-like) transcription factor identified via a gain-of-function *Arabidopsis* mutant *hrd-D* is the *HRD* gene. Overexpression of

this gene improves water use efficiency, drought resistance, and salt tolerance. This mutant has roots showing enhanced strength, increased branching patterns, and more cortical cells, accompanied by increased expression of abiotic stress-associated genes (Karaba et al. 2007). Tolerance to salinity and osmotic stress is also observed in transgenic tobacco expressing *CAP2* (*C. arietinum* AP2), possibly because of a large increase of the root system and LRs (Shukla et al. 2006). In legumes, overexpression of *MtZPT2-1* TF genes, linked to recovery processes in transgenic *Medicago* roots, allows growth under restrictive salt stress conditions (Merchan et al. 2007; de Lorenzo et al. 2007). This gene may activate specific genetic programs linked to the adaptation of legume roots to salt stress. A vascular-specific *bZIP* (basic region/leucine ZIPper motif), representing a novel root-specific transcription factor, is also involved in coordinating gene expression in response to water-deficit stress in *Phaseolus* species (Rodriguez-Uribe and O'Connell 2006).

Endogenous phytohormones and regulatory genes sensing the soil environment may interact to adapt root architecture (Jovanovic et al. 2007). For example, repressing auxin-induced responses together with enhancement of cytokinin sensitivity may have profound effects on recovery responses after salt stress by limiting primary root growth, controlling the emergence of lateral roots or the root apical dominance (Malamy 2005; Aloni et al. 2006; Merchan et al. 2007; Ditengou et al. 2008; Huang et al. 2008; Wolters and Jürgens 2009). A cross-talk between phytohormone signaling and stress responses in roots was observed for the *AtNAC2* TF (He et al. 2005). It is up-regulated by salt stress and its overexpression in transgenic *Arabidopsis* plants results in increased LR formation. This gene is also up-regulated by ethylene, auxin, and ABA, and its induction by salt is compromised in auxin and ethylene signaling mutants. On the other hand, the enhanced drought tolerance conferred by *MYB15* overexpression in *Arabidopsis* seems to be associated to increased ABA biosynthesis and signaling, which results in greater expression of several stress-responsive genes and lower water consumption (Ding et al. 2009). In addition, overexpression of *DREB1*/*CBF* also increases the tolerance of transgenic plants to freezing, drought, and salt stresses (Shinozaki and Yamaguchi-Shinozaki 2000; Sakuma et al. 2002; Fujita et al. 2005) and regulates ABA-independent gene expression in response to drought and cold stress. Abscisic acid and drought stress have similar and probably synergistic effects on LR development. Several *drought inhibition of lateral root growth* (*dig*) mutants have enhanced responses to ABA and are also drought tolerant, whilst others have a reduced LR-inhibition response to ABA and are drought sensitive (Xiong et al. 2006).

### 5.3.1 Spatial Control and Transcriptional Complexity in Response to Stress

Knowledge about responses to abiotic stresses of model plants, such as *Arabidopsis* and *Medicago*, has accumulated during the past decade, based on large-scale mutant analyses and genome-wide transcript profiles at organ or tissue levels.

These approaches give unrefined localization of gene expression and a few data are available to correlate stress-related transcript changes and cell-specific gene expression in an organ.

Ma and Bohnert (2007) analyzed tissue-specific response to stress by integrating diverse large-scale datasets in which cell type-specific and growth stage-specific gene expression in *Arabidopsis* roots was recorded. They combined three types of data analyzing genome-wide expression profiles modulated by a number of stress conditions, regulatory *cis*-elements in promoters, and cell-specific and developmental age-specific root transcripts and their reaction to stress. Among the probes printed on the Affymetrix chip, 12,360 are considered to be present in at least one of the three developmental stages of the root: the root expansion growth region (stage 1), the region of maximum elongation (stage 2), and the root maturation region (stage 3) also dissected in different cell lineages (lateral root cap, epidermis, cortex, endodermis, and stele). Among these genes expressed in roots, 5,963 exhibit statistically significant changes in gene expression during stress. Root-specific genes down-regulated by abiotic stress are highly expressed in stage 1 root cap and epidermis under optimal conditions, whereas other genes up-regulated by stress are expressed in the stage 3 stele, endodermis, and cortex. Thus, complex regulatory mechanisms can be dissected through intersections of stress-responsive and cell-specific profiles to identify how cell files are affected by abiotic stresses. Recently, Dinneny et al. (2008) characterized the transcriptional response to high salinity of different cell layers and developmental stages of the *Arabidopsis* root, showing a highly constraint of transcriptional responses by developmental parameters. Several tissues tend to be highly responsive as 48% of salt-responsive genes are regulated in the cortex, 28% in the stele, and 31% in the epidermis. The transcriptional changes lead to the differential regulation of specific biological functions in subsets of cell layers which, for certain cases, could be correlated with observable physiological changes. Known stress pathways primarily control semi-ubiquitous responses, and mutants disrupting epidermal patterning were used to reveal cell layer-specific and inter-cellular effects.

A major finding arising from these reports is that cell identity determines the gene pool that is regulated during stress, as reflected by the high degree of cell specificity in functional gene categories. This specificity requires maintenance of cell fate during stress, which is probably ensured by a transcript cohort enriched in cell-identity genes that remains unaffected by environmental stress (Laurentius et al. 2008). Environmental stimuli combined with cell- and developmental-stage-specific profiling enable the identification of high-confidence transcriptional modules.

### *5.3.2 Establishing Regulatory Networks: TFs and MicroRNAs*

Even though TFs are central in the regulation of development and stress responses, post-transcriptional events regulated by miRNAs, e.g., mRNA degradation or translational inhibition, have also emerged as playing crucial roles in regulating

gene expression (Sunkar et al. 2007; Voinnet 2009). The interaction of miRNAs and TFs may determine regulatory networks controlling the transcriptome, and examples have been found to affect root developmental and stress responses.

Lateral root emergence is promoted by auxin signals transmitted by the *NAC1* TF (Xie et al. 2000). To study the regulation of the target *NAC1* mRNA by miR164, Guo et al. (2005) manipulated miR164 levels or expressed a miRNA cleavage-resistant version of *NAC1* mRNA in plants. Apparently, miR164 functions as a negative regulator of auxin-mediated lateral root development by controlling *NAC1* mRNA levels and is induced by auxin. This suggests that miR164 mediates the rapid degradation of *NAC1* mRNA to attenuate and terminate auxin signaling. In addition, disrupting miR160 regulation of *ARF17* (Auxine response factor 17) increases the target *ARF17* mRNA levels and leads to severe developmental abnormalities, including root defects (Mallory et al. 2005). This indicates a critical role of miR160-directed regulation of *ARF17* which seems a transcriptional regulator of *GH3*-like early auxin-response genes. The *Arabidopsis* auxin response factors *ARF10* and *ARF16* are also targeted by the miR160 and control root cap cell formation promoting columella cell production (Wang et al. 2005). Indeed, *MIR160* overexpressing plants, in which the expression of *ARF10* and *ARF16* is repressed, and the *arf10-2 arf16-2* double mutants display the same root tip defect. They show uncontrolled cell division and blocked cell differentiation in the root distal region, a tumor-like root apex and loss of gravity sensing. Moreover, auxin and miR160 regulate the expression of *ARF10* and *ARF16* genes independently, generating a pattern consistent with root cap development. Recently, Gifford et al. (2008) report cell-specific data revealing responses that suggest a coordinated cell-specific regulation of a transcriptional circuit mediating LR outgrowth in response to nitrogen via microRNA167 targeting A*RF8*, one of the pericycle-induced *ARF*s. The miR167a, b is expressed specifically in the pericycle and LR cap along with *ARF8*, but, consistent with an antagonist effect on *ARF8*, is repressed in both tissues in response to nitrogen. Thus, *ARF8* offers a link between environmental nutritional inputs and auxin-mediated plasticity of lateral root architecture. In *Medicago*, we mentioned that *MIR166* targets a subset of class-III homeodomain–leucine zipper (HD-ZIP III) TF to regulate LR and root nodule formation (Boualem et al. 2008) as well as affect vascular bundle patterning. Furthermore, Combier et al. (2006) showed that miR169-mediated regulation of *Medicago MtHAP2-1* expression leads to a critical spatial and temporal restriction of this TF to the nodule meristematic zone, thereby allowing correct tissue identity and transition from meristematic to differentiated cells.

## 5.4 Conclusions

The molecular mechanisms controlling root architecture are being unraveled using a variety of approaches combining physiology, genomics, and genetics. Major questions remain to understand how these mechanisms interact with the soil stress

conditions, and the advent of genomic technologies may open new perspectives for the analysis of how roots adapt to the soil environment. This work, mainly done in model systems such as *Arabidopsis* and *M. truncatula*, uncover diverse regulatory genes, notably TFs that participate in abiotic stress responses and genetic programs regulating root growth and architecture. Integration of these data with genomic approaches on different genetic backgrounds will reveal critical regulatory networks and molecular hubs, whose orthologs could then be analyzed in crop plants to establish the generality of these mechanisms and impact agricultural practices.

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# Chapter 6
# Mechanisms of Aluminum Tolerance

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## 6.1 Introduction

### *6.1.1 Scope of Problem*

The Food and Agriculture Organization (FAO) of the United Nations regards Al toxicity as the second largest soil constraint to agriculture, after erosion hazard and affects 14.7% of the world's land area (Bot et al. 2000). In comparison, salinity and sodicity each affects ~3% of the world's land area. Al toxicity is the leading soil constraint to agricultural production in Sub-Saharan Africa, Asia, Oceania, Central and South America and the second largest limitation for North American agriculture (Bot et al. 2000). Nearly one-third of the countries enumerated in a recent FAO survey exhibit Al toxicity on 25% or more of their area (54/166 countries; Bot et al. 2000). Al toxicity exists at soil pH $< 5.5$, at which point rhizotoxic Al cations are solubilized from non-toxic aluminosilicates and other minerals (Kochian 1995; Kochian et al. 2004). While inhibition of root growth and function are early consequences to Al intoxication, increased susceptibility to other stressors and overall diminishment of yield are the latter consequences. For example, Brazil, Argentina, and Colombia are the three largest maize producers in South America. Brazil and Colombia have extensive land area with Al toxicity (63, 56%, respectively) while Argentina has essentially no Al-intoxicated soils (Bot et al. 2000). The 3-year (2004–2006) average maize yields in Colombia were one-third those obtained in Argentina, while Brazil were approximately one-half (NASS 2007). Without the Al stress limitation, significantly higher yields could be achieved on the same arable land, which would promote food security, economic development, and environmental preservation.

### *6.1.2 Brief Overview of Al Tolerance*

Al rhizotoxicity occurs when Al cations reach vulnerable portions of the root, without being detoxified. Al chelation is a common detoxification and can occur outside (Al exclusion) or inside (internal chelation) the root. Al exclusion is the best understood family of tolerance processes and may rely upon malate, citrate, or other small molecules to chelate the Al. Several genes have been identified as major Al tolerance genes in the malate and citrate pathways, while other Al exclusion pathways are less well defined. Al exclusion by chelation requires detection of Al, synthesis and transport of ligands out of the root. The Al tolerance genes identified to date fall into the third (transport) category. Al uptake into the root is nearly unavoidable; plants have mechanisms for internal tolerance to Al stress. Internal tolerance may result from intracellular chelation of Al, reactive oxygen species (ROS) scavenging, modifications to lipid or cell wall synthesis, or other unknown mechanisms.

Our goals for this review are to update recent progress in understanding the molecular processes that underlie the mechanisms of Al tolerance. We have placed emphasis on mechanisms where genes have been identified and confirmed to be important for Al tolerance and devoted less space to the less clearly defined mechanisms. We apologize in advance for any omission.

## 6.2 Al Exclusion by Organic Acid Release

### *6.2.1 Mediated by Malate and* ALMT1*-Type Transporters*

#### 6.2.1.1 Contributions from Wheat

Many discoveries in Al tolerance research were made in wheat, for both physiological mechanisms and their underlying molecular components. The first Al tolerance gene cloned from any species was the *Al activated malate transporter* (hereafter referred to as *TaALMT1*) (Sasaki et al. 2004). This was accomplished via subtractive cDNA library sequencing performed on the ET8 and ES8 near isogenic lines, which differ at the *Alt1* locus found on the long arm of chromosome 4D (Delhaize et al. 1993; Sasaki et al. 2004). *TaALMT1* identifies a gene family with members in *Arabidopsis*, rice, and many other plants (Sasaki et al. 2004). The most striking polymorphism between wheat alleles is at the level of gene expression, where the tolerant ET8 line had much higher levels of expression for *TaALMT1* than the sensitive ES8 (Sasaki et al. 2004). Biophysical analysis demonstrated that the *TaALMT1* protein responds to the presence of extracellular Al and is located within the plasma membrane, consistent with the identification as an Al tolerance gene (Sasaki et al. 2004; Yamaguchi et al. 2005).

Physiological analysis of a collection of wheat cultivars demonstrated that the majority of differences in Al tolerance could be explained by the quantity of malate released (Ryan et al. 1995). The strongly positive correlation suggested that genetic differences in Al tolerance were concentrated within a single major tolerance mechanism ($r^2 = 0.84$, malate efflux to relative root growth) (Ryan et al. 1995). Subsequent molecular analyses of wheat germplasm collections have reinforced the physiological observation; expression of the *TaALMT1* gene is highly correlated with both malate release and overall Al tolerance (Raman et al. 2005). Sequence analysis of the *TaALMT1* promoter region has revealed large structural differences between tolerant and sensitive cultivars, with six clear haplotypes emerging within cultivars that represent a wide range of Al tolerance (Sasaki et al. 2006; Raman et al. 2007). These studies have reaffirmed the relationship between malate release and Al tolerance, as estimated by relative root growth ($r^2 = 0.88$, 0.81 for Sasaki et al. 2006 and Raman et al. 2007, respectively). The importance for any of the motifs within the promoter haplotypes is not yet clear, but it has been hypothesized that one or more motifs found in promoters with low expression have increased in

copy number and rearranged to derive stronger promoters (Delhaize et al. 2007). This hypothesis is intriguing due to similarity with observations made at the $Alt_{SB}$ locus in *Sorghum* (see Sect. 6.2.2 below), but obviously requires additional experimentation. While *TaALMT1* expression is an important determinant for overall Al tolerance, it is not the only one; gene expression differences explain one-half or less of the differences in tolerance observed (Sasaki et al. 2006; Raman et al. 2007).

Multiple lines of genetic evidence support the observation that other factors beyond *TaALMT1* contribute to Al tolerance. First, analysis of the chromosomal arm deletion stocks in the Chinese Spring background (ditelosomic chromosomes) indicated that the loss of three different regions compromised Al tolerance (Papernik et al. 2001). The loss of chromosome 4DL gave reduced root growth, malate release, and increased Al accumulation in the root apex; this is easily explained by the loss of *TaALMT1* (Papernik et al. 2001). However, the loss of the short arms of 5A and 7A also reduce Al tolerance by the same metrics, although not as severely as losing 4DL (Papernik et al. 2001). Thus, at least three factors contribute to Al-activated malate release in the Chinese Spring background. Second, incomplete transfer of Al tolerance from Altas66 into a Chisholm background (Chisholm-T) illustrates that multiple loci are important for the high degree of tolerance observed in Atlas66 (Tang et al. 2002). Malate release in Chisholm-T was approximately half that observed in Atlas66, where the Chisholm-T derivative carries the Atlas66 allele of *TaALMT1* (Tang et al. 2002; Guo et al. 2007a). The Chisholm-T line has higher *TaALMT1* expression than that seen in the sensitive sister near isogenic line (Guo et al. 2007b). RT-PCR or other methods were not used to make the direct comparison between Atlas66 and the Chisholm derivatives, and so it is difficult to assess which degree *cis*-acting and *trans*-acting factors play to determine *TaALMT1* expression. However, it is clear that at least two loci are important for determining the differences in tolerance observed between Atlas66 and Chisholm. Third, while *TaALMT1* represents a major effect QTL in multiple mapping populations, it does not explain all of the variance observed (Raman et al. 2005). Five doubled haploid populations were evaluated for Al tolerance; markers within or tightly linked to *TaALMT1* explained 75–93% of the variance in the trait (Raman et al. 2005). Genome-wide marker scans were not conducted to locate the other, minor QTL that contribute to the remainder of the genetic variance; the authors mention the possible contributions from chromosomes 5AS and 7AS as possible locations for minor QTL. However, the heritability of Al tolerance for these mapping populations was not reported, the component of variance due to genetic factors; it is possible that the heritability of Al tolerance is sufficiently low that *TaALMT1* explains all of the genetically determined differences in Al tolerance by itself.

Other determinants for Al tolerance in wheat may include protein kinases or phosphatases. Reversible protein phosphorylation is a common mechanism for regulating protein activity and is known to be a point of control for many abiotic stress responses, including salt, water, and cold stresses (Liu et al. 2000; Zhu 2001). Malate release in wheat is Al activated, while *TaALMT1* gene expression is not (Sasaki et al. 2004; Raman et al. 2005). This indicates that much of the regulation

for malate release occurs at the protein level. Short pretreatment (30 min) of wheat seedlings with the protein kinase inhibitors K-252a and staurosporine significantly reduced malate efflux after Al challenge, while KN-62, calphostin C, and chelerythrine pretreatments had no effect (Osawa and Matsumoto 2001). Of the protein phosphatase inhibitors tested, only okadaic acid had an effect. Okadaic acid and staurosporine reduced malate release 30–40% while K-252a essentially abolished malate release from treated seedlings (Osawa and Matsumoto 2001). Perhaps the loci found on chromosomes 5AS or 7AS represent these pharmacologically sensitive factors. It is clear that reversible phosphorylation plays a role in the perception of Al, the first step in the Al-activated organic acid release pathway.

From a basic biology perspective, it is clear that Al tolerance research has made great gains in wheat. From the applied biology perspective, two studies are especially noteworthy. First, as *TaALMT1* represents a major Al tolerance QTL, having genotypic information for this locus can allow marker-assisted breeding for Al tolerance. This substitution of low-cost molecular genotyping for field-based phenotyping dramatically accelerates the pace of crop improvement. As a result of germplasm surveys and the concomitant DNA sequence analyses, haplotype-specific DNA markers have been generated for elite *TaALMT1* alleles to support marker-assisted breeding (Raman et al. 2007). This should permit the rapid movement of highly tolerant alleles into elite varieties. Second, *TaALMT1* can be utilized for transgenic crop improvement purposes for species with little variation in Al tolerance. Barley is among the most Al-sensitive economically important cereals; while variation does exist for Al tolerance between barley varieties, it does not provide adequate protection against Al toxicity (Tang et al. 2000). The introduction of *TaALMT1* into barley resulted in dramatic enhancement of Al tolerance (Delhaize et al. 2004). Where 2 μM Al concentrations inhibited root growth 50% for non-transgenic controls and azygous sibling lines grown in hydroponic culture, 40 μM Al was required to achieve the same level of inhibition for transgenic barley (Delhaize et al. 2004). Similar results were also observed for soil-grown plants, although the efficacy of these transgenic events is yet to be evaluated under field conditions.

#### 6.2.1.2 Contributions from *Arabidopsis*

*Arabidopsis thaliana* does not possess a great degree of Al tolerance, unlike wheat (Larsen et al. 1996). However, *Arabidopsis* is an excellent model system for molecular genetic and physiological genomic analyses of Al tolerance. What tolerance exists in *Arabidopsis* is largely due to Al-activated malate release and both plants share at least one homologous protein (Hoekenga et al. 2003; Hoekenga et al. 2006). The existence of a well-annotated and mutagenized genome with the multitude of other genomics-based resources makes study in *Arabidopsis* highly complementary to study in wheat.

*TaALMT1* defines a gene family in *Arabidopsis* with more than a dozen members (Hoekenga et al. 2006). Of these *Arabidopsis*, *ALMT-like* genes (hereafter *AtALMT*), *AtALMT8* is the most similar. The gene family has a diverse pattern of

gene expression according to publicly available gene expression databases, where multiple *AtALMT* are expressed in essentially every tissue tested (Meyers et al. 2004; Kilian et al. 2007). However, mutant analysis indicates that only *AtALMT1* is essential for Al tolerance responses (Hoekenga et al. 2006). An *AtALMT1* knockout mutant lacks Al-activated malate release, but is capable of releasing malate under low pH/phosphate deficiency stress conditions indicating that a second *AtALMT* is likely active under those conditions (Hoekenga et al. 2006). A third locus, *AtALMT9*, encodes a vacuolar malate transporter, expressed in nearly every cell of the plant (Kovermann et al. 2007). *AtALMT9* has a small biophysical response to applied Al as measured when heterologous expressed in oocytes, suggesting that related *AtALMT* proteins share multiple aspects of functionality (Kovermann et al. 2007). Thus, there is clear functional specialization for members of the *AtALMT* family, even if the role for only two members has been identified.

Organic acid release in response to Al stress can be classified as immediate (pattern I) or inducible (pattern II) (Ma et al. 2001). Wheat represents a pattern I style organic acid release; this is consistent with the constitutive expression for the *TaALMT1* gene with responsiveness to Al coming at the protein level (Sasaki et al. 2004). *Arabidopsis* represents a pattern II style plant; this is consistent with the *AtALMT1* gene being strongly induced by Al stress, while also responding at the protein level (Hoekenga et al. 2006). Protein phosphorylation is involved in *AtALMT1* regulation as it is for *TaALMT1* (Kobayashi et al. 2007b). The protein kinase inhibitor K-252a eliminates Al-activated malate release in *Arabidopsis*, much like that seen in wheat. *AtALMT1* gene expression is still enhanced by Al with K-252a co-treatment, suggesting that this drug acts at the protein level to restrict malate release (Kobayashi et al. 2007b). Unlike wheat, staurosporine (a kinase inhibitor) and calyculin A (a phosphatase inhibitor) also reduce malate efflux in Al-treated *Arabidopsis*; *AtALMT1* gene expression does not increase with either of these drugs, suggesting that reversible phosphorylation acts at both the transcriptional and post-transcriptional level to regulate *AtALMT1* (Kobayashi et al. 2007b). Reversible phosphorylation is also important for inactivating *AtALMT1*. Reversal experiments, moving plants from Al-containing media to Al-free solutions, indicate that malate efflux can rapidly be inactivated in *Arabidopsis* (Kobayashi et al. 2007b). Co-treatment with calyculin A prevents the inactivation of *AtALMT1*; malate release rates remain high as in Al-treated plants (Kobayashi et al. 2007b). It would be intriguing to see if *TaALMT1* also requires protein phosphatases for inactivation of transport function. No protein kinases or phosphatases are known to be involved in *AtALMT1* regulation. However, gene expression for *WAK1*, a wall-associated protein kinase is rapidly induced (20 min) by Al treatment. Over-expression of this kinase can also modestly increase Al tolerance in transgenic *Arabidopsis*, but a direct connection to *AtALMT1* is yet to be determined (Sivaguru et al. 2003).

Low pH stress and several other toxic metals can induce *AtALMT1* gene expression to a small degree (5–20%) compared to Al. However, these treatments do not activate *AtALMT1*, which speaks to the specificity of the Al stress response (Kobayashi et al. 2007b). One can imagine commonality of rhizotoxicity between Al and erbium or lanthanum; however, genetic analysis indicates that the tolerance

processes are distinct (Kobayashi et al. 2007a). Low pH and Al stress responses have some degree of overlap, which is not unexpected as Al toxicity is largely predicated by low pH. Proton and Al tolerance can be genetically and experimentally separated (Ikka et al. 2007; Iuchi et al. 2007). This lack of concordance between proton and Al stress tolerance was made several years ago in maize, and the identification of *STOP1* in *Arabidopsis* gives hints to the underlying molecular mechanisms (Poschenrieder et al. 1995; Iuchi et al. 2007). *STOP1* represents a transcription factor required for proton stress tolerance responses; *AtALMT1* expression requires the presence of *STOP1* (Iuchi et al. 2007). The *STOP1* null mutant is hypersensitive to proton stress, but is also susceptible to Al toxicity at doses that do not affect the growth of wild-type plants (Iuchi et al. 2007). It is not yet clear whether *STOP1* activates *AtALMT1* transcription directly or indirectly (e.g., acting at an earlier regulatory level), but this discovery is intriguing in the light of the number of economically important plants that can be classified in pattern II organic acid release. As *ALMT1*-like genes are shared between monocots and dicots as essential Al tolerance genes, perhaps *STOP1*-like transcription factors are also shared (Magalhaes 2006).

#### 6.2.1.3 Contributions from Other Species

Al-activated malate release has been reported for species other than *Arabidopsis* and wheat (Kochian et al. 2004). While many of the advancements in the area of Al-activated malate release have been made in these species, several have not. Two will be mentioned here. First, rapeseed (*Brassica napus*) has been reported on some occasions to release both malate and citrate in response to Al stress (Zheng et al. 1998b). This appears to be cultivar-specific rather than a function of experimental design. Dual organic acid release has also been reported in rye (*Secale cereale*), cowpea (*Vigna unguiculata*), and soybean (*Glycine max*) (Li et al. 2000; Silva et al. 2001; Jemo et al. 2007). Rye is among the most Al tolerant of the cereals; perhaps the dual release of organic acids contributes to its protection and can be exploited as a target for plant improvement. The malate transporter important for Al tolerance in rye has been identified as *ScALMT1*, while the citrate transporter is still unknown (Fontecha et al. 2007). Given that malate and citrate transporters have both been identified, presumably progress can be made in rye, cowpea, soybean, or other species toward the goal of increasing Al tolerance through marker-assisted breeding or biotechnology.

Second, increasing the numbers of organic acid transporters or interfering with signal transduction pathways produces clear effects on organic acid release. Recall that the process of organic acid release can be broken into three components: perception of Al, synthesis of ligand, transport out of the root. The first and third parts of this process can clearly be altered so as to affect Al tolerance. Manipulating the second part of this process, organic acid synthesis, is much less reliable to alter Al tolerance. Success has been reported in alfalfa (*Medicago sativa*) using malate dehydrogenase and in rapeseed with citrate synthase (Tesfaye et al. 2001;

Anoop et al. 2003). However, over-expression of citrate synthase gave inconsistent outcomes in transgenic *Nicotiana* (de la Fuente et al. 1997; Delhaize et al. 2001). Organic acid supplies within the cell may or may not be limiting for effective stress responses. In maize where organic acid release rates differed, no changes occurred in organic acid pools that could be correlated with differential Al tolerance (Pineros et al. 2005). In fact, the efficacy of over-expression of *TaALMT1* in barley would argue that organic acid supplies might not be a limitation to Al tolerance (Delhaize et al. 2004). Perhaps with a more careful and systematic study of the interplay between Al perception, ligand synthesis and release, patterns will emerge to better instruct how these processes can be manipulated to improve Al stress tolerance.

### *6.2.2 Mediated by Citrate and* $\mathbf{Alt_{SB}}$*-Type Transporters*

#### 6.2.2.1 Contributions from *Sorghum*

Several physiological mechanisms of Al tolerance have been proposed but the agronomical efficacy to promote yield stability on acid soils remains at best uncertain. The clear exception to this statement is the utility of Al-induced organic acid release from root apices, which is certainly a major mechanism enabling agriculture on acid, Al toxic soils. The improvement of barley with *TaALMT1* by transformation illustrates this, but only as a proof of concept (Delhaize et al. 2004). A stronger example for the efficacy of improving Al tolerance in crop plants is the discovery and characterization of the major Al tolerance gene in *Sorghum*, $Alt_{SB}$ (Magalhaes et al. 2007). The cultivar SC283 is the best known Al tolerance standard in *Sorghum* and has repeatedly shown superior agronomic performance on acid soils (Duncan et al. 1983; Duncan 1988). Subsequently, using hydroponic culture rather than field-based observations, Al tolerance in cv. SC283 was shown to be largely under the control of a single, semi-dominant gene, $Alt_{SB}$, which was mapped to the end region of *Sorghum* chromosome 3 (Magalhaes et al. 2004). This gene was identified by map-based cloning and shown to underlie Al-induced citrate release, the primary Al tolerance mechanism at work in *Sorghum* (Magalhaes et al. 2007). The fact that segregation of $Alt_{SB}$ was sufficient to explain ~80% of the phenotypic variation for root growth inhibition caused by Al in hydroponic culture strongly suggests that $Alt_{SB}$ is also the major determinant for the superior agronomical performance displayed by SC283 on Al-intoxicated acid soils. It should be noted, however, that Al toxicity is one of the most important but not the only source of abiotic stress on acid soils. Therefore, other genes related to adaptation to the "acid soil syndrome" should also be considered. Nevertheless, recent comparisons for agronomic performance on acid soils and root growth inhibition in hydroponic culture indicated that the two traits are highly correlated in *Sorghum*; genotypes carrying the $Alt_{SB}$ allele from SC283 out-produced sister lines with an inferior $Alt_{SB}$ allele by ~1 mt $ha^{-1}$ (Magalhaes et al., unpublished). The $Alt_{SB}$ gene encodes a member of the Multidrug and Toxic Compound Extrusion (MATE) family; the

gene is Al inducible with maximal levels of expression after several days of Al stress (Magalhaes et al. 2007). In the original mapping population, the $Alt_{SB}$ alleles contained no polymorphisms within the protein coding sequence; rather, significant differences were observed in the promoter region. A MITE-class transposon and sequences immediately flanking it created a repeat unit of 243 bp; the sensitive allele contained three repetitions while the tolerant (SC283) allele contained five (Magalhaes et al. 2007). The number of repetitions is positively correlated with citrate release, root growth, and gene expression. A relatively high level of constitutive expression of $Alt_{SB}$ in a tolerant isogenic line was not accompanied by large and rapid citrate efflux. This suggests the regulation of the gene and the activity of the protein are somewhat more complicated than the typical; perhaps, Al is required to activate transport activity or gene expression does not occur in the epidermis in the absence of Al. Experiments are underway to answer these questions.

#### 6.2.2.2 Contributions from Barley

A locus controlling Al tolerance, *Alp*, was located to chromosome 4H by trisomic analysis (Minella and Sorrells 1997). Subsequently, *Alp* was mapped to the long arm of barley chromosome 4H in a population derived from the cultivar Dayton, and subsequent studies using different mapping populations also identified Al tolerance gene(s) the same chromosome (Tang et al. 2000; Raman et al. 2002; Ma et al. 2004b). In a broader survey with 21 barley varieties, citrate release and Al tolerance were positively correlated, while citrate release and Al content in root apices were negatively correlated, indicating that Al exclusion mediated by citrate was responsible for Al tolerance in barley (Zhao et al. 2003). This conclusion was confirmed and expanded by Ma et al. (2004b), who reported co-localization between the Al tolerance gene on chromosome 4H and rates of citrate release. Complete linkage of a barley homolog of the MATE family (*HvMATE*) with the *Alp* locus was reported in a doubled haploid population (Wang et al. 2007). Expression of *HvMATE* was also correlated to Al tolerance and Al-activated citrate efflux, leading the authors to consider the hypothesis that *HvMATE* underlies the *Alp* locus in barley. Fine-scale genetic mapping and microarray analysis confirmed that a member of the MATE family, *HvAACT1*, confers Al tolerance in barley (Furukawa et al. 2007). The *HvAACT1* gene cloned by Furukawa and co-workers enhanced Al-activated citrate release and Al tolerance in transgenic tobacco. The protein was localized to the epidermal cells of barley root tips and within the plasma membrane, according to GFP translational fusions. In addition, heterologous expression of *HvAACT1* in *Xenopus laevis* oocytes indicated that the protein was permeable to citrate rather than malate (Furukawa et al. 2007).

*Sorghum* and barley have several similarities for the inheritance of Al tolerance; both display rather simple genetic control for Al tolerance and rely on homologous genes. However, barley is considered to be the most sensitive species among the cereals, whereas some *Sorghum* accessions may exhibit extremely high levels of Al tolerance (Wang et al. 2006). A comparison between *HvMATE* and $Alt_{SB}$ protein

sequences uncovers several significant differences, as they are only 65% identical and 79% similar. Also, they possess strikingly different features such as exon/intron structure in addition to apparently different numbers of putative transmembrane domains. Although similarities do exist between the two genes, such as a level of constitutive expression in the absence of Al and a likely Al activation of the *Sorghum* and barley MATE proteins, structural differences may account for the remarkably different levels of Al tolerance encoded by *HvMATE* and $Alt_{SB}$. A third related MATE transporter, *FRD3* from *Arabidopsis*, could also contribute to Al exclusion via citrate release (Durrett et al. 2007). Ectopic expression of *FRD3*, which is normally involved with iron metabolism and transport, is capable of making a modest increase to *Arabidopsis* Al tolerance (Durrett et al. 2007). Comparative analysis between the three MATE proteins, $Alt_{SB}$, *HvMATE*, and *FRD3*, will likely reveal domains and residues important for citrate transport and Al activation.

#### 6.2.2.3 Contributions from Rye

Unlike barley, rye is one of the most Al-tolerant cereals (Aniol and Gustafson 1984). In part, this may result from additive effects of malate and citrate, which are both released when some rye genotypes are exposed to Al (Li et al. 2000). Studies with Triticale, which is a hybrid between wheat and rye, identified that gene(s) on the short arm of rye 3R are required for organic acid release in Triticale (Ma et al. 2000). The pattern of organic acid release in rye involves a lag phase after the addition of Al (pattern II), suggesting induction at the gene or protein level to convey full activity. Interestingly, citrate release as modulated by the *Sorghum* Al tolerance gene $Alt_{SB}$ is also inducible over time of exposure to Al, a response that is paralleled by $Alt_{SB}$ expression (Magalhaes et al. 2007). Given that rye chromosome 3R is homoeologous to *Sorghum* chromosome 3, it is possible that a MATE ortholog of $Alt_{SB}$ is responsible for rye citrate release (Magalhaes et al. 2004).

### *6.2.3 Mediated by Oxalate*

Malate and citrate are not the only organic acid ligands for Al reported in root exudates. Oxalate has also been reported to appear in root exudates from Al-treated plants and is an effective chelate, intermediate between citrate and malate in terms of the dissociation constant for Al binding. Al-activated oxalate release has been reported in buckwheat (*Fagopyrum esculentum*), maize, taro (*Colocasia esculenta*), and alfalfa (*Medicago sativa*) (Ma and Miyasaka 1998; Zheng et al. 1998a; Kidd et al. 2001; Tesfaye et al. 2001). Oxalate is only mentioned in passing in this review due to the lack of identification for an Al-activated oxalate transporter. It is possible that *ALMT1*-type or $Alt_{SB}$-type transporters are permeable to oxalate in addition to malate and citrate, respectively. However, the oxalate transporter may represent a third class of organic acid transporters and is yet to be discovered and described.

## 6.3 Al Exclusion by Non-organic Acid Dependent Mechanisms

Al tolerance is highly correlated with exclusion of Al from the root apex in many species. Al exclusion explained the majority of differences in root growth observed between a small panel of maize varieties (Pineros et al. 2005). However, low and high outliers caused Piñeros and co-workers (2005) to reject the hypothesis that all Al exclusion in maize is mediated by organic acid release. Exclusion could also result from chelation by non-organic acid ligands or increasing rhizosphere pH, which would change the speciation of Al to less or non-toxic forms. Organic acid release does not explain the high degree of tolerance observed in rice or *Brachiaria decumbens* (Wenzl et al. 2001; Ma et al. 2002). Thus, it is likely that other species will be similar to maize, where Al tolerance is dependent upon multiple, independent mechanisms.

### *6.3.1 Al Exclusion Mediated by Other Ligands*

Evidence for Al exclusion mediated by non-organic acid ligands is relatively limited. This may be in part due to the difficulties in detection and identification of root exudates. Presumably, with the advancements in non-targeted metabolomic analysis via mass spectrometry or nuclear magnetic resonance, comprehensive analysis of root exudates will be more common in the future (see (Keurentjes et al. 2006) for example of this methodology).

Beyond organic acids, two classes of compounds have been implicated in Al tolerance. First, inorganic phosphate release was reported to occur concomitantly with malate in wheat (Pellet et al. 1996). Phosphate has high affinity for Al and would therefore make an effective ligand, although an expensive one from a nutritional standpoint. In this wheat study, a constitutive phosphate release was observed in the Al tolerant variety tested that was largely absent from the sensitive variety and from near isogenic derivatives with differing levels of tolerance (Pellet et al. 1996). This suggested that the same transporter did not mediate the release of malate and phosphate. Phosphate, like malate, release was spatially restricted to the root apex; however, the contribution of phosphate release to overall Al tolerance was unclear (Pellet et al. 1996). Phosphate has also been reported to occur in *Arabidopsis*. Like wheat, the Al tolerant variety released more phosphate and again the release was not Al responsive (Hoekenga et al. 2003). In the *Arabidopsis* case, essentially all the differences observed between varieties for Al tolerance were consistent with the differences in malate release, such that it is unclear whether the phosphate release observed made a contribution (Hoekenga et al. 2003). Further analysis will be required to evaluate the importance of phosphate release to Al tolerance relative to it being just coincidental.

Second, phenolic compounds have been implicated in Al exclusion. Both flavanoid phenolics and oxalic acid were observed in root exudates from Al-treated

maize seedlings (Kidd et al. 2001). Similar patterns of oxalate release were observed in three different maize cultivars with varying levels of Al tolerance, suggesting that oxalate release did not correlate with the differences observed in Al tolerance. However, different patterns of catechol, catechin, curcumin, and quercetin release were observed between the three maize varieties, with the magnitude of the catechin release most concordant with the Al tolerance differences (Kidd et al. 2001). Catechin is structurally similar to morin, which is commonly used as an Al-binding dye and means to assess Al absorption (Eggert 1970). Both catechin and morin have high affinity for binding with Al, meeting or exceeding the affinity observed for Al-organic acid complexes. Catechin exudation may represent an Al tolerance mechanism, but validation of this hypothesis requires more evidence.

### *6.3.2 Mediated by pH Change*

Al speciation is pH dependent, with the different cations (e.g., $Al^{3+}$, $Al(OH)^{2+}$) exhibiting different levels of rhizotoxicity (Kinraide 1991). Relatively small differences in rhizosphere pH can shift the balance from a preponderance of highly rhizotoxic $Al^{3+}$ to the less toxic hydroxy-Al compounds. The first evidence that pH gradients at the root surface could confer Al tolerance came in *Arabidopsis* with the identification of a mutant, *alr-104* (Degenhardt et al. 1998). An increased rate of proton influx was observed in the mutant, which led to an alkalinization of the rhizosphere by ~0.15 pH units (Degenhardt et al. 1998). Buffering the pH of the nutrient solution abolished the increased level of Al tolerance. A second, similar mutation was reported in *Arabidopsis* in the form of the *alt1* locus (Gabrielson et al. 2006). While pH buffering of the nutrient solution abolished the increase in Al tolerance observed with *alt1*, rhizosphere pH was not mapped and thus is difficult to compare the two studies directly (Gabrielson et al. 2006). Root surface pH has been measured in maize, to examine whether pH gradients might contribute to Al tolerance differences (Pineros et al. 2005). Differences in root tip pH were observed between varieties in the absence of Al treatment, with the most tolerant variety possessing the most alkaline pH. However, Al treatment collapsed any differences observed along the root surface between varieties and were not restored within 72 h (Pineros et al. 2005). It is still possible that root surface pH differences do contribute to natural variation in Al tolerance, but the proper study system is yet to be identified.

## 6.4 Internal Tolerance

Uncontrolled uptake of Al into the root is essentially inevitable, despite the best efforts of the various Al ligands. Internal tolerance to Al stress is therefore important to some greater or lesser degree for all plant species. In fact, the most highly Al-tolerant species largely or exclusively rely on internal tolerance mechanisms.

For a review on the mechanisms of Al hyperaccumulation, the reader is directed to Watanabe and Osaki (2002). Some internal and external tolerance processes share underlying physiological processes (e.g., chelation) while others are distinct.

### 6.4.1 Internal Chelation

Organic acids are an important source for internal as well as external Al tolerance. Many highly Al-tolerant species, including those considered to be Al hyperaccumulators, utilize organic acid chelation within the root or shoot to achieve Al tolerance. Buckwheat has been reported to use oxalate for both external and internal chelation of Al (Zheng et al. 1998a; Ma and Hiradate 2000; Shen et al. 2002). Oxalate is also the predominant intracellular ligand in tea (*Camellia sinensis*) roots (Morita et al. 2008). On the other hand, citrate is the predominant ligand found in xylem sap, to promote the long distance transport of Al from the root to shoot (Morita et al. 2004). Internal organic acid concentrations respond to Al treatment in *Brachiaria* roots (Wenzl et al. 2002). Organic acid levels increase several fold in the whole root for both tolerant *Brachiaria decumbens* and sensitive *Brachiaria ruziziensis*, where most of the organic acids are concentrated in the root apices. While the tolerant accessions accumulate more than the sensitive ones, the difference is far too small to explain the dramatic differences in Al tolerance (Wenzl et al. 2002). Similarly, citrate content increased in maize root apices due to Al treatment; however, concentrations were equivalent among the six maize inbreds and thus not correlated with Al tolerance (Pineros et al. 2005). It is important to note, however, in spite of the fact that internal organic acid concentrations may not correlate with differences observed in Al tolerance between *Brachiaria* and maize accessions; this does not exclude the possible importance for internal organic acid chelation. Rather, it may be that internal organic acid chelation is essential for Al tolerance but not genetically variable, at least within the accessions that have been studied to date.

Phenolic ligands are often used for long-term storage of Al in cells of hyperaccumulator species. While organic acids are used to transport Al within tea, catechin is the predominant ligand for Al sequestration in tea leaves (Nagata et al. 1992). Delphinidin and chlorogenic acid are associated with Al in *Hydrangea* sepals; association of these pigments with Al influences flower color (Takeda et al. 1990). Delphinidin is an anthocyanin, while chlorogenic acid is a phenylpropanoid; both are related to catechin, a flavanoid. Each of these Al ligands represents separate branches of a phenolic family tree, where early biosynthetic reactions are shared. For example, chalcone synthase (CHS) is the committing step for the synthesis of catechin, chlorogenic acid, and delphinidin. In maize, variation at chalcone synthase loci is significant for resistance to insect herbivores (Szalma et al. 2002). Anthocyanins are induced by abiotic stresses such as cold and high light (Christie et al. 1994; Kimura et al. 2003). Phenylalanine ammonia lyase (PAL), a key gene in primary metabolism, carries out the biosynthetic step prior to CHS and is known to be an Al-inducible gene (Snowden and Gardner 1993).

While some have attributed the induction of PAL and CHS by Al treatment as non-specific stress responses, it is also possible that these changes in gene expression underlie Al tolerance processes dependent upon phenolic ligands. As systems biology approaches are applied to the study of Al tolerance, it should be increasingly possible to identify which stress responses due convey Al tolerance over the noise of non-specific changes in gene, protein, or metabolite expression (Hoekenga et al. 2003, 2006; Yang et al. 2007; Zhang et al. 2007).

Hydroxyamates are another class compounds with potential importance to Al tolerance. Perhaps the best known hydroxyamate is 2,4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) (Frey et al. 1997). DIMBOA is highly effective at controlling insect herbivores and microbial pathogens; the complete synthetic pathway was recently determined (Jonczyk et al. 2008). DIMBOA has also been implicated in other biological processes, including auxin-induced elongation of maize coleoptiles (Park et al. 2001). Poschenrieder and colleagues, who also were the first to make the Al-flavanoid connection in maize, demonstrated intracellular Al tolerance due to DIMBOA–chelation of Al (Poschenrieder et al. 2005). Hydroxyamates are also found in nature as siderophores, ligands used by bacteria to acquire essential metals from the soil solution or to protect against toxins. A siderophore-deficient mutant strain of *Bacillus* has long been known to be sensitive to Al stress (Davis et al. 1971). Al stress elicited siderophore exudation from wild-type *Bacillus* cells, which tolerated Al treatments that completely inhibited growth in the siderophore mutant (Davis et al. 1971). Media supplementation with the *Bacillus* hydroxyamate siderophore, schizokinen, or the *Rhizobium* siderophore, vicibactin, conferred tolerance to Al stress to those species, respectively (Davis et al. 1971; Rogers 1986). As with the phenolic ligands, the application of systems biology approaches to Al stress tolerance will likely demonstrate the efficacy of hydroxyamate and others as contributors to Al tolerance processes across multiple species, genera, and wider evolutionary relationships.

### *6.4.2 Reactive Oxygen Species Scavenging*

Al stress generates ROS, like many other abiotic stressors (Cakmak and Horst 1991). Whether these ROS are a primary or secondary effect of Al toxicity is arguable; however, the damage done to lipids, nucleic acids, and other susceptible molecules is not (Yamamoto et al. 2001). ROS-responsive genes have been detected by gene and protein expression profiling methods in multiple species (Richards et al. 1998; Yang et al. 2007; Zhang et al. 2007). Genetic analysis has not implicated ROS scavenging genes, or their regulators, as responsible for natural variation in Al tolerance. Transgenic experiments that overexpress superoxide dismutase, peroxidase, or glutathione S-transferase do increase Al tolerance by small but significant degrees (Ezaki et al. 2000; Basu et al. 2001). It is certain that ROS scavenging contributes to internal Al tolerance and may be especially important in plants that do not rely upon Al exclusion.

### 6.4.3 Lipid Composition

Lipid peroxidation is an early sign of damage to Al-intoxicated roots (Yamamoto et al. 2001). The degree of lipid peroxidation is variable between Al-tolerant and -sensitive accessions, but it is unclear whether these differences are to due the proximate or ultimate causes of Al tolerance. One can imagine either scenario: (1) lipid composition is variable, thus making some plant less susceptible to peroxidation (a proximate cause of tolerance) or (2) plants with highly effective Al exclusion mechanisms suffer less lipid peroxidation, as less $Al^{3+}$ reaches the plasma membrane (an ultimate cause). A wheat phosphatidylserine synthase gene was capable of increasing Al tolerance in the yeast, *S. cerevisiae* (Delhaize et al. 1999). The transgene dramatically reduced phosphatidylinositol levels while increasing phosphatidylserine, which presumably reduced Al/ROS susceptibility (Delhaize et al. 1999). The result from yeast was not reproduced in plants, as each has specific requirements for functional membranes (Delhaize et al. 1999). This did stimulate the examination of lipid composition between Al-tolerant and -sensitive varieties (Chaffai et al. 2005; da Silva et al. 2006). Lipid profiling in maize root tips suggested that sphingolipid composition might be correlated with Al tolerance; subsequent transgenic experiments with a Δ8 sphingolipid desaturase in maize, *Arabidopsis* and yeast verified this hypothesis (da Silva et al. 2006; Ryan et al. 2007). Together, the lipid profiling and transgenic experiments indicate that lipid composition can be a proximate cause of Al tolerance.

### 6.4.4 Cell Wall Composition

The majority of Al associated with the root (≥80%) can be found in the cell wall, according to estimates from maize and wheat (Ma et al. 2004a; Wang et al. 2004). This association presumably accounts for the reduction in wall extensibility observed with Al-treated plants (Jones et al. 2006; Zakir Hossain et al. 2006). Differences between tolerant and sensitive accessions beg the proximate/ultimate causes question: do tolerant accessions construct cell walls significantly differently than sensitive accessions, or are the differences observed merely due to Al exclusion. Cell wall composition does change in response to Al treatment, especially in the pectin component (Eticha et al. 2005; Zakir Hossain et al. 2006; Yang et al. 2008). This is potentially significant as de-esterification of pectin increases the density of negative charge within the cell wall; increasing the net negative charge could allow greater Al loading onto the cell wall (Cosgrove 2005). Increasing the esterified fraction of pectins has been correlated with increasing cell elongation rates in *Arabidopsis* (Derbyshire et al. 2007). In both maize and wheat, Al tolerant accessions had higher degrees of pectin methyl-esterification and reported lower uptake of Al into the cell wall (Eticha et al. 2005; Yang et al. 2008). Unfortunately, both studies were comparisons between a pair of accessions, one tolerant and one

sensitive. The statistical power for such a comparison is very low, but still the results are intriguing. Additionally, $Al^{3+}$ is a potent inhibitor of expansins, the family of cell wall loosening enzymes responsible for acid-responsive growth (Cosgrove 2000). Cell wall loosening and elongation is diminished or eliminated in the absence of expansin activity; if $Al^{3+}$ inactivates expansins, this could also explain the rapid loss of root growth observed in Al-intoxicated roots. If Al-resistant expansin isoforms exist, they could represent very powerful Al tolerance loci as they could protect cell elongation in the presence of stress.

## 6.5 Concluding Remarks

It is an exciting time to be working in the Al tolerance field. Al tolerance genes have been identified that underlie major QTL of agronomic importance. Given the size and strength of the Al tolerance community, we anticipate many more discoveries of similar magnitude in the coming years. Systems biology approaches that leverage traditional plant physiology against genome sequences and other technologies will permit large improvement in Al tolerance. This should produce outcomes that promote food security, economic development, and environmental protection in acid soil regions.

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# Chapter 7
# Root Responses to Major Abiotic Stresses in Flooded Soils

**Rogerio O. Sousa and Antonio Costa de Oliveira**

**Contents**



## 7.1 Introduction

Soil flooding alters the natural equilibrium of components and organic matter decomposition, unchaining a series of transformations that affect chemical and physical soil attributes. Such changes are beneficial to the rice crop because rice plants present morphological and molecular adaptations in order to survive these environments that lack free molecular oxygen; moreover, most of nutrients increase their availability in flooded conditions (Sousa et al. 2009). However, the soil changes associated to flooding can result in stresses even to the rice crop, which is well adapted to these conditions. These changes generate products such as soluble

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iron and short-chain organic acids which, under proper conditions, can be toxic to rice. In order to achieve a perfect state of growth, the plant must balance the presence of minerals at different concentrations and equate its needs. Among the major stresses faced by rice plants under no tillage cropping systems in South America, iron and organic acid toxicity top the list and will be the focus of this review.

Iron toxicity in the rice crop is a nutritional disorder that occurs in cultivated fields of many countries, mainly in Asia, Africa, and South America. This disorder has been named differently according to each country, symptoms, and occurrence conditions. In Japan, for example, it is denominated "Akagare" type I, in Ceylon and India, it is known as "Bronzing," and in Colombia, it is known as "Anaranjamiento." In Brazil, iron toxicity has already been observed in many rice production areas, especially from the introduction of modern-type cultivars, which occurred in the middle of 1980s (Vieira et al. 1999). Organic acid toxicity is a stress that became important after no tillage cropping started and increasing amounts of organic matter originating from the straw of the previous crop started to take part in the process.

## 7.2 Iron in Flooded Soils

Iron is one of the most abundant elements on earth, contributing to approximately 5% of its total weight (Murad and Fischer 1988), and it is present in all soils, in amounts ranging from 0.7 to 55% (Lindsay 1979). In the soil, iron oxides can be uniformly distributed or concentrated in some profile layers, forming mottles, nodules, concretions, hardpans, plinthites ou laterites. The main forms of iron oxides in soils are hematite, goethite, lepidocrocite, and ferrihydrite, although other oxide/hydroxides may be present (Sousa et al. 2004).

The changes in oxide and reduction states that occur in environments that alternate between dry and flooded conditions, such as lowland soils cultivated with rice, are determinant to the iron oxide and hydroxide forms that predominate (Moormann and Van Breemen 1978). During flooding, a part of the soluble $Fe^{2+}$ ions, which are rapidly oxidized during the following draining period, are precipitated as ill-crystalized $Fe^{3+}$ oxides. If the draining period persists, the $Fe^{3+}$ oxide degree of crystalization can increase, although in a very slow process. When the draining period is over, $Fe^{3+}$ oxides are again reduced and solubilized. The alternation between flooded and non-flooded conditions therefore favors low crystalinity iron oxides. Thus, goethite, lepidocrocite, and ferrihidrite are the most common forms of iron oxides in hydromorphic soils (Allen and Hajek 1989; Schwertmann and Taylor 1989). According to van Breemen (1988), iron can still be present in "green-rust" ($Fe^{3+}$ and $Fe^{2+}$ associated to $Cl^-$, $SO_4^{2-}$ and $CO_3^{2-}$ anions in the interlayers), siderite, pirite, and silicate minerals, such as smectites.

Soil bacteria that reduce $Fe^{3+}$ in the soil have preference for the ill-crystalized iron oxide forms. Thus, the speed and the amount in which iron oxides are reduced and released to soil solution depend, among other factors, from the ratio of crystalized and ill-crystalized soil minerals. As a consequence, the lower the degree of iron

oxide crystalinity, the higher the iron reduction and release into the soil solution (Sousa et al. 2004). However, since the reduction of iron depends on the biological activity, other factors must be considered in this process, as organic matter content and the presence of easily reducible compounds, such as nitrate and manganese oxides. Better fitted regression models were obtained for the prediction of exchangeable $Fe^{2+}$ (Sousa et al. 2004), during the flooding of 32 hydromorphic soils, considering not only the amount of iron oxide, but also the amounts of Mn (extracted with ammonium oxalate at pH 6.0), $NO_3^-$ and organic carbon (Table 7.1).

In Fig. 7.1, the trend of iron concentrations in solution of two flooded soils, a Planossolo (typic Albaqualf, USDA soil taxonomy) which can present iron toxicity

**Table 7.1** Regression fitting models for exchangeable $Fe^{2+}$ and $NO_3^-$, organic-C, and iron and manganese oxides soluble in ammonium oxalate

| Model | $r^2$ |
|---|---|
| $Fe^{2+} = 3.82 + 0.061\ Fe_o$ | 00.17[b] |
| $Fe^{2+} = 1.61 + 0.50\ Fe_o$[a] | 00.50[b] |
| $Fe^{2+} = 2.39 + 0.51\ Fe_o$[a] $- 0.30\ Mn_o$[a] | 00.54[b] |
| $Fe^{2+} = 3.78 + 0.59\ Fe_o$[a] $- 0.37\ Mn_o$[a] $- 0.07\ NO_3$ | 00.59[b] |
| $Fe^{2+} = 4.38 + 0.52\ Fe_o$[a] $- 0.29\ Mn_o$[a] $- 0.11\ NO_3 + 0.42\ C$ | 00.64[b] |

*Source*: Vahl (personal communication)
$Fe_o$ – extracted with ammonium oxalate at pH 3
[a]$Fe_o$ and $Mn_o$ – extracted with ammonium oxalate at pH 6
[b]significant at 1%

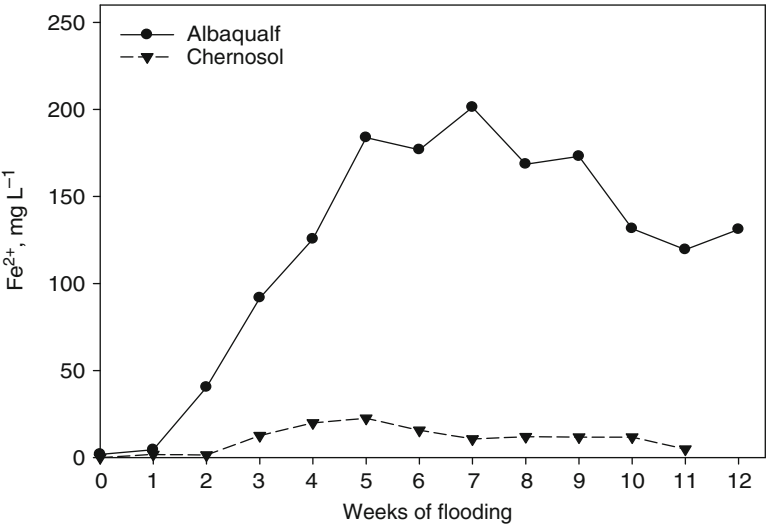


**Fig. 7.1** Iron contents in soil solution from two lowland soils as a function of flooding period. Albaqualf: O.M. = 17g $kg^{-1}$; $Fe_{oxalate}$ = 1.4g $kg^{-1}$ Chernosol: O.M. = 24g $kg^{-1}$; $Fe_{oxalate}$ = 0.4g $kg^{-1}$
*Source*: Adapted from Sousa et al. (2009)

and a Mollisol where this nutritional disorder is not observed. In the Planossolo, in 4 or 5 weeks of flooding, iron concentration peeks high enough and toxicity to plants can be reached. In this soil, the iron concentration peeks normally occur in the stage where rice is most sensitive, which is the end of tillering. In Mollisol, the iron amounts released to the soil solution are lower since this soil has low active iron content (iron extracted with ammonium oxalate), and normally does not present toxicity problems.

Iron toxicity in irrigated rice is commonly associated to some soil traits, such as low pH, high iron oxide content, and low CEC. However, it is common to observe iron toxicity symptoms at different pH, iron oxide contents, and CEC conditions (Sousa et al. 2004).The first idea that one grasps about the toxicity of an element to plants is that high concentrations of this element in the soil lead to an excess absorption and toxicity. However, in iron toxicity, this idea cannot be taken as a common rule, since there have been reports of symptoms occurring in crops growing in low iron soils and no symptoms in crops growing in high iron content soils (Sousa et al. 2004).

The low soil pH is pointed as one factor that favors iron toxicity occurrence. In this condition, iron solubility is higher, soil CEC is lower, and CEC saturation by ($H^+ + Al^{3+}$) is higher. However, reports have described soil samples collected from rice fields showing toxicity symptoms with pH values ranging from 3.8 to 7.5 (Sousa et al. 2004). The high soil iron content, low pH, and low CEC cannot be considered, alone, as obligatory conditions for iron toxicity to occur, since many reports have shown rice fields developing iron toxicity symptoms and showing different iron content, pH, and CEC values. The detection of symptoms depends on different soil and plant attributes, related to toxicity. A soil with high iron content, but high CEC and base saturation, can present high $Fe^{2+}$ content as a consequence of flooding, but this can be low when compared to other cations such as K, Ca, and Mg, as a result of CEC and base saturation values, resulting in healthy plants. Another soil with low iron content, but low CEC, can present low amounts of $Fe^{2+}$ during flooding, but, however, due to the low CEC, the ratio $Fe^{2+}$/other cations can be higher and consequently reach levels toxic to rice plants (Sousa et al. 2004).

Some unpublished results do exist for iron toxicity occurrence in land-leveled areas. The preparation of these areas for rice cropping can give rise to iron toxicity cases due to two factors: exposition of B horizon with higher iron contents, or exposition of E layer, rich in sand and with lower ability to supply nutrients to the plants (Sousa et al. 2004).

### *7.2.1 Iron Toxicity Symptoms*

Iron toxicity is visually divided into two major symptom groups (Fig. 7.2): direct toxicity or bronzing and indirect toxicity or yellowing. Direct toxicity is caused by excessive iron absorption, while indirect toxicity is associated to overall nutrient deficiency, induced by high iron content in the soil solution. These terms have been adopted by the majority of authors in order to define the major iron toxicity-related

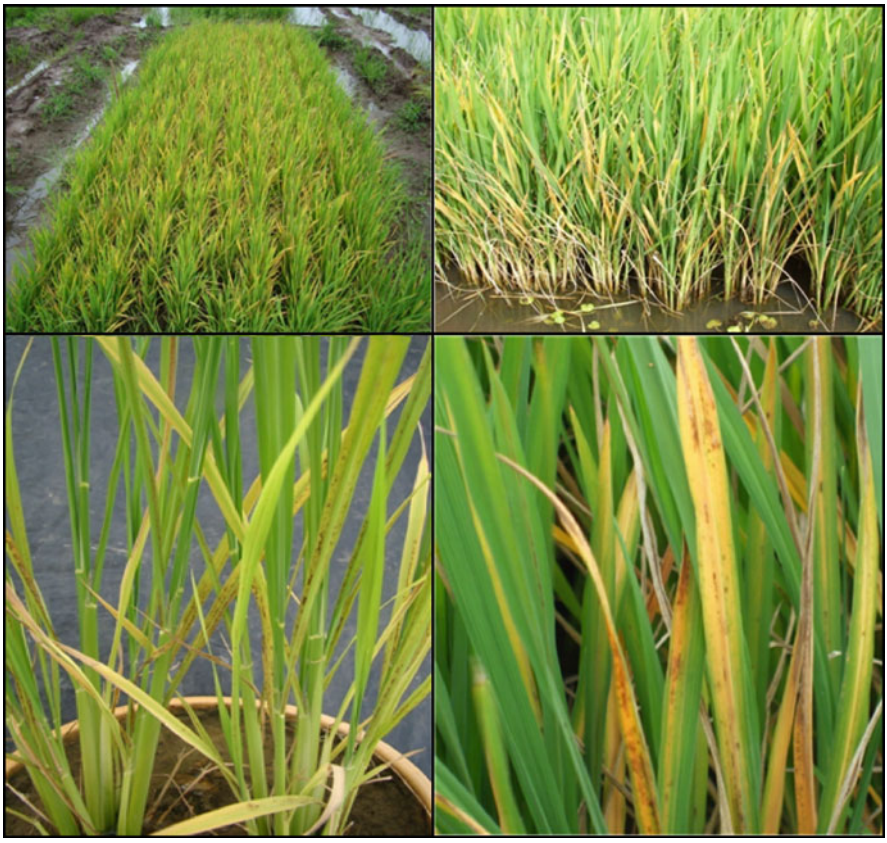

**Fig. 7.2** Regular symptoms of iron toxicity. (**a**) and (**b**) Indirect toxicity; (**c**) direct toxicity; (**d**) direct and indirect toxicity simultaneously.

symptoms. However, Sahrawat (2004) proposed recently the idea of induced toxicity or fake toxicity, when the symptoms are caused by a multiple nutrient deficiency (indirect toxicity) and true toxicity when symptoms are the result of high iron content (direct toxicity).

The symptoms attributed to direct toxicity are composed of many dark brown spots, which initiate in the tips and spread to the base of older leaves (Mengel and Kirkby 1987). Similar symptoms were described in which, at higher iron contents, the dark brown spots fuse, forming large dark brown areas in the leaves (Tanaka et al. 1966). These points match the high iron concentration spots in the leaf. Similar bronzing symptoms have been described (Mengel and Kirkby 1987; Bienfait 1985). Also, it was reported that when the disorder progresses, leaves senesce and die, and more severely injured plants show lower tillering, smaller panicles with high percentage of sterile spikelets and lesser branched roots, with dark brown color (Ponnamperuma et al. 1955; Sousa et al. 2004). Although the degree of toxicity measures has been based on the degree of bronzing (IRRI 1965; Ota 1968), the

phenotype itself and the basis of tolerance are not well understood (Ota 1968; Peng and Yamauchi 1993; Briat and Lobréaux 1997).

Indirect toxicity symptoms initiate with a yellowing of older leaf tips, which evolves toward the base. Subsequently, the younger leaves are also affected and many lower leaves die. In severe cases, leaves acquire an orange or yellow color and may present dark brown stripes (Howeler 1973; Ottow et al. 1983).

Some authors make no distinction between direct or indirect toxicity, describing the symptoms in the following way: iron toxicity is characterized by the development of very small spots in older leaves, which gradually coalesce, giving a purple, brownish red, orange, or yellow color spreading to the leaf base, especially on the edges. These parts, then, become dry and curly toward the center. During the first stages, younger leaves and the unaffected parts of older leaves are green, but later, younger leaves also tend to show small dark brown spots, while older leaves dry completely giving the plant a burned look. The root system is dark brown, thick, and scarce; plant growth is stunted; and there is a high percentage of sterile flowers (Lantin and Neue 1988).

In case the toxicity occurs during the plantlet stage, rice plants remain stunted with a very limited tillering ability (Abraham and Pandey 1989). Toxicity during the vegetative stage is associated with the reduction of plant height and dry matter accumulation (Abu et al. 1989), which is greatly affected by root biomass (Fageria 1988). Tiller formation and number of fertile tillers can be severely reduced (Cheema et al. 1990). When iron toxicity occurs at the end of the vegetative phase or at the reproductive phase, the number of panicles formed decreases (Singh et al. 1992), there is an increase in spikelet sterility (Virmani 1977) and a delay in flowering and maturation. In highly susceptible cultivars, flowering may not occur (Ayotade 1979). Also, root growth can stop and the aerenchyma can senesce and decay, resulting in a decrease of root oxidation ability and formation of $Fe(OH)_3$ compounds on root surface changing it to a darker color (Morel and Machado 1981).

Average yield losses due to iron toxicity range from 35 to 45% (Lantin and Neue 1989; Audebert and Sahrawat 2000). Iron toxicity symptoms can appear in any plant developmental stage. However, the end of tillering and beginning of flowering are the stages in which the symptoms appear more frequently and clear (van Mensroort et al. 1985; Fageria 1984). If iron toxicity occurs in the early stages of development, plants suffer a severe retard in growth; when it is later, vegetative growth is not much affected, but grain yield is reduced due to spikelet sterility (Lantin and Neue 1988). However, some reports state that when iron toxicity occurs in the beginning of the cycle, plant growth can be strongly affected and a total loss of yield can occur (Abifarin 1988).

Analyzing the symptoms described by different authors, one can observe that there is not a unique symptom characterizing iron toxicity, but a range of colors from yellow to orange, with or without dark brown spots. In all descriptions, these symptoms start on older leaves and evolve from tip to base of leaf limb (Sousa et al. 2004).

Leaves become chlorotic because iron is needed for the synthesis of some chlorophyll–protein complexes in the chloroplast. The low mobility of iron is due to its precipitation in older leaves as insoluble oxides or phosphates or the formation

of complexes with phytoferritin, an iron-binding protein (Oh et al. 1996). Iron precipitation decreases the metal's subsequent mobilization inside the phloem. This type of toxicity is less common in Brazilian conditions, but is frequently seen in other climates, where some soils develop extremely high levels of $Fe^{2+}$ when flooded. Indirect toxicity results from the limited absorption of several nutrients such as calcium, magnesium, potassium, phosphorous, and iron itself, due to iron precipitation on rice root epidermis. The formation of an oxide–hydroxide $Fe^{3+}$ layer on the root blocks nutrient absorption, resulting in multiple nutritional deficiencies. Symptoms of this deficiency include plant atrophy, tillering reduction, orange leaves, and the covering of roots by red layers of iron oxides. Besides iron deposition in the roots, changes in leaf peroxidase activity have been described (Peng et al. 1996; Fang and Kao 2000).

The insolubility of iron plus its high reactivity can cause severe damage to the plant cell. The production of reactive species of oxygen, specifically the hydroxyl radical ($OH^{\bullet}$), through the Fenton Reaction, is the major cause for its toxicity inside the cell (Hell and Stephan 2003):

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2\ O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Or:

$$O_2^{\bullet -} + H_2\ O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$$

The entrance of iron into the radicular symplast via the membrane transport systems creates a need to once more protect it from oxygen. Protection is necessary in order to avoid precipitation and the generation of reactive oxygen species. Among the major chelating agents, nicotianamine (NA) appears as the best candidate because it forms poor Fenton reagent stable complexes with iron at both oxidation states, its ubiquitous character, and its correlated localization with iron (Stephan and Scholz 1990; Scholz et al. 1992; Stephan et al. 1996; Herbik et al. 1996; Liu et al. 1998; von Wiren et al. 1999; Pich et al. 2001).

After zinc, iron is the element that most frequently limits rice production, when nutritional disorders in rice caused by micronutrients in Brazilian soils are assessed. Two contrasting scenarios exist: one in dry conditions (upland rice) when the problem is related to iron deficiency and the other in flooded conditions, due to toxicity (irrigated rice). Increases in the $Fe^{2+}/Fe^{3+}$ ratio caused by reduction in the flooded soil are the major cause of toxicity. This reduction can cause an increase of 6,000-fold in soluble iron (600 vs. 0.1 ppm) when soil redox potential reaches 100–300 mV (Brennan and Lindsay 1998).

In Brazilian soils commonly cultivated with flooded rice, soluble iron content after flooding does not reach such high levels as registered in other traditional rice growing countries. Generally, the iron content in Brazilian soils does not exceed

100 ppm. However, these levels are sufficient to cause iron toxicity in rice (Barbosa Filho et al. 1994). The iron content in which toxicity occurs in the soil and plant ranges between 10 and 1,000 ppm and 50 and 1,700 ppm, respectively. Such broad limits illustrate that toxicity development is a complex phenomenon. It does not appear that there is a specific factor in either the soil or the plant that allows a prediction of toxicity (Barbosa Filho et al. 1994).

The predominant and therefore the most important form of toxicity in Brazil is indirect. Toxicity due to the ferric form ($Fe^{2+}$) can cause considerable losses in rice production. This is specially the case in the acid soils of tropical and subtropical areas (Fageria and Rabelo 1987; Wu et al. 1998), as found in southern Brazil. These regions are characterized by their richness in iron and low pH (Silva et al. 2003). Occurrence in rice fields may cause reductions in productivity as high as 80% (Sousa et al. 2004). Iron toxicity was first detected in Brazil during the 1970s. The introduction of modern-type rice cultivars, some of which showed sensitivity to the excess of iron in the soil, revealed the problem. The problem was also seen in the states of Santa Catarina, Minas Gerais, Rio de Janeiro, Espírito Santo, Goiás, and Rio Grande do Sul (Sousa et al. 2004; Vieira et al. 1999).

### *7.2.2 Iron Metabolism*

The stable forms of iron participating in plant metabolism are $Fe^{2+}$ and $Fe^{3+}$ (Staiger 2002). The oxidation of iron-carrying compounds is constantly detected, iron going from $Fe^{2+}$ to $Fe^{3+}$ during the electron transfer and vice versa. The complex compounds formed with iron such as Fe–S proteins are key to electron transfer in the respiratory functions in mitochondria and in the photosynthesis apparatus in the chloroplasts (Balk and Lobreaux 2005). Fe–S clusters also participate in nitrogen fixation, DNA repair, and metabolic pathways. Iron is an essential component of different enzymes involved in electron transfer (redox reactions), such as cytochromes, both heme and non-heme groups, as well as electron carriers and ferredoxin, a substance known to be involved in the photosynthesis electron transfer (Barbosa Filho 1994; Briat et al. 1995; Briat and Lobréaux 1997; Briat et al. 2007). The presence of iron was also observed in plant hormone synthesis as a cofactor (Bouzayen et al. 1991; Siedow 1991). Iron is predominantly present in the chloroplasts as phytoferritin and ferredoxin (ca. 75%) protein complexes which are known to be involved in the photosynthesis electron transfer (Brown et al. 1972).

### *7.2.3 Iron Uptake*

The predominant form of iron is the divalent form $Fe^{2+}$. Its content in the soil ranges from near zero up to 40% in the $Fe_2O_3$ form. In order to cope with the low solubility of ferric ions, an active mechanism to release/absorb iron from $Fe^{3+}$ oxide hydrates

to the soil solution is required. Due to their immobility, plants face a range of iron availability in the environment. Both iron deficiency and toxicity are responsible for severe nutritional disorders deeply affecting their physiology (Ponnamperuma et al. 1955; Chaney et al. 1972). In general, two strategies, one based in reduction and another based in chelation (Kim and Guerinot 2007), have been described for the uptake of iron.

#### 7.2.3.1 Strategy I (Reduction Based)

In this strategy, plants release protons into the surrounding rhyzosphere via a proton-ATPase. Dicot plants improve iron absorption by three reactions: (1) proton efflux via ATPase to acidify the medium and therefore increase $Fe^{3+}$ solubility; (2) reduction of $Fe^{3+}$ by a $Fe^{3+}$-reductase to a more soluble form $Fe^{2+}$; (3) transport of $Fe^{2+}$ by an iron transporter (Römheld and Marschner 1986).

#### 7.2.3.2 Strategy II (Chelation Based)

The organisms using this strategy release phytosiderophores (PSs) that chelate $Fe^{3+}$ at the rhizosphere, allowing specific protein transporters to import the $Fe^{3+}$–PS complexes (Römheld and Marschner 1986; Hell and Stephan 2003). Microorganisms, as well as grasses, use this strategy. Yeast, although not secreting its own siderophores, can recognize and absorb bacterial siderophores such as catecholate or hydroxamate (Yun et al. 2000a; Yun et al. 2000b).

### *7.2.4 Iron Transport and Signaling*

Iron uptake and transport have been described in the model eukaryote *Saccharomyces cerevisiae* (Curie and Briat 2003). In the plasma membrane, reductases reduce $Fe^{3+}$ to $Fe^{2+}$, which is more soluble. A flavocytochrome (Fre1p) reduces $Fe^{3+}$ at the cell surface. Many paralogs of the *FRE* gene have been found (*FRE2 – FRE7*) as a result of yeast genome sequencing (Johnston et al. 1997). *FRE2* encodes a protein related to Fre1p while *FRE3* and *FRE4* genes are involved in the reduction of $Fe^{3+}$-siderophore (Dancis et al. 1990). When the cells are replete with iron, a low-affinity uptake system is responsible for ferrous iron uptake. This is achieved by a plasma membrane transport protein encoded by the *FET4* gene (Dix et al. 1994; Dix et al. 1997). On the other hand, the genes *FET3* and *FTR1* play an important role in high-affinity ferrous uptake, which is induced under iron-deficiency conditions (Askwith et al. 1994; Stearman et al. 1996). *FET3* encodes a trans-membrane protein from a family of multicopper oxidases that has an oxidase catalytic domain located on the cell surface. *FTR1* encodes a plasma membrane permease containing a REGLE motif that has been identified in the ferritin iron-storage protein and seems to be responsible for an iron selective pore. A model for high-affinity iron uptake has

been proposed (Eide 1998). It requires that $Fe^{2+}$ produced by the $Fe^{3+}$ reductases be oxidized outside the cell by the FET3p multicopper oxidase into $Fe^{3+}$, which then binds to a $Fe^{3+}$-binding site on FTR1p. Then, a conformational change is caused by this binding, enabling $Fe^{3+}$ to be transported to the cytoplasm. On another model species, rice, a survey on the iron homeostasis-related genes revealed 18 YS, 2 FRO, 13 ZIP, 8 Nramp, and 2 Ferritin genes (Gross et al. 2003).

The Nramp (Natural Resistance-Associated Macrophage Protein) family of metal transporters is conserved from bacteria to mammals (Gunshin et al. 1997). However, these proteins have also been shown to transport Ni, Zn, Cu, Co, and Cd, as well as Fe and Mn (Gunshin et al. 1997). In order to avoid imbalances in nutrient supply and to meet the nutritional demands for the entire plant, vascular plants employ a strategy of interorgan signaling (Schmidt 2003). The signal for systemic regulation of root responses to iron has been suggested to be ITP1, an iron-binding member of the LEA (late embryogenesis abundant) protein family (Krueger et al. 2002). Transcription factors induced by iron deficiency have been reported, including 14-3-3 and zinc-finger proteins in barley (Negishi et al. 2002). Also, a protein containing a helix-loop-helix domain, FER, was cloned from a tomato mutant (*fer*). This mutant does not respond to iron deficiency and can only survive with a heavy supply of iron chelates (Ling et al. 2002). Nitric oxide (NO) is responsible for the translation of the Fe-deficiency signal, a ubiquitous signal in mammals and plants (Wendehenne et al. 2001).

The transport of iron to the cell interior creates the necessity of a proper storage in order to avoid possible damage due to reactive oxygen species. Iron is stored in the apoplastic space, between the plasmatic membrane and the cell wall of plant cells, in mitochondria (Zancani et al. 2004), in plastids (Seckback 1982), and in the vacuole, in low pH and high organic acid concentrations (Briat and Lobréaux 1997). The vacuole is the place for iron and other metal sequestrations, either as a mechanism of detoxifying the cell or as metal reservoir. Exactly how the vacuole contributes to iron metabolism is not clear. Mutations that affect vacuolar function also affect the assembly of high-affinity transport systems present in the plasma membranes (Urbanowski and Piper 1999). Ferritin, a specialized iron-storage protein, is used to store iron in both mitochondria and plastids. They consist of 24 subunit hollow spheres capable of storing up to 4,500 atoms of iron per molecule in a soluble and bio-available form (Balla et al. 1992; Harrison and Arosio 1996; Connolly and Guerinot 2002). Ferritin forms gated pores, which are highly conserved in ferritins of humans down to bacteria. These pores control iron flow to chelators (Liu and Theil 2005). Iron controls the transcription of plant ferritins in soybean and maize (Fobis-Loisy et al. 1996; Wei and Theil 2000). Also, the accumulation of plant ferritin is regulated post-transcriptionally, since ferritin mRNA accumulates in the maize mutant *ys1* to a similar level as in other genotypes (Fobis-Loisy et al. 1996) but iron accumulation in leaves is lower.

Many genes involved in iron transport have been described. Two ZIP family members that function as root iron transporters, *IRT1* and *IRT2*, are responsible for iron uptake from the soil in *Arabidopsis* (Eide et al. 1996; Guerinot 2000; Connolly et al. 2002; Vert et al. 2002; Varoto et al. 2002). The *OsIRT1* and *OsIRT2* genes from

rice are predominantly expressed in roots and induced in low-Fe conditions (Ishimaru et al. 2006). A root iron-chelate reductase, *FRO2* (homologous to *FRE1*, *FRP1* and *gp91$^{phox}$*), complements the *Arabidopsis frd1* mutant, deficient in root ferric-reductase activity (Robinson et al. 1999). Members of the Nramp family, *Nramp1*, *3*, and *4*, are divalent metal transporters which tend to show increased mRNA accumulation in Fe deficiency (Curie et al. 2000; Thomine et al. 2000). AtNramp3 is a vascular metal transporter involved in plant responses to iron deficiency. It is expressed in the vascular bundles of roots, stems, and leaves under Fe-sufficient conditions, suggesting a function in long-distance metal transport within the plant (Thomine et al. 2003). Mobilization of vacuolar iron is essential for seed germination on low iron and is performed by the products of genes *AtNramp3* and *AtNramp4* (Lanquar et al. 2005). Some iron efflux transporters belonging to the IREG/Ferroportin family have been reported (*IREG 1-3*) and show sequence similarity to mammalian iron efflux transporters (McKie et al. 2000). YS1, a $Fe^{3+}$-phytosiderophore transporter, was cloned in maize from the *ys1* (yellow-striped) mutant (Curie et al. 2001). It was reported as a membrane protein that mediates iron uptake. YS1 is able to translocate iron that is NA or PS bound, and its specificity to iron seems to be several fold higher than that to copper. No evidence was found for YS1 to be active in zinc transport (Roberts et al. 2004). *Arabidopsis* has eight homologues, *YSL 1-8*. *AtYSL1* is an important nicotianamine seed loading. This gene was expressed in the xylem parenchyma of leaves, where it was upregulated in response to iron excess, as well as in pollen and in young siliqua parts (Le Jean et al. 2005). *AtYSL2* is a metal-regulated gene encoding a plasma membrane transporter of nicotianamine–metal complexes that is expressed in many cell types in leaves, roots, and reproductive organs showing a major role in the lateral movement of metals in the vasculature (DiDonato et al. 2004). Rice has 18 putative *YS1*-like genes exhibiting 36–76% sequence similarity to maize *YS1*. From these, *OsYSL2* is strongly induced in rice leaves by iron deficiency (Koike et al. 2004). *TcYSL3* is a Fe/Ni–NA influx transporter and a good candidate for the function of entry of Ni–NA into the symplasmic transport in the root for delivering it into the xylem. It is also important for the unloading of the Ni–NA complexes from the xylem in the leaves and subsequent delivery to storage sites (Gendre et al. 2006).

A member of the LEA family, *ITP*, has a similarity to a $Fe^{3+}$ polypeptide chelating in the phloem (Krueger et al. 2002). A gene belonging to the *cytb5* reductase family, an *NFR* homolog with iron reductase activity in the tonoplast and in the phloem was reported (Bagnaresi et al. 2000; Xoconostle-Cazares et al. 2000). Four genes encode ferritin (*AtFer1-AtFer4*) in *Arabidopsis*. AtFer1 and AtFer3 play important roles in the protection of plant cells from oxidative stress (Petit et al. 2001). *AtFer2* gene expression was detected in mature siliquas and dry seeds, induced by ABA (Briat and Lobréaux 1997). Grasses that utilize strategy II release a low molecular weight chelating compound such as mugineic acid (MA). The phytosiderophore-$Fe^{3+}$ complexes are then transported into the plant (Grotz and Guerinot 2002). In this process, two genes are required for the conversion of S-adenosyl methionine to Nicotianamine (Nicotianamine Synthase, NAS) and NA to deoxymugineic acid (Nicotianamine Aminotransferase, NAAT). A shortage of

NA impairs the functions of metal-requiring proteins, including transcription factors (Takahashi et al. 2003). Maize has two types of NAS proteins based on their expression pattern and subcellular localization (Mizuno et al. 2003). Three genes were found: *ZmNAS1*, *ZmNAS2*, and *ZmNAS3*. The first two are expressed under iron deficiency and the third is downregulated by iron deficiency and induced by iron resupply. Three rice Nicotianamine Synthase genes, *OsNAS1*, *OsNAS2*, and *OsNAS3*, have been shown to be expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *OsNAS1* and *OsNAS2* are expressed in the vascular bundles of green leaves and in all cells showing chlorosis. *OsNAS3* expression is induced in roots but is suppressed in leaves in response to iron deficiency (Inoue et al. 2003). A cDNA macroarray using 36 metal-related genes from rice including metal transporter *(ZIPs*, *NRAMPs*, and *YSLs*) and metal homeostasis (*NAS*, *FER*, *FRO*, *NAAT*, *FDH*, *GSTU*, and *PDR*) genes was developed (Narayanan et al. 2007). The genes *OsIRT1*, *OsZIP1*, *OsZIP5*, *OsZIP8*, *OsYSL5*, *OsYSL6*, *OsYSL7 OsYSL8*, *OsYSL18*, *OsNramp2*, *OsNramp4*, and *OsNramp7* were found to be expressed in all types of leaves (flag and non-flag).

### *7.2.5 Improving high/low Iron Tolerance in Rice*

Rice is a particularly interesting species since it is described as a strategy II plant, but it also absorbs iron through strategy I. This means that it can absorb iron via chelated- and reduction-based strategies. The latter causes an acidification of the medium and increases the ratio of soluble/insoluble iron in the soil (Ishimaru et al. 2006). Thus, rice has the advantage of plasticity regarding growing under normal or submerged conditions. In general, plant species differ regarding the ability to absorb nutrients, the degree of resistance to toxic elements, and efficiency in the use of absorbed nutrients (Clark 1983; Furlani et al. 1986). Shoot length and 9 days of stress were shown to be the best traits for discriminating Brazilian irrigated rice genotypes (Crestani et al. 2009) regarding their genetic response to iron toxicity.

A mapping population consisting of 123 double-haploid (DH) lines was developed from a cross between IR64 and Azucena (Guiderdoni et al. 1992). The parents, 123 DH lines, and 100 DHBC1F1 (DH lines backcrossed to Azucena) were used to find markers associated to seedling tolerance for ferrous iron toxicity (Wu et al. 1997). From a total of 175 cDNA and genomic clones tested, four marker loci on chromosome 1 were identified to be significantly associated with both segregations of tolerance index value (degree of bronzing) and RDSDW (relative decrease in shoot dry weight). A significant association between one marker locus and RDSDW was found. Also, QTLs explaining 32% and 15% of the tolerance index value and 15%, 21%, and 10% of the RDSDW were found (Wu et al. 1997). Another mapping population consisting of 96 backcross inbred lines (BILs) derived from a cross Nipponbare/Kasalath/Nipponbare was developed (Wan et al. 2003). The 96 BIL lines in BC1F9 were phenotyped for iron tolerance. Four QTLs were detected using

RFLP markers on chromosomes 1 and 3 that were significantly associated with leaf bronze index, stem dry weight, tiller number, and root dry weight.

Regarding iron defficiency, rice produces less phytosiderophores than wheat and barley. One strategy has been to increase its PS production. When transgenic rice plants expressing barley NA Aminotransferase were tested, their tolerance was improved, achieving higher vigor and fourfold higher grain yield (Takahashi et al. 2001). The constitutive expression of two $Fe^{3+}$-chelate reductases from yeast in transgenic tobacco resulted in fourfold increase in iron reductase activity and 50% increase in leaf iron content (Samuelsen et al. 1998). Constitutive expression of the *Arabidopsis* NA Synthase gene resulted in a twofold to fourfold increase in leaf iron content of tobacco plants, which grew faster and performed more efficiently under iron-deficient conditions (Douchkov et al. 2001). However, improving iron uptake alone is not sufficient, because of rate-limiting steps further in the pathway. On the other hand, the increase of NA synthesis may be a viable option, although co-supression has been observed in rice transformed with the barley *NAS* gene (Mori et al. 2001).

### *7.2.6 Mutation Inducing*

Another strategy to obtain improved genotypes for iron toxicity tolerance is mutation inducing. Gamma ray was used to generate a collection of rice mutant genotypes from the indica cultivar BR-7 "Taim" (Table 7.1). These mutants were screened for many abiotic stresses, including iron, aluminum, organic acids, and root morphology (Zimmer et al. 2003). Seven variables were analyzed on plants under iron stress: number of roots (NR), main root length (MRL), coleoptile length (CL), shoot length (SL), first leaf insertion (FLI), first leaf length (FLL), second leaf length (SLL). Mutant 6 showed one of the best relative performances being constantly among the three higher values in six of seven evaluated variables (NR, CL, FLI, FLL, SLL, and APL). It also showed the highest values in four variables (FLI, FLL, SLL, and APL), showing great potential as an iron-tolerant genotype. Mutants 4 and 7 were also promising, as both were in the top three values of relative performance in four of seven evaluated characters (FLI, FLL, SLL, and APL; CL, FLI, FLL, and APL, respectively). Mutant 26 was among the three higher values of relative performance in three of seven evaluated characters (NR, MRL, and CL). These mutants show promise for studying iron uptake and metabolism and are being further investigated.

## 7.3 Toxicity of Organic Acids to Irrigated Rice

### *7.3.1 Organic Acid Genesis in Flooded Soils*

Soil flooding decreases gas exchanges between air and soil, since the diffusion of gases in water is ca. 10,000-fold lower than that in the air. As a consequence,

oxygen supply to the soil is very slow and below microorganism needs. In this condition, facultative and obligatory anaerobic bacterial microorganisms proliferate and dominate the biological activity (Ponnamperuma 1972; Sousa et al. 2004).

In the absence of oxygen, biochemical processes responsible for the organic acid metabolism in flooded soils are anaerobic respiration and fermentation. In the anaerobic respiration, microorganisms use the energy released from organic carbon oxidation in their vital processes and from inorganic compounds (nitrate, oxides and manganese hydroxide, iron, and sulphate) such as electron receptors.

In fermentation, media organic compounds or byproducts of metabolic routes are used as donors and acceptors of electrons in the oxirreduction process. These organisms do not use an electron transport chain to oxidate NADH to $NAD^+$, but should work through an alternative form to use this energy and maintain a supply of $NAD^+$. Fermentation is characterized by a smaller generation of $CO_2$ and the formation of short chain and low molecular weight organic compounds. Despite being an inefficient way of breakdown, fermentation promotes the break of complex organic substrates, resulting in a series of substances, many of them transitory and not found in oxidized soils. Many of these substances have the potential of causing toxicity to irrigated rice, especially the short-chain organic acids, such as acetic, propionic, and butyric acids (Rao and Mikkelsen 1977). The anaerobic decomposition of organic compounds happens in successive steps involving different groups of microorganisms which convert complex molecules in simpler forms, those described in Fig. 7.3 (Silva et al. 2008). In the beginning of the process, there is a hydrolysis of organic polymers of plant origin (plant tissue components) into monomers (such as carbohydrates into glycids, lipids into long-chain organic acids, and proteins into aminoacids). This occurs because facultative or obligatory anaerobic microorganisms secrete extracellular enzymes, transforming complex compounds into simpler ones. These simple chain organic compounds are assimilated by these microbes and fermented intracellularly into short-chain organic acids, such as the acetic ($CH_3COOH$), propionic ($CH_3CH_2COOH$), and butyric ($CH_3CH_2CH_2COOH$) acids, in a process called acid formation. Following this process, there is the production of acetic acid, from organic acids with more than two carbons. This step is called ketogenesis and is regulated by anaerobic microorganisms that cannot convert acetic acid into $CH_4$ due to enzymatic limitations. At the end, $CH_4$ is formed from simple compounds generated by ketogenesis as well as formate, $H_2$, methanol, methyl amines, and $CO_2$.

The production of organic acids in flooded soils is directly proportional to degradable carbon availability. Thus, soils rich in organic matter or those soils in which organic residues are added close to the flooding condition tend to present higher production of organic acids. The organic acids can start to accumulate in flooded soils where organic residues have been deposited as soon as day one. Commonly, acid concentration is low at the first few days, reaching maximal values between 2 and 4 weeks of flooding (Sousa et al. 2002). Then, the acid concentrations decrease until stable and low values are found (Fig. 7.4). The peak of acid release varies as a function of soil characteristics, residue amounts, and the type of acid evaluated.

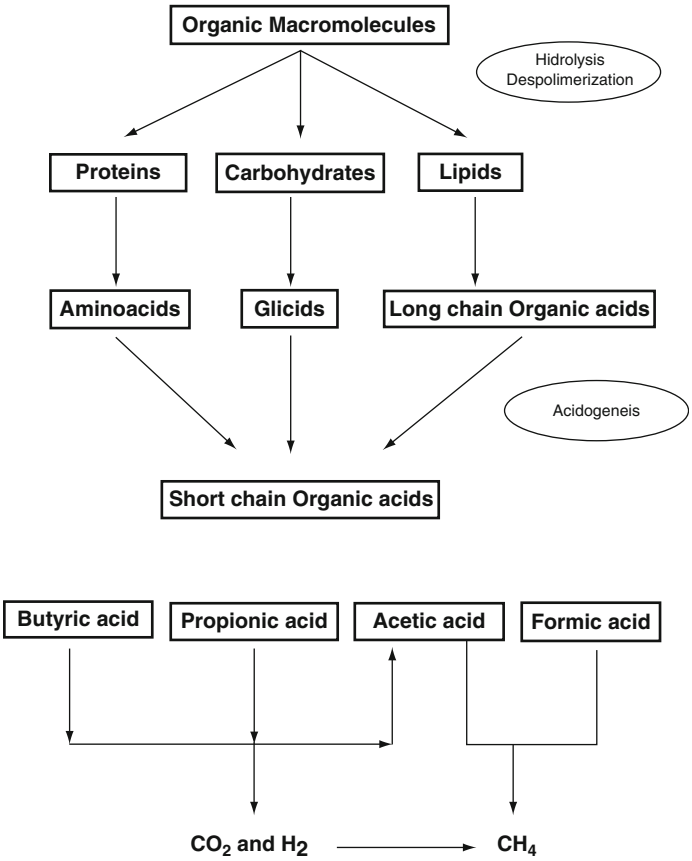


**Fig. 7.3** Scheme showing the degradation of organic matter into simpler compounds in flooded soils. Adapted from Silva et al. (2008)

### *7.3.2 Organic Acid Toxicity Symptoms*

The toxicity by organic acids in rice is observed at early stages of plant development, characterized by a lower germination percentage, lower radicle development, and lower plant height and weight (Sousa and Bortolon 2002). In cases of severe toxicity, plant growth injuries can reflect in other phases, leading to decreases in tillering ability, nutrient absorption, and grain yield (Camargo et al. 1993; Camargo et al. 2001). The higher toxic effect of organic acids occurs in the root system, and concentrations of 2.5 mmol $L^{-1}$ acetic, 1.25 mmol $L^{-1}$ propionic, and 1.00 mmol $L^{-1}$ butyric acid are capable of causing significant reductions on rice growth (Sousa and Bortolon 2002; Schmidt et al. 2007), as can be observed in Fig. 7.5.

The monocarboxylic acids (such as acetic, propionic, and butyric) alter the composition of organic acids on the plasma membrane, decreasing the ratio of

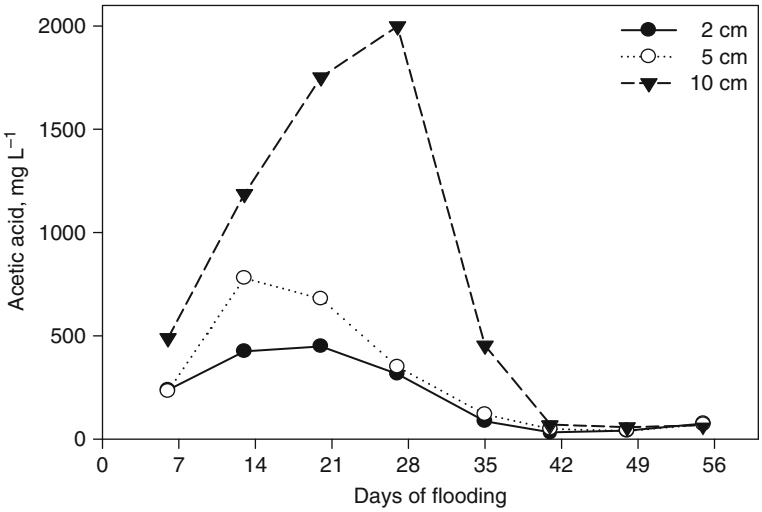


**Fig. 7.4** Acetic acid contents in the soil solution at three depth measures in a flooded Albaqualf, with ammending of ryegrass residues in amounts equivalent to 10 Mg $ha^{-1}$. Adapted from Sousa et al. (2002)

polyunsaturated acids, affecting an important property of the membrane such as selectivity and increasing solute leaking (Marschner 1995). Therefore, organic acids can harm the development of the crop, mainly by inhibiting root elongation and nutrient absorption (Takenaga 1995; Sousa and Bortolon 2002). Organic acids cause root cell division inhibition at the point of contact between root and acid (Armstrong and Armstrong 2001).

Critical levels of organic acid toxicity reported in the literature vary as a function of time of exposure of plants to organic acids, nutrient concentration and nutritive solution pH (Fortes et al. 2008), genotype (Kopp et al. 2008), and acid used, making the establishment of a standard toxic concentration difficult. A concentration of 4.7 mmol $L^{-1}$ of acetic acid causes 50% reductions on root growth of rice cultivar BRS-7 "Taim"(Sousa and Bortolon 2002). A similar result was observed with 1.7 mmol $L^{-1}$ propionic and 2.0 mmol $L^{-1}$ butyric acid (Schmidt et al. 2007). On the other hand, Kopp et al. (2007a) found that concentrations of 10.9 mmol $L^{-1}$, 5.6 mmol $L^{-1}$, and 5.3 mmol $L^{-1}$ of acetic, propionic, and butyric, respectively, were needed to achieve the same 50% reduction of root growth for cultivars BRS-7 "Taim" and SAIBAN.

Even with some differences among authors about the critical levels of organic acid toxicity in irrigated rice, there is a common view that acetic acid, although present in higher amounts in flooded soils, shows less toxicity than propionic and butyric. The increase in the number of carbons on the chain increases the degree of toxicity of the organic acid (Takijima 1964; Rao and Mikkelsen 1977). However, such difference is not so clear when one compares propionic and butyric acid (Schmidt et al. 2007; Kopp et al. 2007a).

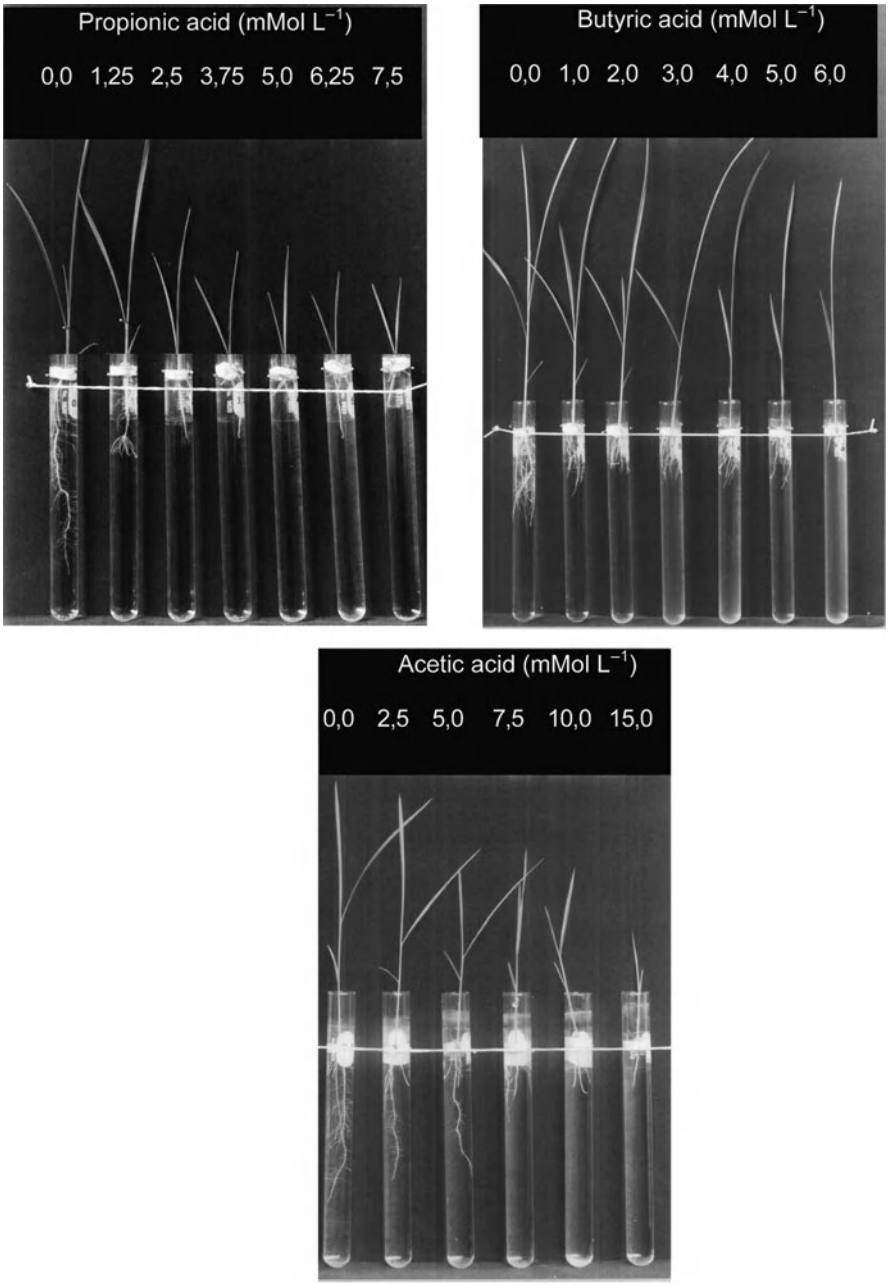


**Fig. 7.5** Rice plants subjected to different organic acid concentrations in nutrient solution for 13 days.

Studies developed in our group regarding organic acid tolerance in rice cultivars and mutants demonstrate that many distinct mechanisms do exist for tolerance to each of the major acids formed in the soil (acetic, propionic, and butyric). It was shown that genotypes tolerant to one acid do not necessarily tolerate the other two (data not shown). However, some genotypes show tolerance to more than one acid or even to the three of them. When these results are compared to studies where all three acids were added simultaneously to form the treatments (Wallace & Whitehand 1980), one observes that the proportion of tolerant genotypes is reduced.

In order to study the genetic variability for tolerance to organic acids in rice, a mutant population was screened (Zimmer et al. 2003; Kopp et al. 2007b, c, d). After cycles of generation advancing, 40 lines were obtained for genetic studies. Lines were divided in 25% tolerant to acetic and propionic and 27.5% tolerant to butyric acid. Also, some very sensitive lines were identified. These results suggest that the mutagen affect some genes related to organic acid response. In Oat, mutants obtained from a gamma Ray induction in the oat cultivar UFRGS 14, which is sensitive to organic acids (Kopp et al. 2006), were shown to vary regarding tolerance to these compounds. The evaluation of 30 mutant lines resulted in 23.3% tolerant genotypes. Further studies regarding mapping and inheritance of these genes are under way.

## 7.4 Conclusion and Perspectives

The genomic analysis of plant roots will enable us to better understand abiotic stresses and improve iron tolerance and/or accumulation as well as organic acid tolerance. Rice is the major staple food for over half of the world's population and understanding the major stresses affecting the rice crop will enable scientists to design better plants with better yields in order to feed the growing population and save the occupation of virgin areas today maintained as ecological reserves. Dealing with iron and organic acids is not a simple task, and a better understanding of the mechanisms by which plants absorb, transport, and store/process these compounds will allow better land use and management. Root genomics is likely to be among the major sciences in this century, since roots have been largely neglected despite its importance on the plant vs. environment interactions.

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# Chapter 8
# Genomics of Root Architecture and Functions in Maize

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**Contents**



## 8.1 Introduction

Twenty-first century agriculture will face formidable challenges to provide mankind with an appropriate level of food security while enhancing the sustainability and profitability of agricultural practices, lowering their environmental impact, and preserving the remaining biodiversity (Borlaug and Dowswell 2005). These challenges will be even more daunting in view of the increased unpredictability of weather patterns as a result of global climate change and the decreased availability of irrigation water required for mitigating the negative effects of drought (Pennisi 2008).

Among the major crops that feed mankind, maize is expected to become the most important by 2030, especially in view of the projected increase in the demand for feed in meat production. More severe and frequent droughts have been forecasted

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for regions where maize represents an important component in the human diet (e.g., tropical Africa and Northeast China) or for biofuel and livestock production (e.g., USA and Eastern Europe). In this challenging scenario, better knowledge of the genetic and functional basis of the processes regulating the development and plasticity of maize roots will allow for a more effective selection to improve yield potential while optimizing water- and nutrient-use efficiency (Guo et al. 2005b; Bohn et al. 2006; de Dorlodot et al. 2007; Osmont et al. 2007; Yu et al. 2007; Desnos 2008; Hochholdinger and Tuberosa 2009). Root traits have been shown to play a major role in the adaptive response of crops to drought and low nutrients (Tuberosa et al. 2003; Lynch 2007), and their selection has often been advocated to mitigate yield losses in crops exposed to water and nutrient deficits (Ludlow and Muchow 1990). This notwithstanding, breeders have largely neglected selecting for roots, not only for the demanding phenotyping but also for the difficulty in identifying a yield-effective ideotype and to effectively select the desirable root architectural features. Other factors that have traditionally discouraged root studies in field-grown plants are the low heritability of root features consequent to high soil heterogeneity and the need to utilize destructive approaches. Maize is no exception to the above.

As an alternative to root surveys in field-grown plants (Fincher et al. 1985; Beck et al. 1987), studies implemented under controlled conditions (e.g., hydroponics, aeroponics, pots) at an early stage facilitate the measurement of root characteristics in a large number of plants (Nass and Zuber 1971; Arihara and Crosbie 1982; Stamp and Kiel 1992; Landi et al. 1998; Sanguineti et al. 1998, 2006). Nonetheless, the unnatural environment in which roots grow and the early growth stage that is usually considered in such studies are major shortcomings that should be cautiously considered before extrapolating the results to field-grown plants. In maize, a significant, albeit weak, positive association was reported between seminal root traits in hydroponics and root-pulling resistance in the field (Landi et al. 2001). Additionally, seminal roots in maize play a prominent role in nutrient acquisition at the seedling stage and thus influence early vigor, a feature particularly relevant under conditions of zero or minimum tillage characterized by low agronomic input. The length and number of seminal roots may be particularly important in the acquisition of immobile nutrients such as phosphorus (Kaeppler et al. 2000; Zhu et al. 2005a, b, c, 2006; Lynch 2007). As the plant reaches flowering, the importance of seminal roots declines as compared to shoot-borne roots, commonly named adventitious nodal roots (Kiesselbach 1949; Hochholdinger et al. 2004b), which have been shown to positively affect grain yield in water-limited conditions (Duchoslav et al. 1989; Navara et al. 1993, 1994; Jesko 2001).

Maize roots show a high level of developmental plasticity in response to external cues (Hose et al. 2000; Ito et al. 2006), a clear example being provided by the interplay between abscisic acid (ABA) and ethylene in sustaining root elongation under conditions of water deficit which inhibit shoot elongation (Sharp and Davies 1985; Saab et al. 1990; Zhang and Davies 1990; Sharp 2002; Sharp et al. 2004; Spollen et al. 2008). Additionally, this plasticity insures the optimization between the allocation of photosynthates to the root and its capacity to (1) capture water and nutrients as a function of the prevailing soil conditions and (2) mitigate the negative

effects of adverse soil conditions. A clear example of the latter is provided by the development of aerenchyma in adventitious roots in response to water-logging conditions (Mano et al. 2005a, b).

Notwithstanding the important role of roots for optimizing maize yield (Bolaños et al. 1993; Hammer et al. 2009), the genetic factors that control root growth have only recently started to be unveiled with the use of mutants and, in some cases, their cloning (Taramino et al. 2007; Hochholdinger et al. 2008). As an example, the cloning of *rtcs* (*rootless concerning crown and seminal roots*) revealed its role in encoding an auxin-inducible transcription factor that controls the early events leading to the initiation and maintenance of seminal and shoot-borne root primordia (Taramino et al. 2007). Nonetheless, because the genetic basis of the variability of root architecture in cultivated maize is prevalently quantitative, the application of suitable genomics approaches is required to identify the relevant quantitative trait loci (QTLs). This, in turn, would enable breeders to apply marker-assisted selection (Varshney and Tuberosa 2007) for tailoring roots according to the ideotype perceived as optimal to maximize crop performance in the target environment (de Dorlodot et al. 2007; Tuberosa et al. 2007).

In this context, the present review surveys the main findings of QTL studies and other genomics approaches aimed at (1) dissecting the genetic basis of the variability in root architecture in maize and (2) investigating and interpreting the effects of this variability on yield and other agronomic traits.

## 8.2 QTLs for Root Architecture and Associated Traits in Maize

The maize root system includes embryonic primary and seminal roots and postembryonic shoot-borne and lateral roots (Hochholdinger et al. 2004b) which have different functions as development progresses. As an example, at flowering, shoot-borne nodal roots play a predominant role in extracting moisture from the more superficial portion of the soil horizon, while primary and seminal roots allow plants to access moisture more deeply stored and useful to avoid desiccation under drought conditions. However, a large root system does not guarantee a high yield as shown in the recurrent selection work in maize carried out at CIMMYT to improve grain yield under severe drought conditions (Bolaños et al. 1993) and by a study conducted testing families derived from the cross of inbred lines (B73 and Mo17) which differed in root characteristics at an early growth stage (Bruce et al. 2002).

A comparative analysis of the results of QTL studies in maize is facilitated by the availability of the UMC (University of Missouri, Columbia) reference map which has been subdivided into 103 sectors (bins) of comparable size (Davis et al. 1999). The boundaries of each bin are defined by flanking markers (RFLPs and SSRs) included in a public set of Core Markers (Gardiner et al. 1993; Davis et al. 1999). The UMC map reports over 15,000 loci and includes genes, probed sites, cytological breakpoints, and QTLs (Schaeffer et al. 2006). Because the bin

framework integrates over 130 independent map sets and includes all mapped loci stored in MaizeGDB (http://www.maizegdb.org), it has been used extensively for the comparison of QTL positions across genetic backgrounds (Lin et al. 1995; Khavkin and Coe 1997; Tuberosa et al. 2002b, 2003, 2005; Chardon et al. 2004; Sawkins et al. 2004; Schaeffer et al. 2006; Wang et al. 2006). Gramene (http://www.gramene.org) is another database that reports information on maize QTLs and allows for comparative searches of maize genomics data with other grasses (Ware et al. 2002). Importantly, the UMC map allows us to compare the map position of mutants (Neuffer et al. 1997) with that of QTLs, thus contributing relevant information for validating Robertson's hypothesis for a specific locus (Robertson 1985).

The first comparative analysis of root QTLs in maize (Tuberosa et al. 2003) highlighted the role of two major QTL regions (on bins 2.04 and 1.06) for their effects on root architecture and other traits, including grain yield, in different genetic backgrounds. In order to more accurately evaluate the effects of these two QTLs on root traits and grain yield, near isogenic lines (NILs) differing for the parental segment at these QTL regions have been developed (for bin 2.04 see Landi et al. 2005; for bin 1.06: Landi et al. 2010). The main results reported to date for these QTL regions are summarized hereafter while the results obtained with the NILs for bin 2.04 are reported in Sect. 8.3.1.

### *8.2.1 Effects of the QTL Region on Bin 2.04*

Lebreton et al. (1995) were the first to report the significant effect of bin 2.04 on root architecture using an $F_2$ population (81 plants in total) derived from the cross between Polj17 and F-2, two lines that were known to differ for root features, especially root-pulling force (RPF) at flowering, and also for the concentration of ABA in the leaf and xylem sap. For all but one of the detected QTLs, the additive effects for ABA concentration and RPF were concurrent. A remarkable correlation ($r = 0.84$) was found between the QTL effects for nodal root number and ABA concentration in the xylem sap. The QTL region on bin 2.04 also showed the strongest effect on leaf ABA concentration (L-ABA); this finding was confirmed by Tuberosa et al. (1998) using a mapping population derived from the cross between Os420 and IABO78, two lines widely different for drought tolerance and L-ABA (Tuberosa et al. 1994; Landi et al. 2001). It is worth noting that none of the major mutants impaired in ABA biosynthesis mapped in bin 2.04, a result that led Tuberosa et al. (1998) to postulate that the effect of the QTL on L-ABA might have been due to a primary effect on root size/architecture, hence on the water status of the plant, the major factor influencing the concentration of ABA in plant tissues (Quarrie 1991). Subsequent studies conducted to further characterize the effects of this QTL in the Os420 × IABO78 background have shown its marked influence on root architecture, root lodging, and grain yield but not on the water status of the plant (Giuliani et al. 2005b; Landi et al. 2007). Further details on the characterization of the bin 2.04 QTL are provided in Sect. 8.3.1. Additionally, the meta-analysis

conducted by Sawkins et al. (2004) has highlighted the effects of bin 2.04 on grain yield under conditions of water stress. Recently, the importance of bin 2.04 in controlling root features has been reported by Trachsel et al. (2009) in an RIL population derived from the cross between CML444 (drought tolerant) and SC-Malawi (drought sensitive) and tested for length of axile and lateral roots at 2, 5, 7, and 9 days after germination. In particular, a QTL region on bin 2.04 affected the elongation rate of lateral roots as well as the elongation and number of axile roots. Additional bins that affected root growth were also reported in bins 1.03, 1.04, 1.08, 2.05, and 7.04. Based on a comparative analysis of their results with those previously published, Trachsel et al. (2009) suggested that root growth at the juvenile stage can be predictive of root morphology at later developmental stages.

### *8.2.2 Effects of the QTL Region on Bin 1.06*

In an experiment conducted in hydroponics using 171 $F_3$ families derived from the cross Lo964 × Lo1016, several QTLs were shown to influence primary root length (R1L), primary root diameter (R1D), primary root weight (R1W), and the weight of the adventitious seminal roots (R2W) (Tuberosa et al. 2002c). Bin 1.06 was the chromosome region with the most sizeable QTL effects (LOD values of 14.7, 6.4, and 8.3 for R1D, R1L, and R2W, respectively). In order to investigate to what extent the QTLs influencing root growth in hydroponics may also regulate root growth in the field, a random sample of 118 (Lo964 × Lo1016) $F_3$ families were tested for root-pulling force (RPF) at flowering in replicated field trials (Landi et al. 2002). Out of the 30 bins with QTLs for RPF and/or number of brace roots, 15 (including bin 1.06) also harbored QTLs for root traits in hydroponics, i.e., a frequency much higher than what would be expected based solely on chance. Subsequent field trials conducted during two growing seasons to measure grain yield (GY) under well-watered (GY-WW) and water-stressed (GY-WS) conditions with the Lo964 × Lo1016 $F_3$ families revealed several QTLs whose peaks overlapped with those for root traits measured in hydroponics (Tuberosa et al. 2002c) and/or in the field (Landi et al. 2002). In particular, QTLs for R2W co-localized with QTLs for GY-WW and/or GY-WS in bins 1.03, 1.06, 1.08, 7.02, 10.04, and 10.07. At five of these six chromosome regions, an increased root weight was associated with a higher GY, a result more likely to be due to pleiotropy rather than linkage, in view of the number of independent chromosome regions involved and the consistency of their effects. Of all regions which concomitantly influenced root traits and GY, the strongest and most consistent effects were confined to a 10 cM interval on bin 1.06 that affected root features in both hydroponics and field conditions and GY under both WW and WS conditions. QTLs for root traits on bin 1.06 have also been reported in Polj17 × F-2 (Lebreton et al. 1995), B73 × Mo17 (Kaeppler et al. 2000), F288 × F271 (Barriere et al. 2001), and Z3 × 87-1 (Liu et al. 2008a). Additionally, it is worth noting that Hirel et al. (2001) reported a

major QTL for nitrogen-use efficiency and GY on bin 1.06, a finding which further highlights the importance of this region for GY.

### 8.2.3 *QTLs for Root Architecture of Maize Grown Under Environmentally Constrained Conditions*

Because drought is the major environmental factor curtailing maize yield (Duvick 2005), a number of reviews have already surveyed QTLs for roots in maize under water-limited conditions and their role in sustaining yield (Tuberosa et al. 2002b, c, 2003, 2007). Here, we summarize the main findings of the studies that have investigated QTLs for roots of maize grown under low temperature, low nutrients, flooding, or in the presence of root worms or in conditions that favor root lodging.

#### 8.2.3.1 Root QTLs at Low Temperature

The development of a vigorous, highly structured root system might be of major importance for growth at low temperature (Hund et al. 2008), especially in no-tillage systems, where low soil temperature becomes a major limiting factor. Genotypic differences in cold tolerance exist for the development of the root (Stamp 1984). QTLs controlling root tolerance to cold at early stages were studied in a set of 168 $F_{2:4}$ families of the Lo964 × Lo1016 cross derived from a corresponding set of $F_{2:3}$ families originally tested for root traits and tolerance to drought (Landi et al. 2002; Tuberosa et al. 2002b). Seedlings were grown at 15/13 °C and evaluated for shoot and root traits (Hund et al. 2004). The analysis of root weight, length, and diameter led to the identification of 38 QTLs, seven of which confirmed QTLs reported by Tuberosa et al. (2002b) for root traits in the same population evaluated in hydroponics at normal temperature. A locus on bin 5.07 for root growth at low temperature was also shown to influence cold tolerance at germination on the same mapping population (Frascaroli et al. unpublished results), thus suggesting that this QTL region plays an important role in controlling cold tolerance at different growth stages.

#### 8.2.3.2 Root QTLs Under Low Nitrogen Conditions

The work of Wiesler and Horst (1994) demonstrated that a deeper root system is essential in maize for utilizing nitrate in deep soils under field conditions and showed that N-efficient maize cultivars had longer roots and larger root surface areas.

An important aspect of maize productivity relates to the capacity of the plant to efficiently absorb soil nitrogen, store it in the vegetative organs, and relocate it

during kernel growth (Wang et al. 2004; Chun et al. 2005; Hirel et al. 2007; Coque et al. 2008). Although QTLs for nitrogen-use efficiency have been described and in some cases accurately characterized in terms of biochemical effects (Agrama et al. 1999; Hirel et al. 2001, 2007; Gallais and Hirel 2004), their possible effects on root architecture and functions remain to be duly investigated. Conversely, QTLs have been identified for root hair length and plasticity in response to low phosphorus, a nutrient that unlike nitrogen shows low mobility in the soil (Chassot and Richner 2002; Zhu and Lynch 2004). By enhancing soil exploration, root hairs play an important role in the uptake of phosphorus.

A paper-roll culture system was used to investigate root hair length (RHL), taproot length, root thickness, and root biomass in a RIL population derived from B73 × Mo17 (Zhu et al. 2005a, b). One QTL was associated with RHL plasticity, three QTLs with RHL under high fertility, and one QTL with RHL under low phosphorus. Six QTLs accounted for 53% of the total variation for seed phosphorus content among RILs. Root biomass plasticity was significantly correlated with RHL induced by low phosphorus, taproot length plasticity, and seed phosphorus reserves.

The only study that has extensively investigated root QTLs under different nitrogen levels was conducted by Liu et al. (2008a) using 94 RILs derived from the cross Z3 × 87-1, a hybrid widely grown in China. The lateral root length (LRL), axial root length (ARL), maximal axial root length (MARL), axial root number (ARN), and average axial root length (AARL) were evaluated under low N (LN) and high N (HN) conditions in a hydroponics system. Of the 17 QTLs that were detected by Liu et al. (2008a), 14 were located on chromosome regions where other authors had previously reported QTLs for root architectural features (Lebreton et al. 1995; Guingo et al. 1998; Landi et al. 2002; Tuberosa et al. 2002b; Hund et al. 2004; Mano et al. 2005a, b; Zhu et al. 2005a, b, 2006). Unexpectedly, among these 17 QTLs, no common loci were found under both LN and HN conditions for any root traits, one possible reason being that the RIL population for QTL detection in this study was very small. A major QTL on bin 1.06 (between *bnlg1025* and *umc2029*) for the AARL under LN explained 44% of the phenotypic variation and co-localized with previously described QTLs for grain yield under low nitrogen (Agrama et al. 1999; Bertin and Gallais 2001) and water-limited (Tuberosa et al. 2002c) conditions as well as for a number of root architectural features (Tuberosa et al. 2002b; Zhu et al. 2006; Landi et al. 2010). Other striking coincidences were identified on (i) chr. 8 between a QTL for LRL at HN (*umc1997*/*umc1724*) and the QTL for LRL at high phosphorus supply (*tpi5*/*umc07*) reported by Zhu et al. (2005b), and (ii) chr. 10, between a QTL for ARN (*umc2043*/*umc1061*) and a QTL for seminal axial root number (*pgamctg300*/*umc49b*/*umc44a*) reported by Hund et al. (2004).

#### 8.2.3.3 Root QTLs Under Low Phosphorus Conditions

Phosphorus (P) deficiency of soils can be a major yield-limiting factor in maize production, particularly in low-input agriculture and in developing countries. In maize, QTL studies have shown the importance of length and number of lateral

and seminal roots in the acquisition of phosphorus (Kaeppler et al. 2000; Zhu et al. 2005a, b, c, 2006; Lynch 2007; Hao et al. 2008). At low soil P concentration, plant growth is affected both by physiological factors inherent to the crop as well as by their interactions with the soil biota. Among the different species colonizing the soil, the role of mycorrhiza in nutrient uptake of crops remains largely unknown. Kaeppler et al. (2000) identified QTLs for growth at low P and response to mycorrhizal fungi in a (B73 × Mo17) RIL population. Three QTLs influenced growth and shoot weight at low P in the absence of mycorrhizae and one QTL on chr. 2 controlled mycorrhizal responsiveness. QTLs for root volume detected for the high-P treatment were not coincident with any of the QTLs detected at low-P concentration.

Following a study of root hair length in hydroponics under low P, Zhu et al. (2005a, b, 2006) identified a major QTL flanked by *npi409-nc007* on chr. 5. Chen et al. (2008) evaluated 241 (Ye107 × 082) $F_{2:3}$ families under normal phosphorus (50 kg P/ha) and low phosphorus (0 kg P/ha) conditions at two sites. A total of 30 and 45 distinct QTLs were shown to influence growth and P efficiency in the two sites. Three regions were found to influence relative root dry weight on bins 5.05 (*mmc0282-phi333597* interval), 5.06 (*umc1680-P5M1/c* interval), and 5.07 (*bnlg1346-bnlg1695* interval) at both sites. Each one of these QTLs explained 13–16% of the variation of relative root dry weight.

Another trait that has been suggested to influence P efficiency is root exudates (Hinsinger 2001; Jones and Hinsinger 2008). Root exudates such as acid phosphatases, organic acid, and $H^+$ compounds may help the mobilization of P from soils. QTLs for P uptake in bean were found to influence $H^+$ and total acid exudation from the root (Yan et al. 2004), two processes capable of mobilizing soil-bound P through soil P desorption or mineralization.

#### 8.2.3.4 Root QTLs Under Flooding Conditions

Root features also play an important role in tolerance to soil flooding or water logging (Ray et al. 1999; Mano et al. 2006a, b). The devastating flood of 1993 that hit most of the corn-producing area in the Midwest USA caused $20 billion damage, curtailing corn production by almost 30% and significantly raising the cost of corn-based goods. Clearly, the availability of hybrids more tolerant to the negative effects of soil anoxia caused by flooding would be beneficial for stabilizing corn production and farmers' income under such adverse conditions. A number of QTL studies have investigated root features in response to flooding and water-logging conditions (Mano et al. 2005a, b; Qiu et al. 2007). One of the major adaptations to soil flooding is the adventitious root formation (ARF) at the soil surface. QTLs for ARF were identified by Mano et al. (2005b) under flooding conditions in 110 $F_2$ plants derived from a cross between the dent line B64 with the tropical Caribbean Flint line Na4. The QTLs for ARF were located on bins 3.07, 3.08, 7.04, 7.05, and 8.05. At all QTLs, the Na4 alleles increased ARF. The comparison of ARF QTLs in the B64 × Na4 population with those in a B64 × teosinte (*Zea mays* ssp.

*huehuetenangensis*) population showed the consistency of the QTLs on chr. 8 (Mano et al. 2005a). *Zea mays* ssp. *huehuetenangensis* contributed all of the favorable QTL alleles for ARF, thus supporting the conclusions of Campos et al. (2004) concerning the value of mining genetic variation from outside cultivated maize to improve its root architecture and functions. On a similar line, QTLs for aerenchyma formation in roots, another important feature for adaptation to water logging, were identified by using an $F_2$ population generated from the B64 × teosinte (*Zea mays* ssp. *nicaraguensis*) cross (Mano et al. 2007; Mano and Omori 2008). Seedlings of *Zea mays* ssp. *Nicaraguensis* clearly formed aerenchyma in the cortex of adventitious roots in non-flooding conditions, whereas the maize inbred line B64 did not. Four QTLs for aerenchyma formation under non-flooding conditions were located on chr. 1 (*Qaer1.02-3* and *Qaer1.07*), chr. 5 (*Qaer5.09*), and chr. 8 (*Qaer8.06-7*); collectively, these regions accounted for 47% of the total phenotypic variance for aerenchyma formation (Mano et al. 2007). Additional QTLs for root aerenchyma under drained conditions have been described in a B73 × teosinte (*Zea luxurians*) population (Mano et al. 2008). Markers linked to QTLs for aerenchyma formation in drained soil conditions could be used to develop maize hybrids with increased flooding tolerance and greater yield stability under such conditions. Additionally, increasing aerenchyma formation might improve soil exploration for a given amount of dry matter invested in the root and might lower the metabolic cost for maintaining root functions. Root and shoot traits were investigated in two experiments to identify QTLs associated with water logging tolerance in an RIL population derived from the cross HZ32 × K12 (Qiu et al. 2007). Several QTLs for shoot dry weigh, root dry weight, total dry weight, plant height, and water logging tolerance mapped on chrs. 4 and 9. These QTLs were consistently detected in both experiments. Secondary and more trait- or environment-specific QTLs influencing water logging tolerance were also identified on chrs. 1, 2, 3, 6, 7, and 10.

#### 8.2.3.5 Root QTLs Under Lodging Conditions

The evaluation of the historical series of maize hybrids released during the past 60 years indicates that modern hybrids are considerably more resistant to lodging than older hybrids, particularly at high planting density, a condition that clearly accentuates this difference (Duvick 2005; Hammer et al. 2009). Lodging resistance is the result of two components: one acting at the level of the root and one at the level of the stalk. Mechanically, root lodging can be caused by strong wind, particularly following heavy rains and/or by a weakened root system following the attack of root worms. It has been shown that root architecture is a major factor influencing root lodging (Ennos et al. 1993). Although a rather large genotypic variability in root lodging has been reported in maize (Melchinger et al. 1986; Stamp and Kiel 1992), the low heritability and unpredictability of root lodging in the field coupled with the high cost required to carry out a large-scale evaluation using artificial devices (Guingo and Hebert 1997) have traditionally hindered the improvement of root lodging. Guingo et al. (1998) measured a number of root traits

for two seasons in 100 field-grown RILs from the cross between F-2 (root-lodging susceptible) and Io (root-lodging resistant). The only QTL that concomitantly influenced a number of root traits (adventitious root number at internodes 7 and 8, and root angle at internode 7) mapped in the *SC343B-C403* interval on bin 5.05. Epistasis was suggested by Guingo et al. (1998) as a possible factor responsible for the small number of QTLs detected in their study. In fact, the detection of epistatic interactions requires the evaluation of a much larger set of RILs (Beavis 1994, 1998). A major QTL affecting root traits and root lodging was described by Giuliani et al. (2005b) and Landi et al. (2005) on bin 2.04. The details for this QTL are reported in Sect. 8.3.1.

## 8.3 Production and Characterization of Near Isogenic Lines for QTLs for Root Traits

For a given genetic background, the accurate characterization of the effects of a QTL requires the production of near isogenic lines (NILs). Their evaluation will remove confounding effects on the investigated trait due to unlinked QTLs for which the parental lines of the RILs may harbour functionally different alleles (Tuberosa et al. 2002a). A common approach for QTL isogenization relies on the identification of $F_4$–$F_5$ plants still heterozygous at the target region and their selfing for a few generations (up to $F_8$–$F_9$) with continued selection for heterozygous plants before deriving the NIL pairs homozygous and contrasted for the target QTL interval (Tuinstra et al. 1997). Ideally, the isogenization of a QTL should be carried out using multiple plants tracing back to different $F_2$ plants. This procedure will insure a more solid evaluation of the effects of the QTL irrespectively of the genomic make-up at other QTLs which may influence the target QTL. Alternatively, each parental line of the original mapping population evaluated for discovering the QTL can be used as recurrent parent in a backcross scheme in which a single plant heterozygous at the QTL in question is utilized as donor of the alternative QTL regions; in this case, the isogenic lines are identified as backcrossed derived lines (BDLs; Alonso-Blanco and Koornneef 2000).

Regardless of the method used to obtain NILs, for a cross-pollinated species like maize that suffers greatly from inbreeding, the evaluation of the effects of a particular QTL on yield or other highly heterotic traits should preferably be carried out in a highly heterozygous background. This is usually achieved by crossing the pairs of NILs with suitable testers. Alternatively, the availability of BDLs allows for the production of near isogenic hybrids (NIHs) which, depending on the BDLs used as parents, are either homozygous or heterozygous at the target QTL region, while being heterozygous for most of the remaining portion of the genome (Giuliani et al. 2005b). Therefore, the evaluation of NIHs as compared to testcrosses allows one to accurately estimate for both additive and dominance effects of the target QTL.

Major drawbacks to a more widespread utilization of NILs are (1) the specificity of their effect to a particular genetic background and (2) the long time required for

their production. Commonly, several years elapse from the identification of a major QTL to its isogenization. This hurdle can be partially overcome with the production of introgression lines (ILs) involving parental lines preferably contrasted for the target trait. An IL library is a collection of backcrossed NILs that differ for a small portion (usually ca. 15–30 cM) of the donor genome. In maize, an adequate coverage of the entire genome requires ca. 80–100 lines. Once the ILs are made available, the fine mapping of any major QTL segregating in the original cross can be readily undertaken (Salvi and Tuberosa 2005). Additionally, the availability of a collection of ILs allows for testing the presence of epistatic interactions between specific QTLs. In maize, ILs have been produced in recent years (Szalma et al. 2007; Hao et al. 2009). At DiSTA, we have developed a library of ILs derived from B73 (recurrent parent) × Gaspé Flint (donor parent) to identify major QTLs influencing, among other traits, root growth and architecture (Ricciolini et al. 2008). Gaspé Flint is an extremely early accession which has been used for the identification and cloning of early flowering QTL alleles (Vladutu et al. 1999; Salvi et al. 2002, 2007). A preliminary evaluation of root features in the ILs has allowed Ricciolini et al. (2008) to identify four bins harbouring QTLs with major effects on root architecture. The fine mapping of one of these QTLs is underway as a prerequisite to its positional cloning.

The positional cloning of a major QTL (Salvi and Tuberosa 2005) requires the availability of (1) a large mapping population (>2,000 plants) derived from the cross of two NILs for the target QTL, (2) the genomic sequence for the physical interval spanning the QTL region (obvious starting points are the web-based genome browser of the target species or at least a contiged genomic BAC library), and (3) forward- and reverse-genetics approaches for validating the identity and testing the effects of candidate sequences (coding and non-coding). Only a handful of the root QTLs reported so far are suitable for a positional cloning approach, the main obstacle being the vast amount of resources needed to accurately measure roots in the thousands of plants to be phenotyped in any QTL cloning project. Additionally, positional cloning in maize is made more complex by its large genome size and functional redundancy. The availability of the annotated sequence of the entire maize genome will facilitate the identification of candidate genes and will streamline the relevant molecular procedures as well as a more effective comparative analysis with the sequence of other species (e.g., sorghum and rice).

### 8.3.1 *Effects of* Root-ABA1 *on Root Architecture, ABA Concentration, Root Lodging, and Grain Yield*

In maize, the most extensive evaluation of NILs for root architecture has been carried out for a major QTL originally mapped for its effects on L-ABA and other drought-related traits on bin 2.04 in the Os420 × IABO78 background (Tuberosa et al. 1998; Sanguineti et al. 1999). Following the production of NILs (Landi et al. 2005), this QTL was shown to influence root architecture, root lodging, grain yield,

and other important agronomic traits (Giuliani et al. 2005b; Landi et al. 2007). In this case, backcrossing was used to obtain pairs of BDLs contrasted for the parental chromosome segments at the target QTL, herein identified as (+/+) and (−/−) for their effects on L-ABA (Landi et al. 2005). When the BDLs were tested under both water-stressed (WS) and well-watered (WW) conditions, the effect of the QTL on L-ABA was fully confirmed. Subsequently, NIHs for the QTL were developed and field tested for 2 years under WW and WS conditions. Relative differences among NIHs for L-ABA and other morpho-physiological traits were not influenced by the level of water supplied through irrigation (Giuliani et al. 2005b). Interestingly, the QTL allele for high L-ABA markedly reduced root lodging. To further characterize the effects of the QTL on root features and L-ABA, plants of two pairs of BDLs were measured in soil columns at three water regimes. The results confirmed the effects of the QTL on L-ABA and highlighted a significant effect on several root architectural features, such as root angle, branching, number, diameter, and dry weight. Based on these and previously published results, Giuliani et al. (2005b) postulated a primary, constitutive effect of the QTL on root architecture and size which, in turn, affects root lodging and also L-ABA. Consequently, the QTL has been identified as *root-ABA1*. The QTL allele for a larger and more superficial root mass was associated with a higher concentration of L-ABA, a finding that Giuliani et al. (2005b) tentatively attributed to the fact that superficial roots are more likely to accumulate ABA that is subsequently translocated to the leaves via xylem flow.

Further validation of the effects of *root-ABA1* on grain yield was sought in different genetic backgrounds. For this purpose, the (+/+) and (−/−) BDLs were crossed with five and 13 inbred lines of different origin, thus originating two sets of testcrosses that were tested in replicated field trials carried out in Italy and China, respectively, under both WW and WS conditions (Landi et al. 2007). In Italy, testcrosses derived from (+/+) BDLs were confirmed as less susceptible to root lodging across both water regimes than the TCs derived from (−/−) BDLs (28 vs. 53%), but were also lower yielding under WS conditions (4.8 vs. 6.3 Mg $ha^{-1}$). The testcrosses derived from (+/+) BDLs were also less productive in China (6.8 vs. 7.5 Mg $ha^{-1}$; average of WW and WS conditions). In both sites, the lower grain yield of the testcrosses derived from (+/+) BDLs was prevalently due to a lower number of both ears/plant and kernels/plant. These results indicate that the (+) *root-ABA1* allele confers a lower susceptibility to root lodging but also a lower grain yield, especially in absence of root lodging. The yield loss associated with the (+) *root-ABA1* allele has tentatively been ascribed to the negative effect of an excessive accumulation of ABA on reproductive fertility (Landi et al. 2007). An alternative explanation might be that *root-ABA1* affects biomass production in response to drought stress. The fine mapping of *root-ABA1* is underway as a preliminary step to its positional cloning. If successful, the positional cloning of *root-ABA1* would allow us to verify whether pleiotropy or linkage is the prevailing cause of the multiple effects ascertained for *root-ABA1*. Additionally, the cloning of *root-ABA1* would pave the way to an accurate profiling of elite germplasm to survey the haplotypes present at the relevant sequence.

Microarray analysis of the transcripts of the contrasting BDLs has been used to investigate the effects of *root-ABA1* on the transcriptome and identify functional markers tightly linked to the QTL (Giuliani et al. 2005a). This study has led to the identification of a number of genes preferentially expressed in one of the two BDLs; among these genes, those which map within the supporting interval of *root-ABA1* are being considered as potential candidates for the QTL effects.

### *8.3.2 Identifying Candidate Genes for Root Features*

When a plausible cause–effect relationship can be postulated between a candidate gene and an overlapping QTL peak, then validation of the former could be attempted through genetic engineering and/or the screening of knockout mutants (e.g., knockouts, TILLING), thus avoiding the time-consuming procedures of the positional cloning approach. Additionally, the option of the candidate gene approach can be pursued even with no *a priori* availability of QTL data. In this case, association mapping through sequencing or EcoTILLING approach carried out on a suitable and sufficiently large panel of accessions provides clues on the association between haplotype variation of the candidate sequence and phenotypic variation for the targeted trait. In view of its very low linkage disequilibrium, maize is particularly suited for an association mapping approach to validate the role of candidate sequences. A compelling example of the power of this approach in maize has been provided by Salvi et al. (2007) through the validation of the role of a 2.3 kb non-coding sequence that positional cloning in a biparental background had highlighted as the causative agent of *Vgt1*, a major QTL for flowering time.

One merit of the candidate gene approach is that candidates can be identified on species other than the one being targeted. A clear example in this direction is offered by several studies conducted in the model species *Arabidopsis* (Scheres and Wolkenfelt 1997; Maggio et al. 2001; Flavell 2005; Malamy 2005; Reymond et al. 2006; Ortega-Martinez et al. 2007; Dello Ioio et al. 2007, 2008; Gonzalez et al. 2009; Iyer-Pascuzzi et al. 2009) and rice (Ismail et al. 2007; Negrao et al. 2008). In these cases, due appreciation should be given to the fact that the morphology and functions of the roots of these species, particularly *Arabidopsis*, are considerably different from those of the maize root. Nonetheless, it is possible that certain core functional/morphological features of root development (e.g., signaling cascades, cell elongation, growth and density of root hairs) may have to a large extent been conserved across species.

The value of using *Arabidopsis* to elucidate the genetic and functional basis of root growth has been shown by testing the possible role in root elongation of the sucrose-splitting enzymes, sucrose synthase and invertase (Sergeeva et al. 2006). Several QTLs affected both invertase activity and root length. The fine mapping of a major QTL for root length revealed consistent co-location with the locus for invertase activity containing a gene coding for a vacuolar invertase. The role of this

invertase gene in root elongation was confirmed by the analysis of a functional knockout line. Another area worthy of exploration relates to the mechanisms regulating the level of gene expression in the root. Also in this case, the model species *Arabidopsis* has provided useful insights. Although several plant microRNAs (miRNAs) have been shown to play a role in plant development, a study in *Arabidopsis* has shown the effect on the root phenotype due to a reduced expression of a miRNA (Guo et al. 2005a). *Arabidopsis thaliana* miR164 was predicted to target five NAC domain-encoding mRNAs, including NAC1, which transduces auxin signals for lateral root emergence. The results of this landmark study indicate that auxin induction of miR164 provides a homeostatic mechanism to clear NAC1 mRNA to down-regulate auxin signals; they also show the value of using *Arabidopsis* as a model for elucidating the complex molecular mechanisms regulating an important feature of root growth. Genome-wide bioinformatic analysis of full-length cDNA databases in *Arabidopsis* has allowed Ben Amor et al. (2009) to show that the adaptive response of root growth to abiotic stress was controlled by a long non-protein coding RNA (npcRNA), an emerging class of riboregulators which either act directly in this long form or are processed to shorter miRNA and siRNA (short interfering RNA). A number of npcRNAs were antisense to protein-coding mRNAs, suggesting their cis-regulatory roles. Ben Amor et al. (2009) proposed npcRNAs as candidate regulators to adapt root growth and development to soil biotic and abiotic interactions. Nonetheless, the candidate gene approach suffers from several notable shortcomings which might make its application risky, particularly with inherently complex traits which are likely to be more "buffered" from a functional standpoint and, as such, less likely to unequivocally show the effects of allelic variation at the candidate locus.

## 8.4 "Omics" of Maize Root Development and Functions

The identification of suitable candidate genes can be facilitated by exploiting platforms that allow us to profile in a high-throughput fashion the transcriptome (Schnable et al. 2004; Giuliani et al. 2005a; Guo et al. 2006), proteome (Hochholdinger et al. 2004a, 2005; Wen et al. 2005; Sauer et al. 2006), and metabolome (Steuer et al. 2003). It should be noted that while microarray platforms allow for the simultaneous analysis of tens of thousands of transcripts in a single experiment, or even the entire genome when the relevant sequences are available, proteomics (Liu et al. 2006) and metabolomics (Fernie and Schauer 2009) can indirectly report changes occurring in only a tiny portion of the genome. Moreover, proteomics is often unable to detect the changes in gene products (e.g., transcription factors) that, despite their low level, can play an important role in root growth and its response to environmental constraints.

Bruce et al. (2001) were first to deploy a high-throughput approach to investigate the root transcriptome in two maize lines characterized by contrasting root features. Among the 13,500 cDNA fragments that were analyzed at two growth stages, 69

showed a twofold or greater difference between the lines at both samplings, suggesting a relationship between these genes and root anchorage traits.

Because maize roots are composed of different tissues and cell types, each with its own peculiar signature at the transcript, protein and metabolic level, physical separation of such cell types can greatly increase our capacity to identify the specific functions of genes whose activity determines the specificity of root architectural features. An important breakthrough in this direction has been made possible through the introduction of laser-capture microdissection (LCM; Schnable et al. 2004; Balestrini and Bonfante 2008; Nelson et al. 2008), which allows for the accurate isolation of a wide variety of cell types from complex organs comprising different cellular types such as the root tip. Transcript profiling of LCM-derived samples of pericycle and root cap cells in the differentiation zone of primary roots has unveiled an unsuspected level of functional complexity that would otherwise have gone undetected (Woll et al. 2005; Jiang et al. 2006; Hochholdinger et al. 2008).

Transcriptome studies are particularly suited to investigate the adaptive response of maize roots to environmental cues as evidenced by Liu et al. (2008b) in their study to investigate the effects on gene expression of local nitrate-induced lateral root formation in maize. These results showed that local nitrate application induced the expression of genes related to nitrate uptake and assimilation, sugar transport and utilization, and cell division and expansion. A similar approach was used by Spollen et al. (2008) to elucidate the mechanisms underlying the adaptation of maize roots to low water potential in the elongation zone of maize primary roots grown under well-watered and water-deficit conditions. This study revealed that the response to water stress in different regions of the maize primary root involves different signaling and metabolic response mechanisms. It is worth noting that the largest functional categories of differentially expressed transcripts were those related to reactive oxygen species (ROS) and carbon metabolism in root tips and membrane transport in the elongation zone (Spollen et al. 2008). Microarray profiling of roots under low-oxygen conditions typically encountered under flooding conditions has shown significant alterations in the expression of 39 miRNAs (Zhang et al. 2008), several of which targeted transcription factors that were also induced upon submergence of the maize roots. Other target genes were related to carbohydrate and energy metabolism, and ROS removal, suggesting that submergence-responsive miRNAs regulate the adaptive response of maize roots post-transcriptionally.

New insights into the regulation of maize root development have also been contributed by proteome profiling studies conducted with complete roots (Hochholdinger et al. 2004c, 2005; Liu et al. 2006; Sauer et al. 2006; Hoecker et al. 2008) or targeting more defined sub-cellular portions (Hachez et al. 2006; Zhu et al. 2006, 2007) of maize roots. Proteome profiling in the elongation zone of the primary root identified a number of cell wall proteins (CWPs: e.g., endo-1,3;1,4-β-D-glucanase and α-L-arabinofuranosidase) involved in cell wall metabolism and cell elongation that had not been previously described in maize (Poroyko et al. 2007; Zhu et al. 2007). Targeting specific cell types via LCM in the primary root of

the mutant *lrt1* which is suppressed in lateral roots initiation, Hochholdinger et al. (2004c) demonstrated the influence of lateral roots on the proteome composition of the maize primary root. Additional comparative work of the proteome profiles of primary roots from the wild-type and the *rum1* mutant (also suppressed in lateral root formation) suggested the involvement of post-transcriptional mechanisms in regulating the mutant phenotype (Liu et al. 2006). Using LCM and combining microarray profiling with suppression subtractive hybridization, EST sequencing, and proteomics, Dembinsky et al. (2007) have identified pericycle-specific genes that appear to be related to the specification of this root cell-type and in lateral root initiation.

## 8.5 Conclusions and Challenges Ahead

As shown by this review, genomics allows us to partially dissect the genetic and functional complexity governing root architecture in maize and its plastic response to environmental cues. On an adaptive basis, the comparison of transcriptome and proteome profiles of roots exposed to water deficit (Zhu et al. 2007), water logging (Zhang et al. 2008), low phosphorous (Li et al. 2007), and low nitrogen (Liu et al. 2008b) has highlighted genes and proteins that might have an adaptive value under such adverse conditions (Bramley et al. 2007), offering new avenues for more targeted breeding activities aimed at mitigating the negative effects of environmental constraints. It is becoming increasingly clear that the response of plant genomes to environmental stress generates both novel genetic and epigenetic (e.g., methylation) polymorphisms that may increase phenotypic diversity and plasticity to abiotic stress (Johannes et al. 2008; Zhang 2008; Chinnusamy and Zhu 2009). Deep sequencing of cDNA libraries of root cell types will produce extensive EST databases and unigene sets to identify candidate genes while providing valuable markers for functional maps (Lister et al. 2009). High-throughput genomic profiling based on the detection of single nucleotide polymorphisms (SNPs) has vastly improved our capacity for allele mining (Ganal et al. 2009; Waugh et al. 2009), a key feature for optimizing the survey of natural variation and the application of association mapping for complex traits (Weber et al. 2008).

From an architectural standpoint, the cloning of major QTLs will eventually shed light on the genetic mechanisms governing the quantitative variability of root structure and its influence on major functions. In this respect, new insights will derive from a better understanding of the role of miRNAs on the modulation of gene expression (Sunkar et al. 2007; Ding et al. 2009). Recent experiments have highlighted the importance of RNA interference for the regulation of the expression of genes and QTLs (Guo et al. 2005a; Lukens and Zhan 2007). From an applicative standpoint, the main challenge remains how to tangibly integrate into extant breeding programs the deluge of molecular information generated through genomics and the "omics" platforms. An equally challenging and limiting factor is our capacity to accurately phenotype roots on the massive scale that genomics studies

usually require (Armengaud et al. 2009). High-throughput phenotyping platforms (Granier et al. 2006; Rajendran et al. 2009; see also the "Plant Accelerator" at http://www.plantphenomics.org/TPA) coupled with non-destructive, advanced technologies promise to alleviate the tedious work of measuring roots, thus opening up new opportunities to deploy more powerful mapping approaches such as nested-associated mapping (NAM; Yu et al. 2008).

The need and urgency to fill the genotype-to-phenotype gap (Yano and Tuberosa 2009) has never been more evident than with the study of root architecture, particularly under drought conditions (Tuberosa and Salvi 2006). The limitations inherent to quantitative trait dissection suggest that only a fraction of the available genotypic variability will be accessible and amenable to a more direct manipulation via marker-assisted selection. Even though positional cloning may become a reality for a handful of major QTLs governing root architecture, the multitude of minor QTLs that control variability in root features will remain undetected even with the most accurate phenotyping platforms and sophisticated statistical approaches. Genome-wide selection bypasses QTL identification (Bernardo and Yu 2007; Bernardo 2008, 2009; Heffner et al. 2009). Nonetheless, also genome-wide selection relies on accurate phenotyping which is often considered the main limiting factor for the dissection of quantitative traits.

Growing attention is being devoted to the opportunities offered by modeling in order to expand our capacity to predict the effects that specific environmental (e.g., water and nutrient availability) and genetic (e.g., QTL effects; Tardieu 2003; Welcker et al. 2007; Collins et al. 2008; Hammer et al. 2009) variables might have on plant growth and final yield. Crop modeling has also the potential to help resolving genotype × environment interactions as well as the genetic basis of traits' plasticity (Chapman et al. 2003; Reymond et al. 2004; Cooper et al. 2009). For this approach to be effective, crop models that are capable of predicting yield differences among genotypes in a population under various environmental conditions are needed (Tardieu 2003; Hammer et al. 2005, 2006; van Eeuwijk et al. 2005; Cooper et al. 2007). The ultimate goal of the modeling approach is to empower an *in silico* selection able to pinpoint the combinations of the desirable alleles at the target loci, including those that dictate root growth and its morphology, thus providing clues on the desired root phenotype. Clearly, integrative and interdisciplinary approaches will be instrumental to advance our understanding of root growth and, eventually, effectively exploit marker-assisted selection and genetic engineering to tailor root architecture in maize for improving yield and its sustainability.

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# Chapter 9
# Phenotyping for Root Traits and Their Improvement Through Biotechnological Approaches for Sustaining Crop Productivity

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**Contents**



## 9.1 How Did the Roots Evolve?

The general structure and function of roots and shoots are so different that the two organs are often conveniently separated for the purposes of research. Functionally, roots absorb water and nutrients, and anchor the plant, while shoots photosynthesize and transpire and are the site of sexual reproduction (Groff and Kaplan 1988). The exact time when root started appearing has been difficult to ascertain, and the fossil records are also less helpful for roots unlike shoots. It is possible that delicate structures such as root caps, root branches, etc. were not properly preserved in fossil remains (Gensel et al. 2001). Evidences suggested that root-like structures appeared sometime during the early Devonian period (Elick et al. 1998). Although the early fossils did indicate the possibility of a root structure positioned to anchor the shoot

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firmly, their role in water- and nutrient-absorption was not clear (Raven and Edwards 2001). Plants colonizing land must have faced powerful evolutionary pressures, which must have forced the roots to increase the absorptive surface to match the development of photosynthetic organs (Brundrett 2002).

In the present scenario, with the continued emission of green house gases, a definite change in the weather, both locally and globally, is expected. Most predictions suggest that this climate change is inevitable and would lead to a significant alteration in the pattern and distribution of rainfall in the warmer world (IPCC report 2007). Hence, water shortage would be the most predominant constraint for achieving potential productivity of crop plants, especially in tropical regions.

## 9.2 Why Are Roots Important for Crop Productivity?

Over two-thirds of the world's human population consumes rice and wheat as staple cereals, which are predominantly grown under irrigated conditions. With the changing scenario, it would be difficult to produce the required cereals through irrigated ecosystems. Furthermore, almost all the pulses, oilseeds, and other crops are cultivated in dry land conditions, where water is the major constraint. Dependence on dry land agriculture is inevitable in arid and semi-arid tropical parts of the world. Ironically, these areas are the most populated locations in the world! Because of the demand from the domestic and industrial sectors, neither expanding area for agriculture nor finding more water for irrigation would be possible. Therefore, increasing the productivity per unit of available water appears to be the only plausible strategy for achieving food security.

Enhancing productivity in the resource-poor dry land conditions is a formidable challenge. Conserving resources through management practices and engineering plants for superior extraction of these resources coupled with an increased efficiency of resource utilization deserve emphasis. Though resource conservation through management practices are equally important, development of superior resource use efficiency as a seed-based technology always has greater acceptance and adaptability.

Roots are essential for higher plants for several important reasons. The firm anchorage of the plant in their soil substratum and the absorption and effective supply of water and nutrients to the shoot are the most important roles of the root system. Furthermore, a number of plant growth hormones, especially cytokinins and ABA, originate in roots, thus having significant influence on growth and development of plants. Ecologically, roots play a pivotal role in weathering of rocks, leading to the formation of soil. A mat-like network of root system prevents soil erosion as well. The evolution of the symbiotic association between roots and microbes such as Rhizobia, Micorhiza, etc. represents yet another spectacular feature of plant root system. From the survival and crop productivity point of view also, roots have a greater role to play. Water mining from deeper soil profiles is considered as one of the important adaptive strategies evolved by plants to

survive water-scarce conditions. Having realized the importance of root traits in crop growth and productivity, improving root traits is worth an effort.

In this chapter, we make an attempt to identify a few root-related traits and review the information describing the relevance of root traits in determining crop growth and productivity, especially under water-limited conditions. Since the major emphasis is on breeding for root traits, we also review the genetic variability in root traits and describe suitable methodology for the assessment of variations in root traits. In the present scenario, greater success in crop improvement can be achieved only through a trait-based approach. Introgression of complex traits is best achieved either by a well-focused molecular breeding strategy or through transgenic technology. A better understanding of the basic mechanisms of root growth and development is necessary for these modern biotechnological approaches to become successful.

## 9.3 Molecular and Hormonal Regulation of Root Growth

In both the dicot and monocot plant species, the short-lived delicate roots perform the function of absorption, while the more long-lived roots help in anchoring the plants. The basic features of root development has been analyzed by dividing the root tip into different parts such as root cap, the meristematic zone, elongation zone, and maturation zone. Root growth occurs due to the division, elongation, and differentiation of the root apical meristematic cells present in the root tip. The lateral growth of roots occurs only after the complete elongation of apical meristem and at a distance away from the root tip (Malamy and Benfey 1997). The pattern of the root growth is strongly controlled by both external and internal factors. While external factors such as soil structure, availability of water and nutrient, etc. determine root growth and patterning, the internal factors are predominantly under hormonal control, which determine the plant's ability to respond to the external stimuli. The internal control of root growth by genes and their regulatory network in root development is partly examined in *Arabidopsis* through global gene expression studies. Many mutants that affect root development have also been identified and characterized, which has led to a clear understanding of the genetic mechanisms of root development (Schiefelbein 2003; Casimiro et al. 2003; Casson and Lindsey 2003; Inukai et al. 2005). These efforts resulted in the discovery of a large number of structural and regulatory genes. Several of the regulatory genes, also referred to as Transcription factors (TFs), have been cloned and characterized and their functional relevance clearly demonstrated. A few of the important genes and their regulatory functions are summarized in Table 9.1.

Further, plant roots show an impressive degree of plasticity in adapting their branching patterns to the ever-changing growth conditions. The adaptation ability depends upon the interaction between hormonal, developmental, and environmental signals. Root growth and development is also influenced by hormones. Research reports accruing in the recent years point towards auxin as one of the prominent

**Table 9.1** Some important transcription factors (TFs) and their role in root growth in plants

| Transcription factor | Phenotypes | Reference |
|---|---|---|
| SLR/IAA14 | Blocks lateral root formations in *Arabidopsis* | Fukaki et al. (2002) |
| CRL1 | Encodes for protein family that govern asymmetric leaves/lateral organ boundaries. Positive regulator for crown and lateral root formation | Inukai et al. (2005) |
| ARL1 | Encodes lateral organ boundaries (LOB); adventitious root formation | Liu et al. (2005) |
| NAC1 | More lateral roots | Xie et al. (2000, 2002) |
| Class III HD-Zip | Promote the meristematic activity, positive regulators of lateral root formation | Hawker and Bowman (2004) |
| SCR-SCARECROW SHR-SHORT-ROOT | Auxin responsive: organization and quiescent center cells and root cap | Wysocka-Diller et al. (2000), Gao et al. (2004), Helariutta et al. (2000) |
| Alfin1 | Over expression enhances root growth under normal and saline conditions in Alfalfa | Winicov (1993, 2000), Bastola et al. (1998), Winicov and Bastola (1999) |
| OsRAA1 | Root development in rice initiation and growth of adventitious roots | Ge et al. (2004) |
| $Ca^{2+}$-dependent protein kinase1 (CDPK1) | Regulates diverse processes including root growth | Ivashuta et al. (2005) |
| CAP2 encoding APETALA2 (AP2) | Over expression of chickpea CAP2 caused drastic increase in the number of lateral roots | Shukla et al. (2006) |
| HARDY (AP2-family) | Better root growth and drought tolerance | Karaba et al. (2007) |
| ARABIDILLO 1 and 2 | Armadillo-related β-catenin-like proteins Over expression increased lateral root formation | Coates et al. (2006) |
| QHB (QC SPECIFIC Homeodomain) | Maintenance of root meristem by inhibiting the differentiation of the adjacent initial cells | Kamiya et al. (2003) |
| MYB77 | Controls lateral root growth through interaction with Auxin response factor (ARF) | Shin et al. (2007) |
| KNAT6 | Member of the knotted-like (*KNOX*) gene family. Prevent production of supernumerary lateral roots | Dean et al. (2004) |

internal controlling factors of root growth (Xu et al. 2005; Lucas et al. 2008). The process of root development can be divided into two successive stages: lateral root initiation and lateral root development/emergence. Auxin controls the emergence of lateral root primordia and also helps in the growth and development of lateral roots (Bhalerao et al. 2002; Casimiro et al. 2001; De Smet et al. 2007; Fukaki et al. 2007). There are experimental evidences to show that the number of lateral roots can be altered either by application of auxin or perturbation of internal auxin levels (Blakely

and Evans 1979; Blakely et al. 1988). The hormone is synthesized mainly in the young apical tissues and then transported downwards to different parts, including roots, by a polar transport system (Muday and DeLong 2001). A number of auxin influx carriers (e.g., AUX1 and LAX gene family) and efflux carriers (e.g., PIN gene family) have been characterized (Bennett et al. 1996; Friml et al. 2002; Reinhardt et al. 2003) and relevance of such auxin-related genes in root development has been demonstrated. For example, *Arabidopsis* mutant called *pin-formed* (*pin1*) fail to establish endogenous auxin gradient and show development disorders in root (Okada et al. 1991; Benková et al. 2003). A gene similar to *Arabidopsis PIN1* has been identified in rice (*OsPIN1*), which, through transgenic approach, has been implicated for altering tiller number and adventitious root development in rice (Xu et al. 2005).

Though a clear implication of auxin is seen in the root growth and development, understanding of the molecular basis of this regulation is not complete (Weijers and Jurgens 2005). However, investigative evidences accruing in the literature have provided significant lead towards understanding the response of root growth to auxin in plants. Transcription factors (TF) called auxin-response factors (ARFs) bind to auxin response elements (AuxREs) in the promoters of auxin-response genes to mediate auxin-induced responses (Ulmasov et al. 1997). The auxin receptor TIR1 (F-box protein), which acts by mediating the degradation of AUX/IAA (AUXIN-RESPONSIVE PROTEIN/INDOLE ACETIC ACID INDUCED PROTEIN) repressor is the most important member involved in the auxin response process promoting the lateral root initiation (Gray et al. 1999, 2001). The F-box gene called CEGENDUO (CEG) negatively regulates auxin-mediated lateral root formation, which is expressed abundantly in vascular tissues of the primary root and is induced by auxin (Dong et al. 2006).

Interaction of growth regulator jasmonate with auxin to regulate lateral root formation has been recently reported (Sun et al. 2009) by characterizing an *Arabidopsis* mutant called jasmonate-induced defective lateral root1 (jdl1/asa1-1). The JDL1 encodes the auxin biosynthetic gene ANTHRANILATE SYNTHASE alpha1 (ASA1), which is required for jasmonate-induced auxin biosynthesis and affects auxin transport (Sun et al. 2009). Jasmonate also has a role in the attenuation of auxin transport in the root and the fine-tuning of local auxin distribution in the root basal meristem.

Cytokinin suppresses the growth of roots as reported in Arabidopsis (Werner et al. 2001) by reducing the size and cell division of roots. The roots of cytokinin-deficient (AtCKK1) plants were larger than those of wild-type, suggesting that the hormone inhibits root growth. Although most studies have reported genes that are directly associated with auxin in root development, a few indicate auxin-independent mechanisms. For instance, a novel gene called ALF4, which appears to be localized in the nucleus, was demonstrated to be required for lateral root formation (DiDonato et al. 2004). The ALF4 functions independent of auxin signaling and has a role in maintaining the pericycle in the mitotically competent state required for lateral root formation.

Most other plant hormones seem to have an indirect effect on root growth through their independent effects on auxin synthesis, transport, and distribution.

For instance, ethylene regulates growth through effects on auxin biosynthesis and auxin distribution through altered transport. ABA, an otherwise growth-retarding hormone, promotes root growth possibly by inhibiting ethylene production (Saab et al. 1990; Sharp 2002).

Genomic approaches have therefore provided immense information about the array of structural and functional genes involved in various aspects of root growth, development, and water and nutrient uptake. Use of these genes in overexpression studies would help validate the utility of these in root trait improvement.

## 9.4 Functions of Root in Uptake of Water and Nutrients

Other than anchorage, the next important function of roots is to take up water and nutrient. This trait of roots enables the plants to tide through different environmental conditions. Terrestrial plants are constantly exposed to an impinging heat load because of the incident solar radiation. To cope with this heat load and to maintain the canopy cool, plants transpire enormous amount of water. Plants recycle over half the amount of global precipitation per annum (Chahine et al. 1992). Hence, the roots must be able to extract water from the soil and supply it to the plant to match the evaporative demand of the canopy.

Vascular tissues and guard cells are mainly involved in conducting water and controlling the transpiration stream. During this, water has to flow in and out of the cells. This flow of water can be across cell walls (apoplastic path), between cells across plasmodesmata (symplastic path), or traversing cell membranes (transcellular path). A better understanding of the conductance of living cells has come from the discovery of a class of water channel proteins called "Aquaporins" (Agre et al. 1998). These are proteins embedded in the cell membrane and regulate the flow of water. These aquaporins are integral membrane proteins belonging to a family of major intrinsic proteins (MIP) that form channels in the membrane for water movement. More than 50% of the water moving across plant cells would traverse aquaporins.

In plants, aquaporins are divided into four subfamilies:

1. Plasma membrane intrinsic protein (MIP)
2. Tonoplast intrinsic protein (TIP)
3. Nodulin-26 like intrinsic protein (NIP)
4. Small basic intrinsic protein (SIP)

However, all the aquaporins have six membrane spanning domains with highly conserved Asn-Pro-Ala motif.

Aquaporins may be involved in a large number of physiological functions in plants such as response to drought or salinity, mineral nutrition, transpiration, cell elongation, etc. (Maurel and Chrispeels 2001). The discovery of aquaporins has showed the importance of membranes in plant–water relations. Further, aquaporins

serve as spatial markers to explore the flow of water and solutes that play a phenomenally important role throughout plant development.

Besides enhancing water uptake, aquaporins also contribute significantly to the total hydraulic conductivity of the roots. A significant reduction in water flux through membranes in the presence of $HgCl_2$ and its reversal with the removal of mercury by an excess of mercaptaethanol provided the initial proof to the involvement of aquaporins to the hydraulic conductivity of roots in tomato (Maggio and Joly 1995). Although transpiration pull sufficiently explains water uptake and distribution in plants, the hydraulic conductivity, the ease with which water moves through the roots, is an equally important factor. The fact that hydraulic properties of roots vary with plant species and environmental conditions has been well-known from a very long time (Brewig 1937; Brouwer 1954). Several factors influence the hydraulic conductivity of plant, viz. number of roots that are absorbing water (Vandeleur et al. 2005), nitrate nutrition (Radin and Boyer 1982), and ABA (Hase et al. 2000).

During evolution, plants have also optimized hydraulic conductivity to enhance their chances of survival under dry and harsh conditions. The evidences to this view were provided recently by Zhao et al. (2005) using wheat lines with different ploidy. They clearly demonstrated that root hydraulic conductivity significantly increased as ploidy level increased during wheat evolution. Since hydraulic conductivity was positively related to plant biomass, the authors opined that increasing water flux into the shoot would enhance photosynthetic efficiency leading to an increase in water use efficiency.

## 9.5 Nutrient

The proper development of roots at all stages will have profound effects on root system architecture as well as nutrient acquisition. The development of roots is particularly sensitive to the changes in the internal and external concentrations of nutrients. Recent information points to the existence of nutrient-specific signal transduction pathways that interpret the external and internal concentrations of nutrients to modify root development. Progress in this field has led to the identification of regulatory genes that play pivotal roles in nutrient-induced changes in root development (Lopz-Bucio et al. 2003).

Nitrogen is an important and critical nutrient that determines crop growth and productivity. For plants, nitrate is the most preferred form of nitrogen and is taken up by active transport through the roots. Changes in nitrate availability has been found to have contrasting effects on lateral root formation and elongation (Zhang and Forde 1998), which is suppressed by both high nitrate and high phosphate availability. Some of the components of the signaling pathways that regulate root-system architecture in response to nutrient availability have been identified. In *Arabidopsis,* the *NITRATE-REGULATED1* (*ANR1*) gene encodes a nitrate-inducible MADS-box transcription factor whose role is speculated in root plasticity in response to nitrate.

In another scenario, crosstalk was found to exist between nodulation and lateral root development in *Lotus japonicus*. It was found that *HAR1*, which encodes a putative serine/threonine receptor kinase that had homology with CLAVATA1, was involved. *HAR1* is required for shoot-controlled regulation of root growth, nodule formation, and nitrate sensitivity of symbiotic development (Nashimura et al. 2002).

Phosphate is one among the least-available macronutrients required by plants and is a constituent of key molecules such as ATP, nucleic acids, and phospholipids. Phosphate deficiency limits plant growth and development, resulting in adaptive stress responses. Over the past decade, many genes including phosphate transporters, phosphatases, RNases, and others of unknown function that help plants adapt to Pi stress have been characterized. SIZ1, a SUMO E3 ligase, was identified to control Pi homeostasis at the posttranslational level through sumoylation (Miura et al. 2005). Earlier, *Phi-2,* coding for a bZIP transcription factor in tobacco was reported to be induced during Pi starvation (Sano and Nagata 2002). Another transcription factor, PHR1, was first reported to play a regulatory role in Pi starvation responses in *Arabidopsis* (Rubio et al. 2001). Similarly, tolerance to phosphate starvation in rice was brought about by OsPTF1, a bHLH transcription factor (Yi et al. 2005). Very recently, the role of *WRKY75* in regulation of Pi starvation responses in *Arabidopsis* was evaluated (Devaiah et al. 2007a). To continue the growing evidence that transcription factors are key components of nutrient regulation, *ZAT6* (zinc finger of *Arabidopsis 6*), a cysteine-2/histidine-2 zinc finger transcription factor, is induced during Pi starvation (Devaiah et al. 2007b).

Sulfur is another nutrient important for plant growth. Under deprived sulfur conditions, plants develop a branched root system. This has been related to the transcriptional activation of the *NITRILASE3* (*NIT3*) gene, a member of nitrilase gene family. It is suggested that NIT3 plays direct role in auxin synthesis and root branching.

Optimum uptake of nutrients from the soil is a very important aspect of nutrient use efficiency. For this, plants require specialized transporters that are at the root/rhizosphere interface to take up nutrients. These comprise of high and low affinity transporters, which allow the plants to transport nutrients from soil to plant. This need of quenching nutrients make plants to modify their organ development to enhance their ability to capture water and nutrients. Many species have evolved mechanisms that allow them to detect nutrient-rich patches in the soil (Zhang and Forde 1998). In *Arabidopsis*, nitrate transporter, NRT1.1 has been identified (Remans et al. 2006). It is seen that NRT1.1 is a key component of the nitrate-sensing system that enables the plants to detect and exploit nitrate-rich soil patches. Likewise, transporters have been identified for phosphate as well. Two different families of transporters have been identified, viz. PHT1 (Liu et al. 2008) and PHT2 (Versaw and Harrison 2002), which influence the allocation of phosphate within the plant under phosphate starvation. More recently, another transporter has been identified recently in *Arabidopsis* called the PHT64;6, which belongs to the family of permeases and is found to be a determinant of salt tolerance

(Cubero et al. 2009). Similarly, a high affinity sulfate transporter has been identified in *Arabidopsis thaliana* called the *HstAt1*.

## 9.6 Relevance of Root Traits in Drought Tolerance

Among a number of stresses that affect crop growth and productivity, drought is perhaps the most prominent stress. A yield loss ranging between 20 and 60% is generally noticed in tropical regions. Hence, improving drought tolerance in crop plants is one of the most essential trusts in global research.

Drought tolerance is the ability of a plant to "avoid" the buildup of stress or to "tolerate" stress effects at the organism level (Levitt 1972; Blum 2005). However, improvement in drought tolerance has largely remained academic owing to the complexity of drought stress and equally complicated crop response to drought. Past research experience point strongly towards trait-based breeding, notwithstanding the significant progress made by selecting for high yield under water limitation.

Avoidance of stress through conservation strategies such as rapid physiological development, sensitive stomatal behaviour, heleonastic movements leaves, etc., though relevant, are normally counter productive. Ability of the plant to explore water source and extracting water from deeper profiles of soil thus has great relevance in maintaining water relation as well as carbon assimilation.

Deep-rooted plants have been shown to be better productive under water-limited conditions (Li et al. 2005; Reynolds and Tuberosa 2008). Such a trend was recently noticed also in $C_4$ crop such as finger millet at our centre (Fig. 9.1). Several of the root-related traits described above have been shown to be related with improved growth under stress.

Hence, improving these component traits has significance in sustaining productivity under water-limited conditions. After having achieved considerable understanding of root growth and development both at the whole plant level and at the molecular level, strategic approaches for crop improvement can be formulated.

Trait improvement can be effectively achieved either by introducing validated genes through transgenic technology or by introgressing desirable alleles through molecular breeding approaches. The traits relevant for drought tolerance and productivity are highly species-specific. While the distance from transition zone to first main lateral root, tap root weight, rapidity of root system development, and root to shoot ratio are important for cotton's (Cook 1985; Pace et al. 1999) ability to penetrate hard pan, root length, basal thick mass, and deep root biomass are important for rice and wheat.

Though deep-rooted plants produced more grains under low water availability, these plants had the risk of exhausting soil water early. Hence, Condon et al. (1993, 2004), Richards et al. (2002), and Sheshshayee et al. (2003) have emphasized that soil factors also need to be considered before attempting to improve root traits.

Despite the realization of the relevance of root traits in imparting drought tolerance and a good understanding of the molecular mechanisms of root growth,

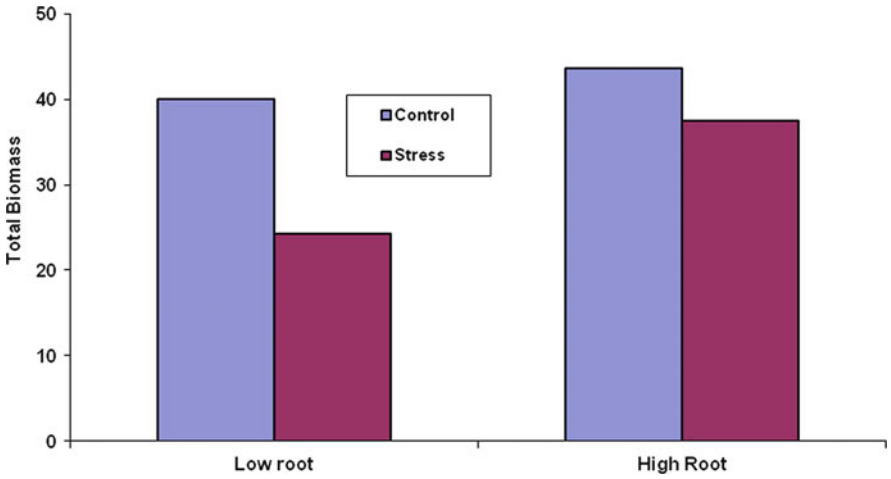


**Fig. 9.1** Differences in total biomass of Finger millet accessions differing in root traits grown under well watered and water limited conditions. Note: Stress was imposed by gravimetric approach and maintained for a period of 45 days between 30 and 75 days after sowing.
*Source*: Shankar (unpublished data)

a thorough exploitation of root traits has not been successfully achieved. Accurate measurement of root traits is one of the most significant constraints in crop improvement programs.

## 9.7 Improving Drought Tolerance Through Exploitation of Root Traits

O'Toole and De Datta (1986) suggest that drought is a syndrome because of the uncertainty of its occurrence, duration of its persistence, and the intensity. The soil characteristics, the crop species, and stages of crop growth all further complicate the process of understanding drought tolerance. Therefore, addressing drought tolerance requires a very comprehensive approach.

From the physiologist's perspective, plant water relations play a very curial role in determining the level of drought tolerance in plants. Root once again occupies the pivotal position through its role in extracting water from deeper soil profiles.

Maintenance of tissue water status is linked with (a) better extraction of water through deep root systems and (b) better water conservation strategies associated with sensitive stomatal behavior and deposition of waxes on the cuticular surface (O'Toole and Chang 1979; Ludlow 1993; Ingram et al. 1994). Though the conservation strategies are very useful under water-limited conditions, most of these traits are counterproductive. Agronomically, any drought tolerance trait would be relevant only when they are also associated with better growth and productivity. Simple

growth models provide the framework for identifying such traits, and one such model was proposed by Passioura (1986). As per this model, yield of any plant is a fraction of the amount of water used, the efficiency of water use for biomass production, and the partitioning of biomass to harvestable parts. Hence, root traits are strongly associated in enhancing crop productivity under water-limited conditions.

Significant developments have been achieved in understanding the physiology of drought resistance and developing physiological screening techniques for drought resistance, which reduce time in selection programs (Blum 1988; Ludlow and Muchow 1990). In recent reviews, Sheshshayee et al. (2003) and Reynolds and Tuberosa (2008) have discussed various traits that deserve exploitation to achieve drought tolerance coupled with sustained productivity under water-limited conditions.

A root system that extends the root zone to fully extract available soil water has the potential to increase yield under drought (Mambani and Lai 1983). Water uptake and transport by rice roots are most important as they affect yield, especially under water-limited conditions (Ingram et al. 1994). Individual root characteristics, such as thickness, depth of rooting, and the ability to penetrate compacted soils, have been associated with drought avoidance (O'Toole and Chang 1979; Yoshida and Hasegawa 1982; Ekanayake et al. 1985). Significant genetic variability in some of these root traits have been demonstrated and implicated for improved drought tolerance in crop plants (O'Toole and De Datta 1986; Thangaraj et al. 1990; Sharma et al. 1994; Sinclair and Muchow 2001). Biomass accumulation in plants is always a function of total water used (Angus and Van Herwerdeen 2001: Passioura 1986). Plants with deep root system hence have the ability to supply water to support a higher transpiration demand, thereby enhancing total biomass (Yadav et al. 1997; Li et al. 2005). In their simulation experiments, Sinclair and Muchow (2001) demonstrated an increase in biomass and yield when root growth was better. These studies emphasized the relevance of breeding to improve root traits to achieve better productivity under water -limited conditions (Reynolds et al. 2007; Reynolds and Tuberosa 2008).

Besides the inherent genetic variability among most plant species in root traits, roots are quite dynamic in responding to both biotic and abiotic stresses as well as soil characteristics. An increase in root length when plants are stressed for water and for nutrients is well known (Pace et al. 1999). However, when stress levels become severe, a significant reduction in root growth becomes inevitable (Prior et al. 1995; Plaut et al. 1996). Variation among genotypes for shifting root distribution downwards in response to drought has been found in cowpea (Matsui and Singh 2003), white clover (Annicchiarico and Piano 2004), and chickpea (Yusuf et al. 2005; Benjamin and Nielsen 2006; Kashiwagi et al. 2006). Absence of suberized hypodermis would permit rapid desiccation of delicate roots, leading to an increased root mortality in drying soils (Shone and Flood 1983; Jupp and Newman 1987; Smucker et al. 1991). A plant's root growth and extension decrease as the soil strength increases. Soil strength greater than 0.3–0.5 MPa and soil bulk density greater than 1.5 g $cm^{-3}$ hamper root growth and penetration below

10–15 cm from the soil surface (Hasegawa et al. 1985; Thangaraj et al. 1990). Presence of compacted soil layers acts as physical and physiological constraints to overall plant growth (Tu and Tan 1991) and impede the downward growth and distribution of the plant root system (Yu et al. 1995). Compacted soil layers reduce leaf area, dry matter accumulation, root elongation rate, transpiration rate, and crop yields (Masle and Passioura 1987; Assaeed et al. 1990; Ludlow et al. 1989; Masle 1992). Mechanical disruption of the compacted soil layers has been done to increase yield in cotton (Camp et al. 1984) and soybean (Khalilian et al. 1991). But mechanical disruption is expensive, and compacted layer often reform in a few years (Busscher et al. 1986).

## 9.8 Measurement of Root Traits

Determination of genetic variability in root traits represents the most difficult challenge in crop improvement programs. Despite the undeniable importance of root traits in better water mining, progress in breeding for these traits has been extremely slow. A few important root-related traits have been enumerated that have direct relevance for maintaining the balance between water relations and carbon assimilation of the plant. Several methodologies have also been developed and are being adopted for studying these root traits.

The simplest and more frequently used method is Hydroponics (Martinez et al. 1998; Tuberosa et al. 2002). This method involves raising of plants in suitable tanks filled with a nutrient solution. This system provides a very convenient approach for assessing the variability in root traits. However, lack of proper aeration to the roots is one of the major disadvantages of this method. Further, large-scale screening of germplasm and breeding lines would be very tedious. Growing plants in mini-rhizotrons (Drouet et al. 2005) or mini-lysimeters (Udayakumar et al. 1998) has also been extensively used for root studies. The rhizotrons can be made of transparent material that readily allows the direct visual monitoring of root growth and patterning. Though very effective, extending this method for large-scale screening is quite difficult. Scientists attempted to raise plants in tubular containers of varying lengths. These tubes are split in half at the time of harvest and the soil is washed off carefully to obtain the entire root mass. Normally, one plant is maintained in each container (Venuprasad et al. 2002; Ayyappa 2004; Giuliani et al. 2005). Plants grown in tubes can also be used for what is often known as "core-break" technique (Taylor et al. 1991). The soil core is taken completely from the tubes and cut into sizes as desired. Each of these pieces is then washed and the roots are taken out carefully.

A few destructive sampling techniques are also being adopted for root trait studies where the root mass is entirely excavated from the soil. Though this method is quite convenient, it is very difficult to completely excavate the roots, and hence this method has a significant random error, which would hamper accuracy of measurements. More sophisticated techniques of determining root growth have also been developed. A capacitance-based method has been used for monitoring

root growth (Van Beem et al. 1998). This method demands wetting of the soil at least up to field capacity and hence cannot be effectively used in assessing root growth in soils with water deficit. X-ray imaging and light transmission imaging are also being adopted for root trait measurements. A more recent technique of scanning the root system was evolved for cotton, where sampling was done for every 10 cm row for a depth of 0–100 cm of soil using a root sampler. The sampled soil was washed and scanned using a hand scanner at 200 dpi. The resultant image was analyzed using a DT-SCAN software (version 1.0; Delta, Inc., UK) to measure root length, average diameter, and surface area (Bouma et al. 2000; Zhang et al. 2005).

Most of the techniques available for root measurements suffer either due to the cumbersomeness of the procedure or due to their inability to screen large number of accessions. Further, root studies using pipes or mini lysimeters do not present the correct phenotypic expression of the root traits as they do not experience interplant competitions. Because the space provided in pipes directs more of a longitudinal growth, lateral root development gets constrained.

Most of these disadvantages can largely be overcome by raising plants in specially constructed "root structures" (Fig. 9.2). Although various dimensions can be adopted, the most suitable would be 5-ft tall, 10-ft wide, and 60-ft long structures built using cement bricks (Fig. 9.2). An additional 5-ft tall wall can be built in the middle of the structure to make two halves, each 5-ft wide, which provide additional strength to the structures. Soil is filled in these structures and compacted to mimic the real field conditions. Crop can be sown or planted in rows, and an exact plant population can be maintained. This approach provides the near-natural condition for phenotyping. Since the plant population is maintained as that in the main field, the plants would experience the interplant completion, which might have an important effect on the phenotypic expression of root growth. Thus, the measurements of the root traits from plants grown in such root structures would be very accurate. At the end of the experiment, the brick walls along the sides can be dismantled with care and the soil washed away using a strong jet of water. The roots are separated carefully

**Fig. 9.2** Specially constructed root structures to assess genetic variability in root traits in large number of accessions. Note: Each of these structures measure 60-ft long, 5-ft tall and 10-ft wide and is constructed using cement bricks. A wall is built in the center for dividing the structures into two halves of 5-ft wide each

from soil particles and then used to record various parameters such as root length, number of primary and secondary roots, root volume, etc. The roots can then be separated from the shoots and oven dried to measure root biomass. Except for the fact that the plants are grown in raised structures, this approach provides an option for determining genetic variability among large number of accessions in several root traits in conditions that are almost natural.

## 9.9 Oxygen Isotope Ratio as a Surrogate for Root Traits

Alteration in the stable isotopic composition of water has been well known to occur during evaporation. Although the theory explaining the phenomenon of oxygen isotopic enrichment during evaporation of water from ocean surface has been known for almost four decades (Craig and Gordon 1965), the application of this theory to predict differences in transpiration rate has been fairly recent (Flanagan et al. 1991, 1994; Farquhar and Lloyd 1993; Bindumadhava et al. 1999). However, discrepancy between the Craig-Gordon prediction and the measured $\delta^{18}O$ of the leaf water has been reported (e.g., White et al. 1994; Buhay et al. 1996). Further, the relationship between stomatal conductance and leaf water $^{18}O$ enrichment has remained equivocal (Farquhar et al. 2007), though increased transpiration has been clearly shown to enrich leaf water $^{18}O$ (Gonfiantini et al. 1965; DeNiro and Epstein 1979). We recently provided experimental evidences and demonstrated that oxygen isotope enrichment is a powerful time-averaged surrogate for transpiration rate (Fig. 9.3) (Sheshshayee et al. 2005)

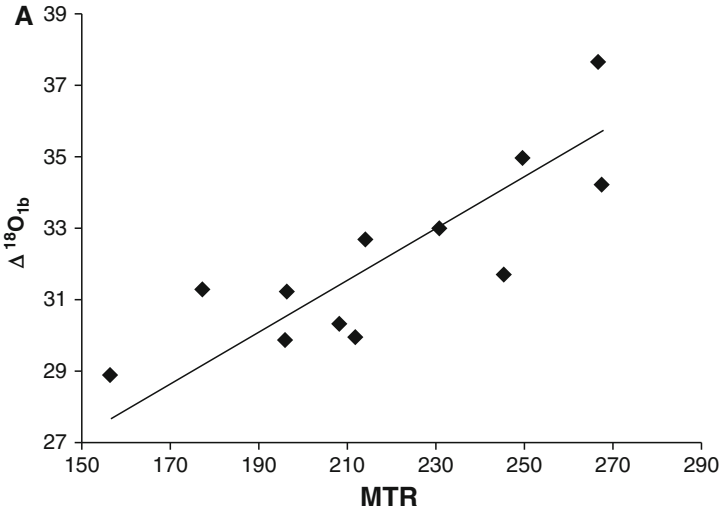


**Fig. 9.3** Relationship between oxygen isotope enrichment ($\Delta^{18}O$) and transpiration rate in rice genotypes (from Sheshshayee et al. 2005)

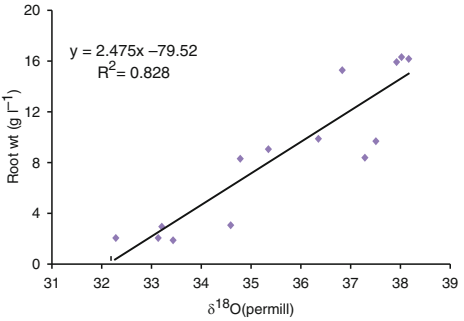


**Fig. 9.4** Oxygen enrichment accurately reflects root biomass in rice. Note: root traits were measured by growing contrasting genotypes in root structure. Leaf samples were taken for the determination of oxygen isotope ratio using IRMS at department of Crop Physiology, UAS, Bangalore (Mohankumar and Sheshshayee – Unpublished data)

In most plant species, the total biomass accumulated is a function of the total water used through transpiration. Total transpiration is further a function of the evaporating surface area of the canopy and the extent of root development to supply water to match the evaporative demand. Hence, transpiration at a given leaf area must be related to root biomass and hence a good indicator of root traits. Oxygen isotope enrichment values at a given leaf area was found to be strongly correlated with root biomass in both annual and perennial crop species (Fig. 9.4). Being high throughput and very accurate, stable isotope ratio is a very useful approach for the determination of root traits in plants.

## 9.10 Genetic Variability in Root Traits and Their Relevance in Improving Crop Growth

Success in breeding for any trait entirely depends on the existence of exploitable genetic variability in that trait with moderate to high heritability. Research on the genetic variation and heritability of rice root traits was reviewed by O'Toole and Bland (1987). Geneticists have estimated both broad sense and narrow sense heritability for root traits and have reported additive gene action and polygenic inheritance of most root traits. Maximum root length, root diameter, root dry weight, root length density, root number, root–shoot ratio, root dry weight, thick root number below 30 cm, and root mass density at different depths of soil layers are a few such traits. Significant genetic variability in these traits has also been reported (Chang et al. 1982; Armenta-Soto et al. 1983; Sharma et al. 1994; Ekanayake et al. 1985).

These traits have significant implication to the growth of plants, especially under water limitation. Root morphology and rooting patterns directly influence the amount and timing of water supplied to the crop canopy (Champoux et al. 1995). Root penetration ability associated with a few thick and long root axes helps to

penetrate the compacted soil layer to reach the water source (Yoshida and Hasegawa1982; Ekanayake et al. 1985; Ingram et al. 1994; Yu et al. 1995; Zheng-Xiang et al. 1998). These long roots through efficient absorption of water have a positive influence on biomass (Yadav et al. 1997). Longer and larger roots have wider xylem diameter, thus contributing to higher hydraulic conductivity and better water uptake (Passioura 1982; Ludlow and Muchow 1990). Variability in root traits among a few important crop species was noticed in various experiments conducted at our center. The mean and range of a few important root traits are indicated in the Table 9.2.

Similarly, several workers reported exploitable genetic variability in many root-related traits (Cook 1985; Pace et al. 1999; AbouKheir et al. 2008) and demonstrated variation in root hair density, hydraulic conductivity, and root length density in chickpea (Kashiwagi et al. 2005, 2006); analysis of the genetic control of these traits has revealed a multigenic inheritance with a predominance of additive gene action. Most of the research on assessing root traits has concentrated on cereals like rice and wheat. Price et al. (1997), while examining both *Indica* and *Japonica* varieties of rice, showed a significant additive and dominance effects on several root-related traits.

## 9.11 Breeding for Drought Tolerance Through Root Traits

Despite the realization of the importance of roots in crop growth and productivity, especially under water-limited conditions, no serious breeding efforts have been initiated till date to improve root traits (Blum 2005). Lack of a proper phenotyping strategy for root traits is perhaps the most important constraint. Though several techniques have been developed and are being used to assess root traits, screening large number of accessions is still a major challenge.

Among all the different abiotic stresses, drought is the most complex and devastating on a global scale (Pennisi 2008). Hence improving drought tolerance of crop plants deserves the greatest emphasis. However, progress towards this endeavour has been show primarily because of an ambiguous definition to drought and drought tolerance in the literatures (Tardieu 2003; Blum 2005; Collins et al. 2008). Remarkable dehydration tolerance has been achieved by adopting genetic engineering strategies that have targeted improvement in a range of processes including cell protection mechanisms (Jenks et al. 2007; Nelson et al. 2007), detoxification of reactive oxygen species that accumulate under stress (Lee et al. 2007; Yang et al. 2007), and hormonal manipulations that regulate adaptive strategies (Rivero et al. 2007). Nevertheless, these approaches have only provided drought tolerance in a laboratory condition while having little yield advantage under a much milder and intermittent drought conditions that are normally encountered in commercially cultivated field conditions (Collins et al. 2008). In contrast, exploration of natural variation in drought-related traits has resulted in a slow but a definite progress in crop performance (Rebetzke et al. 2002; Ribaut et al. 2004; Reynolds and Tuberosa 2008).

**Table 9.2** Genetic variability in a few root traits in several crop and perennial species

| Crop species | Trait | Minimum | Maximum | Mean | SD | Source |
|---|---|---|---|---|---|---|
| Finger millet ($n = 270$) | Root wt | 2.12 | 19.92 | 9.26 | 3.41 | Rajashekar Reddy and Shankar (personal communication) |
| | TDM | 50.25 | 125.7 | 47.45 | 14.12 | |
| | R/S ratio | 0.09 | 0.66 | 0.25 | 0.09 | |
| Rice ($n = 230$) | Root wt | 1.83 | 17.6 | 8.61 | 3.3 | Mohan Kumar and Sheshshayee (unpublished data) |
| | TDM | 12.68 | 84.10 | 40.68 | 14.45 | |
| | R/S ratio | 0.1 | 0.75 | 0.28 | 0.12 | |
| Sunflower ($n = 140$) | Root wt | 5.47 | 137 | 39.8 | 29.5 | Vikarm and Mohan Raju (personal communication) |
| | Root length | 20.33 | 81.25 | 43.78 | 11.88 | |
| | Root volume | 20 | 335 | 112.7 | 63.9 | |
| | TDM | 85.8 | 357 | 184.4 | 64.3 | |
| | R/S ratio | 0.068 | 0.67 | 0.26 | 0.16 | |
| Groundnut ($n = 260$) | Root wt | 0.85 | 3.9 | 1.9 | 0.49 | Namita and Sheshshayee (unpublished data) |
| | Root length | 28.7 | 76.5 | 48.1 | 8.3 | |
| | TDM | 9.4 | 97.10 | 37.8 | 12.3 | |
| | R/S ratio | 23.9 | 123.0 | 54.4 | 14.1 | |
| Mulberry ($n = 175$) | Root wt | 6.53 | 113.9 | 39.0 | 19.2 | Vinoda and Sheshshayee (unpublished data) |
| | Root length | 35 | 116 | 71.3 | 14.9 | |
| | Root volume | – | | | | |
| | TDM | 39.10 | 356.20 | 207 | 31.0 | |
| | R/S ratio | 0.25 | 0.42 | 0.38 | 0.43 | |
| Cotton ($n = 150$) | Root wt | 0.56 | 24.28 | 12.00 | 4.86 | Abou Kheir et al. (2008) |
| | Root length | 51.13 | 123.31 | 77.98 | 12.22 | |
| | Root volume | 6.3 | 100.6 | 50.51 | 19.12 | |
| | TDM | 38.7 | 394.2 | 215.1 | 19.1 | |
| | R/S ratio | 0.021 | 0.15 | 0.08 | 0.01 | |

Note: Germplasm accessions of each of these species were raised in specially designed root structures. Plants were harvested at the grand growth stage (flowering)

Maintenance of transpiration rate is crucial for productivity both under well-watered and water-limited conditions, and hence roots play a pivotal role. Being quantitatively inherited improvements in root traits can be best achieved with the use of molecular marker technology. A number of studies have reported QTLs for root architecture and have investigated their influence on yield under varying moisture regimes in rice (MacMillan et al. 2006; Steele et al. 2006, 2007) and Maize (Tuberosa et al. 2003; Landi et al. 2007). After identifying four major QTLs in rice (Courtois et al. 2000), marker-assisted backcrossing was performed to introgress the alleles for greater root length from Azucena into KalingaIII, an upland *indica* variety (Steele et al. 2006, 2007). Similarly, in maize, a major QTL originally reported for leaf ABA concentration (Tuberosa et al. 1998) was later shown to affect root size and architecture (Giuliani et al. 2005) and grain yield (Landi et al. 2007). These studies clearly demonstrate the possibility of enhancing root traits, thus leading to a better field performance of crop plants under water-limited conditions.

## 9.12 Transgenic Approach for Root Trait Improvement

Transgenic technology has had a great impact on crop improvement. This technology of precision not only allows the validation of identified genes but also helps identification of new genes. In the present scenario as well, this technology could be exploited and utilized to check the efficacy of the identified genes and select the gene(s) that help to obtain the right phenotype.

There are clear overexpression and downregulation studies available on the role of specific TFs and regulatory proteins in root growth and abiotic stress tolerance. A salt stress-inducible gene called Alfin1 (Winicov 1993; Bastola et al. 1998; Winicov and Bastola 1999), which encodes a putative Zn–finger regulatory protein, is predominantly expressed in roots. This TF in alfalfa roots binds to promoter elements of salt-inducible MsPRP2 gene and induces the gene expression (Winicov and Bastola 1999). The Alfin1 from alfalfa shows conservation among diverse plants such as rice and *Arabidopsis* (Bastola et al. 1998). In alfalfa, overexpression of Alfin1 enhances root growth under normal and saline conditions, resulting in salt tolerance (Winicov and Bastola 1999; Winicov 2000). An auxin-induced gene called OsRAA1 was identified and characterized by reverse genetics approach in regulating root development in rice (Ge et al. 2004). OsRAA1 is constitutively expressed in rapidly growing cells such as primordia of the lateral roots, meristem, and division zone of root apex. The expression of OsRAA1 is regulated by auxin, and in transgenic rice plants, overexpressing the gene initiation and growth of adventitious roots were more sensitive to auxin treatment (Ge et al. 2004). It has been suggested that OsRAA1 can be a candidate gene in root development and root response to gravity. Similarly, in another study, $Ca^{2+}$-dependent protein kinase1 (CDPK1) has been predicted to be associated with root development in *Medicago truncatula* (Ivashuta et al. 2005). The TFs that regulate diverse processes of plant development are also shown to be involved in root

growth. For example, CAP2, a gene encoding APETALA2 (AP2)-family TF, has been shown to improve root growth and abiotic (dehydration and salt) stress tolerance (Shukla et al. 2006). Constitutive expression of chickpea (*Cicer arietinum*) CAP2 protein in tobacco caused drastic increase in the number of lateral roots. Overexpression of NAC1 gene enhanced lateral root formation (Xie et al. 2002). Similar to this, overexpression of *Arabidopsis* gene, HARDY, an AP2-family TF, induced better root growth and imparted drought and salt tolerance (Karaba et al. 2007).

Further, components of signaling pathways have also been identified and validated. In plants, $Ca^{2+}$ is a ubiquitous secondary messenger, and changes in cytosolic concentration of $Ca^{2+}$ are associated with plant developmental processes including root growth. $Ca^{2+}$ -dependent protein kinase 1 (CDPK1) is involved in $Ca^{2+}$ signaling events. By using RNA interference-based approach, the importance of CDPK1 in root development was demonstrated (Ivashuta et al. 2005), and the authors suggest that CDPK1 is a key component in signaling pathways. Similarly, Calcineurin B-like proteins (CBL) and CBL-interacting protein kinases (CIPK) mediate a variety response to external stresses in plants. In *Arabidopsis*, CIPK6 is required for growth and development, and tobacco plants expressing a homologous gene (CaCIPK6) from chickpea showed improved abiotic stress tolerance (Tripathi et al. 2009). It has been concluded that CIPK is associated with auxin transport and consequently in root development, and salt-stress response, by regulating the expression of downstream genes. Some of these TFs and regulatory genes can be used to improve abiotic stress tolerance in candidate crops by transgenic approach since some of these genes produced desirable phenotype under overexpressed condition without abnormal phenotype.

Similarly, nutrient acquisition traits can be improved by overexpression of both structural and functional genes involved. Overexpression of ZAT6, a zinc finger transcription factor, resulted in altered root architecture with changes in Pi acquisition (Devaiah et al. 2007a, b).

Therefore, in a broader perspective, transgenic approach could be used to target the modifications of the root systems with the genes involved. This could provide an opportunity to improve the anchorage, hasten the growth of plants by enhancing their exchange abilities, improve the tolerance of plants to drought and salinity, their ability to penetrate compact soils, as well as synthesize important secondary metabolites produced by the root and required by the plants.

## 9.13 Conclusions

Crop improvement for the future requires a very focused and orchestrated strategy through exploitation of genomic resources. With the advent of modern molecular biological tools, genes that regulate the growth and development of roots have been identified. After convincing validation, several candidate genes have also been identified that are being effectively used for improving drought tolerance through

transgenic technology. Though this technique holds tremendous potential in enhancing tolerance to severe stress levels, their field performances have not shown significant advantage. Plants experience much milder stresses under field conditions that are often intermittent. On the other hand, a trait improvement has had a definite improvement in crop performance, albeit with a slower pace. To enhance the field performance under water-limited conditions, introgressing relevant QTLs governing root traits appears to be the most plausible strategy. Based on the stability of their effect across environments, "constitutive" and "adaptive" QTLs have been identified. While the constitutive QTLs are consistently detected across most environments, the adaptive QTLs are detected only in specific environments. With the advent of marker-assisted breeding technologies, it is now possible to introgress relevant QTL for a specific target environment. However, the reliability of a QTL entirely depends on the accurate phenotyping of the root traits in large number of accessions and breeding lines. We have described suitable methodology for such a large-scale screening for traits under field conditions. Phenotyping for root traits in specially constructed root structures would be closest to that observed under field conditions. In this approach, root traits are measured under near-natural field condition, and hence, it is a robust phenotyping technique. Further, a more powerful and high throughput approach based on the oxygen isotope enrichment has been shown to be a good surrogate for root traits at a given leaf area.

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# Chapter 10
# Genomics and Physiological Approaches for Root Trait Breeding to Improve Drought Tolerance in Chickpea (*Cicer arietinum* L.)

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**Contents**



## 10.1 Chickpea Crop

Chickpea is a valuable agricultural crop of South Asia and the third most important pulse crop in the world after dry bean (*Phaseolus vulgaris* L.) and field pea (*Pisum*

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*sativum* L.). Cultivated chickpea, *Cicer arietinum* L., is a self pollinated, diploid ($2n = 2x = 16$) annual pulse crop with a genome size of 750 Mbp (Arumuganathan and Earle 1991). There are two types of chickpea: *desi* (brown colored small seed) and *kabuli* (white or beige colored large seed). *Desi* type covers about 85% of global chickpea area and is predominantly grown in South and East Asia, Iran, Ethiopia, and Australia, and the *kabuli* type is grown mostly in the countries of the Mediterranean regions, West Asia, North Africa, and North America. The wild ancestor of domesticated chickpea is *Cicer reticulatum*. Chickpea originated in southeastern Anatolia (Turkey) and was traditionally cultivated in Asia, the Mediterranean, the Middle East, and northern Africa (Ladizinsky and Adler 1976). In contemporary times, chickpea has become popular throughout the temperate regions in countries such as Mexico, Canada, and Australia (Duke 1981).

Chickpea ranks third among pulses, fifth among grain legumes, and 15th among grain crops of the world. In 2006, the world chickpea cultivation area was 10.7 Mha with over 8 Mha grown in India, Pakistan, and Iran, with a further 1 Mha grown in other countries of Asia, the Middle East, and Canada. Total production was 8.4 Mt, and the average yield was 772 kg/ha (FAOSTAT 2006). Although chickpea is cultivated in about 50 countries, 95% of its area is in the developing countries where South Asia alone covers almost 71% of the world chickpea harvested area. Most of the chickpea harvested is consumed locally and the global trade is about 12% of the total production. The global demand for chickpea is projected to be 11.1 Mt in 2010. Under optimum growing conditions, the yield potential of chickpea is 6 t/ha (Singh 1987), which is much higher than the current global yield average of ~0.8 t/ha (Ahmad et al. 2005).

## 10.2 Drought Stress in Chickpea

The main constraints in chickpea production are the abiotic stresses such as drought, heat, cold, and high-salinity and the biotic stresses such as *Ascochyta* blight, *Fusarium* wilt, and the pod borer. The estimated collective yield losses due to abiotic stresses (6.4 Mt) are higher than that of the biotic stresses (4.8 Mt) (Ryan 1997). In the order of importance, drought, cold, and salinity are the three main abiotic stresses that affect chickpea growth and productivity worldwide (Croser et al. 2003). Drought stress alone causes a 40–50% reduction in yield globally (Ahmad et al. 2005). It is estimated that if the yield loss due to drought stress is alleviated, chickpea production could be improved up to 50%, equivalent to approximately US$ 900 million (Ryan 1997).

As 90% of chickpea crops are cultivated under rainfed conditions, drought is of major concern (Kumar and Abbo 2001), with terminal drought as the major constraint limiting productivity. Terminal drought stress is typical of the post-rainy season crop in the semiarid tropical regions, where the crop grows and matures on a progressively receding soil moisture profile (Ludlow and Muchow 1990; Krishnamurthy et al. 1999), and the intensity of terminal drought varies depending

on previous rainfall, atmospheric evaporative demand, and soil characteristics such as type, depth, structure, and texture. In the arid and semiarid tropics of South and Southeast Asia, chickpea is grown in the winter season immediately after the end of the rainy season. Similarly in the Mediterranean environments, it is grown in spring on stored soil moisture from the winter and early spring rainfall. In both the environments, the soil moisture recedes to deeper soil layers with the advancement in crop growth, and the crop experiences increasing soil moisture deficit at the critical stage of pod filling and seed development (Saxena 1984; Siddique et al. 2000).

## 10.3 Strategies to Tackle Drought Stress

Two main strategies are envisaged to tackle drought stress in chickpea (1) developing early maturing varieties and (2) developing drought tolerant varieties (Gaur et al. 2008a, b). The breeding strategy for development of early maturing cultivars is straight forward. One of the parents used in crosses should be a well-adapted cultivar, and another parent should be an early maturity germplasm accession/cultivar. In segregating generations, plants that flower early, for instance, in 25–30 days at ICRISAT-Patancheru, are selected and their progenies are further evaluated. Selection for time to flower is effective even in early segregating generations as it is controlled by a few major genes. Early flowering is a recessive trait and controlled by a major gene *ppd* in ICC 5810 (Or et al. 1999) and by a major gene *efl-1* in ICCV 2 (Kumar and van Rheenen 2000). Early phenology (early flowering, early podding, and early maturity) is the most important mechanism to escape terminal drought stress. At ICRISAT, the chickpea breeding program has placed high emphasis on development of early maturing varieties for enhancing adaptation of chickpea to environments prone to terminal drought stress (Gaur et al. 2008b). Several varieties (e.g., ICCV 2, ICCC 37, JG 11, and KAK 2) have been developed that mature in 85–100 days at Patancheru, as compared to >110 days taken by the traditional varieties. The short-duration varieties have greatly contributed to the expansion of area and enhancement of productivity of chickpea in terminal drought-prone areas of peninsular India (Gaur et al. 2008b) and Myanmar (Than et al. 2007). Breeding lines have been developed, which are extra-early in maturity (75–80 days at Patancheru) and offer further opportunities for expanding cultivation of chickpea in new niches (Kumar and Rao 1996; Gaur et al. 2008b).

Early maturing varieties that escape terminal drought and heat stress were developed by the breeders and were adopted by farmers with considerable success (Kumar and Abbo 2001). However, this drought escape fixes a ceiling on the potential yield and cannot utilize the opportunities, as and when available, of extended growing periods. Therefore, for achieving high and stable yields under drought, it is necessary to develop drought-tolerant/avoiding varieties (Johansen et al. 1997). Thus, several studies in the recent years have focused on identification of morphological and physiological traits associated with drought tolerance. Cultivated chickpea (*Cicer arietinum*) has a narrow genetic base, making it difficult

for breeders to produce new elite cultivars with durable tolerance to drought stress. In addition, drought tolerance is inherited in a quantitative manner, and the direct yield or biomass assessment under field is prone to confounded environmental effects. Therefore, selection of drought-tolerant plants in the field becomes difficult. Recent advances in genomics can assist crop improvement efforts (Varshney et al. 2005). In fact, marker-assisted selection (MAS) approach has been successfully deployed in developing improved varieties/lines/hybrids in several crop species (see Varshney et al. 2006, 2010). Quantifying the effects of drought stresses, however, involves measurement of various factors like days to flowering and maturity, early shoot growth vigor, yield, shoot biomass production, rooting depth, root length density, root to shoot ratio, total transpiration, and transpiration efficiency. Therefore, developing molecular markers for drought tolerance *per se* is a difficult task. Dissection of such complex traits into components or identification of highly related surrogate traits can enhance the heritability of such traits and facilitate development of molecular markers associated with each of such traits.

### *10.3.1 Targeting Root Traits for Drought Tolerance*

Root traits, such as root depth and root proliferation, have been identified as the most promising traits in chickpea for terminal drought tolerance, as these help in greater extraction of available soil moisture. As these traits are quantifiable under drought stress conditions, it seems feasible to develop molecular markers for these traits and thereby can be used to screen the germplasm for drought tolerance.

One of the important physiological reasons to target root traits under the water-limiting environments is the capability of root systems to absorb relatively more water from deeper soils and/or absorb water relatively rapidly. Chickpea is a crop that is often grown in deeper and heavier soils such as vertisols under progressively receding soil moisture with little precipitation during the crop growth period. Heavier soils are characterized with soil cracking as a consequence of shrinking when dry. These soil cracks aid in enhancing soil evaporation from deeper soil layers, more so under increasing atmospheric evaporative demand coinciding with the reproductive growth stage of the crop. Therefore, it becomes necessary to maximize transpiration over evaporation (Johansen et al. 1994) and to enhance crop growth before the water is lost in cracking heavier soils. More prolific roots at the early stages of growth have been shown to be advantageous for such maximization as the root length density (RLD) values recorded in chickpea were suboptimal (Krishnamurthy et al. 1996; Kashiwagi et al. 2006). However, root prolificacy may not be expected to maximize transpiration in environments where the evaporative demands are too extreme, and also this trait may not help under environments characterized with excessive vegetative growth and poor partitioning. Similarly, deeper rooting or higher proportion of deeper root length can help in mining water from deeper soil profiles, provided the soil profiles are fully saturated in the previous rainy season or the soils are deep enough for the roots to penetrate.

Under such soil conditions, transpiration (T) gets maximized over evaporation, which can increase the total water loss under water-limited conditions. The relationship of grain yield to water-related parameters has been described by Passioura (1977) and Fischer (1981) as:

$$\text{Yield (YLD)} = \text{Transpiration (T)} \times \text{Transpiration Efficiency (TE)} \times \text{Harvest Index (HI)}.$$

The above formula indicates that the grain yield under drought could be improved through improving any one or the combinations of the above components. Also, these yield components have been shown to interact with each other. For example, the timing of water availability is shown to affect the HI. Providing small amounts of water across the growing period in comparison to the application of all the water that is required at one time was shown to favor the wheat yields through improved HI (Passioura 1977). Also, a deeper root system was found to be associated with better HI and seed yield in chickpea (Kashiwagi et al. 2006). As compared to HI, the other two factors, T and TE, can be improved by relatively less efforts. The total shoot biomass can be increased either by increasing T or TE.

In some legume crops, e.g., common bean (White and Castillo 1990), groundnuts (Wright et al. 1991), and soybean (Cortes and Sinclair 1986), deep root systems have already demonstrated to have positive effects on seed yield via improved T. These studies emphasize that the T improvement strategy for better soil moisture absorption through root systems could be applied in drought tolerance breeding program in general or at least in legumes. However, until recently, little breeding effort has been made to improve the root systems for seed yield or shoot biomass under drought environments in chickpea. The reasons include the lack of techniques that allow for large scale screening of genotypes, limited information on genetic variability in root traits, and poor understanding of the genetics of root attributes. It is also important to note that while targeting root traits in several crops has been successful to tackle drought stress in several crops, the root traits may not work in all environments.

At ICRISAT, near Patancheru in southern India (altitude: 545 m above the mean sea level, latitude: 17°27′N, longitude: 78°28′E), a team of multidisciplinary scientists has been working on root traits to improve the chickpea productivity. More than 1,500 chickpea germplasm accessions plus released varieties were evaluated under rainfed as well as irrigated field conditions at ICRISAT to gather information on the yield under terminal drought conditions and potential yields (Saxena 1987, 2003). Some genotypes, e.g., Annigeri, ICC 4958, ICC 10448, ICC 5680, and JG 62, were identified as drought-tolerant lines using a drought-tolerant index in which the effects of early flowering could be removed (Saxena 1987), although each had a different trait/mechanism to cope with the terminal drought. For example, in Annigeri and ICC 10448, narrow (lanceolate) leaves, in ICC 5680 fewer pinnules per leaf and a rapid rate of grain filling through production of twin pods at the early flowering nodes in JG 62 seem to be the mechanism contributing to

drought tolerance. The genotype, ICC 4958, showed the best performance not only at ICRISAT field trials but also at several other locations in India and in the Mediterranean climate in Syria, which was found to possess higher root biomass (ICARDA 1989; Saxena et al. 1993; Krishnamurthy et al. 1996; Ali et al. 1999, 2005). Subsequently, field experiments at ICRISAT with 12 diverse chickpea germplasm including ICC 4958 showed that a prolific root system, especially in the 15–30 cm soil depth, had positive effects on seed yield under moderate terminal drought intensity, and a deeper root system to improved yield under severe terminal drought conditions (Kashiwagi et al. 2006). The large variation in root systems within such a small group of genotypes (Fig. 10.1), and the relation between root length density (RLD) and yield under drought, suggests that an extensive and systematic screening of the chickpea germplasm might offer a promising range of variation for RLD. Furthermore, the RLD was increased under more severe stress conditions, particularly in more tolerant genotypes, and the RLD at the deeper layer was related to yield under more severe drought stress. These data suggest that the dynamics of root growth under drought conditions might be a key factor in understanding the contribution of roots to drought tolerance.


**Fig. 10.1** Comparative root profiles in three chickpea genotypes. The figure shows 35-day-old plants of three chickpea genotypes, namely ICC 4958, KAK 2, and Annigeri. These plants were grown in pots in glasshouse conditions. It is evident from the figure that the root biomass for ICC 4958 is relatively higher than the other two chickpea genotypes. Higher root biomass confers high level of drought tolerance in ICC 4958 genotype.

The research on root systems under field conditions is very laborious, expensive, and time-consuming (Subbarao et al. 1995). To overcome this problem, a modified monolith method was standardized at ICRISAT (Serraj et al. 2004). This method provided systematic field root extraction at a sampling rate of 3.3 root profiles/worker/day. Although this method was fairly reliable to assess the field performance, it still did not provide an adequate sampling rate for large scale screening of genotypes. Although the less cumbersome pot-culture method was tested, the rooting profile could not be estimated in shallow pot grown plants. Thus, extensive efforts were made at ICRISAT to standardize a PVC cylinder-culture system for screening large numbers of genotypes. When the plants were grown in PVC cylinders (18 cm diameter, 120 cm height) filled with a sand–vertisol mixture containing a 70% field capacity soil moisture, the extracted root biomass was significantly correlated with the ones extracted from the field ($r = 0.62$, $p < 0.05$) (Kashiwagi et al. 2006). Moreover, the sampling efficiency of chickpea roots could be improved upto 25 profiles/worker/day. Furthermore, an image capturing and analysis system was introduced to scan the roots and convert the intact root samples into digitalized images for a large number of samples (>150 root samples/day). By using the digital image of roots, the WINRHIZO software (Regent Instruments, Inc., Canada) could generate numerical data, e.g., root length and root diameter, from more than 500 images/day.

### *10.3.2 Physiological Mechanisms of Root Traits*

Plants take up water from soil profile using either an active or a passive water uptake pathway (Hirasawa et al. 1997). In nonstress conditions, i.e., when a plant transpires, the magnitude of active water uptake is far less than that of passive water uptake. Under severe drought conditions, however, the plants close the stomata, so as not to deplete the internal water, and active water uptake becomes more important under such non-transpiration situations. In active water uptake, one of the relevant root-related traits would be osmotic adjustment. However, using such traits is difficult in breeding programs (Turner et al. 2006).

The passive water uptake takes place by gradient of water potential from the roots to shoots, where Vapor Pressure Deficit (VPD) in the air is the principle driving force. Thus, higher VPD causes more transpiration to occur via stomata, which pulls down the leaf water potential. Subsequently, it reduces the xylem pressure potential in the stems and then in the roots. This creates a gradient in water potential, which forces the soil water into the xylem in roots and then to the leaves. Under normal circumstances, this passive water uptake plays a major role in terms of the plant water. Under the passive water uptake, the relevant root traits are root hydraulic conductivity (vertical water flow from roots to leaves) and root permeability (transverse water flow from the root surface to xylem). The root permeability could be further dissected into three different paths (1) apoplastic

(inter-cells), (2) symplastic (cell-to-cell), and (3) transcellular (cell-to-cell) (Steudle 2000). The symplastic path more closely relates to the active water uptake.

Chickpea is known to have varying root distribution across soil depths depending on the soil water availability. It has substantially smaller RLD than that of several cereals, e.g., barley (Thomas et al. 1995), but has an efficient water uptake. The difference for water uptake between chickpea and cereal species has been attributed to the function of root hydraulic conductivity, which is mainly governed by the diameter and the distribution of the meta-xylem vessels (Hamblin and Tennant 1987). Chickpea could develop its root systems upto two to three times greater in the surface soil layer (0–15 cm) at mid-pod filling stage when irrigated. On the other hand, the proportion of RLD distributed at deeper soil layers (115–120 cm) was found higher under receding soil water conditions compared to that of the well-watered condition (Ali et al. 2002). In another study, chickpea had a greater proportion of the root system in the deeper soil layer under dryland environments than field pea (Benjamin and Nielsen 2006). In addition, chickpea possesses greater root surface area to root weight ratio, compared to field pea or soybean. These studies suggest that chickpea plants are better equipped in terms of the soil water uptake to cope with the drought environments. Enhancing root traits would, therefore, be one of the promising approaches to improve drought avoidance in chickpea under terminal drought conditions.

## 10.4 Genetic Dissection of Root Traits

In order to target the root traits in chickpea breeding to improve drought tolerance, understanding the genetics of root traits is crucial. In the first instance, to have a knowledge about the genetic variability of root traits in chickpea germplasm, a mini core collection consisting of 211 chickpea genotypes developed by Upadhyaya and Ortiz (2001) was assessed in the cylinder culture with image capturing and analysis systems in two seasons. A large and significant variation was observed among the accessions of the mini-core collection in terms of root length density (RLD), root dry weight (RDW), rooting depth (RDp), and root to total plant weight ratio (*R/T*) (Krishnamurthy et al. 2004; Kashiwagi et al. 2005). Although a significant genotype × season interaction was observed for RLD and *R/T*, it was a noncrossover type. Therefore, a rank correlation analysis was performed between the accession means of two seasons to identify the contrasting genotypes in terms of root traits. The studies identified two accessions namely, ICC 4958 and ICC 8261, as having large and prolific root systems. In addition, the root traits of ten accessions of annual wild *Cicer* species were also evaluated in one season. The wild relatives had smaller root systems than *C. arietinum* except for the most closely related species *C. reticulatum* whose root systems were similar to that of the average root system of *C. arietinum*. It has to be mentioned here that these findings need further validation keeping in mind the effect of phenology on the timing of root growth.

Most of the wild accessions tested here were late in flowering, and these evaluations have been carried out using 35-day-old plants. As most of the wild *Cicer* species are late in phenology, it may be appropriate to measure the root system differences of wild species accessions at a later growth period.

Subsequently, in a study conducted to estimate the gene effects for root traits, two contrasting pairs of chickpea genotypes, ICC 283 and ICC 1882 (smaller roots) and ICC 8261 and ICC 4958 (larger roots), were identified for developing populations for the genetic analysis (Kashiwagi et al. 2008). In these analyses, the additive gene effect and additive × additive gene interaction have been found to play important roles in determining the RLD and RDW. In addition, the direction of the additive gene effects was consistent and toward increasing the root growth. The results encouraged the ICRISAT team to proceed with the breeding program for root systems in chickpea, although delaying selections until later generations with larger populations was proposed (Kashiwagi et al. 2008).

In order to identify the genomic regions or quantitative trait loci (QTLs) for root traits, three recombinant inbred line (RIL) populations were developed at ICRISAT. The first population consists of 257 RILs from the cross Annigeri × ICC 4958. Two other RIL populations involving parents more genetically and phenotypically distant, selected after screening the mini core collection as mentioned above, were developed: 281 RILs from the cross ICC 283 × ICC 8261 and 264 RILs from the cross ICC 4958 × ICC 1882.

The Annigeri × ICC 4958 RILs were evaluated for two seasons under terminal drought conditions, and approximately 40 molecular markers (SSR) were genotyped in the population. A QTL responsible for 33% of the phenotypic variation for root length and root biomass was detected (Chandra et al. 2004). The root trait phenotyping has been done for the two other mapping populations (ICC 4958 × ICC 1882 and ICC 283 × ICC 8261), and genotyping is underway with a variety of molecular markers. Limited level of polymorphism in intraspecific mapping populations of chickpea is a major constraint in mapping of any trait in chickpea. To aid in mapping, a set of 311 SSR markers have been developed from an SSR-enriched genomic DNA library (Varshney et al. 2007), and a set of 1,344 SSR markers have been developed after mining about 46,270 BAC-end sequences (Nayak et al. 2008). With the existing set of SSR markers in public domain and newly developed markers at ICRISAT (in collaboration with University of California, Davis, CA, USA; University of Frankfurt, Germany) and National Institute of Plant Genome Research (NIPGR), New Delhi, India (Sabhyata Bhatia, pers. commun.), more than 2,000 SSR markers are available in chickpea (Varshney et al. 2008, 2009a; Nayak et al. 2010). An integrated genetic map with 521 loci has been developed by Nayak et al. (2010). In addition to SSR markers, Diversity Arrays Technology (DArT) markers are currently being used for genotyping the two mapping populations (ICC 4958 × ICC 1882 and ICC 283 × ICC 8261). Given the large phenotypic and genotypic contrast between the parents involved in these populations and high density marker genotyping, the chances to identify additional major QTLs for root traits as defined above are high.

## 10.5 Transcriptomics Approaches for Identification of Genes from Root Tissues

Plant stress responses are complex and diverse, and every gene involved, from recognition to signaling to direct involvement, forms part of a coordinated response network. Controlling gene expression is one of the key regulatory mechanisms used by living cells to sustain and execute their functions. Although the final activity of a gene is determined by encoded protein, measurements of mRNA levels have proven to be a valuable molecular tool. In order to obtain a complete picture of a plant's response to stress, it would be ideal to study the expression profiles of all possible genes in its genome or at least those involved in conferring stress tolerance. Traditional approaches for undertaking genome-wide expression studies involve the use of microarray or cDNA macroarrays. Although in chickpea, transcriptomic approaches are not in an advanced stage, they progress in this direction that has already been initiated (Coram and Pang 2007).

The first step toward transcriptomics studies is the identification or cataloging of genes involved in the trait. One of the most simple and straight forward approach is the generation of expressed sequence tags (ESTs), which involves large-scale single-pass sequencing of randomly selected clones from cDNA libraries constructed from mRNA isolated at a particular developmental stage and in response to a particular stress (Sreenivasulu et al. 2002). Functional identification of sequenced clones is becoming easier by the availability of rapidly growing sequence databases, such as Genbank and genome sequence data of several crop species including the three legumes, i.e., *Medicago truncatula*, *Lotus japonicus*, and *Glycine max*.

The EST datasets can be used in gene expression/functional genomics studies to identify putative genes with differential expression and to generate the gene-based functional molecular markers such as EST-SSRs, EST-SNPs, and single feature polymorphisms (SFPs) (Varshney et al. 2005). EST analysis has become a popular method for gene discovery and mapping in cereal crops (Varshney et al. 2006). The first resource of ESTs (ca. 2800) in chickpea was developed at ICRISAT from root tissues challenged by drought stress (Buhariwalla et al. 2005; Jayashree et al. 2005). The EST library was constructed after subtractive suppressive hybridization (SSH) of root tissue from two chickpea genotypes (the landrace ICC 4958 and a popular local variety Annigeri), which were considered to possess important sources of drought tolerance (Saxena et al. 1993; Saxena 2003). A total of 2,179 ESTs were generated with putative identification that resulted into 477 unigenes. A total of 106 EST-based markers were designed from the unigene sequences with functional annotations. To enrich the resource of ESTs involved in drought and salinity stress tolerance (or response), ten different cDNA libraries were constructed from the root tissues of ICC 4958, ICC 1882, JG 11, and ICCV 2 (parental genotypes of the mapping populations segregating for drought and salinity), challenged by different types of drought (chemical induction using polyethylene glycol (PEG), sudden dehydration stress, slow drought stress to potted plants grown in the greenhouse, and prolonged drought stress under field conditions) and salinity stresses (treated

with 80 mM NaCl solution). In summary, a total of 20,162 ESTs have been generated in the study using Sanger sequencing approach at ICRISAT and have been deposited in GenBank (Varshney et al. 2009b). A detailed analysis of ESTs has provided a set of 6,404 unigenes.

In addition, "whole transcriptome sequencing" using Solexa sequencing technology (see Varshney et al. 2009c) has been initiated by ICRISAT in collaboration with colleagues from the National Center for Genome Resources, Santa Fe, New Mexico, USA (Greg May and Andrew Farmer), and the University of California, Davis, USA (Doug Cook). In this approach, the RNA isolated from drought stress challenged root tissues of different stages and were pooled for ICC 4958 and ICC 1882 genotypes separately. Half run of Solexa sequencing on the pooled RNA samples from ICC 4958 and ICC 1882 yielded $5.2 \times 10^6$ and $3.6 \times 10^6$ sequence reads (May et al. 2008), respectively. The preliminary results of the Solexa sequencing are summarized in Table 10.1. Ideally for analyzing the Solexa datasets, genome assembly (reference assembly) of the same species is prerequisite for aligning the short tags (~36 bp). In case of chickpea, however, no genome assembly was available during the analysis. To analyze the generated Solexa datasets, the following three set of sequence resources were used (1) *M. truncatula* (Mt) IMGAG (International Medicago Genome Annotation Group) gene assembly representing 29.5 Mb sequence data, (2) *C. arietinum* transcript assembly (Ca TA) of JCVI (The James Craig Ventor Institute) representing 681 kb sequence data and (3) *C. arietinum* (Ca) BAC-end sequence (Ca BES) data representing 16.4 Mb sequence data. As a result, the Solexa datasets showed matches with 5,886 and 7,338 genes in cases of ICC 4958 and ICC 1882, respectively (Table 10.1). These datasets are being analyzed for identification of gene-based SNPs between ICC 4958 and ICC 1882 so that the polymorphic genes could be integrated in the genetic maps. Such efforts should lead to the identification of drought QTL-associated genes that would be useful for molecular breeding.

Other functional genomics studies using the chickpea/legume-based gene microarrays have also been undertaken for identification of genes for drought tolerance; however, these were not exclusively focused on root traits. For example,

**Table 10.1** Preliminarily gene discovery in two chickpea genotypes by employing the Solexa sequencing technology

| Features | ICC 4958 | ICC 1882 |
|---|---|---|
| Number of reads | 36,15,433 | 52,07,099 |
| Average read length | 36 | 36 |
| Average read quality | 26 | 21 |
| Alignment with TA | | |
| Read aligned | 11,95,622 (33%) | 21,22,069 (41%) |
| Reads uniquely aligned | 5,72,751 (16%) | 9,67,102 (19%) |
| Alignments with BES | | |
| Aligned | 10,48,614 (16%) | 17,88,936 (34%) |
| Uniquely aligned | 5,11,148 (14%) | 8,54,085 (16%) |
| Overall number of gene matches | 5,886 | 7,338 |

Boominathan et al. (2004) carried out a gene expression study of drought adaptation in chickpea using subtractive suppressive hybridization in combination with differential DNA array hybridization and northern blot analysis and identified 101 drought-inducible transcripts. Similarly, Coram and Pang (2006) developed a "Pulse Chip" microarray and applied it to identify the genes expressed in response to abiotic stresses such as drought, cold, and high salinity. In another study, transcript profiling of tolerant and susceptible chickpea genotypes under drought, cold, and high salinity was conducted (Mantri et al. 2007). These studies provide opportunities for illuminating the mechanisms of drought tolerance in chickpea and indicate the molecular pathways used by the plant as well as the function of the candidate genes involved. It would be interesting to see the colocalization of such genes with QTLs related to root trait in chickpea.

## 10.6 Prospects for Molecular Breeding for Root Traits

The role of root traits in conferring drought tolerance in chickpea is well established. A significant challenge to the selection for root traits is the difficulty of evaluating root phenotypes, since many root traits are phenotypically plastic, roots are difficult to extract from the soil, such extraction may change certain traits such as architecture, and many root sampling procedures are destructive. Research on drought tolerance still has to deal with many complicated aspects, especially concerning root functions. The reason is that the root is difficult to visualize and extremely sensitive to the surrounding environmental factors because of the G × E interactions. So, many efforts have been made to characterize and identify varietal differences based on root traits (Kashiwagi et al. 2005). These challenges make the prospects of marker-aided selection an attractive alternative to phenotypic selection.

The availability of appropriate molecular markers is an important prerequisite for marker-assisted selection. The availability of more than 2,000 SSR markers and DArT arrays in chickpea will enable the development of the genetic maps and mapping of traits in intraspecific populations. The integration of the candidate genes showing differential expression as well as SNPs between contrasting genotypes into QTL maps will provide genes and markers associated with root trait QTLs.

After identifying the QTLs, molecular markers associated with these QTLs need to be validated on a range of germplasm to select the most promising QTLs. For introgression of these QTLs, the drought-tolerant (possessing the QTLs) and drought-sensitive lines (showing the polymorphism at QTL with drought tolerant genotypes) are selected. After generating the $F_1$s by crossing the susceptible drought-sensitive varieties (recurrent) with drought-tolerant donor variety, the $F_1$ seeds are raised and backcrossed to the recipient varieties. After raising the $BC_1F_1$ population, these plants are genotyped with the identified molecular marker(s) associated with targeted QTLs. Based on marker genotyping data, the desired plants

are used further for backcrossing to produce the $BC_2F_1$ populations. Similar cycles of backcrossing and selection of lines with molecular markers for making them homozygous for the next generations are continued until the necessary recovery of the recurrent parent genotype is achieved. Many molecular breeding programs do not involve the use of markers in background selection. However, the availability of Diversity Array Technologies (DArTs), a low cost marker system in chickpea, creates the possibility to use DArT markers for background selection. Subsequently, the marker-assisted backcross (MABC) lines are evaluated in replications on-station and on-farm trials for agronomic performance. Eventually, the successful products of MABCs are selected and advanced to release as varieties in targeted environments.

Indeed, the above scheme of introgressing of QTLs/genes into varieties of interest has been successfully utilized in several cereal species (Varshney et al. 2006, 2007). It is anticipated that introgression of root trait QTLs in drought-sensitive chickpea varieties should be feasible in the coming years.

## 10.7 Looking Ahead on Root Trait Research and Applications in Chickpea

This chapter presents the importance of root traits in conferring drought tolerance in chickpea. However, molecular mechanisms of root traits at the physiological and genetic level are yet to be understood. On the one hand, the simple screening methods have been developed for precise phenotyping root traits at a large scale, enabling phenotyping of large segregating populations possible. In parallel, the genomic resources including large number of SSR markers, BAC and BIBAC libraries, BAC-end sequences, ESTs, and Solexa tags have been developed (Varshney et al. 2009a). These resources offer the possibility to develop the dense genetic map, transcript maps, and integrated genetic-physical maps of chickpea. These genomic tools should identify the root trait QTLs at a higher resolution that can be used in molecular breeding for drought tolerance in chickpea.

In order to understand the genetic basis of root traits at the molecular and cellular level, it will be possible to delimit root trait QTLs and dissect them at nucleotide level with the help of genomic resources in chickpea as well as in *M. truncatula*, *L. japonicus*, and *G. max* by using comparative genomics. The approaches like "genetical genomics" or "expression genetics" that involves the analysis of gene expression data together with the phenotyping data should provide the insights on direct involvement or regulation of QTL/gene for root trait on drought tolerance. The function of candidate genes can further be validated by using the chickpea TILLING populations recently developed at Washington State University, USA (Rajesh et al. 2007), and ICRISAT. With such available resources, we envision a more rapid understanding of the genetic and functional basis of root traits for drought tolerance.

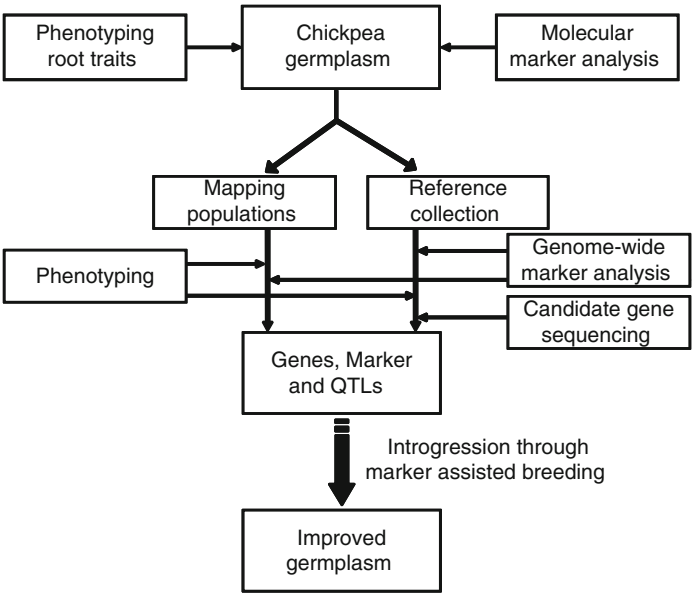


**Fig 10.2** A scheme to utilize the root traits for chickpea improvement. The figure represents the holistic approach combining genomics, physiological, and breeding strategies. For instance, the molecular marker profiling and physiological screening of germplasm provides the contrasting genotypes at genetic as well as physiological level for developing (**a**) the mapping populations and (**b**) the reference collection. The mapping populations can be genotyped with molecular markers and phenotyped for root traits. Linkage analysis together with phenotyping data on the mapping population will provide the QTLs and markers associated with root traits. Similarly, the genome wide molecular genotyping or candidate gene sequencing of the reference collection together with phenotyping data for root traits can be subjected for association genetics and the markers/genes tightly associated with root traits can be identified. Molecular markers/genes identified by linkage analysis or association genetics can be used for marker-assisted breeding to introgress the drought-tolerant genomic regions from drought-tolerant genotypes into drought-sensitive genotypes to develop improved drought-tolerant cultivars of chickpea

Finally, the advancement in chickpea genomics and refinement of root physiology approaches would provide access to agronomically desirable alleles present at QTLs for root traits. A scheme has been proposed in Fig. 10.2, showing the utilization of root traits for chickpea improvement. The combined approach of genomics and physiology in chickpea breeding would enable us to improve the drought tolerance and yield of chickpea under water-limited conditions more effectively.

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# Chapter 11
# Molecular Breeding of Cereals for Aluminum Resistance

**Harsh Raman and Perry Gustafson**

## Contents




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## 11.1 Introduction

Soil acidity limits the production of cereals on over 1.5 billion hectares worldwide and possesses a serious threat to world food security (FAO stat). Crop productivity on acid soils is often restricted due to multiple stresses. On acid soils, there are several limiting factors for plant growth, including toxic levels of aluminum ($Al^{3+}$), manganese, and iron, as well as deficiencies of essential elements, such as phosphorus, nitrogen, potassium, calcium, magnesium, and some micronutrients (2004). Al toxicity is a major factor limiting crop production on highly weathered acid soils (Kochian 1995). The Food and Agriculture Organization of the United Nations (FAO) lists Al toxicity as affecting 14% of all soils worldwide, with the level greater than 50% in many countries (http://www.fao.org/ag/agl/agll/terrastat/wsr.asp#terrastatdb). At low pH (<5), dissolution of Al-containing compounds is enhanced and the release of toxic $Al^{3+}$ cations into soil solution can rapidly inhibit root growth (Delhaize et al. 1993b). Subsequently, Al toxicity may inhibit supply of nutrients, growth hormones, and water mainly due to poorer root penetration (Pan et al. 1989).

A number of key cereal crops such as wheat (*Triticum* ssp.) (Polle et al. 1978), rice (*Oryza sativa* L.) (Nguyen et al. 2001), maize (*Zea mays* L.) (Ceretta 1988; Mazzocato et al. 2002), barley (*Hordeum vulgare* L.) (Reid et al. 1969), sorghum (*Sorghum bicolor* L.) (Blamey et al. 1992), and rye (*Secale cereale* L.) (Gallego and Benito 1997) are sensitive to Al toxicity. The Al resistance order has been reported as maize > rye > triticale (X *Triticosecale* Wittmack) > wheat > barley (Polle and Konzak 1985), rye > oat (*Avena sativa* L.) > millet (*Panicum miliaceum* L.) > bread wheat (*T. aestivum* L.) > barley > durum wheat (*T. turgidum ssp durum* L.) (Bona et al. 1993), and rice > maize > pea > barley (Ishikawa et al. 2000). Most Al-sensitive genotypes show greatly reduced root growth and either die within a few weeks after germination on acidic soils or yield very poorly. The effects of Al toxicity can be more pronounced under drought and heat stress environments.

Symptoms of Al phytotoxicity are not always easily identified in the field; however, the initial and the most dramatic symptom of Al toxicity is inhibition of root elongation, which can occur within minutes of exposure to micromolar toxic concentrations of $Al^{3+}$. Aluminum permeates the plasma membrane and accumulates in the root tips (Samuels et al. 1997). The root apex, where cell division and elongation occurs, is recognized as the main site of Al accumulation and toxicity in sensitive cultivars (Delhaize et al. 1993b; Sivaguru and Horst 1998). However, in maize, distal transition zone is the most Al-sensitive region in the Al-sensitive cultivars such as "Lixis" (Kollmeier et al. 2001; Sivaguru and Horst 1998). Aluminum results from the binding of Al to extracellular and intracellular substances because of the high affinity of Al for oxygen donor compounds. When the root elongation is inhibited by Al, most of the Al is localized on the epidermis and the outer cortex (Jones et al. 2006).

Two strategies have been used to extend cultivation and enhance yield per unit area on acid soils (1) an application of lime for neutralizing the acidity and/or (2)

cultivation of Al-resistant varieties. An application of lime is often not practical because of its slow movement, especially in the deeper layers of subsoils (Foy et al. 1965; Mugwira et al. 1976), and adequate liming may not be economically feasible in many regions of the world (Pandey et al. 1994), especially in low-yielding environments. In addition, heavy application of lime may also have adverse effects on some crops in the rotation or cause deficiencies of certain nutrients (Rao et al. 1993; Whitten 1997). In the literature, both the terms Al tolerance and Al resistance have been used interchangeably. In this review, the term Al resistance is used to cite relevant research on Al resistance/tolerance in cereals.

There are two prerequisites for exploiting resistance genes in breeding programs to develop new varieties (1) presence of genetic variability for Al resistance and (2) understanding the genetic control of Al resistance within the species involved. Genetic variability for Al resistance has been reported among the cultivated and wild germplasm of wheat, barley, rice, rye, oats, sorghum, and maize (Ali et al. 2008; Cançado et al. 1999; Ceretta 1988; Minella and Sorrells 1992; Raman et al. 2008a, b, c; Read and Oram 1995; Reid et al. 1969; Silva et al. 2006) and has been exploited in breeding programs to develop high-yielding varieties with greater resistance to Al toxicity. Crop improvement programs worldwide have developed hundreds of varieties suitable for cultivation on acid soils.

In this chapter, we will describe new advances in understanding of genes and gene complexes conditioning Al resistance in cereals and their further use in molecular breeding via marker-assisted selection and genetic engineering.

## 11.2 Evaluation of Germplasm for Aluminum Resistance

Most methods for testing Al resistance in plants are based upon inhibition of root growth due to Al toxicity. Different methods have been employed for screening germplasm for aluminum resistance including nutrient solution culture (hydroponics), pot assays in the glasshouse, and field evaluation on acidic soils; see review Wang et al. (2006a). However, laboratory and greenhouse-based techniques are widely employed, which are usually nondestructive, and can be performed in early stages of plant development from, only a few days-old seedlings to flowering stage of the plants. There are several advantages of the nutrient solution method over the soil-based assays. In nutrient solution culture, the dose of $Al^{3+}$ and conditions (e.g., pH, light, temperature, humidity, etc.) for screening plants can be precisely controlled. Root measurements from the nutrient solution culture method are much easier as compared with soil assays, as the primary effect of Al toxicity on the plants is the inhibition of root elongation, and the roots are easily observed under nutrient culture. However, root growth measurements are relatively more time-consuming. Root measurements are also dependent upon concentration of particular ions (nutrient status of solution), genetic vigor, and age of seedlings.

Nutrient solution culture-based evaluation is more suited for large-scale screening of germplasm for Al resistance. Several hundred seedlings can be evaluated for

Al resistance, within a week, in a small space, whereas soil-based assays are more labor-intensive, expensive, and require additional glasshouse space. Under nutrient solution culture, Al resistance has been evaluated using hematoxylin (Cançado et al. 1999; Polle et al. 1978), eriochrome cyanine staining (Furukawa et al. 2007; Gruber et al. 2006; Magalhaes et al. 2004), root growth (Raman et al. 2005), and relative root regrowth (Raman et al. 2002, 2005, 2008c). Hematoxylin and eriochrome cyanine stain-based methods are based on the ability of Al-resistant seedlings to continue root growth following a short pulse treatment involving a high Al concentration, while the relative root growth (RRG) method uses the root growth and root resistance index to judge Al resistance over a period of time (usually 2–4 days). Root elongation has been suggested to be one of the most important markers when screening genotypes and cultivars for Al toxicity (Taylor and Foy 1985). Since root growth under Al stress is a combination of root vigor (long roots) and Al resistance, selection of Al resistant genotypes using RRG is preferred as it allows for a better differentiation of genotypes, and it is often used to measure relative level of Al resistance. For example, Hede et al. (2002) evaluated 63 rye accessions from a world spring rye collection for Al resistance using the hematoxylin and the root growth method and demonstrated that the hematoxylin method and the root growth parameter identified genotypes with long root growth under Al stress, but it failed to detect Al resistance in genotypes with poor root vigor. RRG/relative root length or root resistance index has been measured as

$$\text{RRG} = \text{Root growth under Al stress}/\text{control root length } (-\text{ve Al}) \times 100.$$

This technique is very simple to measure and eliminates the genetic difference in root vigor under nutrient culture. Massot et al. (1992) showed that scoring for Al resistance, using root elongation as a single criterion, may avoid the misclassification of genes, which allows for the accumulation of a large amount of Al in shoots. Nutrient culture-based selection for Al resistance has been highly correlated with hematoxylin stain method and field evaluation. Stodart et al. (2007) compared the minimum and maximum root regrowth measures and reported a good relationship between hematoxylin score and the regrowth measure (Fig. 11.1).

Baier et al. (1995) suggested that hydroponic screening of wheat seedlings for Al resistance may be used in breeding programs or in screening germplasm collections. This study correlated root lengths of 43 wheat genotypes grown in Al-containing, acidic hydroponic solutions with root weights from acid-soil experiments and field scores from Brazilian acid-field trials and revealed highly significant correlations ($r = 0.71$–$0.85$) between root length or a root tolerance index in the Al solutions vs. acid-soil experiments and acid-field trials.

Besides the hematoxylin and eriochrome staining methods, Maltais and Houde (2002) reported that nitroblue tetrazolium (NBT) reduction is a simple biochemical marker that is correlated with the degree of Al resistance in wheat, rye, maize, and rice. All the plants were able to grow, demonstrating that this scoring technique is rapid and nondestructive. This Al resistance marker was the first to provide a strong signal in resistant plants rather than in sensitive plants (Bennet 1997; Horst et al. 1997;

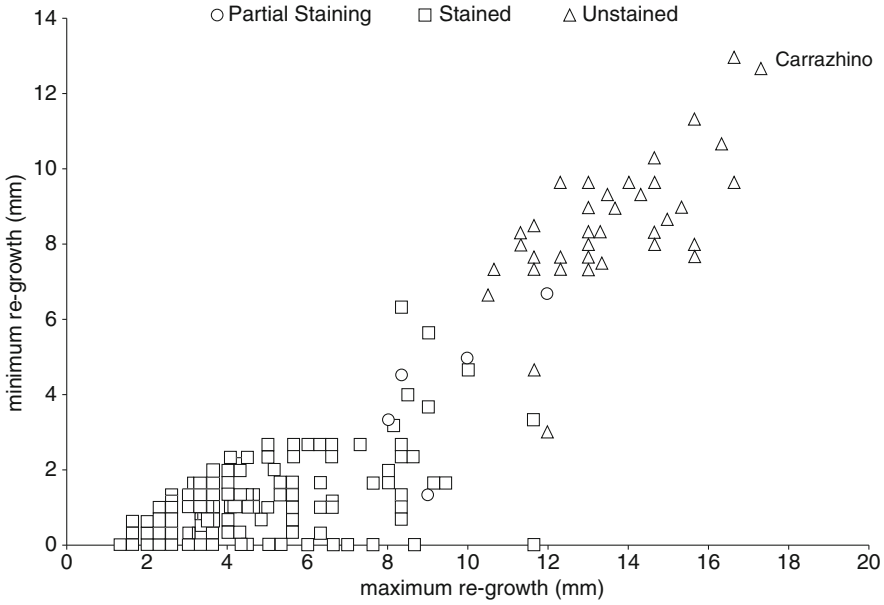


**Fig. 11.1** Comparison of 250 wheat landrace accessions for Al tolerance evaluated using root regrowth measurements and hematoxylin staining (*Unstained*: Al-resistant, *Stained*: Al-sensitive, and *Partial stained*: Intermediate Al-resistant). Al-tolerant reference cultivar, Carrazhino is indicated (after Stodart et al. 2007)

Massot et al. 1992, 1999). NBT test is suitable for screening thousands of plants in a single day/person (Maltais and Houde 2002).

## 11.3 Genetic Variability for Al Resistance

Natural genetic variability for resistance to Al exists within different species of cereals (Bernal and Clark 1997; Bona et al. 1993; Khan and McNeilly 1998; Khatiwada et al. 1996; Pineros et al. 2008; Sivaguru et al. 1992). Among cereal crops, rye is the most resistant cereal (Aniol and Gustafson 1984; Aniol and Madej 1996; Hede et al. 2002; Little 1988; Mugwira et al. 1976), whereas durum wheat is the most Al-sensitive (Bona et al. 1993). In the literature, very limited genetic variability for aluminum resistance has been reported in tetraploid wheats. Raman et al. (2008a) evaluated 408 genotypes of the subspecies *durum*, *dicoccon*, and *carthlicum* of *Tritticum turgidum* ($2n = 4x = 28$, genomes=AABB) for Al resistance using nutrient solution culture techniques. The authors used a new measure "Incremental crop tolerance (ICT)" that reflect the incremental root regrowth between genotypes associated with Al resistance, over and above difference in underlying root vigor. Statistical analysis indicated that three accessions were

Al-resistant in a nutrient solution containing 20 μM of Al. The genetic identity (AABB) of these genotypes was confirmed using genome-specific markers Dgas44, TaALMT1, QSSR (domestication gene-based marker), and gamma gliadin. Despite extensive use of interspecific and intergeneric hybridization to introgress genes for Al resistance, only few wild species have been utilized. Berzonsky and Kimber (1986) evaluated various accessions of goat-grass *Aegilops uniaristata* ($2n = 2x = 14$, genome: NN) and identified useful variation for Al resistance and exploited further to improve Al resistance in wheat (Iqbal et al. 2000).

Rye has been used to introgress superior alleles into wheat, without much success. Triticale, a wheat/rye hybrid, is recognized as particularly Al resistant cereals and is adequate for cultivation in acid soils (Antunes et al. 1996). Among the triticales evaluated, "Arabian" ranked higher in resistance with only 30% reduction in the root growth in contrast with "Beagle," which presented the strongest inhibition (75%). Zhang et al. (1999) performed comparative analyses of genetic variability of Al resistance response in a range of triticale genotypes consisting of six Australian cultivars, eight South African lines, and an Australian control utilizing a solution culture technique and screening under controlled growth cabinet conditions. Results showed that "Tahara," Tahara "S," and "Abacus" were the most Al-resistant triticales among the Australian genotypes in terms of root regrowth characteristics at 10 μg/g Al.

## 11.4 Genetic Control of Al Resistance

The genetics of Al resistance in cereals is reasonably well-understood. Monogenic inheritance for Al resistance has been reported in various populations of wheat (Baier et al. 1995; Delhaize et al. 1993b; Johnson et al. 1997; Kerridge and Kronstad 1968; Luo and Dvorak 1996; Milla and Gustafson 2001; Raman et al. 2005; Somers et al. 1996); barley (Ma et al. 2004; Raman et al. 2003; Reid et al. 1971; Tang et al. 2000; Wang et al. 2006b, 2007), rye (Zhang and Jessop 1998), oats (Wight et al. 2006), sorghum (Gourley et al. 1990; Magalhaes et al. 2004), and maize (Moon et al. 1997; Rhue et al. 1978). Most of the cereals display a range of genetic variation for Al resistance, which seems to be under control of different alleles conditioning different levels of Al resistance (Minella and Sorrells 1992; Raman et al. 2005). However, multigenic inheritance for Al resistance has been observed in wheat, barley, rice, and maize (Berzonsky 1992; Echart et al. 2002; Lima et al. 1992; Nguyen et al. 2001, 2002; Ninamango-Cardenas et al. 2003; Raman et al. 2005). Aniol and Gustafson (1984) associated chromosome arms 6AL, 7AS, 2DL, 3DL, 4DL, 4BL, and 7D with Al resistance in the wheat landrace cultivar "Chinese Spring." A single gene with multiple alleles conditioning various degrees of Al resistance appears to be common in wheat, maize, and rice (Nguyen et al. 2001; Raman et al. 2008c; Sibov et al. 1999).

Al resistance and malate efflux has been reported to be under the control of a single gene in wheat populations derived from ET3/ES3 (Delhaize et al. 1993a),

Diamondbird/Janz and CD87/Currawong (Raman et al. 2005), and locus *XME* involved in malate efflux and *Alt* gene for aluminum resistance has been colocated on the long arm of chromosome 4D (Raman et al. 2005) where Al resistance has been mapped in other populations (Luo and Dvorak 1996; Riede and Anderson 1996). Besides one major QTL on chromosome 4DL, two additional QTLs located on 5AS and 2DL and one region located on chromosome 7AS were identified to contribute Al-resistance in Chinese Spring (Ma et al. 2006; Papernik et al. 2001). In the wheat cultivar "Atlas66," a minor QTL was located on chromosome 3BL (Cai et al. 2008; Zhou et al. 2007).

Rye is an obligate outcrossing species in which Al resistance is conditioned by at least four major independent and dominant loci, *Alt1, Alt2, Alt3*, and *Alt4*, located on chromosome arms 6RS, 3R, 4RL, and 7RS, respectively (Aniol 2004; Aniol and Gustafson 1984; Camargo et al. 2000; Collins et al. 2008; Gallego and Benito 1997; Gallego et al. 1998a, b; Matos et al. 2005; Miftahudin et al. 2002, 2005).

Triticale, the hybrid between wheat and rye, has considerable variation for Al resistance. Zhang et al. (1999) investigated genetic variation in root regrowth characteristics among eight triticale genotypes using root regrowth, following Al stress. Highly significant variation due to both general combining ability and specific combining ability effects indicated that both additive and nonadditive effects were important in explaining the genetic variation for Al resistance. The high estimates of heritability and the predictability ratio for root regrowth revealed the preponderance of additive genetic variance in the inheritance of Al resistance.

Al resistance genes have been reported to be dominant across a range of Al concentration in wheat (Delhaize et al. 1993b; Kerridge and Kronstad 1968). However, incomplete transfer of genes for aluminum resistance has been reported in wheat (Delhaize et al. 1993b). Tang et al. (2002) observed that neither wheat cultivars "Centuary-T" nor "Chisholm-T," which each contain an Al resistance genes from "Atlas 66," possessed the same level of Al resistance as "Atlas 66," as previously suggested (Johnson et al. 1997). Similarly, the reduction of Al resistance genes from rye when they are present in a wheat background was observed. Aniol and Gustafson (1984) suggested that "some genes" are acting as modifiers and thus alter the expression of Al resistance gene. Aniol and Gustafson (1984) also reported that the loss of number of different wheat chromosome arms reduced Al resistance. The loss of the short arm of wheat chromosomes 5A or 7A, or the long arm of chromosome 4D, resulted in a much lower rate of Al-induced malate release from the root apex (Tang et al. 2002). These findings suggest that there are loci located on wheat chromosomes 5A and 7A that have the capacity to modify the expression of Al resistance via malate efflux. Besides one major QTL located on wheat chromosome arm 4DL, two additional QTLs located on wheat chromosome arms 5AS and 2DL and one region located on wheat chromosome arm 7AS were identified to also contribute to Al resistance (Ma et al. 2006).

It has been documented that Al resistance is a dosage-dependent trait (Minella and Sorrells 1997); therefore, there is a need to develop varieties resistant to high concentration of Al.

## 11.5 Molecular Mapping of Al Resistance Loci

The identification of DNA markers diagnostic for Al resistance can accelerate the development of acid-soil-resistant cultivars that can remain productive even under Al stress. Molecular markers have proven to be efficient tools in identifying specific loci controlling qualitative and quantitative traits including for Al resistance. Al resistance loci have also been mapped using cytogenetical (Aniol and Gustafson 1984; Lagos et al. 1991; Minella and Sorrells 1997) and linkage mapping approaches (see reviews Garvin and Carver 2003; Wang et al. 2007). Two methods based upon bulk-segregant analysis and QTL mapping (Ma et al. 2004; Magalhaes et al. 2004; Nguyen et al. 2001, 2002, 2003; Ninamango-Cardenas et al. 2003; Raman et al. 2002, 2005; Tang et al. 2000; Wu et al. 2000) have been used predominantly for locating loci associated with Al resistance in cereals (Table 11.1). Marker-trait linkages are typically determined by linkage and QTL mapping approaches utilizing $F_2$, DH, or recombinant inbred line (RIL) populations developed from contrasting-phenotypic parental genotypes. Al resistance loci (Table 11.1, Fig. 11.2) have been tagged using molecular markers based upon randomly amplified polymorphic DNA-RAPDs (Loarce et al. 1996; Masojć et al. 2001; Philipp et al. 1994; Senft and Wricke 1996), restriction fragment length polymorphism-RFLPs (Riede and Anderson 1996; Tang et al. 2000), simple sequence repeat-SSR (Cai et al. 2008; Ma et al. 2005; Masojć et al. 2001; Raman et al. 2001, 2002, 2006; Saal and Wricke 1999; Wang et al. 2007), amplified fragment length polymorphisms-AFLP (Miftahudin et al. 2002; Raman et al. 2002; Wu et al. 2000), diversity arrays technology-DArT techniques (Wang et al. 2007; Wenzl et al. 2006), and candidate gene markers (Fontecha et al. 2007; Raman et al. 2005, 2008c; Wang et al. 2007) in biparental populations.

**Table 11.1** Wheat and *Aegilops tauschii* genotype by Al resistance phenotype, *TaALMT1* coding allele, and GenBank accession number (adapted from Raman et al. 2005)

| Genotype | Phenotype* | Allele | NCBI genbank accession no. |
|---|---|---|---|
| "ET8" | Al-res | *TaALMT1-1* | DQ072260 |
| "Tasman" | Al-res | *TaALMT1-1* | DQ072270 |
| "Diamondbird" | Al-res | *TaALMT1-1* | – |
| "Halberd" | Al-res | *TaALMT1-2* | DQ072265 |
| "Chinese Spring" | Al-res | *TaALMT1-2* | DQ072262 |
| "Maringa" | Al-res | *TaALMT1-2* | DQ072267 |
| "Embrapa" | Al-res | *TaALMT1-1* | DQ072264 |
| "Currawong" | Al-res | *TaALMT1-2* | – |
| "Cranbrook" | Al-sens | *TaALMT1-1* | DQ072263 |
| "Spica" | Al-sens | *TaALMT1-2* | DQ072268 |
| "Sunco" | Al-sens | *TaALMT1-2* | DQ072269 |
| "Janz" | Al-sens | *TaALMT1-2* | DQ072266 |
| "CD87" | Al-sens | *TaALMT1-2* | – |
| "ES8" | Al-sens | *TaALMT1-2* | DQ072261 |
| *Aegilops tauschii* | Al-sens | *TaALMT1-1* | DQ072271 |

Al-res* and Al-sen* refer to Al-Resistant and Al-Sensitive, respectively

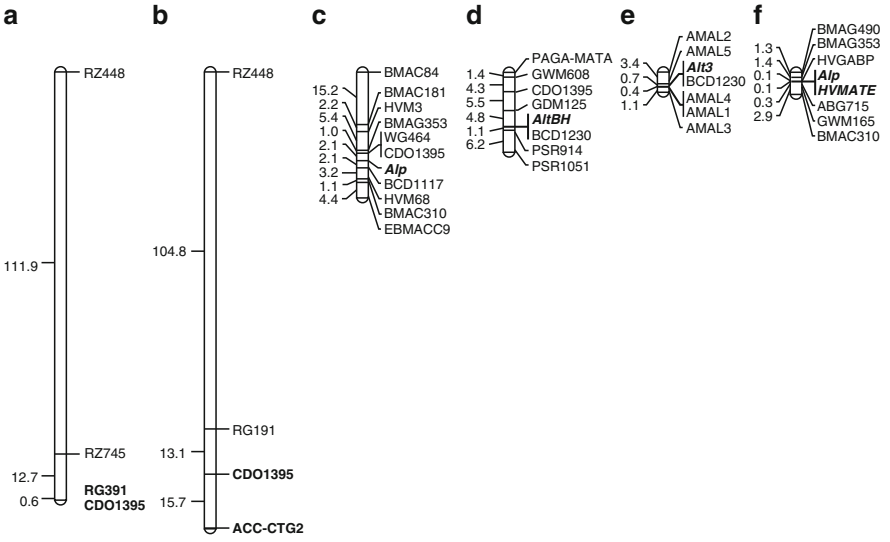


**Fig. 11.2** Location of qualitative and quantitative loci conditioning Al tolerance in wheat, barley, rice, and rye that were mapped on homologous group 4 chromosomes. Bold letters indicate position of loci associated with Al tolerance. (**a**) IR64/*O.rufipogon* (Nguyen et al. 2002), (**b**) IR1552/Azucena (Wu et al. 2000), (**c**) Dayton/Harlan Hybrid (Raman et al. 2003), (**d**) Milla and Gustafson (2001), (**e**) Miftahudin et al. (2002)=Alt4 on 7RS and (**f**) Dayton/Gairdner (Wang et al. 2007)

Association mapping can be utilized for investigating linkage disequilibrium close to Al resistance gene. This technique allows the utilization of germplasm and advanced breeding lines rather than structured segregating populations, which allows for genes associated with traits of interest to be identified by correlating phenotype (Al resistance) with specific alleles at the linked loci. Most of the breeding programs have phenotyping data with Al resistance of the breeding populations/germplasm. Genotyping can be performed using whole genome scanning at marker loci and then correlating with performance of plants under Al stress (e.g., acid soils/nutrient solution).

### *11.5.1 Rye and Triticale*

At least four independent and dominant loci associated with Al resistance, *Alt1*, *Alt2*, *Alt3*, and *Alt4*, located on chromosome arms 6RS, 3R, 4RL, and 7RS, respectively, have been described (Aniol 2004; Aniol and Gustafson 1984; Collins et al. 2008; Fontecha et al. 2007; Gallego and Benito 1997; Gallego et al. 1998a; Ma et al. 2000; Matos et al. 2005; Miftahudin et al. 2002, 2004). Previously, *Alt3* was mapped to the long arm of chromosome 4R (4RL) in the population derived from M39A-1-6 × M77A-1 (Miftahudin et al. 2002, 2004, 2005). More recently, Collins

et al. (2008) confirmed that Al resistance is controlled by an *Alt4* locus that maps on the short arm of chromosome 7R (7RS) instead of 4RL (*Alt3*) in the mapping population from the M39A-1-6 × M77A-1 cross. This location is consistent with a previous report of Benito et al. (2009). Ma et al. (2000) compared 3DS.3RL translocation line (ST22) and a nonsubstitution line (ST2) of triticale for aluminum resistance and suggested the location of Al resistance gene on the short arm of triticale chromosome 3R.

### *11.5.2 Barley*

The cultivar "Dayton" is one of the most Al-resistant genotypes (Minella and Sorrells 1992), and a single locus (*Alp*) on the long arm of the chromosome 4H (4HL) conditions Al resistance in different "Dayton" derived populations (Raman et al. 2003; Tang et al. 2000; Wang et al. 2004a). Stølen and Andersen (1978) reported a dominant allele at *Pht* locus (4HL), which controls high resistance to acidic soils. The same locus conditions Al resistance in other populations (Table 11.1) including those generated from "Harrington"/"Brindabella" (Raman et al. 2001), "Yambla"/"WB229," "Mimosa"/"WB229" (Raman et al. 2002), "Murasakimochi"/"Morex" (Ma et al. 2004), "Ohichi"/"F6ant28B48-16" (Raman et al. 2005), and "F6ant28B48-16"/"Honen" (Wang et al. 2006b). Minor gene effects for Al resistance in barley have also been suggested (Echart et al. 2002; Raman et al. 2005) and require further validation.

### *11.5.3 Oat*

Wight et al. (2006) utilized a mapping population of diploid oat *A. strigosa* Schreb derived from a cross between "CIav 2921" (Al sensitive) and "CIav 9011" (Al resistant) and identified four QTLs. The largest QTL explained 39% of the variation, was associated with the bcd1230 marker, and is possibly orthologous to the major gene found in the *Triticeae* as well as *Alm1* in maize and a minor gene in rice. A second QTL may be orthologous to the *Alm2* gene in maize. Two other QTLs were associated with anonymous markers, which together accounted for 55% of the variation.

### *11.5.4 Rice*

QTL studies have identified 40 Al resistance loci on all 12 rice chromosomes, although the number and locations vary depending on the cross or species used (Ma et al. 2002; Nguyen et al. 2001, 2002, 2003; Wu et al. 2000). Epistatic effects

have also been observed (Wu et al. 2000). However, the greatest effect on Al resistance was associated with genomic regions on chromosome 1 and 3 (Nguyen et al. 2001, 2002, 2003; Wu et al. 2000). One of the QTLs mapped on chromosome 12 was linked with RG9 marker, which was linked with the major QTL for phosphorus uptake efficiency in rice (Ni et al. 1988). Recently, Chuan-zao et al. (2004) identified QTLs for relative root length on chromosomes 1, 9, and 12 and one QTL for root length under Al stress on chromosome 1.

### *11.5.5 Maize and Sorghum*

In maize, at least seven QTLs associated with Al resistance have been found on chromosomes 2, 6, 8, and 10; the number and locations varied depending on the cross (Ninamango-Cardenas et al. 2003; Sibov et al. 1999). In sorghum, a single locus, *AltSB*, was found to control Al resistance in two highly Al resistant sorghum cultivars and was mapped near the end of sorghum chromosome 3 (Magalhaes et al. 2004).

Generally, molecular markers that map within 5 centimorgan from the target gene are recognized as "good" markers for utilization in marker-assisted selection crop improvement programs. However, these markers are of limited value for map-based cloning of the Al resistance gene as it requires very high-density map of the target gene. Furthermore, most of the mapping studies in cereal utilized very small mapping populations to locate loci associated with Al resistance, and their linkage with marker loci may not be very accurate. High-resolution mapping can be achieved by selecting molecular markers flanking "target" gene of interest (Al resistance) from low resolution mapping studies and selecting recombinant plants, which are then selfed and their $F_{2:3}$ progeny are assessed for Al resistance, from a large $F_2$ population comprising 1,000–3,000 individuals. The main advantage of this strategy is to select informative genotypes and to avoid extensive cost and time required in comprehensive and accurate phenotyping. Intercross populations are preferred over the doubled haploid populations as they are more informative due to more accurate recombination frequencies and are easy to generate. This strategy has been used to construct high density map of Al resistance loci in barley, rye, and sorghum (Magalhaes et al. 2007; Wang et al. 2007) and to clone a *AltSb* gene for aluminum resistance in sorghum (Magalhaes et al. 2007). Map-based cloning approach has been used successfully to clone Al resistance genes in cereals. Transposon-tagging strategy has not been feasible due to the lack of an active transposon system in key cereals except in maize.

## 11.6 Molecular Synteny

Comparative mapping studies using molecular markers have revealed extensive synteny or colinearity among the genomes of rice, wheat, barley, rye, oat, maize, and sorghum (Devos and Gale 2000). A conserved genomic region on the long arm

of group 4 chromosomes: wheat 4D, barley 4H, rye 4R and short arm of 7R, and rice 3 exhibit macrosynteny (Devos and Gale 2000; Gale and Devos 1998; Miftahudin et al. 2004; Namuth et al. 1994; Nguyen et al. 2003; Raman et al. 2002; Rognli et al. 1992; Tang et al. 2000; Van Deynze et al. 1995; Vos et al. 1995), and all harbor loci for Al resistance (Luo and Dvorak 1996; Matos et al. 2005; Miftahudin et al. 2002, 2004; Nguyen et al. 2003; Raman et al. 2005; Riede and Anderson 1996; Tang et al. 2000; Wang et al. 2007).

Comparative mapping of the Al resistance loci in cereals can be assessed and validated with a set of common markers linked with different Al resistance loci. For example, Wang et al. (2007) showed that a wheat SSR marker (GWM165-4DL) was located 0.45 cm from the *Alp* locus on the long arm of barley chromosome 4H and has also been located 1.5 cm apart from BCD1117 in the cMap of wheat chromosome 4D (http://rye.pw.usda.gov). Tang et al. (2000) reported that BCD1117 and CDO1395 markers flank the *Alp* locus (Fig. 11.2). Marker CDO1395 that maps on rice chromosome 3S also explained approximately 20–40% of the genetic variation for Al resistance in the rice and wheat populations (Nguyen et al. 2003; Riede and Anderson 1996). The marker BCD1230 exhibited tight linkage with the Al resistance locus *Alt4* in rye (Collins et al. 2008; Miftahudin et al. 2004), and $Alt_{BH}$ in wheat (Riede and Anderson 1996), but was mapped 33 cm away from *Alp* locus in barley. This suggests that a colinearity breakage due to DNA rearrangement between the chromosomes 4H of barley and 4D of wheat (Tang et al. 2000). Milla and Gustafson (2001) reported a high degree of synteny between wheat, rye, barley, rice, maize, and oat in the regions around the BCD1230 locus. This gene encodes for a ribulose 5 phosphate 3 epimerase (R5P3E) gene, which is present in one single copy in barley, rye, rice, and wheat. Interestingly, rye marker B4, which is tightly linked with the *Alt4* locus on 7RS (Collins et al. 2008; Miftahudin et al. 2004), mapped to chromosome 2H in barley instead of the expected 4H (Gruber et al. 2006; Wang et al. 2007). Authors suggested that multiple copies of B4 may exist in the barley genome, or there may be some conservation of genes between chromosomes 2H and 4R. Silva-Navas et al. (2008) reported another *ScAMLT2* gene in rye that showed sequence identities with barley *ALMT1* homolog *HvALMT1* (Delhaize et al. 2007; Gruber et al. 2006) and maps on the long arm of chromosome 2R. This suggests that there may be multiple copies of *TaALMT1* at least in genomes of barley and rye.

If genetic mapping anchors similar traits (such as Al resistance) to the collinear chromosomal regions in different genomes, there is a good reason to suspect that these loci are encoded by different alleles of a single gene (Bennetzen and Freeling 1997). Al-resistance genes from wheat, barley, and sorghum (i.e., *TaALMT1*, *HvMATE*, and *ScMATE*) have been recently isolated and mapped using biparental populations – see Fig. 11.3 (Magalhaes et al. 2007; Sasaki et al. 2004; Wang et al. 2007). *TaALMT1* has shown a complete linkage with Al resistance locus on the long arm of chromosome 4D of wheat (Raman et al. 2005; Ma et al. 2005). Fontecha et al. (2007) reported a *TaALMT1* homolog in rye, *ScALMT1*, and exhibited cosegregation with *Alt4* locus of rye on 7RS, which is consistent with the expected synteny relationships between the wheat 4DL and barley 4HL. Sequence identities between *TaALMT1* gene in wheat on

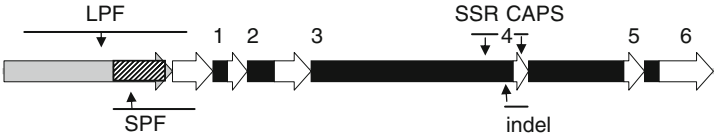


**Fig. 11.3** Structure of the Ta*ALMT1gene* – Adapted from Raman et al. (2005, 2006, 2008c). *White arrows* represent the six exons that are interrupted by five introns (*black blocks*). LPF and SPF represent long and short promoter fragments of the *TaALMT1* gene (Sasaki et al. 2004, 2006). Locations of SSR motif (intron 3) and SNP in exon 4 are indicated with *down arrows*

4DL and *ScALMT1* gene on 7RS indicate that both genes are orthologous. However, *TaALMT1* gene does not show any sequence identities with rice chromosome 3 pseudomolecules but showed an approximately 90% sequence identities to the rice genes located on the long arm of chromosome 4 (Delhaize et al. 2007). This suggests breakdown of macrosynteny among members of triticeae.

In barley, Wang et al. (2007) delineated the *Alp* locus to a 0.2 cm region in the high resolution mapping population by the flanking markers HvGABP and ABG715 on the long arm of barley chromosome 4H. This region is syntenic with 120 kb sequence on chromosome 3 (Wang et al. 2007). Within this region, there were no orthologs of wheat *TaALMT1* gene. Instead, Wang et al. (2007) identified a gene encoding a multidrug and toxic compound extrusion (MATE) within this syntenic region, which showed cosegregation with the *Alp* locus for Al resistance (Fig. 11.3). These results clearly indicate that despite a similar chromosomal location for Al resistance loci in wheat and barley, the genes are likely to encode different proteins and are therefore not orthologous. In rye, ortholog of *HvMATE*, *ScMATE* was mapped within 27.5 cm from *Alt4* locus on chromosome 7RS (Collins et al. 2008). Ryan et al. (2009) also reported a correlation between expression of *TaMATE* gene with citrate efflux involved in Al resistance in an $F_2$ population (Egret/Carazinho) of wheat on chromosome 4B. Existence of *MATE* and associated Al resistance loci on chromosomes 4BL in wheat (Ryan et al. 2009), 4HL in barley (Furukawa et al. 2007; Wang et al. 2007), 7RS in rye (Collins et al. 2008), and 3S in rice (Nguyen et al. 2003) indicates genetic synteny for Al resistance via citrate efflux. Members of the *MATE* family were also shown to facilitate citrate efflux from *Arabidopsis* and sorghum (Durrett et al. 2007; Magalhaes et al. 2007). Al resistance locus in sorghum *AltSB* was not also located within the syntenic region of group 4 chromosomes. Therefore, *AltSB* appears to be different from the major Al resistance loci in the *Triticeae*. Intertribe map comparisons suggest that a major Al resistance rice chromosome 1 QTL is likely to be orthologous to *AltSB*. In maize, Al resistance loci have been identified on chromosomes 2, 6, and 10 (Brondani and Paiva 1996; Sibov et al. 1999). Comparative mapping analysis indicated that the maize QTL region bin 6.05 (Ninamango-Cardenas et al. 2003) is homoeologous to rice chromosome 5, where Nguyen et al. (2001, 2002, 2003) mapped a QTL for Al resistance in rice. Another QTL region (bin 8.07) of Al (Ninamango-Cardenas et al. 2003) was found to be syntenous with rice chromosome 1 and sorghum linkage group G (Magalhaes et al. 2004).

## 11.7 Mechanism of Aluminum Resistance

A number of physiological and biochemical mechanisms underlying aluminum resistance have been proposed; see reviews (Kochian 1995; Larsen et al. 1998; Moroni et al. 1991; Pellet et al. 1996). Ma et al. (2001) proposed two main mechanisms of Al resistance (1) external resistance mechanisms, by which Al is excluded from plant tissues, especially the symplastic portion of the root meristem; and (2) internal resistance mechanisms, allowing plants to tolerate $Al^{3+}$ in the plant symplasm where Al that has permeated the plasmalemma is sequestered or converted into an innocuous form. The details of these mechanisms are reviewed in a separate chapter of this book (see Kochian). Among different mechanisms, Al-activated exudation of low molecular weight organic acids (malate, citrate, and oxalacetate) from root tips is now reasonably well-understood (Table 11.2) and has been tested in an array of germplasm (Furukawa et al. 2007; Miyasaka et al. 1989, 1991; Raman et al. 2008c; Zhao et al. 2003). For example: Al-resistant wheat genotypes release greater amounts of malate from their root apices as compared to Al-sensitive wheat (Christiansen-Weniger et al. 1992; Delhaize and Ryan 1995; Delhaize et al. 1993b; Raman et al. 2005, 2008c; Rincon and Gonzales 1992; Snowden and Gardner 1993; Tang et al. 2002).

Al-activated efflux of organic acids is hypothesized to protect the root apices from Al toxicity by chelating and detoxifying the harmful $Al^{3+}$ cations in the apoplasm or in the soil adjacent to the root apices, the most sensitive part of the root system (Aniol 1996; Basu et al. 2001; Delhaize and Ryan 1995; Kinraide et al. 2005; Miyasaka et al. 1989). This is further supported by studies showing that $Al^{3+}$ ions activate anion currents at the root apices of Al-resistant seedlings (Ryan et al. 1997) via secretion of organic acids such as malate (Zhang et al. 2001). $Al^{3+}$-inducible resistance mechanisms in rye, and triticale, where a lag in Al-activated

**Table 11.2** Examples of organic acid secreted by root apices of the key cereals

| Genotype | Organic acids | Reference |
|---|---|---|
| Bread wheat | Malate | Ishikawa et al. (2000), Papernik et al. (2001), Raman et al. (2005) |
| | Citrate | Ryan et al. (2009) |
| Barley | Citrate | Ma et al. (2004), Wang et al. (2007) |
| Rice | Citrate | Ma et al. (2002) |
| Rye | Citrate and malate | Li et al. (2000), (Ma et al. 2000) |
| Corn | Citrate, malate, and oxalate | Pineros et al. (2002, 2005), Piñeros and Kochian (1999), Kidd et al. (2001), Kollmeier et al. (2001), Mariano and Keltjens (2003), Pellet et al. (1995), Wang et al. (2004b) |
| Oat | Citrate, malate | Zheng et al. (1998a) |
| Sorghum | Citrate | Magalhaes et al. (2007) |
| Triticale | Citrate and malate | Stass et al. (2008), Ma et al. (2000) |
| Buckwheat (*Fagopyrum esculentum* Moench.) | Oxalate | (Ma et al. 1997), Zheng et al. (1998b) |

organic acid efflux and the rate of exudation increases over the first 12–24 h of Al exposure, has been reported (Ellis et al. 2000). However, in wheat, malate exudation is rapidly activated by Al exposure and the rate of efflux does not seem to be increase over time. This is further supported by the presence of different organic acid transporters such as TaALMT1 (Delhaize et al. 2004), HvMATE/HvAACT1 (Furukawa et al. 2007), and SbMATE (Caniato et al. 2007) in wheat, barley, and sorghum, respectively. In addition, other genes such as cysteine synthase have been implicated in Al response in rice. More recently, Ryan et al. (2009) reported a *TaMATE* gene associated with citrate efflux at least in two populations of wheat derived from "Carazinho" – an Al-resistant wheat cultivar from Brazil. This gene was located on the long arm of chromosome 4B. Above evidence suggest that Al resistance is a multigenic trait.

## 11.8 Functional Genomic Approaches in Elucidating and Validating Al Resistance Mechanisms

Whole genome sequencing approaches have allowed sequencing the genomes of more than ten plant species including poplar and papaya. Wheat and barley genomes are being sequenced under international consortia and will provide insights into gene functions, evolution, and the origin of different cultivars. Further research in genomics, sequencing, and bioinformatics platforms will enable us in deciphering and manipulating the aluminum resistance in key cereals. Some of these advancements on gene discovery, high-throughput gene expression using microarray, altering gene expression by transformation technologies, and functional characterization of Al resistance genes are discussed below:

### *11.8.1* TaALMT1 *Gene Family*

Various molecular and physiological studies have provided evidence that organic acid efflux and internal detoxification are the key mechanisms in Al resistance in cereals. During the last 5 years, significant advancements have been made in the discovery of candidate functional genes for Al-resistance such as *TaALMT1* (originally named *ALMT1)* in wheat (Sasaki et al. 2004, 2006; Yamaguchi et al. 2005), *HvAACT1*/*HvMATE* in barley (Furukawa et al. 2007; Wang et al. 2007), and *MATESb* in sorghum (Magalhaes et al. 2007). *ALMT1* members facilitate transport of malate in wheat and rye (Collins et al. 2008; Sasaki et al. 2004), whereas *MATE* proteins transport citrate in Arabidopsis, barley, rye, and sorghum (Furukawa et al. 2007; Wang et al. 2007; Magalhaes et al. 2007; Collins et al. 2008).

*TaALMT1* encodes a membrane-localized transporter (Yamaguchi et al. 2005) that facilitates an Al-activated malate efflux. This gene has been isolated and

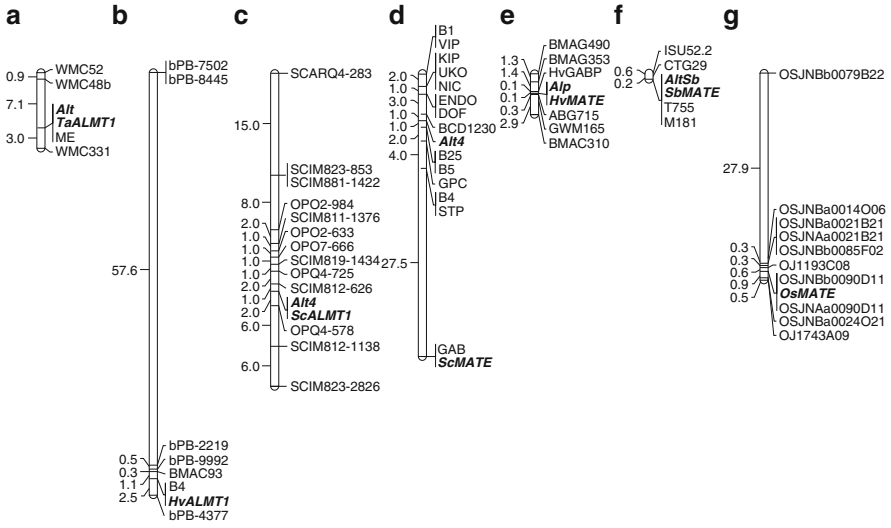


**Fig. 11.4** Molecular mapping of the major genes conditioning Al tolerance in wheat, barley, rye, and Sorghum. (**a**) Diamondbird/Janz (Raman et al. 2005), (**b**) Dayton/Zhepi 2 (Wenzl et al. 2006; Gruber et al. 2006; Wang et al. 2007), (**c**) Ailes/Riodeva (Fontecha et al. 2007), (**d**) Dayton/Zhepi 2 (Wang et al. 2007), (**e**) (Magalhaes et al. 2007), and (**f**) Physical map of rice (http://www.tigr.org), validated on 31st Jan 2008

characterized from different wheat genotypes (Raman et al. 2005; Sasaki et al. 2004). Molecular analysis has indicated that the *TaALMT1* locus harbors two alleles: *TaALMT1-1* and *TaALMT1-2* (Table 11.1). These alleles differ by six nucleotides of which only two nucleotides encode for different amino acids in the predicted protein (Sasaki et al. 2004).

The coding region of *TaALMT1* is interrupted by five introns ranging from 0.1 to 1.8 kb (Fig. 11.4). *TaALMT1* possesses at least 44 SNPs or small insertions/deletions (InDels) (Raman et al. 2005). These polymorphisms in the introns are in addition to six SNPs in the exons. Two of the six SNPs located in exons result in amino acid changes in the predicted protein, and one of these, in exon 4, was used to develop a CAPS marker to distinguish *TaALMT1-1* from *TaALMT1-2* (Sasaki et al. 2004). The intron 3 region is the largest and shows considerable allelic variability (Raman et al. 2005, 2006, 2008c). These variations have been reported to be due to simple sequence repeat motifs (SSR) with variable copy numbers and InDels.

Upstream and downstream sequence of the *TaALMT1* was characterized to identify allelic variations in 69 wheat lines (Sasaki et al. 2006). The first 1,000 bp downstream of *TaALMT1* was conserved among the lines examined apart from the presence of a transposon-like sequence, which did not correlate with Al resistance. However, the first 1,000 bp upstream of the *TaALMT1* coding region was more variable and six different promoter patterns could be discerned (types I–VI). Type I had the simplest structure, while the others had blocks of sequence that were duplicated or triplicated in different arrangements (Sasaki et al. 2006). Besides

six promoter patterns, allelic variants were also reported recently in highly diverse germplasm comprising wheat cultivars, subspecies, and landraces of wheat (Raman et al. 2008c).

### 11.8.2 Homologs and Paralogs of *TaALMT1*

Given that *TaALMT1* encodes Al-activated malate transporter that facilitates Al-activated malate exclusion in roots, it is quite likely that other Al-resistant plant species that secrete organic acids from root apices may harbor "similar" gene. Molecular analysis data has revealed that *TaALMT1* homologs exist in Arabidopsis, wheat, barley, maize rye, lupin, and Brassica.

In barley, Gruber et al. (2006) identified a *TaALMT1* homolog *HvALMT1*. Recently, Fontecha et al. (2007) identified a homolog to wheat *TaALMT1* in rye, *ScALMT,* at the *Alt4* locus for Al resistance on chromosome 7RS in rye. This gene encodes protein with 86% identity to *TaALMT1*. PCR primers were designed from a *TaALMT1*, and this enabled to clone a paralog rye gene designated as *ScALMT1*. This gene was found to cosegregate with the *Alt4* located on 7RS by PCR amplification using the wheat–rye addition lines (Fontecha et al. 2007). SNP polymorphisms for this gene were detected among the parents of three $F_2$ populations that segregate for the *Alt4* locus. Aluminum induces expression of *ScALMT1* particularly to a higher level in root apices of Al-resistant cultivar as compared to a sensitive cultivar.

Recently, Pineros et al. (2008) cloned *ZmALMT1*, a maize gene homologous to the wheat *TaALMT1* and Arabidopsis *AtALMT1* genes. Transient expression of a *ZmALMT1*::GFP chimera confirmed that the protein is targeted to the plant cell plasma membrane. Gene expression data as well as biophysical transport characteristics obtained from *Xenopus* oocytes expressing *ZmALMT1* by Pineros et al. (2008) further indicated that this transporter is implicated in the selective transport of anions involved in mineral nutrition and ion homeostasis processes rather than mediating a specific Al-activated citrate exudation response at the rhizosphere of maize.

A gene from *Arabidopsis* (*AtALMT1*; At1g08430) encoding a *TaALMT1*-like protein is located within an $Al^{3+}$ resistance QTL located on chromosome 1 (Hoekenga et al. 2006). Aluminum not only activates *AtALMT1* to trigger malate efflux but also is required to induce its expression (Gabrielson et al. 2006; Hoekenga et al. 2006). An $Al^{3+}$-sensitive mutant of *Arabidopsis* Columbia ecotype with a disrupted *AtALMT1* gene is reported to lose the capacity for $Al^{3+}$-activated malate efflux (Hoekenga et al. 2006).

In rapeseed *Brassica napus*, two *TaALMT1* paralogs *BnALMT1* and *BnALMT2* encoding proteins with 80% amino acid sequence identity to *AtALMT1* and in *Brassica oleracea* (*BoALMT1*) were cloned (Delhaize et al. 2007; Ligaba et al. 2006). *BnALMTs* conferred an $Al^{3+}$-activated efflux of malate and increased $Al^{3+}$ resistance in tobacco cell suspensions (Ligaba et al. 2006).

### 11.8.3 MATE Gene Family

The multidrug and toxic compound extrusion (MATE) family proteins are proposed to transport small, organic compounds (Omote et al. 2006) and are the members of a large and complex family of transporters. The human genome also contains *MATE1* and *MATE2* genes encoding MATE transporters and is reported to transport various organic cations including toxins (Hiasa et al. 2006; Masuda et al. 2006; Otsuka et al. 2005). In contrast to *MATE* genes in the bacterial and animal kingdom, plants contain more MATE-type transporters. For example, there are 58 MATE orthologs in the genome of *Arabidopsis thaliana* (Omote et al. 2006). However, the functions of most genes are still unknown. The first report of a plant *MATE* transporters concerned *AtALF5*, which was identified from a mutant-defective, aberrant lateral root formation in Arabidopsis (Diener et al. 2001). Heterologous expression of *AtALF5* in yeast conferred resistance to tetramethylammonium, suggesting that its function involved detoxification as an efflux transporter for xenobiotics.

Recently, several MATE transporters conferring Al resistance have been reported. Two independent studies indicated that the *HvMATE* gene conditions Al resistance in barley (Furukawa et al. 2007; Wang et al. 2007). Furukawa et al. (2007) identified essentially the same gene (*HvAACT1*) responsible for the Al-activated citrate secretion by fine mapping combined with microarray analysis, using an Al-resistant barley cultivar, "Murasakimochi," and an Al-sensitive cultivar, "Morex," and found the gene to be localized in barley root tip epidermal cells. The study utilized an $F_4$-derived mapping population from the "Murasakimochi"/"Morex" population (Ma et al. 2004). The Al-resistant cultivar "Murasakimochi" secreted a large amount of citrate from the roots in response to Al while "Morex" did not (Ma et al. 2004). Furukawa et al. (2007) performed a microarray analysis with Barley 1 GeneChip (Affymetrix Co.) to identify up- or downregulated transcripts between "Murasakimochi" and "Morex" with and without Al treatment. This analysis identified the transcript that encodes a member of the multidrug and toxic compound extrusion (MATE) family (Barley1 probe name: Contig9960_at). The homolog of this gene exists on rice chromosome 3, which corresponds to *HvAACT1* in barley. In *Arabidopsis*, MATE family members, *FRDL* showed the highest homology to *HvAACT1* with 59% identity and 86% similarity. The coding region of *HvAACT1* was 1,668 bp long, and the deduced polypeptide was 555 amino acids.

The *MATE* gene also conditions Al resistance in sorghum. Magalhaes et al. (2007) performed high resolution mapping of *Altsb* by screening 4,170 gametes from an $F_2$ population derived from "SC283" (Al resistant) × "BR007" (Al sensitive) and identified a gene encoding a member of the multidrug and toxic compound extrusion (*MATE*) family, an Al-activated citrate transporter, as responsible for the major sorghum Al resistance gene locus. Aluminum-inducible *Altsb* expression was associated with induction of aluminum resistance via enhanced root citrate exudation (Magalhaes et al. 2007).

In white lupins (*Lupinus albus L.*), *LaMATE* is involved in citrate efflux and is highly expressed under phosphorus deficiency (Uhde-Stone et al. 2005). Lupin secretes citrate from the roots in response to phosphorus deficiency, suggesting that *MATE* is also involved in the phosphorus deficiency-induced citrate secretion. The FRD3 – a MATE protein member is also known to be involved in iron nutrition and conferred enhanced Al resistance, presumably due to the increase of root citrate release (Durrett et al. 2007). *AtFRD3* was reported to be involved in the xylem loading of citrate (Durrett et al. 2007) and was localized to the pericycle and cells internal to the pericycle cells in the roots of *Arabidopsis* (Green and Rogers 2004).

Collins et al. (2008) reported on the presence of a cluster of genes homologous to the *TaALMT1*, at the *Alt4* Al-resistance locus of an Al-resistant rye. High-resolution genetic mapping identified two resistant lines resulting from recombination within the gene cluster. It appears that all genes flanking the gene cluster can be excluded as candidates for controlling *Alt4* resistance, including a homolog of the barley *HvMATE* Al-resistance gene. In the recombinants, one hybrid gene containing a chimeric open reading frame and the *ScALMT1-M39.1* gene, each appeared to be sufficient to provide full Al resistance. mRNA splice variation was observed for two of the rye *ALMT1* genes, and one gene contained a ~400 bp insertion in one of its introns.

### *11.8.4 Expression Analysis of* MATE *and* ALMT1 *Homologs*

Although members of the *ALMT* and *MATE* families differ from one another in sequence and structure, they confer $Al^{3+}$ resistance in a similar fashion: by facilitating organic anion efflux from roots. Aluminum resistance in wheat relies on the Al-activated malate efflux from root apices, which appears to be controlled by an Al-activated anion transporter encoded by the *TaALMT1* gene on wheat chromosome 4DL (Sasaki et al. 2006). A strong correlation between malate efflux and Al resistance in wheat (Sasaki et al. 2006) suggested that malate efflux is the primary mechanism for Al resistance. It remains to be established whether (1) Al upregulates malate efflux by interacting with *TaALMT1* protein or via other intermediate steps involved in malate efflux and/or (2) plays the role of a promoter in relation to gene expression and Al resistance (Raman et al. 2008a, b, c).

In rye, the *ScALMT1* gene was found to be primarily expressed in the root apex and upregulated when Al was present in the medium. Fontecha et al. (2007) reported fivefold differences in the expression between the Al-resistant and the Al-nonresistant genotypes. Additionally, much higher expression was detected in the rye genotypes than the moderately resistant "Chinese Spring" wheat. These results suggest that the *Alt4* locus encodes an Al-activated organic acid transporter gene that could be utilized to increase Al resistance in plant species. Collins et al. (2008) reported that Al-tolerant (*M39A-1-6*) and Al-intolerant (*M77A-1*) rye haplotypes contain five and two genes, respectively, of which two (*ScALMT1-M39.1* and *ScALMT1-M39.2*) and one (*ScALMT1-M77.1*) are highly expressed in the root

tip, the main site of Al-tolerance/susceptibility. All three transcripts are upregulated by exposure to Al.

In barley, the relative expression of the *HvMATE* gene was 30-fold greater in "Dayton" (Al resistant) than the Al-sensitive cv. "Gairdner" (Wang et al. 2007). *HvMATE* expression was significantly correlated with Al resistance and Al-activated citrate efflux (Wang et al. 2007). When expressed in *Xenopus* oocytes, HvAACT1/HvMATE protein mediated the efflux of citrate, and it did not mediate malate secretion. *HvAACT1* was presumed to be localized to the plasma membrane. Transgenic tobacco expressing *HvAACT1* showed higher citrate secretion in the presence of Al and exhibited higher resistance to Al, but the citrate secretion was not altered in the absence of Al despite the constitutive promoter in the heterologous host (Furukawa et al. 2007).

In Sorghum, an induction of Al resistance correlated closely with an increase in root citrate exudation over time (over the 6 day period) in Al and the incremental increase in *SbMATE* expression in response to Al (Magalhaes et al. 2007). Citrate release mediated by the *SbMATE* was regulated at multiple levels not only by changes in gene expression but also by a direct effect of $Al^{3+}$ on transporter activity and/or by Al-mediated posttranslational mediation of *SbMATE* (Magalhaes et al. 2007). *SbMATE* expression in a genetically diverse sorghum panel indicated that the variation in Al resistance was due to an allelic series at the *AltSb* locus. Differences in *SbMATE* expression explained over 95% of the phenotypic variation for Al resistance in the panel, providing strong evidence that *SbMATE* underlies *Altsb* and the differences in gene expression constituted the basis for allelic variation at this Al resistance locus. Similarly, (Magalhaes et al. 2007) found a significant correlation between *SbMATE* expression and Al-activated root citrate release and between citrate release and Al resistance, suggesting that differences in expression conditions the Al resistance phenotype primarily by modulating root citrate exudation. Instead, the level of expression of either allele appears to be the major determinant of $Al^{3+}$ resistance in wheat (Raman et al. 2005). *TaALMT1* is constitutively expressed in root apices and the level of expression in different genotypes correlates positively with $Al^{3+}$ resistance (Sasaki et al. 2004).

Comparisons were made among $Al^{3+}$-resistant and -sensitive genotypes of wheat to correlate the level of *TaALMT1* expression with sequences upstream and downstream of the *TaALMT1* coding region, as well as the introns (Raman et al. 2005, 2008c; Sasaki et al. 2004, 2006). Polymorphisms in the introns and downstream sequences did not correlate with $Al^{3+}$ resistance. However, the promoter region upstream of *TaALMT1* was highly polymorphic between genotypes (Raman et al. 2008a, b, c; Sasaki et al. 2006). These studies reported up to seven promoter types in the upstream region of the *TaALMT1* gene. Promoter alleles differ from one another in a number of arrangements of tandem repeats, which are thought to influence the level of *TaALMT1* expression and Al resistance (Raman et al. 2008a, b, c). The origin of these tandem repeats is unclear but may have originated by inadvertent replication of genomic DNA by the "rolling circle" machinery used by some viruses and transposons (Piffanelli et al. 2004) as suggested for the *Mlo* locus in barley. Promoter that possess three tandem repeats but are otherwise

identical to those with two tandem repeats as shown in a study by Raman et al. (2008a, b, c) could have arisen by unequal cross over events during recombination. .

MATE proteins are known to facilitate citrate efflux from *Arabidopsis*, barley, and sorghum (Durrett et al. 2007; Magalhaes et al. 2007; Wang et al. 2007). Wang et al. (2007) measured *HvMATE* gene expression in the root apices of "Dayton" (Al-resistant) and "Gairdner" (Al-sensitive), using qRT-PCR, and found that the relative expression of the *HvMATE* gene in "Dayton" was 30-fold higher than "Gairdner." Expression of the *HvMATE* gene was correlated with Al resistance and Al-activated citrate efflux in an $F_{2:3}$-derived population from Dayton/Gairdner (Wang et al. 2007), and the expression of *HvMATE* was significantly correlated with Al resistance and Al-activated citrate efflux. Of the $F_{2:3}$ families assayed, *HvMATE* expression and citrate efflux were greater in the homozygous Al-resistant families than in homozygous Al-sensitive families, while families heterozygous for Al resistance were generally intermediate for expression and citrate efflux.

## 11.9 Discovery of Candidate Genes Expressed Under Al Stress

Aluminum has to affect numerous physiological parameters in order to reach the plasma membrane and the cytosol in less than 30 min (Lazof et al. 1994). Aluminum may induce several genes associated with oxidative stress (Richards et al. 1998) including those regulating the organic acid pathway featuring the citrate synthase gene (Anoop et al. 2003; Garvin and Carver 2003.; Raman et al. 2005), or the antioxidant pathway with genes for superoxide dismutase and glutathione peroxidase (Milla et al. 2002; Richards et al. 1998), or the pathogen defense pathway genes such as β-1,3-glucanase and phenylalanine ammonia-lyase (Cruz-Ortega et al. 1997; Snowden and Gardner 1993), or signal transduction genes such as cell wall-associated receptor kinase 1 gene (Sivaguru et al. 2003), or the general stress-responsive pathway genes such as blue copper-binding protein gene (Richards et al. 1998; Milla et al. 2002). However, most of these genes can also be induced by other biotic and abiotic stresses. Furthermore, identification of these genes was based on comparisons of gene expression levels using a single genotype under Al-stressed vs. nonstressed conditions, or between two genotypes with different genetic backgrounds under Al-stressed conditions. Recently, a gene encoding a putative ABC transporter (*ALS3*) was found to be contributing to an Al resistance mechanism in *Arabidopsis*, possibly by facilitating the redistribution of absorbed Al away from sensitive root tissues (Larsen et al. 2005). Seven different genes termed *wali1–wali7*, whose expression is induced by Al stress, were isolated from root tips of Al-treated wheat (Richards et al. 1994; Snowden and Gardner 1993). These gene sequences exhibited high similarities to *rali2* (Gallego et al. 1998b).

With the availability of various genomic tools, it is possible to study transcript abundance of many genes simultaneously on a genome-wide scale with respect to their structure and function. Such studies have identified and characterized a set of

genes and families identified by traditional and genomic studies (Furukawa et al. 2007; Guo et al. 2007). To understand the mechanisms of Al resistance and to identify genes responsible for Al resistance in wheat, Guo et al. (2007) constructed suppression subtractive hybridization libraries from Al-stressed roots for two wheat near-isogenic lines (NILs), "Chisholm-T" (Al-resistant) and "Chisholm-S" (Al-sensitive). Relative gene expression levels between "Chisholm-T" and "Chisholm-S" were compared at seven time points of Al stress: 15 min to 7 days. Twenty-eight genes including genes for Al-activated malate transporter-1, entkaurenoic acid oxidase-1, β-glucosidase, lectin, histidine kinase, and phospoenolpyruvate carboxylase showed more abundant transcripts in "Chisholm-T" and therefore may facilitate Al resistance. In addition, genes related to senescence and starvation of nitrogen, iron, and sulfur, such as copper chaperone homolog, nitrogen regulatory gene-2, yellow stripe-1, and methyl-thioribose kinase, were highly expressed in "Chisholm-S" under Al stress. The results suggested that Al resistance is probably coregulated by multiple genes with diverse functions in enhancing Al resistance and protecting root growth under Al stress. The highly expressed genes in "Chisholm-S" under Al stress may be symptomatic of root growth repression and restricted uptake of essential nutrient elements, leading to root senescence.

## 11.10 Molecular Breeding for Al Resistance Using Genetic Transformation

Several research studies indicate that Al resistance can be manipulated using various candidate genes either involved in organic acid biosynthesis or stress responsive genes. For example, aluminum resistance in canola (*Brassica napus*) (Anoop et al. 2003; Basu et al. 2001), *Arabidopsis thaliana (*Koyama et al. 2000*)*, tobacco (*Nicotiana tabacum*) (de la Fuente et al. 1997), papaya (*Carica papaya* L) (de la Fuente et al. 1997), and alfalfa (*Medicago sativum L.*) (Tesfaye et al. 2001) has been reported to be enhanced by increasing organic acid biosynthesis through overexpression of citrate synthase or malate dehydrogenase genes. *TaALMT1* has shown to increase Al resistance in root of transgenic tobacco cells and barley via Al-activated malate efflux (Delhaize et al. 2004; Sasaki et al. 2004). *SbMATE* conferred an Al-activated citrate efflux that results in Al resistance in wheat and *Arabidopsis* (Magalhaes et al. 2007). Furukawa et al. (2007) reported the heterologous expression of *HvAACT1* in Xenopus oocytes and showed efflux activity for 14C-labeled citrate but not for malate. Overexpression of this gene in tobacco enhanced citrate secretion and Al resistance compared with the wild-type plants. A good correlation was found between the expression of *HvAACT1* and citrate secretion in ten barley cultivars differing in Al resistance. Findings of Wang et al. (2007) and of Furukawa et al. (2007) suggest that *HvAACT1*/*HvMATE* Al-activated citrate transporter conditions Al resistance in barley.

Overexpression of genes induced with Al stress has also been reported to enhance Al resistance in Arabidopsis, tobacco, and canola (Basu et al. 2001; Ermolayev et al. 2003; Ezaki et al. 2000; Sivaguru et al. 2003).

In highly acidic soils, toxicity of $Fe^{2+}$ and $Mn^{2+}$ can occur as a result of an excess of these elements in associations with Al toxicity. Genetic variability for $Fe^{2+}$ and $Mn^{2+}$ toxicities has been reported in wheat (Camargo et al. 1989, 1992, 2000; Camargo and Ramos 1989; Moroni et al. 1991). Advanced breeding lines that showed resistance to $Al^{3+}$, $Mn^{2+}$, and $Fe^{2+}$ toxicities, under acid soil conditions, exhibited a high grain yield as compared with the control (Camargo et al. 1989, 2000). Genomic region associated with Al resistance on chromosome 1 has also been related to the ability of the rice root to exclude excessible $Fe^{2+}$ toxicity (Wu et al. 1998). Pyramiding QTLs associated with resistance to Fe and Mn and Al toxicity would allow to develop cereal germplasm for resistance to acid soils. As an experimental proof, the overexpression of *AtFRD3*, which enhanced exudation of citrate and malate from roots of transgenic Arabidopsis, led to the higher resistance to aluminum (Durrett et al. 2007). MATE family members are of particular importance as they have a wide range of transport functions including anthocyanin uptake, iron translocation, and aluminum resistance. Furthermore, *FRD3* does not require $Al^{3+}$ for activation; therefore, it can be manipulated to improve the phosphorus efficiency as outlined by Delhaize et al. (2007).

## 11.11 Molecular Breeding for Al Resistance Using Marker-Assisted Selection

While genetic transformation enables us to increase Al resistance in plant species that are generally "Al-sensitive," DNA markers allows to fast-track genes conditioning Al resistance including in transgenics. Table 11.3 describes the linkage between markers based upon RFLP, AFLP, SSR, DArT, and SNP and Al resistance loci in different cereals. Some of these Al resistance-marker associations have been validated in different genetic backgrounds (Raman et al. 2002, 2005, 2008c; Wang et al. 2006b, 2007).

Among different marker systems, SSR and SNP markers appear to be suited to marker-assisted selection (MAS) as they are abundant in plant genome, highly reproducible and polymorphic, more amenable for high throughput marker screenings. However, the use of markers in breeding programs depends upon the cost of phenotyping, genotyping, number of lines to screen, and time. With the revolution of technologies for genotyping such as capillary electrophoresis and SNP-typing, the cost of marker screening is becoming more affordable. Molecular markers for Al resistance have been applied in various cereal breeding programs in Australia and elsewhere and have monitored the expression of desirable alleles in genetic backgrounds. The Department of Agriculture and Food, Western Australia (DAFWA), is planning to release an Al resistance barley variety developed using

**Table 11.3** Linkage of aluminum resistance loci with PCR-based markers suitable for marker assisted selection in cereal crops

| Screening method | Population | Chromosome | Markers location | Reference |
|---|---|---|---|---|
| Barley (*Hordeum vulgare* L.) | | | | |
| RG | Yambla/WB229 WB229/Mimosa | 4HL | Bmag353, Bmac310 HVM68 | Raman et al. (2002) |
| | Harrington/ Brindabella | 4HL | Bmag353, Bmac310 | Raman et al. (2001) |
| | Ohichi/ F6ant28B48-16 | 4HL | Bmag353, Bmac310 | Raman et al. (2005) |
| | Dayton/Zhepi2 | 4HL | Bmag353, HvMATE, HVM68, HvMATE, GBM1071 | Wang et al. (2007) |
| | Dayton/ F6ant28B48-16 | 4HL | Bmag353, Bmac310, HVM68 | Raman et al. (2003) |
| RRG | Dayton/Zhepi2 | 4HL | HvGABP, Bmag353, HVM68, HvMATE, GBM1071 | Wang et al. (2007) |
| Hematoxylin staining | Dayton/Harlan Hybrid | 4HL | Bmag353, Bmac310, HVM68 | Raman et al. (2003) |
| Eriochrome cyanine | Dayton/Zhepi2 | 4HL | HvGABP, Bmag353, HVM68, HvMATE, GBM1071 | Wang et al. (2007) |
| | Dayton/Gairdner | 4HL | HvGABP, ABG715, GWM165, Bmag353 | Wang et al. (2007) |
| | F6ant28B48-16/ Honen | 4HL | Bmag353, HVM68 | Wang et al. (2006b) |
| Root/shoot fresh wt ratio | Murasakimochi/ Morex | 4HL | Bmag353 | Ma et al. (2004) |
| Wheat (*Triticum aestivum* L.) | | | | |
| Hematoxylin | Diamondbird/ Janz | 4DL | TaALMT1, WMC331 | Raman et al. (2003, 2008c, 2005) |
| | Currawong/CD87 | 4DL | TaALMT1, WMC331 | Raman et al. (2008c, 2005) |
| | Spica/Maringa | 4DL | TaALMT1, GWM165 | Raman et al. (2008c, 2005) |
| | Atlas66/Century | 4DL | WMC125, GDM125, TaALMT1 | Ma et al. (2005) |
| Root growth | BH1146/ Anahuac | 4DL | BCD1230, GDM125, TaALMT1 | Riede and Anderson (1996), Milla and Gustafson (2001), Raman et al. (2008c) |
| RRG | Diamondbird/ Janz | 4DL | TaALMT1, WMC331 | Raman et al. (2005) |
| | Cranbrook/ Halberd | | TaALMT1 | Raman et al. (2005, 2008) |
| | Sunco/Tasman | | TaALMT1 | |

(*continued*)

**Table 11.3** (continued)

| Screening method | Population | Chromosome | Markers location | Reference |
|---|---|---|---|---|
| | | | | Raman et al. (2005, 2008) |
| | Atlas66/Century | | WMC125, GDM125, TaALMT1 | Ma et al. (2005) |
| | Atlas66/ Chisholm | 4DL | TaALMT1, WMC331, GDM125 | Zhou et al. (2007) |
| | | 3BL | BARC164 | Zhou et al. (2007) |
| | FSW/ND35 | 4DL | TaALMT1 | Cai et al. (2008) |
| | | 3B | BARC164, BARC344 | Cai et al. (2008) |
| | | 2A | GWM515, GWM296 | Cai et al. (2008) |
| | Carazhino/EGA-Burke | 4BL | GWM495, GWM513 | Ryan et al. (2009) |
| Rice (*Oryza sativa* L.) | | | | |
| RG, RRG | IR64/O rufipogon | QTLs | RFLP/SSR markers | (Nguyen et al. 2003) |
| | CT9933/IR62266 | QTLs | RFLP/SSR markers | (Nguyen et al. 2002) |
| Rye (*Secale cereale* L.) | | | | |
| RG,RRG | Ailes × Riodeva *(Alt1)* | 6RS | $ScR01_{600}$, $ScB15_{790}$ | Gallego and Benito (1997) |
| | (AR6-17, AR1-13) | | *SCR01600* | Gallego et al. (1998a, b) |
| | Ailes × Riodeva (*Alt3*) | 4RL | $ScOPS17_{705}$ | Benito et al. (2009) |
| | M39A-1-6 × M77A-1 (Alt4) | 7RS | B1, B4, B11, B25, B26, B27, BCD1230 | Collins et al. (2008), Miftahudin et al. (2002, 2004, 2005) |
| | Ailes x Riodeva *(Alt4)* | 7RS | B1, B4, B26, ScALMT1, | Benito et al. (2009), Fontecha et al. (2007) |
| Oats (*Avena sativa* L.) | | | | |
| | CIav2921/ CIav9011 | – | SCA08 and calretB1_3 | Wight et al. (2006 #646) |
| Maize *(Zea mays L.)* | | | | |
| | L53/L1327 | QTLs | SSR | Ninamango-Cardenas et al. (2003) |

*RRG*/*RRE* Relative root growth, *RG*/*RE* Root growth, *RFLP* Restriction fragment length polymorphism

MAS (Reg Lance, per communication). So far, no epistatic or pleiotropic effects of Al resistance loci are known except in rice. Major genes/QTL effects for Al resistance can be tested via the association mapping approach. It is expected that advanced breeding lines and cultivars will have high linkage disequilibrium as compared to wide diverse germplasm, and that flanking markers will be better suitable for MAS as compared to single markers associated with the trait of interest. Potential MAS schemes include selection of the parental genotypes for making crosses, allele enrichment among $F_1$ individuals, selection of marker alleles during

recurrent backcrossing and intercross populations, and genome-wide selection for restored background genotype. The usefulness of markers in the breeding programs will depend upon polymorphic content (PIC) value of the markers. The higher the PIC, the more useful will be linked markers, which makes it important to test the polymorphism on a suite of markers linked with the locus of interest. In order to increase selection efficiency of MAS for Al resistance, diagnostic markers based on functionally associated variation in the candidate genes for Al resistance, such as *TaALMT1, HvMATE*, and *SbMATE*, have been developed, but unfortunately, some of these markers were not found to be "diagnostic" (Raman et al. 2005). Nevertheless, these candidate gene-based functional markers are preferred for association-mapping studies and MAS as compared with whole genome marker scans. Association mapping approach circumvents the need for construction of linkage maps and linkage analysis in the biparental populations.

## 11.12 Allele Mining

The gene pool of cereals present in nature has tremendous allelic variation for different traits of agronomic importance. Considerable genetic variation for Al resistance also exists in all key cereals including wheat, rice, and barley (Aniol 1996; Bona et al. 1993; Camargo et al. 1991; Caniato et al. 2007; Ceretta 1988; Mazzocato et al. 2002; Minella and Sorrells 1992; Mugwira et al. 1976; Raman et al. 2008c; Reid et al. 1969; Stodart et al. 2007; Wu et al. 2000; Xu et al. 2004). An array of molecular techniques is available to detect and understand the overall diversity for Al resistance genes within species including RFLP, RAPDS, SSRs, DArT, AFLP, and SNP. Furthermore, genomic approaches also provide the mean to access genes directly via gene discovery programs. Functional gene-specific markers are more suited for allele mining, as they are functionally relevant directly to the trait of interest.

Among various cereals, at least three candidate genes, *TaALMT1*, *HvMATE*, and *SbMATE*, have been correlated with Al resistance in wheat, barley, and sorghum, respectively, and could be utilized to determine allelic diversity within the genes conditioning Al-resistance. Raman et al. (2008a, b, c) characterized more than 300 genotypes of wheat for aluminum resistance using a two-step approach that involves (1) screening of germplasm using hematoxylin staining method, and (2) reevaluation of Al-resistant germplasm using the *TaALMT1* gene-specific markers for exon 4 (Sasaki et al. 2004), intron 3 (Raman et al. 2006), and long/short promoter sequence. Analysis of *TaALMT1* exon sequences has identified two alleles neither of which is diagnostic of Al resistance (Sasaki et al. 2004; Raman et al. 2005). By contrast, intronic regions display significant polymorphisms (Raman et al. 2005). Among the different introns, three regions show considerable allelic variability (Raman et al. 2006). These variations are due to SSR motifs with variable copy numbers and Indels (Raman et al. 2005; 2006). These markers identify at least eight alleles (Raman et al. 2006, 2008a, b, c). Analysis of upstream region of *TaALMT1* (Sasaki et al. 2006) revealed at least six types of promoter region (Sasaki et al. 2006).

Raman et al. (2008c) utilized *TaALMT1* gene-specific markers to characterize over 400 cultivars, landraces, and subspecies of bread wheat and found that at least 23 haplotypes. Among different haplotypes, promoter V was present in most of the Al-resistant germplasm. Correlation of gene haplotype structure and phenotypic variation provided the basis for a new paradigm in wheat marker-assisted breeding based on direct selection of superior alleles. Magalhaes et al. (2007) also found that the large differences in aluminum resistance in sorghum are largely due to an allelic series at the *AltSb*. Molecular markers have proved to be useful in understanding the origin and distribution of Al resistance. Raman et al. (2008c) demonstrated that markers based on *TaALMT1* intron, exon, and promoter regions can trace the inheritance of the Al resistance locus within wheat pedigrees and track Al resistance in breeding programs. Molecular and pedigree analysis suggested that Al resistance in modern wheat germplasm has been derived from several independent sources and that most of the promoter alleles associated with Al resistance preexisted in Europe, the Middle East, and Asia prior to the dispersal of domesticated germplasm around the world.

Genetically diverse sorghum accessions indicated that the Al resistance related mutations are located in regulatory regions of *AltSb*, and this may be due to regulatory *MITE* sequences (Magalhaes et al. 2007). Further analysis of sorghum near isogenic lines indicated that significant allelic variation occurs at the *AltSb* loci for the lines in the 1.9 kb *MITE* insertion class, and they have been shown to possess alleles that encode significant different Al resistance levels (Caniato et al. 2007).

For plant species that do not display significant variability for aluminum resistance such as barley, rice, and durum wheat, there is an urgent need to broaden the gene pool for enhancing Al resistance. It is well known that wild species and landraces have unique alleles that are not found in the cultivated gene pool and can be especially potent sources of abiotic stress resistance traits (Ellis et al. 2000). Discovery of such alleles in landraces and wild progenitors such as *Aegilops uniaristata*, and *A tauschii*, conditioning Al resistance, would provide new means to develop varieties suitable for cultivation on acidic soils. Novel alleles from resistant landraces/wild species can be introgressed/backcrossed into adapted high-yielding genetic backgrounds to ensure “optimum” yield required for local adaptation.

## 11.13 Conclusions

Significant achievements have been made in the identification and utilization of genetic variability for Al resistance in cereals. Thousand years of untargeted selection by early farmers and targeted selection by the modern breeding programs have narrowed down genetic variation in cereal germplasm. Genepools including landraces and wild relatives need to be exploited by screening, intercrossing, and subsequently introgressing desirable gene complexes, minus any associated linkage drag, into the target species. Further efforts need to be focused upon screening plant

germplasm for better sources for Al resistance, identifying new sources of Al resistance, understanding origin and transmission of superior alleles from cultivated and wild relatives, and understanding other mechanisms involved with Al resistance (besides organic acid efflux) and regulatory networks associated with Al resistance, in conjunction with value-added trait genes involved in yield and other abiotic stresses functioning under acid soil conditions. New data on regulatory pathways involved in Al stress response generated using functional genomic approaches is becoming available and may be useful to develop and enhance level of Al resistance in crop species. There is no doubt that genomics-assisted breeding will accelerate the development of stable Al-resistant crop varieties. A higher degree of Al resistance might also be achieved by pyramiding multiple copies of gene complexes conditioning Al resistance (such as 4DL, 4BL, 3BL, and 2AL in wheat) into selected germplasm and/or by genetic manipulating expression of endogenous genes or by expressing foreign genes in desired germplasm. In order to make MAS and GMO approach more effective, careful establishment of breeding strategy is required.

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# Chapter 12
# Molecular Breeding of Rice for Problem Soils

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**Contents**



## 12.1 Introduction

Problem soils are globally widespread and seriously constrain agriculture production. These soils generally contain toxic amounts of minerals or are deficient in some essential plant nutrients. They are generally of limited agricultural use because of variable factors, including toxic levels of salts or elements such as iron, aluminum, and heavy metals, as well as deficiency in other essential nutrients, such as phosphorus, iron, and zinc. Both acid and alkaline soils have low productivity. Globally, acid soils constitute about 2,500 Mha, of which over 1,700 Mha are in the tropics. These soils provide great potential for agriculture expansion if effectively utilized. Soil acidification problems are also likely to increase with rising $CO_2$ levels in the atmosphere, continuous use of ammonium-based nitrogenous fertilizers, removal of nutrients in farm products without replenishment, and nitrate leaching. The highly weathered acid soils of the tropics are inherently low in productivity with high Al and Fe and low in phosphorus.

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Problem soils constitute a considerable proportion of rice production areas, which are mostly inhabited by poor communities because of limited opportunities. Vast areas of lands suitable for rice production in South and Southeast Asia, South America, and Africa are currently underused or entirely unused because of these soil problems; however, these soils remain potential targets for extra food production and can significantly contribute to the elimination of global hunger and poverty if sufficiently exploited. For example, in rice-producing areas, high-yielding varieties with sufficient tolerance of predominant soil problems are expected to provide a yield advantage of over 2 tons per hectare on these problem soils (Ponnamperuma 1994).

Problem soils bring about their primary effects on plant growth and productivity essentially via plant roots. These effects either directly or indirectly suppress root growth, with considerable negative impacts on water and nutrient uptake, as well as on plant growth and productivity. Mineral toxicities (excess salts, soil acidity, Fe and Al toxicity, and numerous heavy metals) as well as deficiencies (Zn, P, and Fe) can have direct effects on root growth. These factors can also lead to indirect responses in growth and yield exerted through roots in problem soils, such as excessive uptake of cations and anions when these elements are in abundance, causing toxic effects in relatively more sensitive plant tissues as young leaves, growing tissues, and reproductive organs. Moreover, even mild soil problems can result in chemical and/or hydraulic signals, which induce responses in shoots that can negatively impact growth and productivity. Multiple abiotic stresses are commonly experienced in these soils, such as P- and Zn-deficiencies and Fe and Al-toxicities in acid and alkaline soils. Tolerance of these stresses involves a plethora of complex traits and mechanisms, and this complexity has slowed previous breeding efforts to develop high-yielding varieties with sufficient adaptation to such conditions (Ismail et al. 2007). These challenges forced breeders to search for innovative strategies to make further progress on the seemingly intractable problems that have continued to hamper conventional breeding efforts. New approaches are necessary to genetically dissect and incorporate these complex adaptive traits into high-yielding rice varieties while simultaneously retaining their good agronomic and quality attributes. This could be achieved if the genes responsible for tolerance of these stresses were identified and exploited for crop improvement using modern breeding and biotechnological approaches.

Recent developments in genomics and molecular biology and the advances in molecular marker techniques have made it possible to unravel the genetic determinants of complex traits underlying stress tolerance in crop plants. Genetic linkage mapping of polygenic traits has led to the identification of quantitative trait loci (QTLs) that control complex traits in plants. Furthermore, by using natural genetic variation to investigate the intricate systems plants have developed to deal with the multitude of abiotic stresses in natural ecosystems, geneticists can now identify superior tolerance alleles and transfer them into high-yielding varieties that are intolerant of a particular stress. These efforts led to the development of marker-assisted breeding systems for cultivar improvement through the transfer of major QTLs into popular varieties and advanced breeding lines. While transgenic

approaches will ultimately play an important role in developing abiotic stress-tolerant plants, a marker-assisted approach provides a useful alternative when the required traits are available within the species gene pool and particularly in instances where genetically modified food crops such as rice are still far from being widely accepted.

In this chapter, we will present cases where molecular markers are being used as tools for dissecting complex traits associated with tolerance of abiotic stresses encountered in problem soils. We will then provide several cases where these techniques are currently being used in rice to identify and transfer major loci associated with tolerance, particularly those that are directly or indirectly mediated through plant roots. We also present some insights into how future advances in marker development and high throughput genotyping could impact progress in breeding to more efficiently develop high-yielding, stress-tolerant varieties to enhance and stabilize productivity in problem soils as well as in other areas facing similar abiotic stresses.

## 12.2 Abiotic Stresses Affecting Root Growth in Problem Soils

Adaptation to unfavorable soil conditions generally involves several complex and interrelated physiological and morphological tolerance mechanisms, most of them expressed at the root level. Understanding the mechanisms that are involved in these processes and how they are integrated and regulated will ultimately speed the efforts to improve the performance of crop plants for problem soils. Traits that allow traditional cultivars to survive and produce well under such extreme conditions need to be incorporated into popular varieties and elite breeding lines, without substantial changes in their adaptive and quality traits. This will require a systematic analysis of the genetics and physiology of such characters, together with a thorough evaluation of the target environments to select for relevant traits. Mechanisms associated with tolerance of various abiotic stresses encountered in problem soils can now be dissected into component elements that can then be targeted for molecular breeding through the use of molecular markers that are linked to genes controlling each specific trait component. Research over the past few decades uncovered many agronomically useful characters within and between cultivated and wild rice germplasm, making genetic improvement more feasible. We will highlight the progress made in several abiotic stresses common to rice problem soils, particularly those affecting root growth and function.

### *12.2.1 Salt Stress*

Excessive salt stress is a major constraint for crop production in vast areas of the world, affecting over 12 million ha of rice land in Asia. Salinity in coastal areas fluctuates within the year, being high during the dry season because of tidal inundation and intrusion from saline shallow water tables but decreasing with the freshwater flush during the rainy season. Secondary salinization can also occur as a result of misuse

of irrigation water with poor drainage, and recently this has become an alarming problem in inland areas worldwide, steadily leading to soil deterioration and eventual abandonment by poor farmers. About 10 million ha of agricultural lands in the world are believed to be lost annually to salinization (Pessarakli and Szabolcs 1999).

#### 12.2.1.1 Salt Stress Tolerance in Rice

Rice is moderately sensitive to salt stress, yet it is still preferred as an initial crop during soil reclamation because of its unique ability to thrive in standing water. Sensitivity also varies with the climate and the stage of development, with poor association between tolerance at the two most sensitive stages, early seedling, and reproduction (Moradi et al. 2003; Peng and Ismail 2004). Considerable genetic variation in salinity tolerance was reported in rice (Flowers and Yeo 1981), and progress has been made in developing elite breeding lines with a reasonable level of tolerance, some of which were released as commercial varieties (Gregorio et al. 2002; Senadhira et al. 2002; Salam et al. 2007). Salinity tolerance in rice is complex and involves several physiological and adaptive mechanisms (Yeo and Flowers 1986; Peng and Ismail 2004; Ismail et al. 2007). The physiological bases of salt tolerance during early seedling stage are fairly well understood, involving key traits such as high seedling vigor to dilute salt concentration in plant tissues, selective ion uptake by roots, compartmentation of harmful ions in structural and older tissues (particularly older leaves, stems, leaf sheath and roots), responsive stomata that regulate water and salt uptake in response to increasing salt stress in the rhizosphere, high tissue tolerance through sequestering salts in the apoplast, and recirculation of sodium back to roots to avoid accumulation of toxic concentrations in the cytoplasm. The latter is probably achieved through a set of active processes involving a gene family of ion transporters such as $Na^+/H^+$ antiporters that sequester salt in vacuoles (Blumwald et al. 2000) or move it out of the cell cytoplasm and recirculate it back to the roots (Berthomieu et al. 2003). Responsive stomata that quickly close upon initial exposure to salt stress, probably in response to signals from roots, but partially reopen after a period of acclimation could also contribute to tolerance by minimizing salt uptake. Antioxidant scavenging systems also seem to play an important role through neutralizing toxic radicals generated during stress (Moradi and Ismail 2007). Overexpression of superoxide dismutase, a key enzyme in the ascorbate–glutathione pathway, conferred tolerance of salinity in *Arabidopsis* (Gao et al. 2003). During reproductive development, tolerant genotypes also tend to exclude salt from flag leaves and developing panicles (Yeo and Flowers 1986; Khatun et al. 1995).

#### 12.2.1.2 Germplasm Improvement for Salt Stress Tolerance

Despite the fact that traits associated with salinity tolerance in rice are seemingly independent, all known salt-tolerant landraces are superior in only one or a few of them, and significant genetic variation exists for each particular trait. This suggests

the possibility of identifying better donors that can provide superior combinations of alleles at useful genes. Combining the traits that are effective at seedling and reproductive stages will then ensure the development of rice cultivars with higher levels of salt tolerance. Selection could essentially be made in parallel for individual traits, which can then be combined through multiple crosses. Moreover, identifying and fine-mapping major QTLs and cloning of genes underlying these traits will particularly help speed the breeding process by precise targeting of useful alleles using marker-assisted backcrossing (MABC). By reducing linkage drag, MABC has allowed the precise introgression of agronomically useful traits into popular varieties without changing their adaptive or quality traits. A good example is the transfer of the *SUB1A* locus into numerous rice varieties, making them extremely tolerant of submergence (Neeraja et al. 2007; Septiningsih et al. 2009; Singh et al. 2009).

Considerable progress was recently made in deciphering genes associated with salt stress tolerance in plants. For example, numerous cases demonstrated the role of sodium transporters in maintaining ion homeostasis in plants under salt stress through mechanisms that remove sodium from the cytoplasm by either compartmenting it into vacuoles or extruding it out of the cell (Horie and Schroeder 2004). The salt overly sensitive (SOS) signaling pathway was characterized in *Arabidopsis* as being involved in signal perception and ion homeostasis (Zhu 2003), and the role of this system in controlling salt stress tolerance in rice was established recently (Martinez-Atienza et al. 2007). The HKT family of transporters was also shown to have significant roles in sodium and potassium uptake and homeostasis in a number of plant species including rice (Horie et al. 2001; Golldack et al. 2002), and the cloning of the rice QTL *SKC1*, originally detected by its effect on $K^+$ concentration, identified the causal gene as the sodium transporter OsHKT8 (Ren et al. 2005). Discovery of the genes underlying tolerance of salt stress will help in designing functional markers for more accurate and efficient use in MABC.

Several studies have identified QTLs associated with salinity tolerance in rice (Table 12.1). A major QTL for salt tolerance was tagged with an RFLP marker on chromosome 7 using an $F_2$ population derived from salt-tolerant japonica rice mutant M-20 and the sensitive original variety 77-170 (Zhang et al. 1995). Using a cross of an *indica* variety of moderate tolerance (IR64) with a sensitive *japonica* variety (Azucena), seven QTLs for seedling traits associated with salt stress tolerance were mapped, though all explained less than 20% of the variation (Prasad et al. 2000). Using a cross between two moderately tolerant elite *indica* breeding lines, one of which had Pokkali in its pedigree, several QTLs were identified, of which the QTL with the largest effect was for $K^+$ uptake on chromosome 9, explaining 19.6% of the variation (Koyama et al. 2001). A study employing the highly tolerant *indica* variety Nona Bokra with the susceptible japonica Koshihikari identified several QTLs of much larger effect, including the *SKC1* QTL for shoot $K^+$ concentration on chromosome 1 and a QTL for shoot $Na^+$ concentration on chromosome 7 (Lin et al. 2004). Furthermore, using a population of 80 recombinant inbred lines (RILs) generated from a cross between sensitive variety IR29 and a tolerant landrace, Pokkali, QTLs were identified on chromosomes 1, 3, 4, 10, and 12 for salinity

**Table 12.1** Comparison of the number of QTLs and size of the largest QTL detected across different mapping studies for tolerance to various problem soils

| Trait | Total QTLs | Largest QTL | Max. $R^2$ | Tolerant donors | References |
|---|---|---|---|---|---|
| Salinity tolerance | >20 | Chr. 1 (Gregorio 1997) | 64%[a] (Na/K ratio) | Pokkali, IR64, Nona Bokra | Zhang et al. (1995), Gregorio (1997), Prasad et al. (2000), Koyama et al. (2001), Bonilla et al. (2002), Lin et al. (2004) |
| P-deficiency tolerance | 10 | Chr. 12 (Ni et al. 1998) | 61% (Shoot dry weight) | Kasalath, IR20 | Wissuwa et al. (1998), Ni et al. (1998), Shimizu et al. (2004) |
| Zn-deficiency tolerance | 6 | Chr. 12 (Wissuwa et al. 2006) | 24% (Mortality) | Jalmagna | Wissuwa et al. (2006) |
| Fe-toxicity tolerance | 9 | Chr. 3 (Wan et al. 2003) | 48% (Stem dry weight) | Azucena, Kasalath | Wu et al. (1997, 1998), Wan et al. (2003) |
| Al-toxicity tolerance | >30 | Chr. 8 (Nguyen et al. 2002) | 28% (Ratio of root length of stress vs. control) | Azucena, Chiembau, CT9993, *O. rufipoogon*, Koshihikari, Asominori | Wu et al. (2000), Nguyen et al. (2001, 2002, 2003), Ma et al. (2002), Xue et al. (2006, 2007) |

[a]*Note*: This $R^2$ value was obtained using phenotypic extremes for the QTL analysis

tolerance during seedling stage, including a major QTL designated *Saltol* mapped on chromosome 1 explaining 64% of the variation for seedling shoot $Na^+/K^+$ ratio using phenotypic extremes and 43% of the variation in a subsequent study (Gregorio 1997; Bonilla et al. 2002). Current efforts at IRRI include fine mapping of the *Saltol* QTL, using MABC to incorporate this QTL into popular varieties sensitive to salt stress, and targeting additional QTLs for salinity tolerance at different growth stages for combining multiple QTLs to increase the level of tolerance in salt-stressed environments. As with the *SUB1* QTL, *Saltol* is being transferred into popular rice varieties using MABC to precisely incorporate the Pokkali introgression conferring tolerance while reducing any unwanted DNA segments that may contain negative characters.

### *12.2.2 Mineral Deficiency*

Nutrient deficiency induced by problem soils is wide spread in rice production areas, particularly in soils with high fixing capacity as in acid and calcareous/sodic soils. This induced deficiency is causing considerable reductions in grain yield, and is further being worsened by the high demand for these nutrients under the newly evolving intensive farming systems using modern varieties. The rate of plant nutrient removal from the soil by modern high-yielding rice varieties is about three times that of traditional varieties (De Datta et al. 1990), which further aggravates the problem. Phosphorus and zinc are the most widely encountered deficiencies in rice soils caused by their immobilization in forms that are not readily available for plant roots. Varieties with greater ability to mobilize and use these nutrients will be more efficient in these soils, especially where farmers are resource-poor and adding sufficient nutrients to overcome these deficiencies is out of their reach.

#### 12.2.2.1 Phosphorus Deficiency

Phosphorus is the most important inorganic plant nutrient after nitrogen but the least available in soils because of its limited mobility and the tendency of most soils to fix it into forms that are hardly available for plant roots, as in most alkaline and acid soils. This tight binding of P in the soil rather than a low total P content is often the primary cause of its deficiency. As a result, phosphorus deficiency is widespread in both upland and lowland rice-growing areas. In most of these areas, phosphorus fertilizers are not always available or affordable for resource-poor farmers and the tendency of soils to rapidly fix it reduces fertilizer use efficiency.

Breeding cultivars capable of efficient mining of the large pool of P already existing in most rice soils will help increase and sustain yields in low-input agricultural systems. Large variability among lowland and upland rice cultivars in their ability to utilize soil P was observed (Wissuwa and Ae 2001a); however, no

formal breeding program has yet been in place to develop P-efficient varieties. The concentration of available P in soils is usually very low and coupled with its extremely slow mobility, particularly in highly weathered soils, suggesting that its acquisition must occur against a steep concentration gradient involving active uptake. So far, two main types of phosphate transport systems were identified in rice, high-affinity and low-affinity transport systems. The low-affinity transport system appears to be expressed constitutively, whereas the high-affinity uptake system is strongly enhanced when phosphorus is limiting (Vance et al. 2003).

In plants, two types of mechanisms are involved in P deficiency tolerance, internal mechanisms associated with the efficient use of P by plant tissue and the external mechanisms that allow greater P uptake by plant roots. Genetic variation in internal P efficiency was observed in rice but is mostly associated with low P uptake. External efficiency is probably the most important mechanism underlying tolerance to P deficiency in rice. However, mechanisms responsible for this efficiency still await further studies. The main external mechanisms observed in plants involve (a) the ability to develop long, fine hairy roots to maximize exposure to the rhizosphere, (b) the ability to mobilize P through pH changes or the release of ligands or chelating agents such as organic acids, (c) the ability to utilize soil organic P through release of phosphate enzymes, and (d) the ability to associate with mycorrhizal fungi (Kirk et al. 1993; Hedley et al. 1994). Mycorrhizae are expected to be less effective in fine-rooted crops such as cereals, especially in anaerobic flooded soils.

## Root Characteristics Associated with High P-Uptake

Because of its slow mobility in the soil, root morphological characteristics such as length, surface area, fitness, and intensity of root hairs are found to be important for P uptake in numerous crops (Otani and Ae 1996; Kirk and Du 1997). Using rice cultivars of different origins, Wissuwa and Ae (2001a) observed a strong relation between tolerance to P deficiency with both root size and root uptake efficiency but with stronger association with the root size. A large root system may therefore be adaptive and may provide a more reliable criterion to identify genotypes with tolerance of P deficiency. The ability of rice cultivars to solubilize P fixed in the soil has been suggested (Hedley et al. 1994; Saleque and Kirk 1995; Kirk et al. 1999). This could involve acidification of soils by roots, and changes of over two pH units had been reported in the immediate vicinity of roots in flooded soils (Saleque and Kirk 1995). Under aerobic soil conditions, mechanisms involved in remobilization of P are expected to be different and could involve the secretion of low molecular weight organic acids such as citrate (Kirk et al. 1999). Organic acids may act as chelating agents for aluminum and iron to free P in soil solution, and high rates of excretion of P-solubilizing organic acid anions from roots was reported in rice in response to P-deficiency (Kirk et al. 1993).

## Germplasm Improvement for Higher P-Uptake Efficiency

Genotypic differences in P deficiency tolerance in rice were reported long ago; however, breeding efforts were limited to screening available cultivars and advanced breeding lines for superior performance in P deficient soils rather than developing genotypes with higher efficiency of P uptake (Fageria et al. 1988; Hedley et al. 1994; Ismail et al. 2007). Traditional landraces are more efficient in P uptake than modern high-yielding varieties (Wissuwa and Ae 2001a). These landraces will therefore provide potential donors of P-deficiency tolerance for cultivar improvement using conventional approaches and also could serve as sources of agronomically important QTLs and genes identified through mapping and subsequent cloning.

Tolerance of P deficiency is quantitatively inherited in rice, with both additive and dominant genetic effects. QTLs associated with P-deficiency tolerance were identified in two mapping studies (Wissuwa et al. 1998; Ni et al. 1998). Wissuwa et al. (1998) used a backcross inbred population with the recurrent parent Nipponbare (japonica, sensitive) and the landrace Kasalath (aus, tolerant) and identified four QTLs for P uptake on chromosomes 2, 6, 10, and 12, including a major QTL on chromosome 12 that controls most of the variation in P-deficiency tolerance. For P uptake, this QTL had an LOD score of 10.7 and explained about 28% of the phenotypic variation. Ni et al. (1998), using RILs from the cross of IR20 (tolerant) with IR55178-3B-9-3 (sensitive), found a similarly strong QTL in the same location. They measured P uptake efficiency as relative tillering ability, relative shoot dry weight, and relative root dry weight. Moreover, an intermediate QTL on chromosome 6 and several other minor QTLs were mapped to several chromosomes. The QTL on chromosome 6 explained 25–34% of the variance for the above traits in the Ni et al. (1998) study but had a much lower effect ($R^2 = 9.8\%$) in the field study of Wissuwa et al. (1998). The QTL on the long arm of chromosome 6 was also identified in another independent mapping study using a population developed from a cross of the tolerant Kasalath and the intolerant Gimbozu and was found to be associated with phosphorus deficiency-induced root elongation (Shimizu et al. 2004). Recently, the position of this QTL, named *qREP-6* for root elongation under phosphorus deficiency, as well as its role were confirmed using chromosome segment substitution lines developed in the background of Nipponbare (Shimizu et al. 2008). The substitution line carrying *qREP-6* had higher tillering ability on P-deficient soils and also higher phosphorus concentration in the shoot, suggesting that this QTL will potentially be important in breeding cultivars with better root traits for P-deficient soils. The *qREP-6* was fine-mapped in an $F_2$ population and a total of 37 genes were annotated in the region (Shimizu et al. 2008), paving the way for its subsequent positional cloning. This will further enhance our understanding of its mechanistic role and quantify its effects in improving adaptation to phosphorus deficiency stress.

The major QTL on chromosome 12, named *Pup1* for P uptake 1, controls most of the variation in P-deficiency tolerance in the Nipponbare/Kasalath population. *Pup1* substantially increased P uptake from P-deficient soils but has no apparent

effect when P is not limiting. Transferring *Pup1* to intolerant genotypes increased P uptake, plant biomass, and grain yield by over threefold on a P-fixing soil (Wissuwa and Ae 2001b). Near isogenic lines containing *Pup1* maintained relatively higher root growth and root surface area in P-deficient soils than their counterparts lacking the *Pup1* introgression. Carbohydrate supply from leaves to roots did not explain the reduction in root growth in lines missing the *Pup1* introgression under P deficiency as root starch concentration increased in P-deficient roots (Wissuwa 2005). However, model simulations suggested that only small changes in root growth are necessary to account for the large effects of *Pup1* in enhancing P uptake from P-deficient soils, and these differences were mainly due to variation in root external uptake efficiency (Wissuwa 2003, 2005). These studies suggest that the genes involved are probably expressed in root tissue where they either lead to higher root growth per unit P or improve P uptake per unit root size or surface area.

*Pup1* was recently fine-mapped to the long arm of chromosome 12 within the physical interval of 15.31–15.47 Mb (Heuer et al. 2009). The genes in this locus were initially annotated based on Nipponbare reference genome sequence; however, this annotation did not unveil obvious candidates for P-uptake efficiency. Subsequently, the locus was sequenced in the original donor parent Kasalath, and this revealed significant variation with the reference sequences of both Nipponbare and 93-11, with considerable distinction in size differences caused by insertions and deletions, together with a large number of transposon and retrotransposon-related sequences (Heuer et al. 2009). This variation highlighted the significance of sequencing QTL regions in the donor parent targeted for cloning, as the underlying genes might be lacking in the two reference genomes that are currently available. Similar observations were also made when cloning the *SUB1* gene associated with tolerance of submergence in rice (Xu et al. 2006). Several of the newly annotated genes using Kasalath sequence are novel and are mainly located within the insertion–deletion regions. Detailed analysis of these genes annotated from the Kasalath sequence is ongoing and their potential role in tolerance of P deficiency is being depicted based on physiological evidence and sequence analyses. Identifying and cloning of *Pup1* will help in designing precise gene-based markers for use in breeding and for revealing its physiological and molecular bases, particularly its effects on root growth under P-deficiency. This information could also be important for enhancing tolerance in other crop species by identifying *Pup1* homologs.

A marker-assisted breeding system to introgress *Pup1* into popular varieties is also being developed, and its contribution for enhancing tolerance of P deficiency in a wider range of genetic backgrounds and under natural field conditions is being further quantified. SSR markers linked to the *Pup1* locus were identified and tested in a few accessions, and some of them were found to be specific to Kasalath donor parent alleles, suggesting their potential use for monitoring the *Pup1* introgression during backcrossing (Collard et al. 2006). PCR-based markers were also developed based on the genes annotated at the *Pup1* locus and are currently being used to transfer *Pup1* into a few upland and lowland popular varieties using MABC,

following the strategy used for *SUB1* locus (Septiningsih et al. 2009). Cloning of the gene responsible for *Pup1* action will accelerate the development of this marker system. Combining *Pup1* with *qREP-6* into the background of popular varieties and advanced breeding lines could significantly enhance their performance under P-deficient soil conditions.

#### 12.2.2.2 Zinc Deficiency

Zinc deficiency is a widespread soil constraint for rice production, with about 50% of lowland rice soils believed to be Zn-deficient. Zn deficiency can result from low total soil–Zn content, but it is more frequently caused by Zn immobilization in the soil. A range of soil conditions have been associated with binding it in forms that are less readily available for plants, such as alkaline pH, prolonged submergence and low redox potential, high organic matter and bicarbonate content, high Mg:Ca ratio, and high available P (Yoshida et al. 1973; Forno et al. 1975; Neue and Lantin 1994; White and Zasoski 1999). High soil pH and bicarbonate appear to be the main factors associated with the widespread Zn deficiency in calcareous soils as the case of the Indo-Gangetic Plains of India and Pakistan (Qadar 2002), whereas perennial wetness and low redox potential are the major causes of Zn deficiency in peat and coastal saline soils (Neue and Lantin 1994; Quijano-Guerta et al. 2002). Similar to P solubilization under flooded soils, rice roots can solubilize Zn through acidification of the rhizosphere in the vicinity of the roots (Kirk and Bajita 1995) through the release of $H^+$ from the roots or during oxidation of iron by $O_2$ released from roots.

Zinc deficiency can be effectively eliminated by using Zn fertilizers; however, the high cost associated with applying sufficient Zn places a considerable burden on farmers, particularly in rainfed areas of Asia, where most soils demand high Zn application as a consequence of its immobilization in the soil. Breeding efforts to develop rice cultivars that are more efficient in Zn uptake from these soils should therefore be intensified to improve tolerance of Zn deficiency in rice (Quijano-Guerta et al. 2002; Ismail et al. 2007). Incorporating tolerance of Zn deficiency also seems to improve performance under other abiotic stresses such as alkaline soils, salinity, P deficiency, and peat soils (Singh et al. 2004; Quijano-Guerta and Kirk 2002; Quijano-Guerta et al. 2002). However, the mechanisms of this cross-tolerance still awaits further investigation and may be attributed solely to better Zn acquisition when Zn is most limiting, with the consequent improvements in root health and growth.

The major mechanisms associated with Zn deficiency tolerance in plants are still poorly understood and several mechanisms were suggested (Hacisalihoglu and Kochian 2003); however, the effectiveness of these different traits as well as their physiological and molecular bases are still incomplete. Multiple symptoms are generally observed in rice in Zn-deficient soils, including development of brown spots on leaves that eventually entirely cover older leaves (leaf bronzing), stunted plant growth and poor root development, and seedling mortality in severe conditions. Flowering is normally delayed or even hindered and grain yield substantially decreases (Ismail et al. 2007). These symptoms are largely under independent

genetic control as different QTLs were associated with traits such as leaf bronzing and plant mortality (Wissuwa et al. 2006). The results largely suggest multiple tolerance mechanisms that can either operate in root or shoot. Mechanisms associated with Zn uptake and root growth obviously reside in roots, whereas mechanisms associated with reduced leaf bronzing likely occur within leaf tissue. Our recent studies suggested that tolerance to Zn deficiency in flooded Zn-deficient soils was associated with rhizosphere processes that enhance availability and uptake of Zn rather than with shoot traits or internal efficiency (Wissuwa et al. 2006).

## Effects of Zn Deficiency on Root Growth in Rice

Zinc uptake into roots is either as $Zn^{2+}$ ion or as a Zn-phytosiderophore complex, and as for most cations, its transport is mediated by a low-affinity transport system and a high-affinity system, with the latter dominating under Zn deficiency (Hacisalihoglu et al. 2001). However, the molecular nature of these systems remains poorly understood. In conditions when Zn availability is low due to binding of Zn in the soil, adaptive root mechanisms that increase Zn availability through desorption of Zn from binding sites in the soil are likely more important than transmembrane transport systems. Release of Zn from soil-bound forms has been linked with two classes of compounds secreted from plant roots, phytosiderophores, and nonprotein amino acids that chelate a number of micronutrients (Rengel et al. 1998; Suzuki et al. 2006) and organic acids such as citrate and malate, which were also thought to be involved in both Zn and P deficiency tolerance in rice. The involvement of a rhizosphere effect in maintaining Zn uptake under field conditions was further supported by the observation that increasing the plant density per hill increased shoot dry matter and Zn uptake, with no apparent symptoms of Zn deficiency (Hoffland et al. 2006).

Root growth in rice is severely inhibited under Zn deficiency, and tolerant genotypes tend to maintain their ability to regenerate new roots and maintain higher root biomass in Zn-deficient soils. In both calcareous and heavily submerged soils, Zn deficiency typically coincides with high bicarbonate concentration in the soil solution, and sensitive genotypes showed strong suppression in root growth in response to bicarbonate, with consequent reduction in Zn acquisition. The negative effect of bicarbonate is probably caused by excess accumulation of organic acids within the roots of sensitive cultivars, whereas tolerant genotypes avoid this effect by maintaining higher rates of organic acid excretion. This might also help in mobilizing Zn in soil solution and enhance its accessibility by plant roots, resulting in further root growth in tolerant genotypes, commonly seen as early as 2 weeks after transplanting in Zn-deficient soils (Hajiboland et al. 2005; Ismail et al. 2007).

## Germplasm Improvement for Zn Efficiency Tolerance

Genetic variability in the ability to grow under low Zn conditions has been observed in rice (Quijano-Guerta et al. 2002; Yang et al. 1994). However, despite this genetic

variability and the dire need to develop Zn-efficient varieties, no formal breeding program has yet been initiated to develop more Zn-efficient varieties. Limited progress was achieved indirectly when selecting for tolerance of other soil problems as in alkaline soils of north India (Singh et al. 2004). Our recent efforts aimed to identify genotypes contrasting in their tolerance of Zn deficiency under natural field conditions to understand the mechanisms of tolerance and to develop strategies to incorporate tolerance through breeding.

Identification of QTLs with reasonably large effects on Zn deficiency tolerance is a crucial first step that will allow the eventual incorporation through MABC as well as the identification of tolerance genes after further fine-mapping and subsequent positional cloning. Using a mapping population developed from the indica genotype IR74 (sensitive) and Jalmagna (tolerant), several QTLs associated with plant mortality, leaf bronzing, and biomass were detected on a Zn-deficient field, with only one minor QTL for plant mortality colocalized with a QTL for leaf bronzing (Wissuwa et al. 2006). QTLs for plant mortality acted in a purely additive manner, whereas digenic interactions were important for leaf bronzing and for shoot biomass, and in both cases, the epistatic interactions involved the main QTL for plant mortality mapped on chromosome 12. Currently, several of these QTLs are being targeted for fine-mapping for further genetic dissection and for use in breeding. Advancing our knowledge of the mechanisms of tolerance together with the identification of genes responsible for the mapped QTL regions will enable a precise MABC strategy to speed up breeding for tolerance of Zn deficiency.

### *12.2.3 Mineral Toxicity*

Approximately 30% of the earth's lands are classified as acidic and about half of the potentially arable land is acidic (von Uexkull and Mutert 1995). Soil acidity limits crop production through a combination of nutrient toxicities and deficiencies. These soils constitute a serious constraint across vast portions of rice-growing areas of the tropics. Besides mostly being deficient in major plant nutrients such as P, they also contain toxic concentrations of other elements such as aluminum and iron, as both $Al^{3+}$ and $Fe^{2+}$ ions become soluble under low pH. These in turn damage the root system, and their excessive uptake leads to toxicity within the plant, leading to decreased growth and yield. Research on the genetic control of tolerance of the stresses encountered in acid soils in rice is still in its early stages despite their enormous effects on rice production in affected areas.

#### 12.2.3.1 Aluminum Toxicity

Aluminum is the most abundant metal in the earth's crust, constituting approximately 7% of the soil and is predominately found in clays. Under low pH (<5), it is solubilized as $Al^{3+}$ in soil solutions, which is highly toxic to plants. Aluminum

toxicity is the main factor limiting the productivity of crop plants in acid soils, particularly in the tropics and subtropics. A high concentration of $Al^{3+}$ severely hampers root growth, with consequent inhibition of water and nutrient uptake, resulting in severe reduction in growth and productivity. Al toxicity has been extensively studied in several plant species and particularly in grasses, including wheat, sorghum, maize, and rye (Kochian et al. 2004, 2005). The primary mechanism of tolerance identified in most of these crops involves the exudation of organic acids from the root apex, which in turn binds aluminum and excludes it from entering the root, as was first identified in wheat (Delhaize et al. 1993). Several organic acid exudates were documented in several plant species such as malate exudation in wheat and *Arabidopsis*, citrate exudation in maize, sorghum, and soybean, and both citrate and malate in rye, *Triticale*, and oilseed rape (Kochian et al. 2004). Another potential mechanism involves tolerance of high Al accumulation in roots and shoots tissue through internal detoxification (Ma et al. 1998). Recently, genes that control tolerance of Al toxicity were cloned from wheat and sorghum (Sasaki et al. 2004; Magalhaes et al. 2007), and in both crops, tolerance of Al toxicity was attributed to the exudation of organic acids by roots to serve as chelates and detoxify $Al^{3+}$ in the rhizosphere, particularly around the actively growing root tips, which are the main site of Al toxicity.

Aluminum toxicity is a major limitation to rice production in both rainfed lowland and upland soils. Rice is the most tolerant cereal; however, little is known regarding the physiology of this tolerance. Mechanisms of tolerance in rice are expected to act differently compared with other cereals due to the low organic acid excretion by rice roots, which is unlikely to play a major role in Al detoxification in the rhizosphere. A few reports have suggested exclusion of excess Al at the root tip to be involved in rice tolerance of Al toxicity; however, these studies were limited to only two genotypes, one tolerant and one sensitive (Ma et al. 2002; Yang et al. 2008). Apparently, novel mechanisms are probably involved in the high levels of Al toxicity tolerance in rice. Understanding these mechanisms and the gene(s) underlying the tolerance traits will facilitate further improvement of rice varieties and development of varieties of other cereals with higher tolerance of Al toxicity than the existing varieties.

Numerous studies have identified QTLs associated with Al toxicity tolerance in rice (Table 12.1). For example, Wu et al. (2000) identified several QTLs associated with Al tolerance in a recombinant inbred mapping population derived from Azucena and IR1552. Nguyen et al. (2001) also detected five QTLs for Al toxicity tolerance distributed on five chromosomes, with a major QTL located on chromosome 1. Using a double haploid population developed from CT9993 (tolerant) and IR62266 (sensitive), Nguyen et al (2002) identified 20 QTLs controlling root growth under Al toxicity stress and control conditions, distributed over ten chromosomes, with the two largest QTLs identified on Chromosomes 1 and 8. The region on chromosome 1 was found to be conserved across several genetic backgrounds, and therefore, could be targeted for use in breeding as well as for subsequent cloning. Using a backcross population derived from Koshihikari (tolerant) and

Kasalath (intolerant), Ma et al. (2002) identified three QTLs on chromosomes 1, 2, and 6, collectively explaining about 27% of the phenotypic variability in Al toxicity tolerance in this population. In an RIL population derived from the cross of *Oryza sativa* (IR64, sensitive) and *Oryza rufipogon* (tolerant), three QTLs were identified for root length under Al toxicity stress and five for relative root length. *O. rufipogon* contributed all favorable alleles for each of the five QTLs for relative root length as the most important trait affected by Al toxicity. Individually, these QTLs explained 9–25% of the phenotypic variation. The QTLs for relative root length on chromosomes 1 and 9 were observed to be consistent among different rice populations. The major QTL explaining 25% of the phenotypic variation was on chromosome 3 of rice, and was conserved across cereals, suggesting the potential for its use in breeding (Nguyen et al. 2003). Recently, Xue et al. (2006) identified three QTLs on chromosomes 1, 9, and 11, using an RIL population derived from a *japonica* cultivar Asominori (tolerant) and an *indica* cultivar IR24 (sensitive), with phenotypic variance of 13–18%; the two QTLs on chromosome 1 and 9 also were found to be consistent among different rice populations. In a subsequent study, the QTL on chromosome 9 was fine-mapped using a high-resolution physical map, and linked markers that cosegregated with this QTL were identified (Xue et al. 2007). These studies indicated the complexity of Al toxicity tolerance in rice; however, identification of similar QTLs across different populations and backgrounds suggested that these QTLs could be targeted for breeding through MABC. Subsequent studies are also needed to advance our knowledge beyond the identification of QTL loci.

#### 12.2.3.2 Iron Toxicity

Iron toxicity is a nutrient disorder, caused by excessive uptake of ferrous ions in amounts that disrupts metabolic processes, resulting in injury and reduced growth and yield. It commonly occurs in highly reduced soils when toxic concentrations of ferrous iron accumulate in soil solution, or when inflow carries soluble iron from upper slopes into highly reduced low lying areas. It is also a common problem in acid sulfate rice soils, as in Vietnam, Thailand, Bangladesh, and Indonesia. In West Africa, iron toxicity is widespread throughout the humid tropics, affecting about 30–40% of the cultivated lowlands.

##### Iron Toxicity in Rice and Bases of Tolerance

Iron toxicity was first reported in rice by Ponnamperuma et al. (1955) when they attributed the bronzing disease of lowland rice to high concentration of ferrous iron in soil solution and its subsequent excessive uptake and accumulation in plant tissues. Since then, iron toxicity has been recognized as one of the most widely spread micronutrient disorders, especially in the humid tropics of Asia, West and

Central Africa, and South America, particularly in acid, acid sulfate, and peat soils (Dobermann and Fairhurst 2000; Balasubramanian et al. 2007; Fageria et al. 2008). Large areas of these wetlands ideally suited for rice production remain underused or even unused in severe cases. In West Africa, yield losses of 12–100% were reported, depending on the severity of the stress and the extent of tolerance of the varieties being grown. Symptoms of damage are expressed as rusty leaf spots (bronzing), stained leaf edges, and dark-brown rigid and poorly developed roots. The typical visual symptom in rice is the bronzing of leaves, and the yield losses associated with the appearance of these bronzing symptoms commonly range from 15 to 30%; however, severe stress can cause complete crop failure (Audebert and Sahrawat 2000).

The physiological basis of tolerance of iron toxicity in rice has been studied by various investigators, and a few strategies were proposed (1) exclusion of ferrous irons by roots through root selectivity to avoid excessive uptake; (2) proper compartmentation through apoplastic immobilization or storage in less active tissues such as older leaves, leaf sheaths, old roots, and stems; and (3) high tissue tolerance, probably through sequestration in vacuoles or enzymatic detoxification in the symplast. Formation of iron plaque on the root surface in soils containing high concentrations of ferrous iron in solution could also be another strategy to reduce its uptake. Plaque formation is caused by oxidation of ferrous irons by oxygen that leaks from rice roots to form the insoluble ferric irons, which then precipitate on the root surface. Presumably, several mechanisms could be involved in enhancing tolerance of rice to iron toxicity; however, the genetic and molecular bases of these mechanisms are still not well understood.

## Germplasm Improvement for Iron Toxicity Tolerance

Substantial genetic variation has been reported in rice in response to high ferrous iron concentration in soils or in hydroponics (Gunawardena et al. 1982; Sahrawat et al. 1996; Fageria et al. 2008). This makes it possible to breed rice cultivars with greater tolerance of iron toxicity, which could substantially enhance rice production in affected areas. However, despite this genetic variability, still little progress was made in developing tolerant varieties that are high-yielding. Several studies have identified QTLs associated with tolerance in rice. Wu et al. (1997) identified three QTLs, two of them on chromosome 1 and one on chromosome 8, using a mapping population derived from the tolerant *japonica* Azucena and the moderately sensitive *indica* variety IR64. The phenotypic contribution of these QTLs ranges from 10 to 32%. Using a backcross population developed from Nipponbare and Kasalath, Wan et al. (2003) identified four QTLs for various traits associated with Fe toxicity tolerance, three of them were on chromosome 1, and one on chromosome 3. These QTLs has LOD scores between 3.17 and 7.03, and phenotypic effects ranging from 20 to 48%. Thus QTLs of major effects on Fe(II) toxicity tolerance are present in rice and provide future targets for MABC to introgress them into popular varieties and breeding lines for use in target areas.

## 12.3 Current and Future Prospects of Marker Assisted Backcrossing for Breeding Varieties Adapted to Problem Soils

Genetic linkage maps have made it possible to study the chromosomal locations of genes for improving yield and other complex agronomic and adaptive traits important in agriculture (Tanksley and McCouch 1997). Genetic mapping studies have led to over 8,500 QTLs identified for many different traits in rice, including tolerance to abiotic stresses (www.gramene.org). At the same time, advances in physiology and genomics have led to a more detailed insight into the responses of rice to soil stresses. While many previous studies explored differential gene expression between stress and control conditions through microarrays and RT–PCR (Walia et al. 2005, 2007), a deeper understanding of plant responses to abiotic stresses is now being investigated through proteomic and metabolomic profiling (Bohnert et al. 2006; Torabi et al. 2008) and by studying small RNAs (Sunkar et al. 2007). Future techniques in high throughput sequencing will only make these studies faster and more powerful (Sunkar et al. 2008). By integrating genomic methods to study key traits with genetic tools such as NIL development and QTL cloning, a better understanding of key tolerance mechanisms will lead to more efficient methods for breeding more tolerant varieties (Varshney et al. 2005; Salvi and Tuberosa 2005). For example, important QTLs and loci identified through association mapping for different traits can now be combined through marker-assisted breeding for crop improvement (Takeda and Matsuoka 2008). Furthermore, as more genomic sequence and SNP data becomes available through resequencing (McNally et al. 2006) and de novo whole genome sequencing, the genetic variation of tolerance can be investigated on a scale never before possible. Having more genome sequence data will be important when dealing with *indica* varieties as the tolerant donors, since the gene content between *indica* and the *japonica* Nipponbare reference sequence can be significantly different, as was shown by the recent study at the *Pup1* locus (Heuer et al. 2009). Moreover, high-density SNP arrays will lead to more powerful association genetic studies that will help explore the useful genetic variation that is captured in rice germplasm collections. High throughput SNP genotyping platforms will also enable more efficient MABC by reducing the cost per marker and by speeding up the process through multiplexing. As more SNP markers in rice are characterized, then subsets of SNPs that are optimized for different breeding applications can be selected. For example, a small number of targeted SNPs at gene loci, including functional SNPs and key SNP haplotypes, can be used for foreground selection in breeding programs for traits where the QTLs have already been cloned. In addition, QTL mapping and background selection can employ low-cost multiplexed sets of 384 SNPs, while QTL fine-mapping and more precise tracking of introgressions may require larger multiplexed sets of 1,536 SNPs or even 10,000 SNP chip platforms that are becoming available. By offering rapid, cost-effective, and robust genotyping, these new technologies will allow the wider use of the valuable QTLs that have already been identified, and will ultimately bring marker-assisted selection into mainstream breeding efforts.

## 12.4 Conclusions

The stresses encountered in problem soils are generally complex, where several abiotic factors are commonly encountered. This complexity coupled with the multitude of traits required for plants to withstand a particular stress has made improvement through conventional breeding a challenging undertaking, as witnessed by the slow progress in previous efforts. New approaches are therefore necessary to identify the suite of adaptive traits and mechanisms of tolerance, followed by swift incorporation into varieties and elite material that lack these traits but meet farmers' expectations. Considerable progress was made in understanding signaling and response pathways for most of the major soil-related problems, and the recent developments in genomics have provided powerful tools for genetic dissection of these traits. Despite the complexity of most soil problems, tolerance of some of them was attributed to a few QTLs of large effects (Table 12.1), and the recent developments in marker technologies made it possible to tag and incorporate these major QTLs into high-yielding varieties. Preliminary efforts to incorporate some of these QTLs have demonstrated measurable effects on the performance of rice varieties under stress. The recent developments in high throughput genotyping systems also hold great potential in overcoming the obstacles encountered in MABC. Complementing conventional methods with MABC will continue to help accelerate the development of more resilient varieties that could positively impact productivity of rice on problem soils.

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