ALUMINIUM-INDUCED GENE EXPRESSION IN SUGARCANE ROOTS

by

NATALIE JANE GRAHAM

SUBMITTED IN FULFILMENT OF THE ACADEMIC REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN THE SCHOOL OF LIFE AND ENVIRONMENTAL SCIENCES, UNIVERSITY OF NATAL, DURBAN.

DECEMBER 2002

ABSTRACT

Due to the increasing prevalence and severity of Al phytoxicity in certain regions of the South African sugar industry, a research programme has been initiated at SASEX to elucidate the molecular mechanisms by which sugarcane detects and responds to the metal. As part of this larger investigation, the current study aimed to assess the response of a reportedly Al tolerant cultivar, *Saccharum* spp. hybrid *cv.* N12, to phytotoxic levels of Al. Hydroponically-grown plants of this commercial genotype were used in Al inhibition studies, the results of which indicated that exposure of plants to 250μM Al for 24 hours resulted in maximum reduction of root elongation. Under these conditions, root growth was inhibited by approximately 36%, compared with only 4% for the 50μM Al treatment. Subsequently, this exposure regime was used to gather the terminal 5 to 10mm of root tips, the site of the primary Al lesion, of challenged and control, unchallenged plants for molecular analysis.

Total RNA was extracted from the Al challenged and control root tips, from which mRNA was subsequently isolated, reverse transcribed and converted to double-stranded cDNA. The two populations of cDNA were reciprocally subtracted from each other and used to construct subtractive cDNA libraries in Lambda ZAP®II phages. Randomly selected clones, 576 representatives from each of the libraries, were screened using membrane-based array technology. Results indicated that only 33% (190) of the Al-treatment specific library cDNAs were found to be more highly expressed under conditions of Al stress than under control conditions. Of these potentially Al response-related cDNAs, 25 were sequenced and submitted to sequence databases for the assignment of putative identities. No genic sequences known to be directly associated with the Al stress response were identified, however, several were found to be related to pathogenesis or general stress pathways. Although further Northern hybridisation work is required to validate these results, they suggest that the induction of general stress response pathways may be involved in the aluminium stress response of this sugarcane cultivar. Such Al stress-related sequences could have applications in marker-assisted breeding programmes and as candidate genes for the genetic engineering of tolerant genotypes.

PREFACE

The experimental work described in this dissertation was carried out in the Biotechnology Department of the South African Sugar Association Experiment Station (SASEX), Mount Edgecombe, from January 2000 to January 2002, under the supervision of Dr Derek Watt.

These studies represent original work by the author and have not otherwise been submitted in any form for any other degree or diploma to any other tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

ACKNOWLEDGEMENTS

The author would like to thank the staff and students of the Biotechnology Department at the South African Sugar Association Experiment Station for their expert guidance and friendship, especially Dr Barbara Huckett, Dr Derek Watt and Dr Deborah Carson.

The author would also like to gratefully acknowledge financial support from the South African Sugar Association Experiment Station as well as the Sugar Industry Trust Fund for Education (SITFE).

The author also gratefully thanks her parents for their support, both financial and emotional.

And lastly, but far from least, the author thanks her husband for his enormous patience.

TABLE OF CONTENTS

	Page
1. INTRODUCTION	**1**
2. LITERATURE REVIEW	**5**
2.1 Chemistry of aluminium in soils	**5**
2.2 Aluminium as a phytotoxin	**6**
2.2.1 Phytotoxic species of aluminium	
2.2.2 Effects of aluminium on plant growth and physiology	
2.2.3 The impact of aluminium on global food security	
2.3 Plant tolerance of aluminium	**15**
2.3.1 Physiology of detoxification	
a. Exclusion of aluminium	
b. Internal mechanisms of aluminium detoxification	
2.3.2 Genetics of aluminium tolerance	
2.4 Approaches to minimise aluminium toxicity	**24**
2.4.1 Agronomic and plant breeding strategies	
2.4.2 Molecular strategies	
2.5 Aluminium phytotoxicity in the South African sugar industry	**29**
2.6 Current study	**33**
3. MATERIALS AND METHODS	**35**
3.1 Plant materials	**35**
3.1.1 Cultivar	
3.1.2 Propagation and growth	
3.2 Aluminium challenge	**37**
3.2.1 Root elongation assay	
a. Preparation and acclimation of plantlets and challenge with Al	
b. Root growth measurements	
c. Calculations and statistics	
3.2.2 Challenge conditions for library construction	
a. Preparation and acclimation of plantlets and challenge with Al	
b. Harvesting and storage of root tips	
3.3 Construction of subtractive cDNA library	**38**
3.3.1 RNA isolation	
a. Precautions	

b. Tissue disruption

c. Total RNA extraction and purification

d. Quantification and quality assessment

e. Poly A^+ isolation

3.3.2 cDNA synthesis

a. First strand synthesis

b. Second strand synthesis

3.3.3 cDNA processing

a. Digestion with restriction enzymes

b. Adaptor ligation

3.3.4 cDNA subtraction

a. PCR amplification

b. Synthesis of tracer cDNA and biotinylation of driver cDNA

c. Hybridisation of tracer and driver cDNA

d. Isolation of subtracted tracer cDNA

3.3.5 cDNA cloning

a. Adaptor ligation

b. cDNA phosphorylation

c. cDNA digestion with restriction enzyme

d. Ligation into cloning vector

e. Packaging

3.4 Characterisation of the subtractive libraries **48**

3.4.1 Preparation of bacterial cell line

3.4.2 Plating of phage libraries

3.4.3 Assessment of insert sizes

3.4.4 Amplification of phage libraries

3.5 Analysis of library cDNA inserts **50**

3.5.1 Reverse Northern hybridisation analysis

a. Probe synthesis

b. Array printing

c. Preparation of labelled total cDNA populations

d. Array querying

e. Array analysis

3.5.2 Northern hybridisation analysis

a. Total RNA size fractionation

b. RNA blotting

c. cDNA probe labelling

d. Hybridisation

e. Visualisation

3.5.3 Sequencing

a. Phagemid rescue and quantification

b. Capillary electrophoresis

c. Sequence editing and homology searches

4. RESULTS **57**

4.1 Hydroponic culture of sugarcane **57**

4.2 Effect of aluminium on root growth **57**

4.3 Isolation of aluminium-challenge-associated cDNA **59**

4.3.1 Quantity and quality of RNA

4.3.2 Conversion of mRNA into complementary DNA (cDNA)

4.3.3 Isolation of cDNAs associated with the aluminium phytotoxic response

4.3.4 Cloning of subtracted cDNAs

4.4 General analysis of aluminium-challenge-associated cDNA library **64**

4.5 Expression analysis of aluminium-challenge-associated cDNA libraries **65**

4.5.1 Reverse Northern hybridisation analysis

a. Array querying

b. Array analysis

4.5.2 Confirmation of differential expression patterns: Northern hybridisation analysis

4.6 Clone identification **69**

5. DISCUSSION **75**

5.1 Performance of hydroponically-grown sugarcane under conditions of aluminium stress **75**

5.2 Evaluation of molecular strategies **77**

5.2.1 Isolation and manipulation of genetic material

5.2.2 Applicability of array technology to this study

5.2.3 Inconclusiveness of Northern hybridisation analyses

5.3 Effect of aluminium on gene expression in sugarcane roots **80**

5.4 Future work **83**

5.5 Conclusions **86**

REFERENCES **87**

LIST OF TABLES

Table 1: Selected studies reporting secretion of organic acids as Al^{3+} chelating agents. **20**

Table 2: Some selected examples of aluminium-induced genes, isolated from a variety of plant species. **25**

Table 3: Molecular techniques that have been employed in various gene search programmes. **27**

Table 4: Hydroponic nutrient medium composition (after Hewitt, 1966). **36**

Table 5: MINTEQ analysis of Al^{3+} activity in 1mM $CaCl_2$. **37**

Table 6: Characteristics of Lambda ZAP®II subtractive libraries prepared from mRNA isolated from N12 root tips. Sub A – Al exposure treatment (250μM $AlCl_3$ [in 1mM $CaCl_2$] for 24 hours); Sub B – control treatment (1mM $CaCl_2$ for 24 hours). **64**

Table 7: Putative identities and characteristics of selected sequences expressed in N12 root tips in response to challenge with 250μM Al for 24 hours in hydroponics. **73**

Table 8: Possible roles of clones putatively identified through sequence homology with the NCBI non-redundant protein database entries (BLASTx), expressed in sugarcane roots in response to Al-induced stress. **85**

LIST OF FIGURES

Fig. 1: Schematic representation of a single hydroponic vessel (a), and position of Neoprene®collar (foam tubing) on plantlets (b). **36**

Fig. 2: Generalised subtraction scheme. Tracer cDNA from the Al-exposed root tips (+) was hybridised to >10-fold excess of driver cDNA from the control treatment root tips (-). The resulting hybrids and excess driver were removed to enrich for sequences specific to the tracer cDNA population. (after Patel and Sive, 1996). **43**

Fig. 3: Sequence of subtractions used for the isolation of A-specific and B-specific genes (after Patel and Sive, 1996). **44**

Fig. 4: Array design for subtractive libraries A and B, showing addresses of PCR products on array membrane. **52**

Fig 5: Cultivation of *Saccharum* spp. hybrid N12 (N12). (a) View of hydroponically grown plants in glasshouse. (b) Root system of hydroponically grown plants. **58**

Fig 6: Average percentage root growth inhibition (%RGI) of hydroponically-cultivated N12 plants as a result of exposure to aluminium ($AlCl_3$ in 1mM $CaCl_2$ at pH 4.15) (a) supplied at various concentrations for 24 hours, and (b) supplied at 250µM over a 72 hour period. **60**

Fig. 7: Fractionation of representative RNA samples via denaturing agarose gel (1.2% [w/v]) electrophoresis, as described by Ingelbrecht *et al.*, 1998, and visualised via ethidium bromide staining and short-wavelength UV radiation. Lane 1: RNA size ladder 1 (Roche); Lane 2 & 3: 10µg RNA samples isolated from root tips exposed to 250µM Al ($AlCl_3$ in 1mM $CaCl_2$) for 24 hours using a hydroponics system. **62**

Fig. 8: Progressive removal of common abundant sequences from two populations of cDNA over the first four cycles of hybridisation (a: 1st cycle; b: 2nd cycle; c: 3rd cycle; and d: 4th cycle) via PCR based subtraction. (Lanes 1: Marker 3 [λDNA digested with *Eco*RI and *Hind*III]; Lanes 2: Tracer cDNA A [Al-exposed treatment]; Lanes 3: Tracer cDNA B [control treatment]; Lanes 4: Marker 5 [pBR322 digested with *Hae*III]. **63**

Fig. 9: A subset of PCR amplified plaques from subtractive library A, fractionated via agarose 1.2% (w/v} gel electrophoresis. cDNA library was prepared from mRNA isolated from N12 root tips exposed to 250μM Al for 24 hours in hydroponics. Note multiple banding in lanes 12, 13 and 14. Such samples were discarded. **65**

Fig. 10: Probing of total mRNA, isolated from (a) Al-exposed (250μM Al in 1mM $CaCl_2$ for 24 hours) N12 root tips and (b) from control treatment (1mM $CaCl_2$ for 24 hours) root tips, with subtractive library A (control treatment cDNA subtracted from Al challenge cDNA) array. **66**

Fig. 11: Superimposition of array image results from the hybridisation of the Sub A (Al-specific) library array to control treatment and to Al-exposure cDNA, using QuantArray® Microarray Analysis Software (version 3.0, Packard Bioscience). Regions of green signal indicate signal from control treatment cDNA hybridisation, regions of red signal indicate signal from Al-exposure cDNA hybridisation, and yellow indicates regions of overlap between the red and green signals. **67**

Fig. 12: Pie representation of the relative expression of array clones under conditions of Al-stress (red) and control treatments (green) using QuantArray® Microarray Analysis Software (version 3.0, Packard Bioscience). Smaller pie charts indicate data points below the brightness threshold of 25; larger pie charts indicate data points above the brightness threshold of 25. **68**

Fig. 13: Proportion of clones preferentially expressed under conditions of Al challenge (250μM Al in 1mM $CaCl_2$ for 24 hours) and under control conditions (1mM $CaCl_2$ for 24 hours). Values allocated to inner pie-segments indicate the proportion each segment represents of combined expression in both challenge and control treatments. Segments represent groupings of clones falling within 10% intervals of percentage expression under Al challenge conditions. **70**

Fig. 14: Representative phosphor-image from Northern hybridisation analyses, using putative Al-induced genic fragment to probe membrane bearing 10μg total RNA isolated from Al-challenged (+Al) (250μM Al in 1mM $CaCl_2$ for 24 hours in hydroponics) N12 root tips and 10μg total RNA from control (-Al) (1mM $CaCl_2$ for 24 hours in hydroponics) root tips. **71**

Fig. 15: Differential expression of an N12-derived root specific cDNA fragment in cultivars N19 and N17. (a) Fractionated RNA samples, stained with ethidium bromide (b) Northern hybridisation analysis of root, leaf and callus RNA using root specific cDNA fragment as probe. (+Al: Al stress treatment; +D: Diamide stress treatment; – : Control treatment). **72**

LIST OF ABBREVIATIONS

λ	lambda
μg	microgram
μl	microlitre
μM	micromolar
°C	degrees Celsius
A	adenine
AFLP	amplified fragment length polymorphism
Al	aluminium
ATA	aurintricarboxylic acid
dNTP	deoxynucleotide triphosphate
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CEC	cation exchange capacity
cm	centimetre
cv.	cultivar
dATP	2'-deoxyadenosine 5'-triphosphate
dbEST	EST database
dCTP	2'-deoxythymidine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
cDNA	complementary DNA
dNTP	2'-deoxynucleotide 5'-triphosphate
dT	deoxythymidine
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylene diamine tetraaccetic acid

EST	expressed sequence tag
E value	expect value
g	gram
g	relative centrifugal force
GTP	guanosine triphosphate
H^+	proton
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani
M	molar
MAS	marker assisted selection
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
ng	nanogram
NIL	near isogenic line
nr	non redundant protein database
NZY	NZ amine (casein hydrolysate) and yeast
PAS	ρ-amino salicylic acid
PCR	polymerase chain reaction
PEG	polyethylene glycol
PM	plasma membrane
PVP	polyvinylpyrolidine
RFLP	restriction fragment length polymorphism
RGI	root growth inhibition
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SSH	suppression subtractive hybridisation
SubA	subtractive library A (Al treatment)

SubB	subtractive library B (control treatment)
TBE	Tris borate EDTA
TE	Tris EDTA
Tris	Tris[hydroxymethyl]aminomethane
Tris-HCl	Tris hydrochloric acid
UV	ultraviolet
V	volt
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

INTRODUCTION

Soil degradation and acidification are of major global concern, primarily due to the marked influence these processes have on agricultural productivity. Poor agronomic practices such as the excessive use of ammoniacal fertilizers, coupled with intensive continuous monocropping, are the main contributors to the increasing prevalence of acidic soils in the agricultural sector. The negative consequences of soil acidification on plant growth are primarily effected by the increased solubility of aluminium, a major phytotoxin at low soil pH values. While soil acidification is reportedly a common occurrence during the cultivation of annual cycle crops, such as wheat and maize, it is particularly acute for sugarcane, which is a vegetatively propagated crop that is grown continuously over several cycles. In the South African sugar industry, this tendency towards soil acidification is exacerbated by the widespread use of urea, the most cost-effective source of inorganic nitrogen readily available to sugarcane growers. A recent survey conducted by the SA Sugar Association Experiment Station (SASEX), noted that the rate of acidification in the industry appears to be accelerating, with the percentage of fields considered to be strongly acidic (below pH 5.0) increasing from 18% to 43% over the last twenty years (Schumann, 1998).

In several sugarcane industries around the world, an apparent sugar yield plateau has been experienced over the past two to three decades. It is not surprising, therefore, that much attention has been devoted to the elucidation of the factors that may be contributing to this yield phenomenon, with particular focus on the effects of soil degradation. The exact quantification of this latter process on yield has, however, proved to be an elusive goal. Nevertheless, the evidence gathered to date strongly suggests that the degradation of soil has placed a significant constraint on production.

Currently, approaches in the South African sugar industry to counteract the negative effects of soil acidity and thus aluminium phytotoxicity on cane production have focused primarily on the alleviation of acidity through the application of lime. Concomitant to this have been attempts to enhance organic matter content of the soil through the practice of trash blanketing, whereby non-millable portions of the cane are retained in the field. However, these approaches may not be sustainable in the long-term due to several drawbacks associated therewith, particularly the restriction of the beneficial effects of the treatments to the upper soil horizons, as well as high

costs in the case of liming. The latter is an important consideration in light of the increasing number of small-scale growers who struggle to maintain economic viability under difficult circumstances. Consequently, as part of SASEX's research efforts, alternative, more sustainable approaches are being sought to counteract the negative effects of aluminium phytotoxicity on sugarcane production.

There are many instances in which conventional crop breeding strategies have successfully produced cultivars with increased tolerance to specific environmental stresses, but only when genotypic diversity for the tolerance trait exists within the species. Such diversity has been demonstrated for aluminium tolerance in sugarcane, in that the two primary ancestral species to modern hybrid cultivars, *viz. S. officinarum* L. and *S. spontaneum* L., are reported to have different degrees of tolerance to the metal, with the latter being the more susceptible (Landell, 1989). It is not surprising, therefore, that liming field trials have demonstrated different responses to soil acidity alleviation amongst local sugarcane cultivars (Schroeder *et al.*, 1994), thus indirectly confirming the presence of genotypic diversity in the trait for aluminium tolerance. Given such diversity, selection for the aluminium tolerance phenotype during the breeding programme would, therefore, appear to be a feasible and attractive goal.

Accurate quantification of the phytotoxic effects of aluminium is technically demanding, as the first obvious effect of the metal is an inhibition of root elongation, a symptom not easily measurable under field conditions. Consequently, primarily for reasons of practicality, no attempts have been made to introduce aluminium tolerance as a selection criterion within the industry's breeding programme. It is possible that this barrier to breeding for aluminium tolerance may be overcome through the discovery of molecular markers linked to either the tolerant or susceptible phenotype. The availability of such markers would allow for the selection of suitable parental germplasm to be used in specific crosses aimed at producing cultivars tolerant to the metal. Ultimately, such markers could be used to screen for the presence of the desired trait amongst the progeny of specific crosses.

Work conducted at SASEX has demonstrated that the potential for the identification of molecular markers depicting a particular trait in sugarcane is enhanced when the markers are functionally involved in the expression of the trait (Thokoane and Rutherford, 2001). In the study by Thokoane and Rutherford (2001), the use of such 'perfect' markers in combination with a number of phenotypically well-characterised non-sibling genotypes allowed for the identification of several DNA sequences significantly linked to resistance or susceptibility to

infection by sugarcane smut (*Ustilago scitaminea* Sydow) and/or infestation by the sugarcane stalk borer (*Eldana saccharina* Walker). However, there are two important resources essential for the implementation of such an associative genetic approach to marker discovery, *viz.*DNA sequences encoding the desired phenotype and a panel of genotypes well-characterised for the trait.

Recent progress in molecular technology has provided plant physiologists with a number of tools to isolate DNA sequences expressed in response to particular stresses, both biotic and abiotic. Of these technologies, cDNA subtractive hybridisation has proven to be particularly effective at providing insight into gene expression in response to defined environmental conditions. In addition, advances in the analytical power of DNA array technology has allowed for the verification of the efficiency of the subtractive hybridisation, thereby enabling the quantification of this complex and somewhat unpredictable subtractive process. Thus, the combination of cDNA subtractive and array technologies allows for the isolation of genic fragments expressed in response to specific environmental stresses, thereby fulfilling an essential requirement for marker discovery. In addition to their role as resources in marker identification, genes associated with the aluminium phytotoxic response may also have a potential role as transgenes in the engineering of tolerance phenotypes. This approach is not without precedent, with Ezaki and co-workers (2000) successfully enhancing the aluminium tolerance of *Arabidopsis thaliana* through the individual expression of several aluminium tolerance-related cDNAs.

To date, methods to determine the aluminium tolerance status of local cultivars have been based on growth and yield performances subsequent to application of lime. However, due to the complex influence of pH on soil nutrient availabilities, only indirect deductions have been possible regarding the aluminium tolerance of cultivars. Ideally, to accurately assess the phytotoxic effect of aluminium on sugarcane growth, the complex interactions between the metal and the host of ions and organic molecules present in the rhizosphere should be minimised. This has been most often achieved for other species through the culture of plants on a liquid media of defined chemical composition. A further advantage of such hydroponic systems is that they provide unrestricted access to the roots of the plant, which is important if the effects of aluminium on root elongation are to be measured. Thus, to provide the set of phenotypically characterised sugarcane genotypes necessary for the isolation of molecular markers linked to aluminium tolerance, the development of a system for the hydroponic culture of local cultivars would be essential.

This study aimed to address two of the primary challenges associated with the discovery of an aluminium tolerance related genic sequence in sugarcane. In the first instance, a major consideration was the requirement for a rapid and reliable method for the quantification of the phytotoxic effects of aluminium on sugarcane. Hence, the initial phase of this investigation focused on the development of a hydroponics culture system that not only permitted the analysis of sugarcane growth and performance under defined conditions of aluminium stress, but which also provided rapid access to the root system for subsequent molecular analyses. It is of note that such a system has potential applications beyond the scope of the current study, in that it may be used for the future screening and phenotypic characterisation of a panel of genotypes for aluminium tolerance, thus providing one of the primary resources required for the implementation of the associative genetic approach to marker discovery. The second challenge addressed in this study was the appropriate application of complex DNA technologies to complex molecular analyses with the view to identify aluminium-induced alterations to genetic expression patterns of a reportedly aluminium tolerant cultivar. In using such an approach, sequences displaying apparently enhanced expression under conditions of aluminium stress could reflect aluminium tolerance related responses and possibly be responsible for the tolerant phenotype. Thus, in fulfilling the second requirement for this approach to marker discovery, i.e. genic sequences associated with the tolerant phenotype, the future identification and manipulation of aluminium tolerance characteristics in sugarcane has become a more attainable goal.

CHAPTER TWO
LITERATURE REVIEW

2.1 CHEMISTRY OF ALUMINIUM IN SOILS

Aluminium: the most abundant metal and third most abundant element in the earth's crust and yet required by neither plant nor animal. Comprising almost 8% by weight of the outer crust (Driscoll and Schecher, 1990) aluminium (Al) exists primarily in the form of insoluble alumino-silicates and oxides (Gallego and Benito, 1997) and is therefore largely unavailable for uptake by plants. In the aqueous phase, Al may be associated with a number of organic and inorganic ligands, such as humic acid, citrate, malate, OH^-, F^-, SO_4^{2-}, PO_4^{3-}, H_4SiO_4 and HCO_3^- (Driscoll and Schecher, 1990; Ezaki *et al.*, 1995). The extent of complexation of Al by these ligands is regarded as a function of a number of variables, such as Al availability, ligand concentration, soil pH, ionic strength and temperature of the soil solution. Cycling of Al in the lithosphere is very complicated and the aqueous chemistry of this element is rather poorly understood.

Aluminium is a strong hydrolysing metal and relatively insoluble in the near neutral pH range of most soils (Driscoll and Schecher, 1990). However, the availability of Al is dramatically increased under conditions of acidity (pH < 6.0) and alkalinity (pH > 8.0). At a pH less than 4.0 the hexa-aquo Al(III) ion $Al(H_2O)^{3+}$ (commonly referred to as Al^{3+}) tends to predominate, while above pH 7.5 the aluminate ion complex $Al(OH)_4^-$ becomes the major ion in solution (Smith, 1972). Aqueous Al can exist in a plethora of chemical forms and, at pH values between the two above-mentioned extremes, one can never be sure of the exact concentration of the various species (Shann and Birch, 1993).

Soil pH not only affects the solubility of Al, a potential soil phytotoxin, but it also has a notable impact on soil chemistry and the availability of plant nutrients. A vast proportion of the world's arable lands (>70%) are considered acidic, making soil acidification an issue of global agricultural importance (Hamel *et al.,* 1998). The phenomenon of soil acidification is considered one of the major symptoms of the degradation of our soil resources, and is regarded along with declining genetic diversity and pest and disease problems as one of the most serious threats to sustainable agriculture (Meyer *et al.*, 1996).

Low pH soils (<5.0) are found in many volcanic and tropical regions as a result of natural soil weathering (Gallego and Benito, 1997). However, anthropogenic effects have had a rather

severe impact on this otherwise slow and natural process through poor agricultural practices and acid rain. Accelerated acidification of soils under cultivation can largely be attributed to the oxidation of ammoniacal fertilisers to nitric acid, the removal of basic cations from the soil by crops and through leaching, and to the mineralisation of organic matter (Van Antwerpen and Meyer, 1996), aggravated through continuous and intensive cropping (Schroeder *et al.*, 1994). These factors outstrip the buffering capacities of soils, leading to the release of toxic levels of Al and manganese, and to deficiencies in important nutrients, such as Ca, P, Fe and Mo (Snowden and Gardner, 1993; Schumann, 1998).

There is increasing evidence that there is a global increase in soil acidification, which is exhibiting a marked effect on agricultural productivity. Not only is it necessary to amend current soil management strategies to slow the deterioration of soil quality, but also to find other ways of coping with already acidic conditions.

2.2 ALUMINIUM AS A PHYTOTOXIN

2.2.1 Phytotoxic species of aluminium

Not considered as either an animal or plant nutrient, Al generally does not accumulate in living tissues and is known to be toxic to a wide variety of organisms when it does (Driscoll and Schecher, 1990). While there is little dispute regarding the potent toxicity of Al, there has been some debate as to which species is responsible for the observed damaging effects.

Unlike biologically important elements, such as Ca, Mg and K, Al is distinguished by the low solubility of its hydroxide and myriad hydrolysis products (Bennet and Breen, 1991). The solubility of Al is normally too low to be available for uptake by plants, yet as the pH decreases below 5.0 the amount of Al available in the soil solution can increase exponentially and become extremely toxic (Andersson, 1988; Miller *et al.*, 1997). The exact chemical speciation in solution is very difficult to determine as soluble Al can exist in many different ionic forms (Kochian, 1995). Activities of the various ions are usually calculated using computer speciation programmes, such as GEOCHEM-PC (Parker *et al.*, 1995) and MINTEQA2/PRODEFA2 (Allison *et al.*, 1990) and based on assumed equilibrium conditions. However, the more complex the Al-containing aqueous solutions, the more unreliable these computational predictions become, as Al speciation is strongly influenced by even the smallest of pH changes,

ionic strength and by the activity of other ions present (De la Fuente-Martínez and Herrera-Estrella, 1999).

The strong association between soil acidity and Al toxicity lends support to the notion that it is the low-pH soluble Al^{3+} ion that is responsible for this phenomenon (Ezaki *et al.*, 1995). It has also been shown that the symptoms of Al toxicity are more closely linked to the activity of monomeric Al^{3+} than to the total level of Al in the soil (Hue *et al.*, 1986) and it is known to be toxic at even micromolar concentrations (Delhaize and Ryan, 1995; De la Fuente-Martínez and Herrera-Estrella, 1999).

As the pH increases, Al^{3+} (which exists as the octahedral hexahydrate $Al(H_2O)_6^{3+}$) undergoes successive deprotonations to form $Al(OH)^{2+}$ and $Al(OH)_2^+$ (Kochian; 1995; De la Fuente-Martínez and Herrera-Estrella, 1999). At near-neutral pH, the relatively insoluble gibbsite [$Al(OH)_3$] tends to predominate, limiting the solubility of other Al monomers. Further alkalinisation of the soil solution to pH values commonly found in the cytoplasm (*c.* 7.4) results in aluminate ($Al(OH)_4^-$) becoming the most abundant form (Kochian, 1995; De la Fuente-Martínez and Herrera-Estrella, 1999).

Recent work, however, has also shown that the polynuclear Al cations, the most important of which is $AlO_4Al_{12}(OH)_{24}(H_2O)_{12}^{7+}$, commonly referred to as Al_{13}, are also extremely toxic, possibly even more so than the monomeric Al^{3+}(Kochian, 1995; Masion and Bertsch, 1997). These cations seem to form under conditions of increased total Al activity and partial solution neutrality. While Al_{13} has been detected in the laboratory, it is not clear whether this cation occurs naturally and its contribution to phytoxicity in soils is thus unresolved (Delhaize and Ryan, 1995; Kochian, 1995).

However, since trivalent ions are generally known to be more toxic to plants than divalent and monovalent ions, and because Al toxicity is strongly associated with low soil pH conditions, it is commonly assumed that Al^{3+} is the major species involved in the phytotoxic responses of plants to this metal (Delhaize and Ryan, 1995; De la Fuente-Martínez and Herrera-Estrella, 1999).

2.2.2 Effects of aluminium on plant growth and physiology

Treatment of plants with increased levels of Al has been found to cause a variety of adverse effects. Aluminium appears to act at a number of levels, the severity and permanence of the effects being proportional to the Al concentration and duration of the exposure (Hairiah *et al.*, 1993; Jones *et al.*, 1995). Symptoms of Al toxicity are not always readily identified as being Al-related. For instance, some plants display foliar symptoms resembling P deficiencies, with general stunting and delayed maturation, small, dark green leaves, purpling of stems, leaves and leaf veins, and yellowing and death of leaf tips (Foy, 1983; Taylor and Foy, 1985a; Andersson, 1988). Aluminium toxicity may also manifest foliarly as an induced Ca deficiency, with younger leaves exhibiting a tendency to curl or roll and the collapse of growing points and petioles (Foy, 1983; Andersson, 1988; Aniol and Gustafson, 1990). Some of the younger leaves may also suffer from interveinal chlorosis, symptomatic of an Fe deficiency (Cambraia *et al.*, 1983; Taylor and Foy, 1985a). The effects of Al are generally far more pronounced in the roots than in the shoots of affected plants, with far higher concentrations and lengthier exposures required before symptoms become visible in aerial parts of the plant (Taylor and Foy, 1985a; Andersson, 1988). Many of the effects observed in the foliage can in fact be indirectly attributed to the damage caused by Al on the rooting system of the plant.

Inhibition of root growth is considered the initial and most dramatic symptom of Al phytotoxicity, and is usually detectable within minutes of exposure (Delhaize and Ryan, 1995; De la Fuente-Martínez and Herrera-Estrella, 1999). Aluminium challenged roots generally develop much less vigorously, with diminished elongation of the main root axis, and lateral roots often failing to develop (Andersson, 1988). Roots appear stubby, swollen, gnarled and brittle, with bent, necrotic tips, closely resembling the symptoms of nematode predation (Cambraia *et al.*, 1983; Taylor and Foy, 1985a; Andersson, 1988; Verkleij and Schat, 1990; Gascho *et al.*, 1993). The vascularisation of the roots may also be disturbed, with root systems thus frequently restricted to the upper soil horizons (Andersson, 1988, Aniol and Gustafson, 1990). Therefore, plants suffering from Al toxicity also tend to display increased susceptibility to pathogens, drought and nutrient stresses (Andersson, 1988; Aniol and Gustafson, 1990; Zhang and Jessop, 1998).

Despite the vast body of research conducted in the field of Al toxicity, there remains much confusion and controversy regarding the fundamental mechanisms involved. Several hypotheses have been proposed to explain the observed symptoms in Al-exposed plants (Kochian, 1995; De

la Fuente-Martínez and Herrera-Estrella, 1999) yet for each of these there exists evidence both supportive and contradictory.

Since the principal effect of Al is recognised as the inhibition of root growth, it is generally accepted that the primary site of Al toxicity is the apical meristem of the root apex (Verkleij and Schat, 1990; Marienfeld and Stelzer, 1993; Budíková, 1999). Studies have shown that only exposure of this region to Al resulted in inhibition, whereas selective exposure of other regions on the root had no impact on growth (Delhaize and Ryan, 1995; De la Fuente-Martínez and Herrera-Estrella, 1999).

There is no consensus as to whether Al has to enter the cell to be toxic or whether it can act via external elements on the plasma membrane or cell wall (De la Fuente-Martínez and Herrera-Estrella, 1999). Aluminium is known to interact with a number of both extra- and intracellular structures in the root apex, which suggests that there may in fact be several different mechanisms of toxicity (Kochian, 1995). Once Al has entered the symplasm, the presence of suitable ligands, combined with a marked increase in pH, would serve to drastically reduce the activity of Al to the nanomolar range (Delhaize and Ryan, 1995; De la Fuente-Martínez and Herrera-Estrella, 1999). However, the destructive potential of Al, even at these low concentrations, remains high enough to cause damage due to the extremely high binding affinity of the metal for cytoskeletal elements and several metabolically important molecules, such as DNA and RNA, purine residues (ATP and GTP), enzymes and calmodulin (Delhaize and Ryan, 1995; Blancaflor *et al.*, 1998; De la Fuente-Martínez and Herrera-Estrella, 1999).

One of the means by which Al is thought to reduce root growth rates is through interference with cell division in root tips and lateral roots (Foy, 1983; Kochian, 1995; Crawford *et al.*, 1998). Aluminium has also been shown to be closely associated with the nuclei of root tip cells, binding directly to DNA and/or RNA (Crawford *et al.*, 1998; Richards *et* al., 1998; Silva *et al.*, 2000) which could have a severe impact on critical physico-chemical and biological functions in the cell, such as cell division and elongation, and synthesis of DNA and RNA (Foy, 1983; Andersson, 1988; Kochian, 1995; Espino *et al.*, 1998). It has been postulated that inhibition of nucleic acid synthesis could occur directly as a result of Al binding to phosphate groups in the DNA backbone. It has, however, since been demonstrated that Al is more likely to be bound to protein structures strongly associated with DNA, such as histones, than to be bound to these phosphate moieties within the actual DNA structure (Kochian, 1995). But because the onset of root growth inhibition is so rapid, it becomes more plausible to suspect reduced cell elongation

than affected cell division and DNA synthesis as the primary cause (Bennet and Breen, 1991; Larsen *et al.*, 1998). In the long term, however, blockage of both mitotic activity and cell elongation processes are likely to be important contributing factors to root growth inhibition.

Growth studies have revealed that the site of Al toxicity in the root apex is associated with the elongation zone (Blancaflor *et al.*, 1998). There have been suggestions that the growth inhibition and morphological changes observed in this region may be attributable to Al-induced effects on the cytoskeleton (Grabski and Schindler, 1995; Kochian, 1995; Blancaflor *et al.*, 1998). Links have been found in animals between several Al-induced neurological disorders and cytoskeletal abnormalities (Grabski and Schindler, 1995; Blancaflor *et al.*, 1998). Aluminium is reported to bind 10^7 times more effectively than magnesium to ATP- and GTP-binding sites present on actin and tubulin, and the rates of hydrolysis of these Al complexes are in the region of 10^5 times slower than for the physiological Mg^{2+} complexes (Grabski and Schindler, 1995; Blancaflor *et al.*, 1998). These sites are essential for microfilament and microtubule assembly, and the interference of Al can therefore disrupt the intricate dynamics of these processes.

In mammalian and some plant systems Al has been known to negatively affect the expression of certain cytoskeletal regulatory genes, such as fimbrin (Cruz-Ortega *et al.*, 1997) as well as affect the phosphorylation of cytoskeletal proteins and the production of secondary messengers responsible for the regulation of cytoskeletal processes (Grabski and Schindler, 1995; Blancaflor *et al.*, 1998). In soybean (*Glycine max* L.) root cells, Al was shown to induce a rapid and dramatic increase in the rigidity of the actin network (Grabski and Schindler, 1995). Studies in maize (*Zea mays* L.) have revealed that changes in the organisation and stability of cytoskeletal elements were correlated with the symptoms of Al toxicity (Blancaflor *et al.*, 1998). Results suggested that the stabilisation of the microtubular network in the outer cortical cells could play an important role in the retardation of root elongation. It has yet to be established whether these changes in the cytoskeleton are direct or indirect symptoms of Al toxicity, however, the rapidity and close correlation of the response with the onset of growth inhibition make the disruption of cytoskeletal dynamics a strong candidate for the primary phytotoxic response (Blancaflor *et al.*, 1998).

A more recently proposed mechanism for Al toxicity in plants is through the inhibition of intercellular transport of various molecules, including small ions, peptides, nucleic acids and hormones (Sivaguru *et al.*, 2000). Symplasmic transport of these molecules between adjacent

cells is achieved via the plasmodesmata, and any disruption of these cytoplasmic channels can have severe consequences for intercellular communication.

Aluminium is known to induce the synthesis of callose (1-3-β-glucan) possibly through Al-induced elevations of intracellular Ca levels in intact roots, which activates the plasma membrane-bound enzyme 1-3-β-glucan synthase, located in the plasmodesmata (Staβ and Horst, 1995; Wagatsuma *et al.*, 1995; Budíková, 1999; Sivaguru *et al.*, 2000). Aluminium is capable of eliciting rapid closure of plasmodesmata in root cells, primarily through the induction of callose synthesis (Sivaguru *et al.*, 2000). This Al-induced callose production impedes molecular transport and communication via the plasmodesmata, a response also associated with physical stress, pathogen attack and wounding (Sivaguru *et al.*, 2000). The trafficking of important signalling molecules, such as hormones, are also effectively blocked, with pivotal implications for basipetal auxin transport, and thus root growth regulation (Bennet and Breen, 1991; Sivaguru *et al.*, 2000).

There has also been much support for the notion of the plasma membrane as the primary target in the Al phytotoxic response (Kochian, 1995; Sasaki *et al.*, 1995; Staβ and Horst, 1995; Wagatsuma *et al.*, 1995). Aluminium is known to negatively affect the influx of nutrients, such as P, K, Ca, Mg, Zn and Fe, into the root (Verkleij and Schat, 1990; Baligar *et al.*, 1993; Jones *et al.*, 1995; Calba and Jaillard, 1997) presumably as a result of the disruption of the plasma membrane and associated transport processes (Cumming and Taylor, 1990; Marschner, 1991; Crawford *et al.*, 1998). Combined with a poorly developed rooting system, this can have rather detrimental effects on a plant, through the manifestation of severe mineral nutrient deficiencies (Andersson, 1988; Bennet and Breen, 1991; Kidd and Proctor, 2000).

The maintenance of membrane integrity is vital to the functioning of the cell, with the regulation of cytoplasmic ion levels of essential importance for normal physiological function, repair and maintenance processes, and growth. Studies have shown that Al reduces membrane fluidity (Vierstra and Haug, 1978; Suhayda and Haug, 1986) and membrane permeability (Cumming and Taylor, 1990; Sasaki *et al.;* 1995; Staβ and Horst, 1995) possibly through binding of the metal to the hydrophilic regions of phospholipids, thus altering protein-lipid interactions (Cumming and Taylor, 1990; Delhaize and Ryan, 1995). Other suggested potential mechanisms include the binding of Al to cell wall proteins or pectic residues, or displacement of other ions from critical sites on the cell wall or membranes, as well as via direct interactions with membrane-bound proteins, such as ion channels, thus disrupting nutrient transport and possibly

disturbing the electrical potential of the plasma membrane (Sasaki *et al.*, 1994; Delhaize and Ryan, 1995; Sasaki *et al.*, 1995; Richards *et al.*, 1998). This may also have deleterious implications for intracellular communications via second messenger pathways (Delhaize and Ryan, 1995; Kochian, 1995).

Many research groups have reported a reduction in net K efflux from root tip cells of soybean and wheat in response to exposure to toxic levels of Al (Sasaki *et al.*, 1994; Staβ and Horst, 1995). This has been suggested to be linked to reduced H^+-ATPase activity, which would result in decreased fluxes of several ions, including H^+ influx/K^+efflux, thus upsetting H^+ homeostasis and the electrical membrane potential (Cumming and Taylor, 1990; Sasaki *et al.*, 1995). There are, however, other reports which claim Al had either no detectable effect on K transport across the plasma membrane (Calba and Jaillard, 1997) or even induced K leakage and reduced potassium concentrations in root cap and apical meristem cells (Kochian, 1995; Wagatsuma *et al.*, 1995). These discrepancies in the literature may have arisen from the choice of either cultured cells or intact roots in the respective studies, or possibly due to simple interspecies and intervarietal differences in response to Al.

Despite the apparent lack of characterisation and understanding of the exact mechanisms involved, there is, however, little dispute that Al does indeed disturb cellular metabolism (Aniol and Gustafson, 1990). This is perhaps most clearly demonstrated by the effects of Al on Ca homeostasis in the cell, processes which can be linked to nearly all vital aspects of normal cellular functioning and maintenance, from cell division, to cytoskeletal organisation and inter- and intracellular communication networks (Delhaize and Ryan, 1995; Rengel *et al.*, 1995).

Many polyvalent cations (e.g. La^{3+}; Ga^{3+} and Gd^{3+}) are known to inhibit the transport of Ca, and there is also extensive evidence demonstrating the reduced uptake and translocation of Ca by Al^{3+} (Verkleij and Schat, 1990; Delhaize and Ryan, 1995; Jones *et al.*, 1995; Sasaki *et al.*, 1995; Kidd and Proctor, 2000).

In view of the close correlation between the Al phytotoxic response and the inhibition of Ca uptake with regards to site (root apex) and time-scale (measurable within minutes) it was strongly suggested that Al toxicity was directly related to the disruption of Ca transport and homeostasis (Verkleij and Schat, 1990; Delhaize and Ryan, 1995; Rengel *et al.*, 1995). It has, however, since been shown that at low concentrations, Al remains capable of inhibiting root

growth without affecting Ca fluxes (Jones *et al.*, 1995), which suggests an alternative mechanism for the primary phytotoxic response.

It was first proposed by Siegel and Haug (1983) that one of the initial targets for Al might be the Ca-binding protein, calmodulin. Calmodulin is a key regulatory protein that undergoes a conformational change when bound to Ca, allowing it to form complexes with, and thus activate, certain enzymes, such as the kinases responsible for phosphorylation. Aluminium, though, has been shown to bind to the Ca-binding sites in calmodulin with an affinity ten times higher than Ca^{2+}, and elicits rather drastic conformational changes in this protein, thus affecting its ability to activate other enzymes (Suhayda and Haug, 1986; Kochian, 1995). However, subsequent research has found that Al had very little impact on calmodulin in the range of pH values found in the cell (pH 5.5 – 6.5) and suggested that Al may directly affect the enzymes, and not via any interactions with calmodulin (Kochian, 1995). Aluminium is known to affect phosphodiesterase, hexokinase and ATPase activity (Viola *et al.*, 1980; Siegel and Haug, 1983; Sasaki *et al.*, 1995), so it is quite possible that Al may have inhibitory affects on the vital functions of a number of physiologically important enzymes (Delhaize and Ryan, 1995).

Aluminium has also been shown to affect cellular respiration and electron transport pathways, possibly due to the high affinity of Al^{3+} for oxygen donor (electron rich) ligands (Kochian, 1995). Aluminium has been shown to reduce respiration in root cells, as well as affect the rate of protein synthesis (Cambraia *et al.*, 1983; Barnabas *et al.*, 2000). It has also been observed that Al has an adverse effect on photosynthesis as a result of it lowering the chlorophyll content, and thus reducing electron flow (Barnabas *et al.*, 2000). Loper and co-workers (1993) proposed that the interference of Al with reduction processes of the plasma membrane was probably attributable to a direct interaction of the ion with some component of the electron transport system. A reduction in the respiratory activity of the mitochondria, another membrane associated process, has been reported by de Lima and Copeland (1994). It was suggested that Al affects electron transport through cytochrome Complexes I and II, and may also interact with other mitochondrial sites following more prolonged exposure, with severe implications for cellular growth and maintenance.

While most of the research in the field of Al phytotoxicity has tended to focus on symplasmic targets, there remains support for the apoplasm as the primary site of toxicity. As mentioned previously, concentrations of Al are reduced to nanomolar values upon entering the cell, due to cytoplasmic pH and the abundance of potential ligands that bind Al (Delhaize and Ryan, 1995;

De la Fuente-Martínez and Herrera-Estrella, 1999). Aluminium has been known to interact with the cell wall (Crawford *et al.*, 1998) and access to the apoplasm is both easy and rapid (Kochian, 1995). A number of hypotheses suggest that Al-induced alterations in the cell wall may be responsible for the phytotoxic response, such as the displacement of Ca and Mg by Al from critical binding sites in the apoplasm (Delhaize and Ryan, 1995; Godbold and Jentschke, 1998).

Aluminium has also been thought to accumulate in the apoplast as Al phosphate precipitates, which may result in reduced PO_4^{3-} transport and thus account for the phosphate deficiencies often associated with Al stress (Foy, 1983; Godbold and Jentschke, 1998). The addition of phosphates in solution can help ameliorate toxicity in Al stressed roots (Hairiah *et al.*, 1993; Pellet *et al.*, 1997) but whether this occurs as a result of Al precipitation in the growth medium, thus reducing Al activity and minimising uptake by the roots, or by overcoming phosphate deficiencies, has not been resolved.

Another hypothesis suggests the binding of Al to negatively charged sites on cell wall pectins (Foy, 1983; Godbold and Jentschke, 1998; Watanabe *et al.*, 1998) with consequences for cell wall extensibility and conductivity. These pectins are also thought to play a major role in establishing the cation exchange capacity (CEC) of the cell wall. As roots mature, the cell walls undergo lignification and decrease the relative proportion of pectin, which may account for the reduced sensitivity of older roots to Al stress (Godbold and Jentschke, 1998). A disturbance of the cell wall CEC, however, could drastically affect the ability of the root to take up valuable nutrients.

While consensus on the cellular site of Al toxicity has yet to be reached, it is generally agreed that the effects of Al interactions with the apoplasm are much less significant than the effects of Al in the symplasm, particularly on the plasma membrane structure and function (Marschner, 1991). Despite intensive research, the biochemical and molecular basis for Al toxicity is not yet fully understood. It is, however, becoming increasingly accepted that numerous processes associated with both symplasmic and apoplasmic elements are likely to be targeted by Al, each forming an integral part of a multiple response system (Bennet and Granger, 2000).

2.2.3 The impact of aluminium on global food security

Among all the high profile pests and diseases that plague the world's agriculture, there is another devastating candidate that receives much less attention: Al. One of the major factors

limiting crop productivity, Al-induced inhibition of root growth results in the inability of crop roots to penetrate the subsoil (Sumner, 1970). The consequences of this are poor water and nutrient acquisition, resulting in nutrient deficiencies and possibly Al-induced drought, all combining to reduce crop yields (Kochian, 1995). Since most plant species are sensitive to extremely low concentrations of Al, the potential for this metal to be a problem for agriculture is enormous.

In many of the crop-producing areas in the world there have been reports of yield declines and losses in soil productivity (Van Antwerpen and Meyer, 1996). More than 70% of the world's arable lands are considered acidic, a condition which increases the availability of phytotoxic Al species (Hamel *et al.*, 1998). Indeed, for some economically important crops, such as maize, Al is second only to drought in its restriction of crop yields, in some cases reducing productivity by as much as 80% (Barinaga, 1997). Maximising yields from these areas is thus of prime importance if increasing global demands on food supplies are to be met (Sivaguru *et al.*, 2000).

Overturning the trends of yield declines, as well as expanding food production into those areas where soils are less favourable, are becoming the major challenges currently facing modern agriculture (Aniol and Gustafson, 1990). With the problem of escalating soil acidification, it is becoming increasingly urgent that the matter of Al phytotoxicity be addressed, not only through the amendment of soil management practices, but also through the breeding and selection of resistant genotypes.

2.3 PLANT TOLERANCE OF ALUMINIUM

2.3.1 Physiology of detoxification

Despite the presence of toxic levels of Al in many of the world's soils, numerous species still inhabit and even thrive in these environments. It therefore seems clear that there must exist some means by which these species can mitigate the damaging effects of Al. While the physiology and chemistry behind the phytotoxic effects of this metal have yet to be resolved, a number of mechanisms have been proposed to account for the apparent tolerance of these species.

Strategies for coping with phytotoxic concentrations of Al can be broadly classified into two categories, based primarily on the site at which detoxification is effected. Exclusion or

apoplastic mechanisms prevent Al from crossing the plasma membrane and entering the cell, thus prohibiting the metal from accessing sensitive intracellular sites. Conversely, internal or symplastic mechanisms do not prevent entry into the cell, but rather serve to immobilise or detoxify Al only once it has penetrated the symplasm.

a. Exclusion of aluminium

Plants have developed a number of Al tolerance features that can be termed exclusory mechanisms. Evidence seems to indicate that tolerant plants tend to restrict the transport of Al from the roots to other parts of the plant (Baker and Walker, 1990), preferably excluding the metal from the symplasm, and thus accumulating significantly lower levels of Al in the roots than more sensitive genotypes (Andersson, 1988; Verkleij and Schatt, 1990; Taylor, 1991; Lazof *et al.*, 1994; Sasaki *et al.*, 1994; Jorge and Arruda, 1997; Kochian, 1995; Pellet *et al.*, 1997). It has long been suggested that some of these plants are capable of metabolically excluding Al as was evidenced by the enhanced uptake of Al following the application of a metabolic inhibitor, 2,4-dinitrophenol (DNP) (Taylor, 1991).

Since the availability of Al in the soil solution is largely a function of the prevailing pH, it has been hypothesised that tolerant plants may be able to modify their rhizospheric pH to reduce Al solubility. In some instances, the pH of the rhizosphere has been shown to vary by as much as 2 units from that of the bulk soil (Marschner, 1991). This suggests that the plant may very well be capable of setting up a pH barrier within which the activity of Al is drastically reduced (Taylor, 1991; Kochian, 1995). An increase of only 0.1 pH unit from 4.5 to 4.6 has been shown to result in a 26% decrease in Al availability, which suggests that variations in rhizospheric pH must be of some biological significance (Blamey *et al.*, 1983). In rice (*Oryza sativa* L.) seedlings, it has been demonstrated that the degree of tolerance displayed by a cultivar was related to the ability of that cultivar to modify its rhizospheric pH from an acidic to a neutral range (Sivaguru and Paliwal, 1993). Similarly in wheat (*Triticum aestivum* L.), tolerant cultivars were shown to consistently maintain a higher solution pH than sensitive cultivars (Taylor, 1988a).

The nitrogen usage of a plant (cation vs. anion uptake) is considered to have a significant impact on the ability of the plant to resist acidification of the root medium (Taylor and Foy, 1985b; Taylor and Foy, 1985c; Taylor, 1988a). Species that favour the basic ammonium ions (NH_4^+) over nitrate ions (NO_3^-) tend to display lower rhizospheric pH values and thus greater Al sensitivity than species with preferential nitrate uptake (Foy, 1983; Taylor, 1988b). However,

the relative tolerance of some cultivars of wheat were largely unaffected by pH or NH_4^+/NO_3^- ratios in the supplied medium, which suggests that the superior Al tolerance of certain cultivars could not be exclusively attributed to the inherent ability to maintain a high solution pH (Taylor, 1988b; Lazof *et al.*, 1994). Aluminium has also been shown to inhibit the uptake of nitrates in both sensitive and tolerant plant species (Keltjens, 1988; Durieux *et al.*, 1993; Lazof *et al.*, 1994). It thus seems that while the pattern of nitrogen usage may affect rhizospheric pH, it is not likely to be solely responsible for Al tolerance, but may serve to augment other tolerance mechanisms.

An extremely large proportion of the Al bound to root cells is associated with the apoplast and is therefore probably unrelated to the observed symptoms of Al stress (Lazof *et al.*, 1994). However, differences in the permeability and chemistry of the cell wall have been suggested to account for enhanced tolerance in some species (Masion and Bertsch, 1997), possibly by means of increased binding of Al in the cell wall, thus preventing it from entering the symplasm (Taylor, 1991). A reduced cation exchange capacity (CEC) and metal-binding properties of the cell wall have been proposed to affect the relative tolerance of some plants by reducing the amount of Al in the cell wall (Verkleij and Schat, 1990; Kochian, 1995). This model assumes that binding to the cell wall is the initial step leading to Al uptake in the cell, and thus resulting in the phytotoxic response of the plant to the metal (Taylor, 1991; Kochian, 1995; Mugai *et al.*, 2000). However, there are many discrepancies associated with the evidence relating Al with cell wall chemistry and several tolerant genotypes seem to have a substantially higher CEC than many sensitive cultivars. While further research and evaluation of the techniques employed are necessary if conclusive results are to be achieved, current work seems to indicate a relatively minor role for the root CEC in any Al resistance mechanisms.

The high profile of the plasma membrane (PM) as a potential primary site for Al toxicity has resulted in much research being focused on its possible involvement in Al tolerance. It has been suggested that tolerance in some species is conferred via the alteration of the PM and permeability, thus reducing the uptake of Al into the cell (Verkleij and Schat, 1990). The ability to maintain PM function and integrity under conditions of Al stress has been widely accepted as having a role in a tolerance response system (Miyasaka *et al.*, 1989; Cumming and Taylor, 1990; Sasaki *et al.*, 1994; Sasaki *et al.*, 1995; Wagatsuma *et al.*, 1995). Aluminium is known to bind to the PM, thus affecting the structure and function thereof, including the electronegativity, which may account for the effect of Al on cation-uptake pathways in the roots (Taylor, 1991). Tolerant sorghum (*Sorghum bicolor* L. Moench.) plants have demonstrated superior influx of

nutrients into the roots, such as P, K, Ca, Mg, Zn and Fe, when compared with sensitive cultivars (Baligar *et al.*, 1993). Differential blockage of root plasma membrane Ca channels has also been suggested to account for observed differential tolerances to Al (Rengel *et al.*, 1995). Other research has shown that tolerant wheat cultivars were capable of maintaining normal ion fluxes and membrane potentials in the presence of Al, whereas sensitive cultivars could not (Miyasaka *et al.*, 1989). The extent to which K^+ and H^+ pumping activity was affected has also been related to the relative tolerance of the cultivar concerned (Sasaki *et al.*, 1994; Sasaki *et al.*, 1995; Wagatsuma *et al.*, 1995). Increased nutrient uptake and transport efficiency have also been proposed to account for an increased tolerance to Al in species such as rice (Sivaguru and Paliwal, 1993) and sorghum (Baligar *et al.*, 1993). It thus appears that the role of the plasma membrane in any tolerance systems is potentially great, as it is at this site that many of the toxicity effects are thought to be exerted.

It is widely believed that enhanced tolerance to Al is achieved via the secretion of substances from the root apex. It has been suggested that Al tolerance may be derived from factors relating to root cap mucilages, with elevated secretory activity of the cap resulting in a more tolerant phenotype (Blamey *et al.*, 1990; Bennet and Breen, 1991; Lazof *et al.*, 1994). Mucilage is continuously produced by growing roots and generally displays a high binding capacity for polyvalent cations, such as Al^{3+} (Marschner, 1991). While tolerance was associated with a higher mucilage production rate in soybean cultivars, the concentration of Al in the mucilage was lower than for sensitive genotypes (Lazof *et al.*, 1994). Root border cells, which form a sheath of detached somatic cells around the root tip, are known to influence the chemical and physical properties of the rhizosphere through the production of specific metabolites, including mucilage (Hawes *et al.*, 2000). Within 2 hours of exposure to Al, the layer of mucilage surrounding each border cell can increase in diameter from virtually undetectable to wider than the cell itself. After the establishment of this mucilaginous layer, the rate of border cell deaths returns rapidly to pre-exposure levels, suggesting that these cells possess the capacity to restrict the damaging effects of Al via the synthesis of an inducible extracellular layer (Hawes *et al.*, 2000). Although evidence suggests a possible connection between root cap mucilage activity, mucilage production and Al tolerance, nothing conclusive has as yet been determined.

The concept that exudations originating from the root apex may enhance tolerance to Al has received much support from the scientific community. Aluminium has been shown to induce or enhance exudation of certain polypeptides in roots of tolerant wheat cultivars exposed to Al (Basu *et al.*, 1994b). In contrast, no significant changes were observed in the polypeptide profile

derived from sensitive cultivars. High levels of Al were also found to be associated with these polypeptides, suggesting a preferential binding of Al and a possible chelatory role for the polypeptides.

Phosphates in the soil solution are known to not only form compounds with Al but also to bind to protons in the rhizosphere, thus increasing pH and effectively decreasing Al^{3+} activity (Pellet *et al.*, 1997). It has been proposed that Al-stimulated efflux of PO_4^{3-} may serve to immobilise Al in the rhizosphere, thus preventing its entrance into the root symplasm (Ownby, 1993). However, most acidic soils are considered phosphate deficient, and an Al tolerance mechanism based on the exudation of a limiting essential nutrient is thus questionable (Pellet *et al.*, 1997).

Organic acids, on the other hand, are well recognised for their role in the amelioration of Al toxicity and have long been the focus of much intensive research (Cambraia *et al.*, 1983; Hue *et al.,* 1986; Marschner, 1991; Harper *et al.*, 1995; Ostatek-Boczynski *et al.*, 1995; Jorge and Arruda, 1997; Zheng *et al.*, 1998; Koyama *et al.*, 1999). Several different organic acids are capable of binding Al, and have been classified as weak (e.g. succinic, formic, acetic and pthalic acids) intermediate (e.g. malic, malonic and salicylic acids) or strong (e.g. oxalic, tartaric and citric acids) complexers of Al (Hue *et al.*, 1986). The detoxification properties of these short-chain carboxylic acids have been correlated with the positions of the hydroxyl and carboxyl groups on the main carbon chain that favoured the formation of stable 5- or 6-bond ring structures with Al (Hue *et al.*, 1986; Pintro *et al.*, 1997).

Higher molecular weight organic acids, such as humic and fulvic acid, are known to form complexes with Al of even greater stability than the shorter chain molecules, such as citric and oxalic acids (Harper *et al.*, 1995). These longer chain molecules are also significantly less susceptible to microbial degradation than the shorter chain acids, providing a more permanent means for the amelioration of Al toxicity (Marschner, 1991; Harper *et al.*, 1995). Despite this, most species and cultivars that employ the organic acid strategy for overcoming Al stress appear to favour the lower molecular weight compounds, such as citrate and malate (Miyasaka *et al.,* 1991; Delhaize *et al.*, 1993b; Jorge and Arruda, 1997; Cocker *et al.*, 1998; Larsen *et al.*, 1998; Yang and Zhang, 1998; Mugai *et al.*, 2000; Yang *et al.*, 2000).

While the type and amount of organic acid released may differ from species to species (see Table 1) the increased exudation of these compounds has been clearly demonstrated by several research groups to confer Al tolerance (Cambraia *et al.*, 1983; Jorge and Arruda, 1997; Pellet *et*

al., 1997; Pintro *et al.*, 1997; Zheng *et al.*, 1998; Koyama *et al.*, 1999). Work conducted by Miyasaka *et al.* (1991) showed that the root system of an Al tolerant cultivar of snapbean (*Phaseolus vulgaris* L.) grown in Al-containing solutions released 70 times as much citrate as when grown in the absence of the ion, and 10 times more citrate than a sensitive cultivar grown under similar conditions. Furthermore, the insertion and over-expression of a bacterial citrate synthase gene in tobacco (*Nicotiana tabacum* L.) and papaya (*Carica papaya* L.) plants significantly improved the performance of these transgenics under conditions of Al stress (De la Fuente *et al.*, 1997).

There is the concern that the metabolic cost of continual production and release of these organic acids, effectively resulting in the loss of fixed carbon, could drastically impact on the productivity of the plant (Taylor, 1991; Barinaga, 1997). However, since only the root apex is vulnerable to Al, and therefore only this small region from which organic acids need be exuded, it becomes much less energetically demanding and a more worthwhile investment for the plant, especially when weighed up against the potentially damaging effects of Al (Delhaize *et al.*, 1993b). Several groups have shown that organic acid exudations are restricted to the root tip, with undetectable or negligible releases from other portions of the root (Delhaize *et al.*, 1993b; Pellet *et al.*, 1997). It has also been suggested that the mucilaginous coatings of the root tip region may help prevent dilution of these exudates, increasing their effectiveness by concentrating them around the sensitive portions of the roots and thus decreasing the metabolic demand on the plant (Jorge and Arruda, 1997).

Table 1: Selected studies reporting secretion of organic acids as Al^{3+} chelating agents.

Organic acid	Species	Reference
Citrate	*Phaseolus vulgaris* (snapbean)	Miyasaka *et al.*, 1991
	Zea mays (maize)	Jorge and Arruda, 1997
Succinate	*Triticum aestivum* (wheat)	Christiansen-Weniger *et al.*, 1992
Malate	*Triticum aestivum* (wheat)	Delhaize *et al.*, 1993b
		Cocker *et al.*, 1998
	Zea mays (maize)	Jorge and Arruda, 1997
	Sorghum bicolor (sorghum)	Cambraia *et al.*, 1983

What is interesting to note is that nitrogen usage has been shown to affect the nature and composition of root exudations in a plant (Taylor, 1988b). Individuals that primarily received nitrates tended to increase the organic acid content, while polyamines seemed to predominate in plants fed ammonium ions. This suggests a link between NO_3^-/NH_4^+ assimilation, plant-induced pH changes, and cultivar tolerance due to exudations of Al-chelating compounds. While current evidence most strongly supports the organic acid strategy, one cannot dismiss the involvement of other mechanisms, both exclusory and internal, that combine to produce an Al tolerant phenotype.

b. Internal mechanisms of aluminium detoxification

If the mechanisms for excluding Al are ineffective or incomplete, the potential for cytoplasmic lesions remains a risk for the plant, despite the extremely low solubility of the ion at intracellular pH levels (Taylor, 1991). Although a large proportion of the Al associated with a plant root cell is apoplastic, the extreme sensitivity of symplastic sites warrants the consideration of internal detoxification systems. Some species are also known to accumulate high concentrations of Al and must surely possess some effective mechanisms for its amelioration within the cell symplasm (Verkleij and Schat, 1990; Bennet and Breen, 1991; Delhaize and Ryan, 1995). Several internal mechanisms have thus been proposed, although most are speculative and conclusive substantiating evidence has yet to be published.

Aluminium is known to adversely affect photosynthetic and respiratory rates, as well as protein synthesis (Cambraia *et al.*, 1983; Aniol and Gustafson, 1990; Barnabas *et al.*, 2000). It has therefore been proposed that tolerant species and cultivars have increased levels of sensitive enzymes to overcome Al-induced inhibition, or developed Al tolerant enzymes, or even evolved alternate metabolic pathways to overcome the effects of Al (Taylor and Foy, 1985b; Taylor, 1991; Pintro *et al.*, 1997). There is, however, very little evidence to support such hypotheses.

Another mechanism, by which some plants have been proposed to deal with Al internally, is vacuolar sequestration or compartmentation, restricting toxic Al^{3+} ions from sensitive cytoplasmic sites (Verkleij and Schat, 1990; Marschner, 1991; Taylor, 1991). Species that accumulate large amounts of Al in the aerial parts of the plant are known to apportion most of this into the apoplast or vacuoles of the leaf cells, thus restricting the toxicity of the metal to the plant (De la Fuente-Martínez and Herrera-Estrella, 1999).

Perhaps the most promising hypothesis for an internal tolerance mechanism remains the production of Al-induced proteins. It has been suggested that tolerant plants may be capable of synthesising metal-binding proteins in response to Al stress, similar to metallothionein-like proteins, which have the ability to bind a number of metals and may be a common factor in metal tolerance (Aniol and Gustafson, 1990; Bennet and Breen, 1991; Delhaize and Ryan, 1995). Protein bands enhanced under conditions of Al stress have been isolated from the roots of a tolerant wheat cultivar, which may be involved in the mediation of some resistance mechanism (Basu *et al.*, 1994a). These proteins were found to be concentrated in the root apical region, and their expression was not inducible by temperature stress (heating and freezing) or exposure to other metals, such as Cu, Mn and Zn. Aluminium stress has also been shown to induce the synthesis of certain proteins in mungbean (*Phaseolus aureus* Roxb.) seedlings, and these are believed to be linked to Al tolerance in these cultivars (Yang and Zhang, 1998). Although Al has been clearly demonstrated to induce the synthesis of a range of proteins in both tolerant and sensitive cultivars in a number of species, conclusive evidence linking these to a tolerance mechanism is still lacking.

Each of these proposed strategies have strengths and weaknesses, with evidence both consistent and inconsistent with each hypotheses. As a result, no single hypothesis has been accepted by the scientific community as conferring Al tolerance. However, the probability remains open that they may form part of a complex multigenic system, involving both apoplastic and symplastic components.

2.3.2 Genetics of aluminium tolerance

Considerable progress has been achieved in the breeding of cultivars that display superior tolerance to several soil mineral stresses, suggesting the existence of some genetic basis for these traits (Foy, 1983). There is an abundance of information on the genetic variability associated with plant responses to Al, yet the understanding of the physiology behind the mechanisms and systems involved remains limited and fragmentary.

It is strongly believed that Al tolerance must be a complex dominant trait under the control of a few major genes and several minor genes (Foy, 1983; Kochian, 1995; Aniol, 1996; Pellet *et al.*, 1997). There have, however, been some reports where Al tolerance has been correlated with single genes. For example, a phosphatidylserine synthase gene isolated from an Al tolerant wheat cultivar was shown to confer increased resistance to Al toxicity when expressed in yeast

(*Saccharomyces cerevisiae*) cells (Delhaize *et al.*, 1999). Similarly, a DNA fragment isolated from an Al tolerant strain of the soil bacterium *Arthrobacter viscosus*, was shown to significantly enhance the tolerance of transformed *Escherichia coli* cells to Al (Jo *et al.*, 1997).

Moon and co-workers (1997) demonstrated that tolerance in an inbred maize line was also under the control of a single nuclear, semi-dominant gene, which was designated *Alm 1* (aluminium maize tolerance). More recently, it has been shown that there are in fact two distinct loci responsible for Al tolerance in this particular line: the *Alm 1* locus, and a second locus *Alm 2*, although the phenotypic contribution of this second gene was considerably less than for *Alm 1* (Sibov *et al.*, 1999).

From Al-treated wheat, Snowden and Gardner (1993) isolated five different cDNA fragments (termed *wali 1* to *wali 5*, wheat aluminium induced) the expression of which was induced by Al stress in the root tips of the plants. Although the level of expression of these transcripts was dose-dependent, the genes were expressed in both tolerant and sensitive cultivars. This suggested that these genes were involved in more general stress responses to Al, and probably did not confer any particular advantages for overcoming Al stress (Hamel *et al.*, 1998). However, the exclusion of Al from wheat root apices has been consistently linked with another locus, termed *Alt 1* (aluminium tolerance). Isolated from a near-isogenic wheat line, differing in tolerance to Al, this locus has been proposed to encode an Al tolerance mechanism based on Al-stimulated exudation of malic acid into the rhizosphere (Delhaize *et al.,* 1993a; Delhaize *et al.,* 1993b).

Much work has been focused on attempts to elucidate the genetic systems responsible for Al tolerance in a number of agronomically important crop species, including wheat (Hamel *et al.*, 1998; Delhaize *et al.*, 1993a), maize (Sibov *et al.*, 1999), tobacco (Ezaki *et al.*, 1995), triticale (*Triticum* spp. x *Secale cereale* L. hybrid) (Zhang *et al.*, 1999) and rye (*Secale cereale* L.) (Gallego and Benito, 1997). Currently over 20 genes induced by Al stress have been isolated from a range of plant species (see Table 2). The assignment of putative identities has indicated that these are mostly general stress genes, inducible by a wide range of stress conditions, such as oxidative stress (Richards *et al.*, 1998), pathogen attack (Cruz-Ortega *et al.*, 1997) and phosphate starvation (Ezaki *et al.*, 1995). A selection of these Al-induced genes have been expressed in transgenic *Arabidopsis thaliana* L. plants and have demonstrated their ability to alleviate Al stress, regardless of the species of origin (Ezaki *et al.*, 2000).

Although the biological role of Al-induced genes in plants has not yet been fully resolved, and the activation of these genes has not been proven to be a prerequisite for tolerance, positive links between these genetic systems and some degree of tolerance to Al stress have been established in several species. However, further research is still needed. Once obtained, information regarding more precise patterns and sites of expression may contribute substantially to the understanding of the physiology behind Al toxicity mechanisms, as well as to the development of genetic markers and increased success in the breeding of Al tolerant phenotypes.

2.4 APPROACHES TO MINIMISE ALUMINIUM TOXICITY

2.4.1 Agronomic and plant breeding strategies

The occurrence of Al toxicity is largely unpredictable, with the nature of the soil concerned inherited to some extent from the parent rock (Sumner and Meyer, 1971). The distribution of soluble Al is also affected by factors such as the internal drainage of a soil, its position in the landscape, the effective rainfall, and the movement of base-rich waters through the soil (Sumner and Meyer, 1971).

Low soil pH (below 5) is frequently associated with sandy soils, as well as many volcanic and tropical soils (Snowden and Gardner, 1993). However, naturally acidic soils can be further acidified through the oxidation of applied ammoniacal fertilisers to nitric acid (Schroeder *et al.*, 1994). Poor farming practices, such as the removal of basic cations from the soil during harvesting or as a result of acid rain, further exacerbate the problem of soil acidification and thus Al toxicity (Meyer *et al.*, 1996).

Considered the most important phytotoxic metal in agriculture, Al is most commonly ameliorated by soil management strategies, with remedial measures primarily involving the direct modification of the soil. The practice of liming, whereby lime (calcium carbonate) or less frequently gypsum (calcium sulphate) are ploughed into the soil, serves to elevate the pH of the soil to levels where Al solubility and activity are significantly reduced (Bennet, 1995). Although successes in the alleviation of Al toxicity have been reported, regular application is required at approximately 1.5 tonnes per hectare at least once every five years (Schumann, 1999). Careful monitoring of the soil is also required, as the application of lime where it is not needed is not only an unnecessary expense, but may also result in deficiencies of certain trace elements, such

as potassium and zinc (Schumann, 1998). Liming has also been shown to not address the problem of subsoil acidity, only effecting a favourable pH change in the upper soil horizons (Zhang and Jessop, 1998). Another drawback associated with the application of lime or gypsum is the potential for the polluting of run-off waters (De la Fuente *et al.*, 1997) as well as the leaching of already scarce nutrients from sandy soils (Aniol and Gustafson, 1990). Furthermore, liming materials are not readily available in many areas, and costs can often be prohibitive.

Tolerance to Al may thus be economically justified as a major breeding objective for plant breeding programmes. It has been well documented that many plant species exhibit a remarkable degree of genetic variability in their responses to Al stress, suggesting that one may specifically select for the dominant trait of Al tolerance. Using traditional breeding methods, crop researchers have had much success in boosting the tolerance of some food crops, most notably wheat (Carver *et al.*, 1993; Barinaga, 1997; Scott *et al.*, 2001).

Table 2: Some selected examples of aluminium-induced genes, isolated from a variety of plant species.

Gene	Identity	Species of origin	Reference
wali 5	Bowman-Birk protease inhibitor	*Triticum aestivum* (wheat)	Snowden and Gardner, 1993
war 4.2	Peroxidase	*Triticum aestivum* (wheat)	Hamel *et al.*, 1998
war 5.2	Cysteine proteinase		
war 7.2	Phenylalanine-ammonia lyase		
war 13.2	Oxalate oxidase		
Glc 1	B-1,3-glucanase	*Triticum aestivum* (wheat)	Cruz-Ortega *et al.*, 1997
AtBPI	Bowman-Birk protease inhibitor	*Arabidopsis thaliana*	Richards *et al.*, 1998
AtBCB	Blue copper binding protein		
AtPOX	Peroxidase		
parA	No homologue	*Nicotiana tabacum* (tobacco)	Ezaki *et al.*, 1995
parB	Glutathione-s-transferase		

The success of any breeding programme hinges on the availability of appropriate genetic variation and tolerance to Al in a particular crop, with a well-established mode of inheritance in the species. An inexpensive and rapid screening procedure for the identification of tolerant genotypes from a large pool of potential candidates is also essential. However, tolerant crops with which to perform crosses are few and traditional plant breeding is a very slow process due to the size and complexity of most crop genomes (Moffat, 1999). Aluminium tolerance is also hard to quantify and screening for this trait is generally very imprecise (Taylor and Foy, 1985a; Bennet, 1995).

2.4.2 Molecular strategies

In the past, very few breeding programmes have specifically selected for resistance to Al, and existing tolerant cultivars may have arisen indirectly as a result of selection for other agronomic characteristics, but performed on acidic soils (Wenzl *et al.*, 2001). Furthermore, a definitive understanding of the mechanism and genetics behind a tolerance response are still lacking, yet could significantly improve the success of plant breeding and allow for substantially more accurate and efficient screening of putatively tolerant lines. There are also many instances where plants have already undergone intensive selection for high yield and pest resistance but are Al sensitive, and in such cases it would be extremely useful to insert a tolerance gene into these otherwise superior genotypes using biotechnological techniques.

As a result, much research effort has been focused on the elucidation and isolation of a specific gene or set of genes with the ability to confer Al tolerance. There are a number of molecular strategies available for the isolation of genes associated with agronomically relevant characteristics (see Table 3), each with varying success rates.

The isolation of tolerance-related genes is significantly easier when encoded by a single major gene, or a small number of genes, than when the trait is polygenic. This, however, is a problem with many crops, as it is widely believed that Al tolerance is under the intricate control of a whole suite of genes (Taylor, 1995; Aniol, 1996; Pellet *et al.*, 1997).

The availability of closely related, or near-isogenic lines (NILs) differing only in tolerance to a specific metal, can also significantly improve the success rate of any gene search programme (Aniol, 1996). However, most crop species, including sugarcane, are genetically complex with

NILs frequently unavailable, necessitating the development of alternative strategies (Taylor, 1991).

The approaches taken for the isolation of many metal tolerance genes are generally divided into those dealing with the screening of cDNA libraries, and those with the analysis of differentially expressed proteins (Robinson, 1990). The libraries are prepared from messenger RNA isolated from metal-exposed tolerant cells, thus representing the genes expressed under such stress conditions, and screened for clones containing sequences preferentially expressed in exposed tolerant cells as opposed to unexposed sensitive cells. The advantage of this approach is that no prior information on specific sequences is required to isolate target fragments. However, the success of this approach is limited when the sequence conferring tolerance is expressed at very low levels, or the difference in expression between tolerant and sensitive plants is very small and thus difficult to detect. With approximately 99% of the different 20 000 to 30 000 mRNA transcripts present in a cell classified as rare, many are present at levels as low as only one to two copies per cell (Sargent, 1987; Sabelli, 1996; Appel *et al.*, 1999). The implementation of subtracted cDNA libraries, whereby common sequences are removed and rarer stress-specific sequences enriched for, has improved the limits of detection of less common gene fragments (Sargent, 1987; Wilson *et al.*, 1994).

Table 3: Molecular techniques that have been employed in various gene search programmes.

Technique	Aim	Reference
Chromosomal DNA library expressed in Al sensitive *E.coli* cultured on Al-containing medium	Isolation of a gene from soil bacterium *Arthrobacter viscosus* encoding a protein conferring Al tolerance	Jo *et al.*, 1997
Molecular mapping (RFLP) and bulked segregant analysis for Al tolerance	Isolation of genes linked to Al tolerance in *Zea mays* (maize)	Sibov *et al.*, 1999
Expression of cDNA library in yeast cultured on Al-containing medium	Cloning of *Triticum aestivum* (wheat) cDNAs conferring resistance to Al	Delhaize *et al.*, 1999
PCR amplification of homologous sequences using primers designed from known genes	Isolation of cDNA clones from *Ricinus communis* with close homology to hexose carriers isolated from *Arabidopsis thaliana*	Weig *et al.*, 1994

Differential display in cDNA libraries	Isolation of heavy metal responsive genes in *Zea mays* (maize)	Didierjean *et al.*, 1996
AFLP mRNA fingerprinting	Isolation of genes differentially expressed in white and red *Ipomoea purpurea* (morning glory) flowers	Habu *et al.*, 1997
cDNA-AFLP display	Isolation of genes expressed during oomycete infection of *Arabidopsis thaliana*	Van der Biezen *et al.*, 2000
Suppression subtractive hybridisation (SSH)	Isolation of molecular markers for ozone stress in *Pisum sativum* (pea)	Sävenstrad *et al.*, 2000
Subtractive cDNA libraries, bacterial arrays	Isolation of phase-specific cDNAs from sporophytic and gametophytic generations of *Porphyra purpurea*.	Liu *et al*, 1994
	Isolation of cDNAs preferentially expressed in sugarcane leaf roll (meristematic region) tissue	Carson and Botha, 2000
Differential screening of cDNA library	Cloning cDNAs induced by Al treatment and P_i starvation in cultured *Nicotiana tabacum* cells	Ezaki *et al.*, 1995
	Isolation of genes expressed in response to osmotic, salt and heavy metal stresses in *Cicer arietinum*	Muñoz *et al.*, 1998
	Identification of transcripts expressed in *Lycopersicon esculentum* (tomato) roots after nematode infection	Lambert *et al.*, 1999
	Isolation of Al-induced cDNA clones in *Triticum aestivum* (wheat)	Cruz-Ortega *et al.*, 1997; Snowden and Gardner, 1993
	Isolation of Al-regulated genes in *Triticum aestivum* (wheat)	Hamel *et al.*, 1998
	Isolation of Al-induced genes in *Arabidopsis thaliana* linked to oxidative stress responses	Richards *et al.*, 1998

The direct approach to isolating metal tolerance genes is based more on biochemical evidence, whereby differentially expressed proteins are isolated as potential candidates for conferring tolerance (Robinson, 1990; Basu *et al.*, 1999). These proteins are subsequently sequenced and appropriate probes designed, or antibodies raised, and used for the screening of expression libraries. Positive results are confirmed using the corresponding cDNA sequences as probe material in Northern hybridisation analyses. This approach was successfully employed by Cruz-Ortega and co-workers (1997), whereby an Al-induced protein Tal-18 was partially sequenced and a degenerate oligonucleotide probe designed. This probe was then used to probe a cDNA library constructed from mRNA isolated from the Al-exposed roots of an Al sensitive wheat cultivar. The probe hybridised to several clones, one of which was sequenced and identified as a novel 1,3-β-glucanase. While protein expression data is generally more informative, it is often technically more difficult to obtain and applications of this technique thus remain limited (Bouchez and Höfte, 1998).

In the event that an Al tolerance related gene or molecular marker should be isolated and shown to consistently map with tolerant populations, such sequences could be used as candidate genes for genetic engineering, or have applications in marker-assisted selection (MAS) in crop improvement (Young, 1999). Sequences tightly linked to resistance to soybean cyst nematode have been isolated (Mudge *et al.*, 1997), and have subsequently formed the basis of several commercial breeding efforts in this crop.

Although the field of MAS is still relatively young, the advantages of this technology are multiple. Not only does it provide the opportunity to select desirable lines at the seedling stage, but it also offers the potential to screen for multiple characteristics that would normally be difficult to analyse separately. This would enable the streamlining of breeding programmes to a substantial degree, especially with regards to traits that are traditionally phenotypically difficult to score, such as Al tolerance (Butterfield, 1995; Young, 1999).

2.5 ALUMINIUM PHYTOTOXICITY IN THE SOUTH AFRICAN SUGAR INDUSTRY

Over 425 000 hectares of South African agricultural lands are under sugarcane cultivation, producing an average of 2.5 million tonnes of sugar per season (Anon., 2001a; Anon., 2001b). According to figures for 2000/2001, the sugar industry generated approximately R5 billion last season, contributing R1.9 billion to the country's foreign exchange earnings through exports to

27 destinations around the world (Anon., 1999). In South Africa, approximately one million people are dependent on the sugar industry, through both the direct and indirect employment of an estimated 550 000 people (Anon., 2001b). The sugar industry is thus a very important part of the South African economy and, as such, needs to remain internationally competitive to retain its reputation as one of the leading cost-competitive producers of high quality sugar in the world (Anon., 2001b. Of the economic concerns facing the industry, many can be linked to changes in the structure and chemical properties of the soil, including acidification, as sugarcane production is widely believed to have a deleterious effect on soil quality. The South African sugar industry appears to have reached a yield plateau in the last three decades, despite the introduction of higher yielding cultivars (Meyer, 1996; Van Antwerpen and Meyer, 2001), and is most likely due to soil acidification and increased availability of Al (Schroeder *et al.*, 1994). Fallowing is not a common practice in sugar farming, and continuous monocropping has aggravated the problem of soil degradation. However, with an average of eight ratoon crops before field replanting is required, it is not usually feasible to break the cycle of sugarcane production (Van Antwerpen and Meyer, 1996).

Results from a ten-year trial, monitoring pH and available Al in the soil of a sugarcane field on the northern KwaZulu Natal coast, have indicated an average acidification rate of –0.2 units per annum (Schumann, 1999). The rate of acidification has been accelerating, with the percentage of sugarcane fields considered strongly acidic (below pH 5.0) increasing from 18% to 43% in the last twenty years (Schumann, 1998). Acidification is most prevalent in the KwaZulu Natal Midlands and South Coast regions, where an estimated 50% of the soil samples taken from sugarcane plantations have a pH below 5.0 (Schumann, 1998).

The concern over soil acidification and associated Al toxicity in the South African sugar industry is thus warranted. Current short-term approaches to alleviate the problem focus largely on the application of lime (Turner *et al.*, 1992; Schroeder *et al.*, 1994). Long-term measures include the careful monitoring of nitrogenous fertiliser usage, as nitrification of ammoniacal fertilisers generates nitric acid and thus strong acidity (Meyer *et al.*, 1996). It is also recommended practice to actively increase the nutrient and organic matter content of the soil via the recycling of mill residues into the soil, such as filtercakes and molasses, as well as other agricultural by-products, such as poultry litter (Schumann, 1998; Meyer, 1999). However, success based solely on amelioration of problematic soils is limited and is in many cases uneconomical. It thus becomes necessary to incorporate crop breeding techniques into solving the problem of Al phytotoxicity, as a more long term and cost-effective approach.

Sugarcane is one of the oldest cultivated plants in the world, and breeding programmes have been producing new sugarcane varieties for over 100 years, with some remarkable achievements along the way. For example, a 300% increase in sugar produced per hectare of cane was achieved in Java over the period 1885 to 1925, and this was largely attributable to breeding (Stevenson, 1965). However, it is doubtful that such dramatic results will be achieved as easily as in the past, and long term improvements in cane quality and yield will need to be achieved through the incorporation of modern technology, especially biotechnology, to supplement and facilitate conventional breeding (Heinz, 1987).

Most of the economically important traits in sugarcane are controlled by multiple genes, such as yield, juice quality and drought resistance, and the molecular and genetic basis for most of these, including Al tolerance, are not well understood (Berding and Skinner, 1987; Legendre and Burner, 1997). It thus becomes exceedingly difficult to obtain a suitable combination of all the favourable genes in one cultivar, with the simultaneous elimination of all unfavourable ones, especially when one considers the size and complexity of the sugarcane genome. Sugarcane is a very difficult crop to breed, with its low fertility and unpredictable inheritance of certain traits (Gallo-Meagher and Irvine, 1996). Until recently, relatively little was known about the sugarcane genome, due to its high ploidy and frequent aneuploidy and the substantial complexity of its chromosomal composition (Roach and Daniels, 1987; Legendre and Burner, 1997). This resulted in sugarcane genomic research being both slow and costly, and thus lagging behind other genetically well-characterised crops such as maize, wheat and tobacco (Märländer, 2000). Recent work has, however, shown considerable improvements in the understanding of sugarcane at the molecular level. For example, chromosomal walking within the sugarcane genome has now been successfully undertaken by D'Hont and co-workers (2001), with the view towards isolating a gene encoding resistance to a major sugarcane pathogen.

Standard selection programmes for sugarcane generally run for 12 to 14 years, from the production of seed to the commercial release of a new variety (Barnes *et al.*, 1997; Legendre and Burner, 1997). This prolonged selection period is due to the difficulty in accurately evaluating varieties without extensive field trials, as a result of the strong influence of the environmental conditions under which sugarcane is grown on the phenotypic characteristics of the plants (Barnes *et al.*, 1997; Gallo-Meagher and Irvine, 1997). Marker-assisted selection would, however, allow for the selection for traits at a genetic level, independent of environmental interactions, and for the early elimination of seedlings not possessing the trait of interest (Huckett, 1995; Barnes *et al.*, 1997). Markers linked to an undesirable phenotype are

also valuable, in that seedlings containing such sequences in their genomes could also be identified and removed from breeding programmes at an early stage. This would result in a substantial reduction in the number of undesirable varieties that progressed to field trials, and enhance the precision of selection in the remaining plants (Butterfield, 1995; Moore, 1999).

An additional application for genetic information regarding agronomically important features would be in the area of genetic engineering. Should a gene for a particular attribute be isolated, it can be engineered into an existing variety that lacks that attribute, with the result that breeding for such a trait becomes unnecessary (Butterfield, 1995). This would then further increase the efficiency of selection programmes as the number of characteristics being selected for at any one time would be reduced. This also eliminates the need to perform numerous crossings to separate desirable genes from undesirable genes, which can be very costly and time-consuming, and without the guarantee of success (Mirkov, 2001).

Molecular markers that appear to be linked to fibre traits, an important characteristic in sugarcane, have been identified, although linkage has not been confirmed (Msomi and Botha, 1994). Several putative markers for the prediction of disease and pest resistance have been identified in sugarcane, with the potential for incorporation into breeding programmes (Barnes *et al.*, 1997; Barnes and Botha, 1998; D'Hont *et al.*, 2001). Molecular maps are also currently under construction with the view to identifying markers linked to priority traits in sugarcane, including sugar-related characteristics, fibre, suckering and disease resistance (McIntyre *et al.*, 2001). Markers putatively associated with fibre and sucrose content, among others, are also under analysis for linkage with these important traits (Da Silva *et al.*, 2001).

Protocols for genetically engineering sugarcane have been developed, with transgenic plants resistant to various pests and pathogens successfully produced through genetic modification (Braga *et al.*, 2001; McQualter *et al.*, 2001). Genetically modified sugarcane plants resistant to a number of different herbicides have also been reported (Gallo-Meagher and Irvine, 1997; Snyman *et al.*, 1998; Mirkov, 2001). Although the field of genetics and transgenics in sugarcane has advanced rapidly in recent years, a genetic basis for Al tolerance in sugarcane has not yet been determined, and a gene or marker sequence for utilisation in MAS or for genetic engineering purposes is thus not available. The employment of genetic engineering and MAS with the view to improving Al tolerance in sugarcane therefore remain long-term goals.

2.6 CURRENT STUDY

The problem of Al phytotoxicity has until recently not received a significant amount of attention in the South African sugar industry. However, with notable yield increases observed in other crops specifically bred or engineered for tolerance (Miller *et al.,* 1997) and the increasing extent and severity of the problem in the industry, further research into the phenomenon of Al toxicity and tolerance in sugarcane is warranted. Although sugarcane (*Saccharum* spp. hybrid) is generally considered one of the more Al tolerant crops (Sumner and Meyer, 1971; Hetherington *et al.*, 1986; Nuss, 1987), some cultivars that possess several other desirable traits still display considerable sensitivity to the metal (Turner *et al.*, 1992).

The isolation of a gene fragment linked to the tolerant phenotype could have potential applications in the genetic engineering of such Al-sensitive germplasm, as well as in the design of molecular markers for use in MAS-assisted breeding programmes. However, it should be noted that these are long-term objectives, both requiring an extensive repertoire of resources and further research prior to the successful implementation of such strategies. A basic requirement for the development of MAS in any crop species is a population segregating for the trait of interest, whereas Al tolerance in sugarcane has neither been well characterized nor has a population segregating for the trait been identified for the purposes of establishing linkage. Should a putative Al-tolerance transgene be inserted into sugarcane cultivar, a suitable method for analysing the resultant genotypes after transgene insertion needs to be in place. The difficulty associated with the rating and thus quantifying of tolerance thus poses an obstacle, in that both techniques require a reliable and efficient rating system to confirm that Al tolerance is indeed linked with a candidate gene or marker. Due to the complex interactions of Al in the soil, the effect of the metal on sugarcane performance is currently measured indirectly, usually by means of general growth characteristics that can easily be influenced by other factors. Most studies on other crops utilise direct measurements, usually in the form of root elongation assays (De la Fuente *et al.*, 1997; Hamel *et al.*, 1998), however, no practical system presently exists for the measurement of root growth inhibition by Al in sugarcane.

It has become increasingly popular to use genic fragments, or expressed sequence tags (ESTs), as probes over anonymous DNA sequences when searching for markers linked to specific traits. Thokoane and Rutherford (2001) showed that genetic markers could successfully be obtained via the use of differentially expressed ESTs as probes in sugarcane. There is the advantage that the EST may directly affect the trait of interest, if shown to be genetically associated with the

trait (Cato *et al.*, 2000). Furthermore, because ESTs are derived from the coding regions of the genome, a higher degree of sequence conservation is expected, with potential applications across several sugarcane cultivars and possibly even other closely related species. These markers are thus more valuable than those derived from potentially non-coding regions, such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNA (RAPDs) and simple sequence repeats (SSRs), which frequently display a greater degree of polymorphisms across cultivars and species (Cato *et al.*, 2000). The use of EST sequences associated with known traits can also be used as probes in the construction of genome maps (Carson and Botha, 2000), which is of great importance for crops such as sugarcane in which mapping programmes are frequently hindered by the genetic complexity of the species.

This study therefore aimed to investigate the genetic response of sugarcane to Al, with the view to isolating a genic fragment putatively associated with tolerance to the metal. The first step towards the isolation of these EST markers was the development of a suitable method for the exposure of sugarcane roots to Al under controlled conditions, as well as a means for the quantification of the effect of the metal on the plants. Thereafter, a genotype, identified as tolerant based on available field data, was used for the isolation of genic fragments potentially associated with the trait of Al tolerance in this genotype. This involved the construction of subtractive cDNA libraries, which were then analysed via membrane-based arrays, Northern hybridisation analyses, and the sequencing and identification of selected clones.

CHAPTER 3
MATERIALS AND METHODS

3.1 PLANT MATERIALS

3.1.1 Cultivar

Single-budded setts (short segments of the stem with a bud at the node) of *Saccharum* spp. hybrid *cv.* N12 (N12) were germinated in washed graded silica medium (B&E Silica, [PTY] Ltd, Delmas) in polymer-coated (Styro-Seal; Hygrotech Seeds [PTY] Ltd, Silverton) 72-place seedling trays under glasshouse conditions. During this time, the germinating setts were watered twice daily and supplemented weekly with a nutrient medium (Hydroponic Nutrient Mix, Hygrotech Seeds [PTY] Ltd, Silverton). After the first flush of shoot roots were established, at approximately 5 weeks after bud-break, the plantlets were transferred to a hydroponics system. Prior to introduction into the hydroponics vessels, the remaining portions of the original setts were removed.

3.1.2 Propagation and growth

Commercially available 10 litre plastic buckets served as vessels for the hydroponics system, with aeration and agitation of the nutrient medium provided by a Hailipai Aquarium air-pump ACO-9630 (approximately 0.5 litres air per vessel per minute). To accommodate the plants, four holes (2.5cm radius) were cut in the lid of each vessel, through each of which a single 5 week old plant was inserted and supported by a Neoprene® collar. A 32cm length of polycarbonate tubing (internal diameter 1.1cm, external diameter 1.2cm) was inserted through the centre of each lid, which served to introduce air from the diaphragm pump to the bottom of each vessel (Fig. 1).

The four plants within each vessel were supplied with ten litres of half strength Long Ashton solution (Hewitt, 1966), modified to contain 2mM NH_4Cl, 0.09mM Fe EDTA and 0.0033mM $CuSO_4$ (Table 4). The pH of the nutrient medium was adjusted to a value of 5.5 with concentrated H_3PO_4. Fresh nutrient solutions were supplied on a weekly basis.

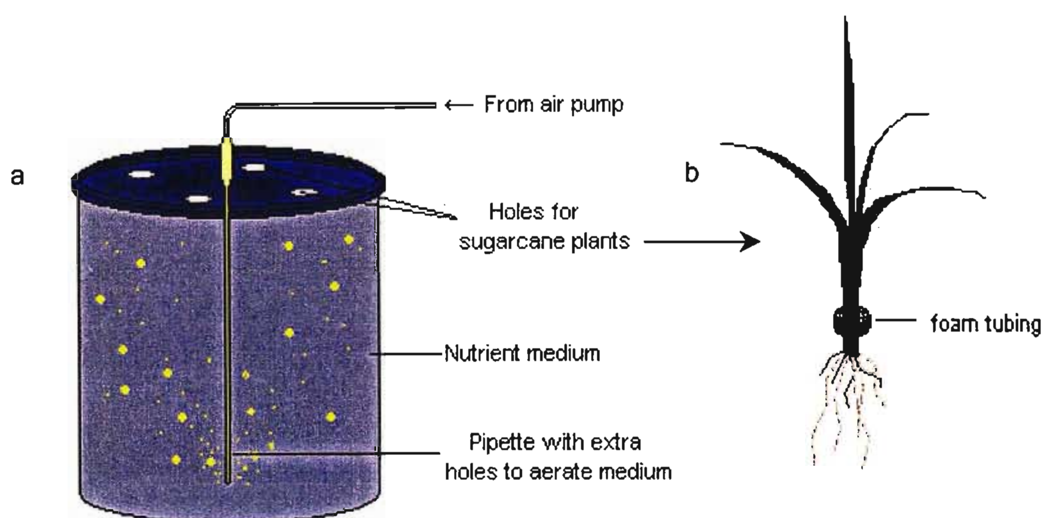

Fig. 1: Schematic representation of a single hydroponic vessel (a), and position of Neoprene®collar (foam tubing) on plantlets (b).

Table 4: Hydroponic nutrient medium composition (after Hewitt, 1966).

Compound	Final concentration (mM)
Macro-nutrients	
$MgSO_4.7H_2O$	1.5
K_2SO_4	2.0
$CaCl_2.2H_2O$	4.0
Phosphate source	
$NaH_2PO_4.2\ H_2O$	1.87
Na_2HPO_4	0.13
Micro-nutrients	
H_3BO_3	0.1388
$MnSO_4.4H_2O$	0.0208
$ZnSO_4.7H_2O$	0.0023
$CuSO_4$ (anhydrous)	0.0033
$Na_2MoO_4.2H_2O$	0.0002
Iron source	
Fe EDTA	0.09
Nitrogen source	
NH_4NO_3	2.0

3.2 ALUMINIUM CHALLENGE

3.2.1 Root elongation assay

a. Preparation and acclimation of plantlets and challenge with Al

Twenty-four hours prior to exposure to Al, plants were supplied with fresh nutrient media (Table 4). In preparation of the medium for Al challenge, a 0.1M $AlCl_3$ stock was prepared by adding an appropriate amount of the chemical to polished water that had been acidified to a pH value of 3.0 with concentrated HCl (Hamel *et al.*, 1998). The appropriate amount of this $AlCl_3$ stock was added to 1mM $CaCl_2$ (pH (4.50) to give Al of concentrations 0μM, 50μM, 100μM, 250μM, 500μM and 1000μM. The final pH of these solutions was adjusted to 4.15 with concentrated HCl. The activity of the Al^{3+} ion at each concentration was calculated with the aid of the ion speciation programme MINTEQA2/PRODEFA2 (Allison *et al.*, 1990) (Table 5).

Table 5: MINTEQ analysis of Al^{3+} activity in 1mM $CaCl_2$.

$AlCl_3$ concentration (μM)	Al^{3+} activity (μM)	% of total Al concentration
50	45.21	90.42
100	87.99	87.99
250	220.9	88.36
500	443.6	88.7
1000	896.9	89.6

b. Root growth measurements

Before exposure to the Al media, roots were rinsed in distilled water to remove traces of nutrient media. The distal 10mm region of each root (ranging from 4 to 32 roots per plant) was demarcated with indelible ink. Increase in length of the demarcated tip of each root was measured after 24, 48, and 72 hours.

c. Calculations and statistics

The average increase in root length for each treatment was expressed as a relative root growth inhibition index (%RGI), calculated according to the following equation (Hamel *et al.*, 1998):

$$\% \text{ RGI} = 100 \times \left(1 - \frac{\text{average root length in treatment}}{\text{average root length in control}}\right)$$

The significance of the effects of the Al treatments on retardation of root elongation was assessed by means of an unpaired student t-test (SigmaPlot® version 4.0, Jandel Scientific).

3.2.2 Challenge conditions for library construction

a. Preparation and acclimation of plantlets and challenge with Al

Plants were treated as for the aluminium trial, with fresh nutrient medium supplied 24 hours before commencement of the challenge, and roots rinsed thrice in distilled water before exposure to the Al-containing medium. Aluminium was supplied at a concentration and for a duration shown to have maximum inhibitory effect during root elongation assays (section 3.2.1).

b. Harvesting and storage of root tips

After Al challenge, root tips (terminal 10 to 15mm portions) were excised and immediately flash frozen in liquid nitrogen and stored at –80°C until required for the extraction of RNA.

3.3 CONSTRUCTION OF SUBTRACTIVE cDNA LIBRARY

3.3.1 RNA isolation

a. Precautions

Several precautions were taken to prevent RNase contamination and resultant RNA degradation (Sambrook and Russel, 2001). Disposable gloves were worn at all times during preparative procedures and RNA extraction. In addition, gloves and bench surfaces were wiped at regular intervals with RNase Away (Molecular BioProducts) to prevent RNase cross-contamination. Where possible, sterile disposable plastic-ware was used, including sterile aerosol-resistant disposable pipette tips. Non-disposable items, such as glassware, mortars and pestles, scalpels and spatulas, were autoclaved prior to use. Solutions were prepared with water treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC). Chemicals used for the preparation of solutions were dedicated to RNA work to avoid the risk of possible RNase contamination. All solutions and samples were kept chilled on ice during the extraction procedure. Electrophoresis apparatus, including combs, gel-casting trays and tanks, were cleaned with detergent, dried with ethanol, wiped with RNase Away and rinsed with DEPC-treated water prior to use.

b. Tissue disruption

Frozen root tips (1-2 g) were placed in a pre-chilled, sterile mortar containing liquid nitrogen and ground to a fine powder using a sterile pestle. The powder was placed in sterile 50ml polypropylene centrifuge tubes (Corning®) and retained on ice.

c. Total RNA extraction and purification

Aliquots (4 ml each) of RNA extraction buffer (1% (w/v) SDS; 1mM ATA; 4% PAS; 10mM Tris (pH7.5); 1mM EDTA; 2% (v/v) 2-mercaptoethanol) and phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v) were added to the ground root tips. Samples were immediately homogenised for 4 minutes with an Ultra-Turrax T25 homogeniser (Janke & Kunkel Ika® - Labortechnik). The phases of the mixture were separated by centrifugation (4361*g*, 20 min, 4°C). The upper aqueous phase (approximately 4ml) was recovered and transferred to a sterile 15ml volume polypropylene tube (Corning®) containing 20 μl of 100mM ATA and 660 μl of 12M LiCl (final concentrations of 2M LiCl and 1mM ATA). The RNA was allowed to precipitate overnight at 4°C.

The precipitated RNA, which was collected by centrifugation (4361*g*, 20 min, 4°C), was resuspended in 1ml of 50 μM ATA and transferred to sterile 1.5 ml microfuge tubes. To eliminate contaminating particulate matter, samples were then centrifuged for 2 min at 5200*g*, and the supernatants transferred to clean microfuge tubes. To these tubes, 170μl of 12M LiCl was added and RNA allowed to precipitate overnight at 4°C.

After RNA sedimentation by centrifugation (9700*g*, 10 min, 4°C), the supernatant was discarded and the RNA pellets rinsed by immersion in 70% (v/v) ethanol and subsequent centrifugation (9700*g*, 10 min, 4°C). The washed RNA was resuspended in 250 μl of 50μM ATA. Where particulate matter contamination was persistent, a further centrifugation step (5200*g*, 2 min, 4°C) was required. In a final precipitation step, 125 μl of 7.5M ammonium acetate (final concentration 2.5M) and 750μl of 95% (v/v) ethanol were added to the samples, which were then incubated for at least one hour at -20°C. Samples were then centrifuged at 9700*g* for 30 min at 4°C, after which the RNA pellets were dried for 5 minutes in a Savant SpeedVac SC-110, followed by resuspension in 150 μl of 50μM ATA.

d. Quantification and quality assessment

The extracted RNA was quantified by means of ultra-violet spectrophotometry, at a wavelength of 260nm (Beckman DU-7500 spectrophotometer). Quality of the samples was determined by calculation of the ratio between absorbance values at 260nm and 280nm (Sambrook and Russel, 2001) and via denaturing agarose gel electrophoresis (Ingelbrecht *et al.*, 1998). In the latter method, 10μg RNA was denatured at 65°C for 5 minutes in the presence of 20mM MOPS (pH 7.0), 5mM sodium acetate, 1mM EDTA, 50%(v/v) formamide, and 2.2M formaldehyde (final volume of 30μl). Samples were immediately quenched on ice and 3 μl of gel loading buffer (50% [v/v] glycerol; 1mM EDTA [pH 8.0]; 0.25% [v/v] bromophenol blue) added. RNA was then fractionated on a 1.2%(w/v) agarose gel, containing 20mM MOPS (pH 7.0), 5mM sodium acetate, 1mM EDTA and 2.2M formaldehyde, using a tank buffer containing 20mM MOPS (pH 7.0), 5mM sodium acetate, 1mM EDTA and 0.45M formaldehyde, at a voltage of $5V.cm^{-1}$. The fractionated RNA was stained with $1\mu g.ml^{-1}$ ethidium bromide, destained in DEPC-treated water, and visualized with a short-wavelength ultraviolet trans-illuminator (Hoefer).

e. Poly A^+ isolation

The poly A^+ RNA (mRNA) was isolated from total RNA (75μg) by means of a Dynabeads mRNA Purification kit (Dynal®) according to the manufacturer's instructions. At the recommendation of the manufacturer, an additional step in the procedure was included to eliminate rRNA contamination. Final elution from the magnetic beads was in a 10μl volume.

3.3.2 cDNA synthesis

a. First strand synthesis

Messenger RNA was used to generate double stranded cDNA, according to the protocol supplied with the Expand™ Reverse Transcriptase kit (Roche). For first strand synthesis, each mRNA sample (~500ng) was heated at 65°C for 10 minutes with oligo-$(dT)_{15}$ primer (2.5μM final concentration, Promega) and distilled water to a final volume of 8μl. After immediate quenching on ice, the following components were added to a final volume of 20.μl: 4μl Expand™ reverse transcriptase buffer (5X); 2μl DTT (100mM); 2μl dNTP mix (10mM); 0.5 units RNase inhibitor (Roche); and 2.5μl Expand™ Reverse Transcriptase. Tubes were incubated for 45 minutes at 42°C and then placed on ice.

b. Second strand synthesis

Second strand synthesis followed immediately after first strand synthesis, with the addition of the following components to the tube containing the first strand reaction (final volume of 100µl): 10µl second strand synthesis buffer (10X: 500mM Tris-HCl pH 7.6; 1M KCl; 50mM $MgCl_2$); 5µl DTT (100mM); 1µl ATP (100mM); 1µl ammonium sulphate (1M); 0.8 units RNase H (Roche); 23 units DNA polymerase 1 (Roche); 0.5 units DNA ligase (Roche); 5µl BSA ($1mg.ml^{-1}$). Tubes were incubated for 2 hours at 14°C, followed by 10 minutes at 70°C. After brief centrifugation to collect tube contents, samples were placed on ice, 2 units T4 DNA polymerase (Roche) added, and the tubes incubated at 37°C for 10 minutes. A 2 µl aliquot of 0.5M EDTA was then added to terminate the reaction, and the cDNA purified via a phenol:chloroform:isoamyl alcohol (25:24:1) extraction, followed by a chloroform:isoamyl alcohol (24:1) extraction. Residual traces of the oligo-$(dT)_{15}$ primer were removed using a QIAquick® PCR Purification kit (QIAGEN) according to manufacturer's instructions, and eluted in 40 µl TE buffer (pH 7.6) (10mM Tris.Cl [pH 7.6], 1mM EDTA [pH 8.0]).

The concentration of the double-stranded cDNA was assessed fluorometrically (Hoefer® DyNAQuant 200) with calf thymus DNA (Sigma) as a standard.

3.3.3 cDNA processing

a. Digestion with restriction enzymes

Each of the double stranded cDNA populations (A: Aluminium-exposed; B: Control) were digested with restriction endonucleases: one population with 10 units *Alu 1* (Roche) alone and the other with 10 units each of *Alu 1* and *Rsa 1* (blunt-end cutters) (Roche). Approximately 30 ng of cDNA served as starting material for each restriction reaction. To ensure complete digestion, samples were incubated at 37°C overnight, followed by a heat inactivation of the enzymes (≥10 minutes at 65°C).

b. Adaptor ligation

Oligonucleotide adaptors were prepared for each set of cDNA populations: a1/a2 adaptors for the A cDNA population, and b1/b2 adaptors for the B cDNA population. The oligonucleotides were synthesized by Roche, according to sequences given by Patel and Sive (1996). To prepare these adaptors for subsequent ligation reactions, 1.5µl of a 3 $µg.µl^{-1}$ stock of oligonucleotides

a1 (5'-TAG TCC GAA TTC AAG CAA GAG CAC-3') and b1 (5'-ATG CTG GAT ATC TTG GTA CTC TTC-3') were phosphorylated by the addition of the following components (final volume of 25μl): 2.5 μl ATP (10mM); 2.5 μl T4 polynucleotide kinase buffer (10X, Roche); 0.5μl T4 polynucleotide kinase (10U. μl^{-1}, Roche). Incubation proceeded for 60 minutes at 37°C, followed by heat inactivation of the enzyme (20 minutes at 65°C). To the appropriate tube, 1.5μl of a 2.5μl stock of oligonucleotide a2 (5'-CTC TTG CTT GAA TTC GGA CTA-3') or b2 (5'-GAG TAC CAA GAT ATC CAG CAT-3') were added to create a1/a2 or b1/b2 adaptors respectively. After incubation at 45°C for 10 minutes, adaptors were stored at -20°C until required.

The adaptors were ligated onto the digested cDNA fragments under the following reaction conditions (final volume of 130μl): 13μl T4 DNA ligase buffer (10X, Roche); 30μl 40%(w/v) PEG 8000; 1μl ATP (15mM); 10μl *Alu 1*-digested cDNA; 10μl *Alu 1* and *Rsa 1*-digested cDNA; 2μl a1/a2 or b1/b2 adaptor (as appropriate). After 2 hours of incubation at 16°C, reactions were chilled on ice for a minimum of ten minutes. To each tube, 1μl aliquots of 75mM ATP and T4 polynucleotide kinase ($10U.\mu l^{-1}$, Roche) were added and incubated for 30 minutes at 37°C, followed by phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v) and chloroform:isoamyl alcohol (24:1; v/v) extractions. The reaction mixtures were then size fractionated (Quick Spin ™ Columns [TE] Linkers 6 [Roche]) to remove unligated adaptors.

3.3.4 cDNA subtraction

a. PCR amplification

To obtain large amounts of cDNA for the subtraction process, the ligated cDNA was PCR amplified. The PCR reaction mix was as follows (50μl final volume): 5μl *Taq* DNA polymerase buffer (10X, Promega); 3μl $MgCl_2$ (25mM); 1μl dNTP mix (10mM); 0.5μl oligonucleotide a2 or b2 ($2.5\mu g.\mu l^{-1}$); 5μl ligated A cDNA or B cDNA; 0.5μl *Taq* DNA polymerase ($5U.\mu l^{-1}$, Promega). The cDNA was amplified using the following thermal cycling parameters (GeneAmp® PCR System 9700, Applied Biosystems): 30 cycles of 1 minute at 94°C (denaturation); 1 minute at 50°C (annealing); 2 minutes at 72°C (extension) with 25 seconds auto-extension at 72°C. The cDNA populations yielded by this amplification step were termed A_0 and B_0. Small aliquots (10μl) of each population were fractionated by means of agarose gel (1.5% [w/v]) electrophoresis to determine the size ranges of the amplified cDNAs.

b. Synthesis of tracer cDNA and biotinylation of driver cDNA

The subtraction scheme (Fig. 2) allowed for the removal of sequences common to both tracer and driver cDNA populations, resulting in the enrichment for tracer-specific sequences. As a reciprocal subtraction was performed (A - B and B - A), two populations of subtracted cDNA sequences were obtained, one set enriched for sequences specific to the control treatment (B) and the other set for sequences specific to the Al challenge (A) (Fig. 3).

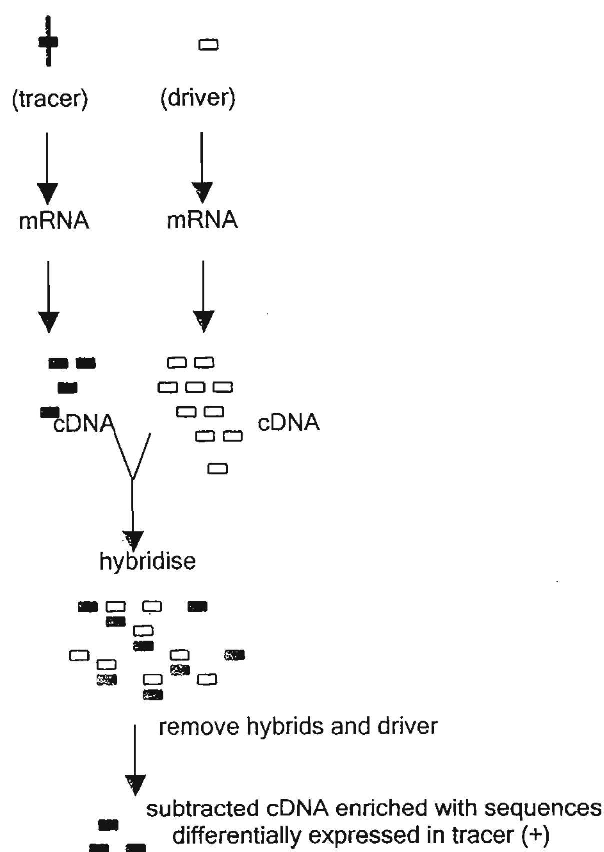

Fig. 2: Generalised subtraction scheme. Tracer cDNA from the Al-exposed root tips (+) is hybridised to >10-fold excess of driver cDNA from the control treatment root tips (-). The resulting hybrids and excess driver are removed to enrich for sequences specific to the tracer cDNA population (after Patel and Sive, 1996).

To enable the removal of driver/driver and driver/tracer hybrids via streptavidin binding and phenol extraction, driver cDNA was biotinylated. For the PCR synthesis of driver cDNA, four sets of the following reaction were set up per cDNA population (100µl final volume): 10µl *Taq* DNA polymerase buffer (10X, Promega); 6µl $MgCl_2$ (25mM); 6.7µl driver dNTP mix (0.5mM bio-11-dUTP (Enzo Diagnostics), 1.5mM each dATP, dCTP and dGTP, 1.0mM dTTP); 1µl

oligonucleotide a2 or b2 (2.5μg.μl^{-1}); 1μl A_0 or B_0 cDNA; 1μl *Taq* DNA polymerase (5U.μl^{-1}, Promega). For both sets of amplified cDNAs, the following tracer synthesis PCR reaction was set up (100μl final volume): 10μl *Taq* DNA polymerase buffer (10X, Promega); 6μl $MgCl_2$ (25mM); 2μl dNTP mix (10mM); 1μl oligonucleotide a2 or b2 (2.5μg.μl^{-1}); 2μl A_0 or B_0

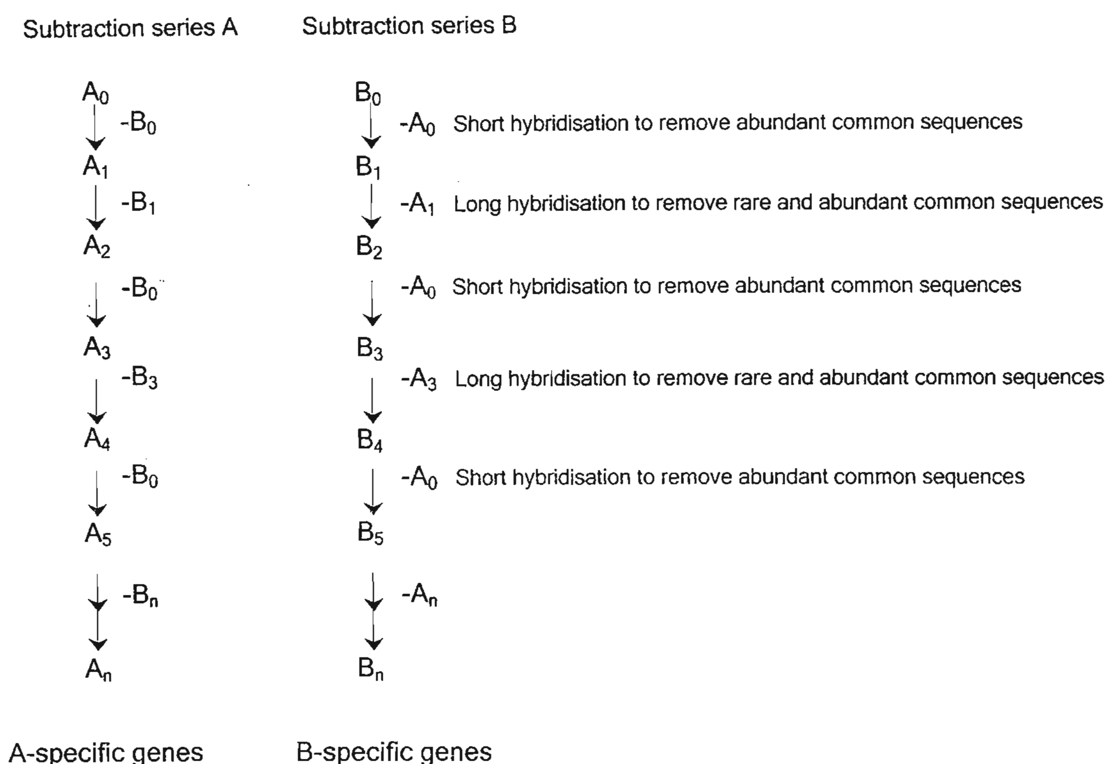

Fig. 3: Sequence of subtractions used for the isolation of A-specific and B-specific genes (after Patel and Sive, 1996).

cDNA; 1μl *Taq* DNA polymerase (5U.μl^{-1}, Promega). Thermal cycling parameters were the same as for the initial amplification of ligated cDNA for the synthesis of A_0 cDNA and B_0 cDNA (section 3.3.4 *a*). However, due to the larger reaction volume, reaction mixtures were overlaid with sterile PCR-grade mineral oil and amplified in a Hybaid OmniGene thermal cycler.

Amplified cDNA products were purified away from unincorporated nucleotides, primer, and salts, using a QIAquick® PCR Purification kit (QIAGEN), as directed by the manufacturer. Yields were determined spectrophotometrically using a Beckman DU-7500 spectrophotometer, and a 5μl aliquot of each product was fractionated on a 1.2% (w/v) agarose gel at 5V.cm^{-1} to monitor the size range of the amplified cDNA fragments.

c. Hybridisation of tracer and driver cDNA

Two hybridisation reactions were set up: bio-A_0 (driver) + B_0 (tracer) and bio- B_0 (driver) + A_0 (tracer). For each reaction, 1μg of tracer and 20μg biotinylated driver were ethanol precipitated together (5mM $MgCl_2$ in 70% ethanol) in a 1.5ml silanised microfuge tube (No-Stick®, Eppendorf), without freezing. The pellets were air dried and resuspended by gentle pipetting in 5μl HEPES buffer (100mM HEPES pH 7.3; 1mM EDTA). Resuspended DNA was transferred to a 0.6ml PCR tube, to which 5μl of 68°C 2X hybridisation buffer (50mM HEPES pH 7.3; 10mM EDTA; 0.2% (w/v) SDS; 1.5M NaCl) was added. After gentle mixing, a few drops of sterile PCR-grade mineral oil were added, and the tubes briefly centrifuged. Tubes were then incubated for 10 minutes at 95°C and allowed to cool slowly over one hour to 68°C, whereafter the temperature was maintained at 68°C for a further two hours. This constituted a short hybridisation cycle. (For long hybridisation cycles, the temperature was maintained at 68°C for a further 30 to 40 hours).

d. Isolation of subtracted tracer cDNA

Tracer/driver and driver/driver hybrids, as well as single stranded biotinylated driver cDNA, were removed through the addition of streptavidin, followed by extraction with phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol. To each hybridisation mixture, 7μl of 1M NaCl and 140μl HEPES buffer (premixed and warmed to 68°C) were added. After cooling to room temperature, 15.5μl aliquots of streptavidin solution ($2\mu g.\mu l^{-1}$ streptavidin and 0.15M NaCl in HEPES buffer) were added to each tube, which were then vortexed and incubated for 5 minutes at room temperature. Equal volumes of phenol:chloroform:isoamyl alcohol were then used to extract the streptavidin:biotinylated cDNA hybrid complexes. The aqueous phases containing the non-biotinylated, and thus rarer sequences, were retained and transferred to fresh tubes. A second round of streptavidin binding (10.6μl) followed, with two rounds of phenol:chloroform:isoamyl alcohol extraction and two additional chloroform:isoamyl alcohol extractions. The resulting subtracted cDNAs were referred to as A_1 and B_1 cDNA, or A_n and B_n cDNA, as per the number of rounds of subtraction performed.

Further subtractions were then performed using subtracted cDNA from the previous round as template for the driver and tracer synthesis reactions. Hybridisation steps alternated between long and short cycles, continually enriching for differentially expressed genic sequences. For

short hybridisations, A_0 and B_0 cDNA drivers were used, whereas A_n and B_n drivers were used for the long hybridisation cycles (Fig. 3), with the amount of cDNA template used for tracer synthesis reduced to approximately 5 to 10ng for subsequent rounds. After each cycle of short or long hybridisation, small aliquots (5μl) of each of the PCR amplified products were fractionated on a 1.2% (w/v) agarose gel to confirm subtraction, as indicated by changes in the size range of the cDNA fragments.

3.3.5 cDNA cloning

After six rounds of subtraction (three short and three long hybridisation cycles) the cDNA was amplified as per normal tracer synthesis conditions (section 3.3.4 *b*), using 5μl A_6 or B_6 cDNA. Amplifications products were purified using a QIAquick-spin PCR Purification kit (QIAGEN).

a. Adaptor ligation

The a1/a2 adaptors contain an internal *Eco*RI restriction site, whereas the b1/b2 adaptors contain an internal *Eco*RV and not *Eco*RI restriction site. The cloning vector into which the cDNA was to be cloned was pre-digested with *Eco*RI (Lambda ZAP® II Pre-digested *Eco*R1/CIAP-Treated Vector Kit, Stratagene). It was thus necessary to ligate *Eco*R1 adaptors onto the amplified B cDNA in order to allow for cloning of this population.

Firstly, it was necessary to ensure that the B cDNA was blunt-ended prior to the ligation of vector-compatible adaptors. A Klenow reaction was thus set up according to the Promega Protocols and Applications Guide (1990) (10μl final volume): B_6 cDNA (~300ng); 10X nick translation buffer (0.5M Tris-HCl pH 7.5; 0.1M $MgSO_4$; 1mM DTT; 500μg.ml^{-1} BSA); 4 units Klenow enzyme (Promega). After incubation for one hour at 37°C, the enzyme was heat inactivated at 70°C for 10 minutes, followed by the ligation of pre-digested *Eco*R1 adaptors (30μl final volume): Klenow reaction (10μl); 3μl ligation buffer (10X, Roche); 3μl BSA (1mg.ml^{-1}); 5μl *Eco*R1 adaptors (10pM); 30 units T4 DNA ligase (Roche). The adaptors were supplied at a 50-fold molar excess, based on the assumption that the average cDNA fragment was approximately 400 base pairs in size. The ligation reaction mixture was incubated at 15°C overnight, followed by 10 minutes at 70°C.

b. cDNA phosphorylation

Since the Lambda ZAP® II cloning vector was dephosphorylated, it was necessary to phosphorylate the cDNA via a kinase reaction (40μl final volume): 4μl polynucleotide kinase buffer (10X, Roche); 2μl ATP (0.1mM); 30 units T4 polynucleotide kinase (Roche). Reactions were incubated for 30 minutes at 37°C, followed by phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol extractions, and size fractionation through a Sephacryl® S-400 spin column (Promega). The samples were then precipitated (5mM $MgCl_2$ in 70% ethanol) and resuspended in 2.5μl water.

c. cDNA digestion with restriction enzyme

The *Eco*R1 adaptors ligated onto the B cDNA was pre-digested with *Eco*R1, however the restriction site for this enzyme in the a1/a2 adaptors ligated onto the A cDNA was an internal site. It was thus necessary to first restrict the A cDNA before proceeding with the cloning procedure (20μl final volume): 200ng A_6 cDNA; 10 units *Eco*R1 (Roche); 2μl Buffer H (10X, Roche). After 60 minutes incubation at 37°C, a further 10 units of *Eco*R1 were added, followed by a further 2 hours incubation at 37°C. The restriction enzyme was then heat inactivated (70°C for 10 minutes) and the cDNA ethanol precipitated (5mM $MgCl_2$ in 70% ethanol) and resuspended in 2.5μl water.

d. Ligation into cloning vector

Ligation reactions were set up for each of the cDNA populations as per manufacturer's instructions (Stratagene) and incubated overnight at 14°C (5μl final volume): 2.5μl cDNA (~200ng); 1μl Lambda ZAP® II vector arms ($1\mu g.\mu l^{-1}$); 0.5μl ligation buffer (10X, Roche); 5 units T4 DNA ligase (Roche).

e. Packaging

Aliquots of each ligation reaction were packaged using Gigapack® III Gold Packaging Extract (Stratagene) as per manufacturer's recommendations. Briefly, 4 μl of each ligation reaction were added to tubes of packaging extract that had just begun to thaw, and mixed gently using a pipette tip. The tubes were spun for a few seconds and then incubated at 22°C for 2 hours,

followed by the addition of 500μl of SM buffer (0.58% (w/v) NaCl; 0.2% (w/v) $MgSO_4.7H_20$; 5% (v/v) Tris-HCl pH 7.5; 0.01% (w/v) gelatin) and 20μl chloroform. After gentle mixing and the sedimentation of cell debris, the supernatant containing the primary phage libraries (hereafter termed SubA and SubB) were stored at 4°C.

3.4 CHARACTERISATION OF THE SUBTRACTIVE LIBRARIES

3.4.1 Preparation of bacterial cell line

Streak plates were prepared from bacterial cell line XL1-Blue MRF', supplied with the Lambda ZAP® II cloning kit (Stratagene), using Luria Bertani (LB) plates (1% (w/v) tryptone; 1% (w/v) NaCl; 0.5% (w/v) yeast extract; 2% (w/v) agar; pH 7.0) containing $12.5mg.l^{-1}$ tetracycline. Cultures (50ml) were initiated from these streak plates (stored for a maximum of two weeks at 4°C) using LB broth supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$. These cultures were grown to an optical density (600nm) of between 0.5 and 1.0 to ensure that cells were in the logarithmic phase of growth, thus providing the maximum number of viable cells for phage infection. Bacterial cells were then pelleted (500g for 10 minutes at 4°C) and gently resuspended in approximately 10ml of sterile 10mM $MgSO_4$, and stored for a maximum of one week at 4°C. Prior to use, cells were diluted with sterile 10mM $MgSO_4$ to an optical density (600nm) of 0.5 and used immediately.

3.4.2 Plating of phage libraries

Serial dilutions were prepared of the primary libraries (assumed to fall within the range of 10^7 to 10^8 plaque-forming units (pfu) per ml) using SM buffer, and 1μl aliquots of each dilution were combined with 200μl of diluted XL1-Blue MRF' cells and incubated for 15 minutes at 37°C. The cells were immediately plated onto 90 mm diameter NZY plates (0.5% [w/v] NaCl; 0.2% [w/v] $MgSO_4.7H_20$; 0.5% [w/v] yeast extract; 1% [w/v] casein hydrolysate; 1.5% [w/v] agar; pH 7.5) using approximately 3ml NZY top agar (agar replaced with 0.7% [w/v] agarose) containing 15μl of 0.5M isopropyl-β-D-thiogalactopyranoside (IPTG) and 50μl of $250mg.ml^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D- thiogalactopyranoside (X-Gal) (in dimethyl formamide). The addition of these two chemicals to the NZY agar allowed for blue-white colour selection and thus the determination of the ratio of recombinants to non-recombinants, the latter appearing

blue. Plates were incubated at 37°C overnight and then analysed for percentage non-recombinants, as well as for titre determination ($pfu.ml^{-1}$).

3.4.3 Assessment of insert sizes

Random plaques, including a blue non-recombinant plaque, were punched out using sterile glass Pasteur pipettes, and allowed to diffuse overnight into 500µl SM buffer. Using the universal M13 primer pair, cDNA inserts contained in the phages from each plaque were PCR amplified using the following reaction mixture (15µl final volume): 1µl plaque suspension; 1.5µl *Taq* DNA polymerase buffer (10X, Promega); 0.6µl $MgCl_2$ (25mM); 0.15µl dNTP mix (10mM); 0.5µl M13 Forward Primer (6µM); 0.5µl M13 Reverse Primer (6µM); 0.2µl *Taq* DNA polymerase ($5U.\mu l^{-1}$, Promega). Reaction mixtures were cycled according to the following thermal cycling parameters (GeneAmp® PCR System 9700, Applied Biosystems): 1 cycle of 1 minute at 94°C; 10 cycles of 30 seconds at 94°C, 1 minute at 50°C, and 2 minutes at 72°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 45°C, and 2 minutes at 72°C; 1 cycle of 2 minutes at 72°C. PCR amplification products were fractionated on a 1.2% (w/v) agarose gel containing $0.5\mu g.ml^{-1}$ ethidium bromide at $5V.cm^{-1}$, and viewed under ultraviolet light.

3.4.4 Amplification of the phage libraries

As primary libraries are unstable, they were amplified, as recommended by the manufacturers of the Lambda ZAP® II cloning kit (Stratagene), to prepare large stable quantities of high titre recombinant phage library stocks for long-term storage. To this end, aliquots of the library suspensions containing approximately 5×10^4 pfu of bacteriophage were combined with 600µl of host cells (XL1-Blue MRF', diluted to an optical density of 0.5 absorbance units at 600nm) and incubated for 15 minutes at 37 °C. Cells were then plated onto 10mm square NZY plates, using NZY top agar (*c.* 6ml, no IPTG or X-Gal) and incubated at 37°C for 6 to 8 hours until plaques were 1-2mm in diameter. Plates were then overlaid with 8ml of SM buffer and placed at 4°C overnight, allowing the phage to diffuse into the buffer. The bacteriophage suspensions were then recovered and pooled into sterile 15ml tubes, and the plates rinsed with an additional 2ml of buffer. To each tube, chloroform was added to a final concentration of 5% (v/v) and incubated with the suspension for 15 minutes at room temperature, followed by centrifugation for 10 minutes at 2000g to remove cell debris. The supernatants were dispensed into aliquots and stored in 7% (v/v) dimethyl sulfoxide at -80°C. The titre of the amplified libraries was

determined in the same manner as previously outlined for the primary libraries (section 3.4.2), with the omission of IPTG and X-Gal from the growth medium.

3.5 ANALYSIS OF LIBRARY cDNA INSERTS

3.5.1 Reverse Northern hybridisation analysis

a. Probe synthesis

Plaques were punched out randomly from plates of each library, allowed to diffuse overnight into 500μl SM buffer, and PCR amplified as previously described (section 3.4.3). To confirm amplification, 3μl of each sample was fractionated on a 1.2% (w/v) agarose gel at $5V.cm^{-1}$, stained with ethidium bromide ($10\mu g.ml^{-1}$) and viewed under ultraviolet light. Samples that contained clones that did not amplify well or contained multiple clones were discarded.

b. Array printing

Samples were denatured prior to arraying via the addition of NaOH to a final concentration of 0.2N. A manual gridding device (V&P Scientific, Inc.) was then used to transfer 0.6μl aliquots of the denatured PCR products onto a 150 x 100mm positively charged nylon membrane (Hybond™-N+, Amersham). For replication, aliquots of each PCR product were delivered to two separate addresses on the array. Six 96-well PCR plates of amplified products were incorporated into the arrays for each of the libraries, resulting in the representation of 576 clones per library, arrayed in a 4x3 format (Fig. 4). The double-stranded DNA PCR products were cross-linked to the membranes by means of short-wavelength UV-radiation (120 000 $J.cm^{-1}$ for 2 minutes, Hoefer UV-Crosslinker), air-dried and stored at 4°C until required.

c. Preparation of labelled total cDNA populations

Unsubtracted total cDNA from each of the target populations, namely Al-challenged and control treatment, were used to query arrays bearing PCR amplified inserts from both subtraction libraries. This was done to determine the efficiency of the subtractive process. Labelling of the total cDNA was based on a combination of the protocols outlined by Sambrook and Russel (2001) and by the technical bulletin accompanying the Expand™ Reverse Transcriptase kit (Roche). Approximately 1μg of poly-A^+ RNA (mRNA) was heated

for five minutes at 70°C, and then chilled on ice. In a separate tube, 2.5μl random hexamer primers (5 μg.μl^{-1}), 1μl dNTP mix (20mM dCTP, dGTP and dTTP), 1μl dATP (120μM) and 2μl ddCTP (100 μM) were mixed and dried in a centrifugal sample evaporator (Savant SpeedVac SC-110). To this tube, 1.5μl DEPC-treated water, 4μl Expand™ buffer (5X) and 2μl DTT (100mM) were added. The contents were allowed to resuspend for a few minutes, whereafter 20 units RNase inhibitor (Roche), the mRNA sample (5μl), 5 units Expand™ Reverse transcriptase (Roche) and 5μl [α-^{33}P] dATP (10 μCi.μl^{-1}) (AEC Amersham) were added. The contents were then incubated for 10 minutes at 30°C, followed by 45 minutes at 42°C. A 1μl aliquot of 0.5M EDTA (pH 8.0) was then added, as well as 1μl of 10% SDS. After mixing the contents of the tube, 3μl of 3N NaOH was added, followed by a 30 minute incubation at 68°C to hydrolyse the RNA. After cooling to room temperature, 10μl of 1M Tris-HCl (pH 7.4) was added, mixed well, and 3μl of 2N HCl added. The labelled cDNA was then purified by means of a standard phenol:chloroform:isoamyl alcohol extraction, followed by a chloroform:isoamyl alcohol extraction.

The efficiency of target cDNA labelling was tested via paper chromatography. A small aliquot of the labelled mixture (2μl) was spotted onto a piece of filter paper, the end of which was placed in 0.75M Na_2PO_4. The mobile phase front was allowed to migrate for a distance of 5 to 10cm, after which the chromatogram was exposed to a phosphor screen (Cyclone™) for 1 to 2 hours. The autoradiographic image was captured and viewed by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard).

Unincorporated dNTPs were removed from the labelled total cDNA populations via ethanol precipitation and washing. After the addition of 3 volumes of 95% (v/v) ethanol, and a minimum of 2 hours incubation at –20°C, the target cDNA was centrifuged for 30 minutes (15 800*g*, 4°C). The supernatant was decanted and the pellet allowed to air dry prior to resuspension in 50μl TE buffer. The labelled targets were heat denatured (100°C, 5 min) and quenched on ice before addition to the hybridisation buffer (section 3.5.1 *d)*.

d. Array querying

The array membranes were incubated for 8 –18 hours in 30ml of a modified Church and Gilbert buffer (0.5M sodium phosphate pH 7.2; 7% (w/v) SDS; 0.94mM EDTA) (Church and Gilbert, 1984) containing 10μg.ml^{-1} denatured fragmented salmon sperm DNA. Incubation was

conducted at 65°C in 300ml volume hybridisation bottles within a rotary hybridisation oven (Hybaid Micro-4). After prehybridisation, an aliquot (30ml) of fresh hybridisation buffer containing the denatured cDNA target was added. Membranes were incubated overnight at 65°C.

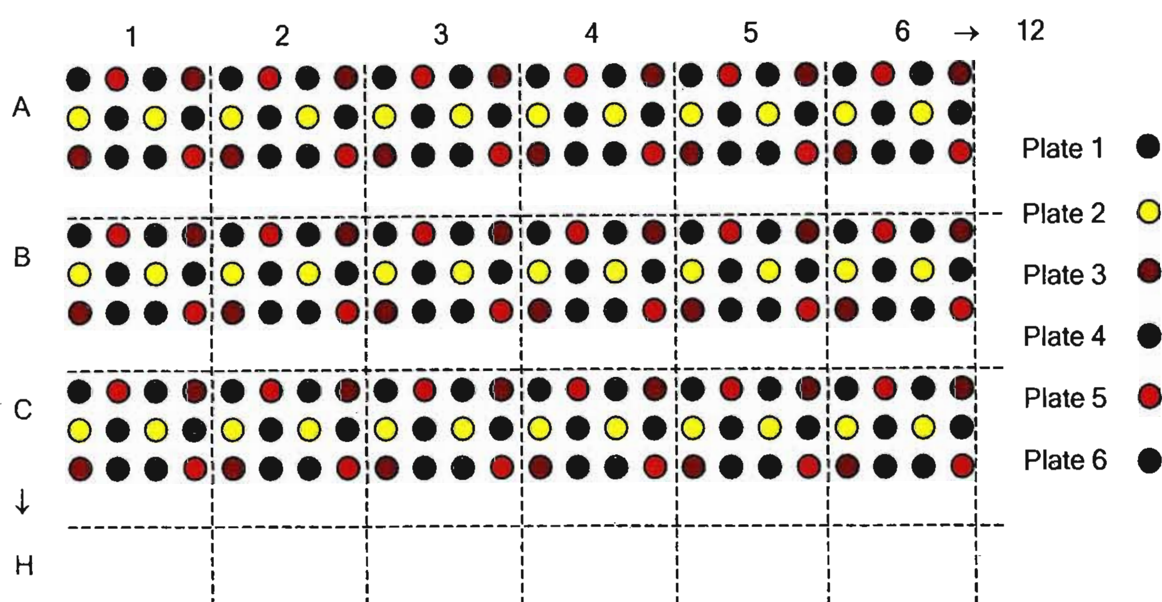

Fig. 4: Array design for subtractive libraries A and B, showing addresses of PCR products on array membrane.

Following overnight hybridisation, the membranes were washed with increasingly stringent washes to remove traces of unbound target: two washes with 1X SSC (15mM trisodium citrate; 150mM NaCl) for 20 minutes each; two washes with 0.5X SSC for 20 minutes each; one wash with 0.1X SSC for 10 minutes. Membranes were then sealed in polyethylene film and exposed to high-resolution phosphor screens (Cyclone ™). After between 24 and 56 hours of exposure, the images on the phosphor screens were captured and viewed by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard).

e. Array analysis

Array images were analysed using QuantArray® Microarray Analysis Software (Version 3.0, Packard Bioscience). This software facilitated the quantification of hybridisation intensity to each of the probes contained on the array membranes in response to each querying event, in terms of spot diameters and intensities, background, and signal to noise ratios. Furthermore, it

allowed for the calculation of the proportional hybridisation intensities to each probe between different target cDNA populations.

3.5.2 Northern hybridisation analysis

a. Total RNA size fractionation

Total RNA from both Al-challenged and control root tips (section 3.3.1) were purified using an RNeasy® Plant Mini Kit (QIAGEN), as per manufacturer's instructions. After spectrophotometric requantification (section 3.3.1 *d)*, 10μg RNA from both Al-challenged and control root tips were fractionated under denaturing conditions according to the method of Ingelbrecht *et al.* (1998). RNA samples were incubated at 65°C for five minutes in the presence of 25μl RNA incubation buffer (20mM MOPS pH 7.0; 5mM sodium acetate; 1mM EDTA; 50%(v/v) de-ionised formamide; 2.2M formaldehyde). Samples were immediately chilled on ice and 2.5μl of gel loading buffer (50% glycerol; 1mM EDTA pH 8.0; 0.25% bromophenol blue) added. Fractionation was performed on a 1.2% agarose gel containing 1X MOPS and 17% (v/v) formaldehyde (37%), at $5V.cm^{-1}$ with 1X MOPS as the running buffer. Duplicate sample sets were fractionated simultaneously, and one set stained immediately with ethidium bromide ($1\mu g.ml^{-1}$), destained with DEPC-treated water, and viewed under ultraviolet light to confirm the integrity and loading consistency of the RNA samples.

b. RNA blotting

The remaining portion of the gel was subjected to downward capillary blotting, using 50mM NaOH as the transfer medium. All wicks and paper supports were prepared from Whatman 3MM filter paper and were pre-wet in transfer medium. Transfer to a positively charged nylon membrane (Hybond™-N+, Amersham) was allowed to proceed overnight, whereafter the gel was stained with ethidium bromide (as in section 3.6.1) to confirm that transfer of RNA was complete. After transfer, the membrane was allowed to air-dry, and then neutralised via a brief rinse in DEPC-treated 2X SSC (30mM trisodium citrate; 0.3M NaCl). Once the membrane was dry, it was stored at 4°C until required.

c. cDNA probe labelling

Probe labelling was performed via random priming using the Megaprime ™ DNA labelling system (AEC Amersham). A 5μl aliquot of random nonomer primer solution ($5\mu g.\mu l^{-1}$) and

20ng of purified (QIAquick® PCR Purification kit (QIAGEN) cDNA fragment, amplified as described in section 3.4.3, were mixed to a final volume of 26μl and then heated to 100°C for 5 minutes. After a brief centrifugation step to collect the contents of the tube, 10μl of labelling buffer (10mM each of dGTP, dTTP and dATP), 5μl [α-^{32}P]-dCTP (3000 $Ci.mmol^{-1}$) and 2 μl Exo(-) Klenow enzyme ($5U.\mu l^{-1}$) were added. The components were thoroughly mixed and incubated for one hour at 37°C. The labelling reaction was terminated by the addition of 25μl of 50mM EDTA.

Unincorporated radiolabelled dNTPs were removed from the probe using a NucTrap® Purification Column (Stratagene), as per manufacturers instructions. The degree of radiolabel incorporation into probe DNA was tested using paper chromatography, using filter paper and 0.75M Na_2PO_4 buffer as the mobile phase. After the mobile phase front had migrated 5 to 10cm the chromatogram was exposed to X-ray film (Amersham Hyperfilm™-MP) for 15 minutes, and then developed. The probe was denatured at 100°C for 5 minutes, quenched on ice, and then added to hybridisation buffer.

d. Hybridisation

Membranes were prehybridised at 65°C for a minimum of 2 hours in 30ml of hybridisation buffer (5X filtered SSPE (900mM NaCl; 50mM NaH_2PO_4; 5mM EDTA pH 7.7); 5X Denhardt's reagent (0.1% (w/v) each of BSA, Ficoll™400 and PVP); 0.5% (w/v) filtered SDS; $20\mu g.ml^{-1}$ denatured fragmented salmon sperm DNA). Hybridisation solution was then replaced with a fresh 15ml aliquot of hybridisation buffer without salmon sperm DNA, to which the denatured labelled probe was added. The labelled cDNA probe was allowed to hybridise overnight to the membrane-bound fractionated RNA at 65°C in a rotary hybridisation oven (Hybaid Micro-4).

e. Visualisation

After hybridisation, the membranes were washed according to the following protocol of increasing stringency: 2 washes of 25 minutes each with 3X SSC and 0.1% SDS (68 °C); two washes of 20 minutes each with 1X SSC and 0.1% SDS (68°C); one final wash for 30 minutes with 0.1X SSC and 0.1% SDS (55°C). The washed membranes were then sealed in polyethylene film and exposed to high-resolution phosphor screens (Cyclone™ Storage

Phosphor Screen [Packard]). After between 24 and 56 hours of exposure, the images on the phosphor screens were captured and viewed by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard).

3.5.3 Sequencing

a. Phagemid rescue and quantification

Phagemids were excised from the Uni-ZAP® XR vector using the ExAssist helper phage, as per manufacturer's instructions (Stratagene). Phagemids (plasmids) were then introduced into bacterial host cells (*E. coli* strain SOLR™), from which overnight cultures were prepared and plasmids extracted using QIAprep® Spin Miniprep kit (QIAGEN), as per manufacturer's instructions.

b. Capillary electrophoresis

Samples were quantified fluorometrically (Hoefer® DyNAQuant 200) and prepared for single-run partial sequencing reactions as described in the protocol supplied with the ABI Prism ® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Reactions contained 3.2pmol of the M13 reverse primer (5') only and 500ng recombinant plasmid DNA. After amplification, unincorporated dye terminators were removed via an ethanol precipitation step, as recommended by the manufacturer (PE Applied Biosystems), and resuspended in 18μl of Template Suppression Reagent (supplied with the kit). Samples were then heat denatured (95°C for 2 minutes), chilled on ice for 1 minute, and analysed via capillary electrophoresis on an ABI Prism® 310 Genetic Analyzer.

c. Sequence editing and homology searches

Sequences were edited (Sequence Navigator 1.0.1) to remove vector and ambiguous sequences. The edited sequences were submitted to the BLAST (Basic Local Alignment Search Tool) program (Altschul *et al*, 1990) for comparative sequence analysis against the National Centre for Biotechnological Information (NCBI) non-redundant protein and dbEST databases, using the BLASTx and BLASTn algorithms respectively.

The Expect (E) value decreases exponentially with the bit score obtained for each sequence hit, with higher bit scores resulting in lower E-values, indicative of a more statistically significant

hit. The bit scores are, however, not considered as reliable an indication of sequence homology as the E-values, which also take into account the lengths of the sequence being compared. The E-value can be related to the p-value (indicating statistical significance) by the following equation:

$$\text{p-value} = 1 - e^{-\text{E-value}}$$

Thus as the E-value tends towards zero, the E-value and p-value may converge. Thus in cases where E-values were less than 0.01 were obtained for a particular sequence, these were considered homologous proteins for the sequence submitted (Altschul *et al*, 1990). Where several protein homologies were assigned to a sequence, the EST was assigned the identity of the protein showing the lowest p-value. Where all the alignment scores were greater than 0.01, it was assumed that there was no significant homology with an EST or protein in the NCBI database and the sequence remained unidentified.

CHAPTER 4
RESULTS

4.1 HYDROPONIC CULTURE OF SUGARCANE

As conclusive evidence exists indicating the root tip to be the primary site at which the phytotoxic effects of Al are exerted, the development of a method for the controlled exposure of root tips to Al was a major requirement for this study. Conducting root growth and response studies in the soil is technically very difficult. The heterogeneous nature of most soils means that the feasibility of isolating and studying one factor at a time is limited. As a result, it was not possible to use field-grown material, due to the difficulties associated with the controlled application of aluminium stress in a heterogeneous soil and the retrieval of intact root tips from deep beneath the soil surface. An alternative approach was thus adopted in which a hydroponics system was used for Al stress application. This method not only allowed for the rapid and accurate manipulation of Al concentration in the root environment, but also allowed for easy access to the roots for both physiological analysis and harvesting.

As sugarcane is a vegetatively propagated crop, plant production from seed is not routine, a characteristic that necessitated the development of a novel hydroponics system. For example, most classical hydroponics systems consist of a raft or mesh to support the plants that is positioned in such a way that facilitates the contact of roots with a reservoir of nutrient medium. However, as single bud sett-derived sugarcane plantlets remain attached to a nodal segment of the parental stalk, the traditional hydroponics design required modification. To this end, five-week old plants, with well-developed plant root systems, served as starting material for initiation of the hydroponics system used in this study (Figs 1 and 5).

4.2 EFFECT OF ALUMINIUM ON ROOT GROWTH

As little is known about the response of South African sugarcane varieties to direct Al challenge, the initial objective of this study was to determine the effects on root growth of exposure to Al at various concentrations for different periods of time. Sugarcane cultivar N12 was selected for this study as it is one of the cultivars most widely grown on acid soils within the industry (Turner *et al.*, 1992). Since the primary symptom of Al toxicity is the retardation of root growth, the effect of Al on root elongation was used as an indicator of the phytotoxic effect

Fig. 5: Cultivation of *Saccharum* spp. hybrid N12 (N12). (a) View of hydroponically gown plants in glasshouse. (b) Root system of hydroponically grown plants.

of the metal on the plant. Root growth measurements were taken from plants exposed to Al at various concentrations (0, 50, 100, 250, 500 and 1000μM) after various periods of exposure (24, 48, and 72 hours). Exposure to Al did not exceed 72 hours to circumvent the effects of possible root branching on root elongation measurements.

Exposure of roots to Al resulted in visible symptoms of phytotoxicity, including the appearance of brown necrotic lesions and mucilage exudation (results not shown). In general, root growth over a 24 hour period was inhibited in a manner approximately proportional to Al concentration (Fig. 6a), with 50 and 1000μM Al resulting in relative root growth inhibition of 4 and 48% respectively. Similar trials conducted with Al tolerant wheat cultivars showed that inhibition at 50μM Al resulted in approximately 45% inhibition, and reached a plateau of approximately 70% inhibition at 250μM Al (Hamel *et al.*, 1998). Sugarcane is, however, considered fairly Al tolerant, and thus this study included a treatment with Al supplied at 1000μM. The minimum exposure period over which the inhibitory effect of Al on root elongation could be quantified was identified as 24 hours and was thus used for the initial dose-response experiments. As the negative effects of the metal on root growth reached a plateau at approximately 250μM, this concentration of Al was selected for further experimentation. For technical reasons, data generated by root growth inhibition trials were variable and experiments were, therefore, repeated thrice. The mean inhibition of root growth over the three independent experiments was then averaged to determine the %RGI for each set of treatment conditions, as shown in Figure 6b. This problem of variability has also been reported in maize root growth studies, where

precise evaluations are difficult to obtain due to the enormous variability observed (Sibov *et al.*, 1999). This problem was similarly overcome in the maize studies through increasing the number of repetitions or treatments. However, for the purposes of this study, the results were sufficiently reliable to allow for the selection of a concentration and exposure duration for the Al challenge for the generation of root material for molecular analysis. Maximum inhibition in response to the metal was apparent at a concentration of 250μM or more, and did not increase significantly after the initial 24-hour period. It was therefore deemed unnecessary to use an Al concentration greater than this value, or to deprive the plants of nutrients for more than 24 hours and risk the complication of nutrient deficiencies.

4.3 ISOLATION OF ALUMINIUM-CHALLENGE-ASSOCIATED cDNA

4.3.1 Quantity and quality of RNA

Plant tissue is generally considered one of the more difficult tissues from which to isolate RNA. This is largely due to the problems associated with the co-purification of plant polysaccharides and phenolics, as well as the ubiquitous presence of ribonucleases (RNases), which are highly prolific in meristematic regions such as the root tip (Sambrook and Russel, 2001). Most protocols thus include measures to minimise these factors, such as the inclusion of RNase inhibitors (e.g. ATA) and protein denaturants, such as SDS and β-mercaptoethanol.

RNA was successfully extracted from frozen root tips, using a method optimised for sugarcane (Carson and Botha, 2000), with yields varying from 120μg to 750μg RNA per gram of frozen root material. Quality analysis was performed via spectrophotometric means as well as agarose gel electrophoresis with ethidium bromide staining (Fig. 7) The integrity of the samples was confirmed by the visible presence of two ribosomal bands, 28S ands 18S (Wilkinson, 1991).

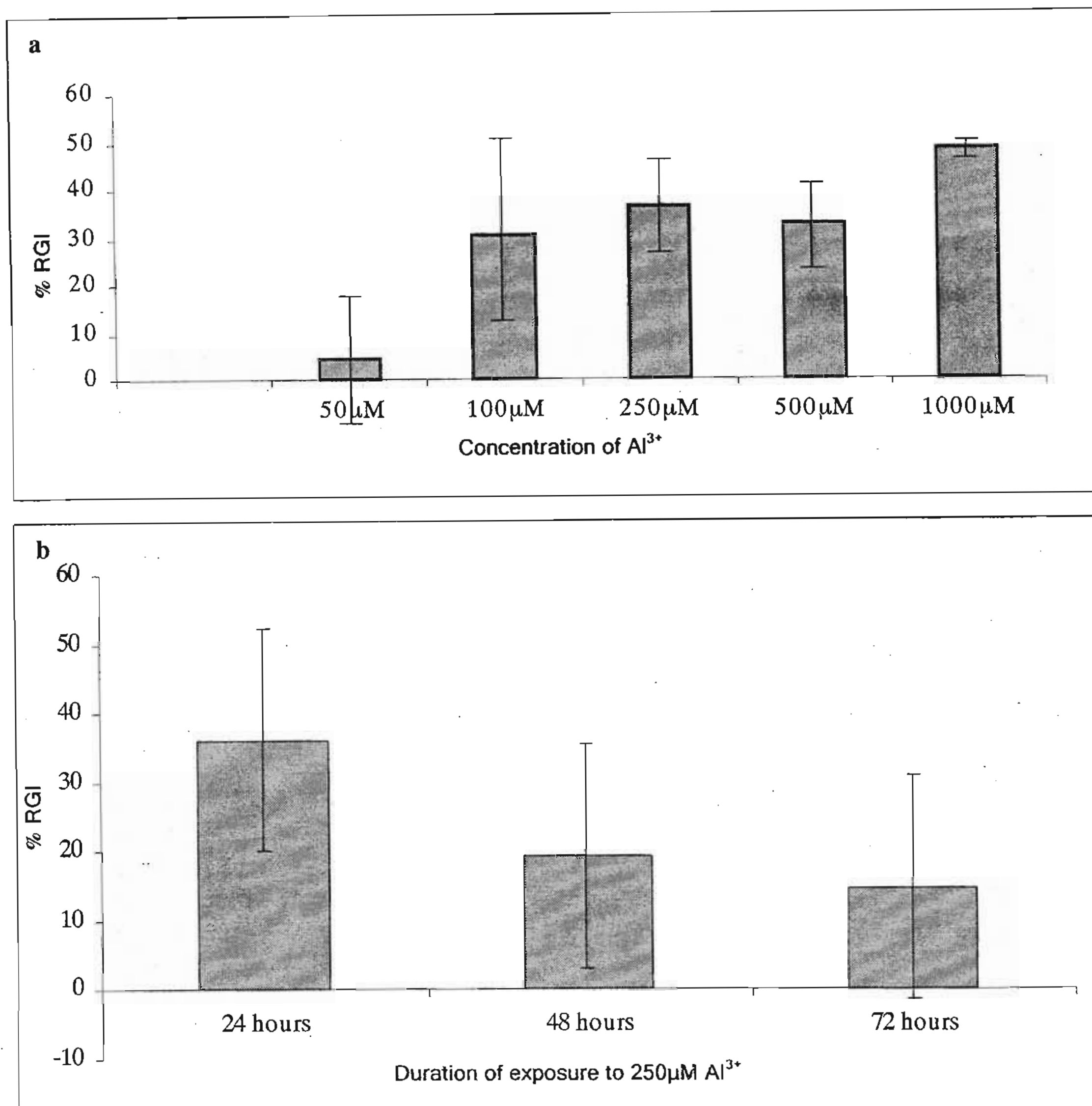

Fig. 6: Average % root growth inhibition (RGI) of hydroponically-cultivated N12 plants as a result of exposure to aluminium ($AlCl_3$ in 1mM $CaCl_2$ at pH 4.15) (a) supplied at various concentrations for 24 hours, and (b) supplied at 250µM over a 72 hour period.

The bulk of RNA within a cell consists of ribosomal RNAs, mostly in the form of the 18S and 28S rRNA, with poly-A^+ RNA (mRNA) comprising only up to 5% (Wilkinson, 1991). Hence, to increase the sensitivity of detection of less abundant transcripts, such as those differentially expressed under Al stress, rRNA sequences were removed from the pool of total RNA.

As most messenger RNA sequences contain long portions of adenylic acid residues, located on the 3'-end of intact transcripts (Davis *et al.*, 1986), the presence of this poly A^+-tail, typically 50 –100 bases long, allows for the purification of mRNA from the total RNA pool by means of

affinity chromatography (Wilkinson, 1991). Isolation of mRNA from total RNA preparations were based on a method in which oligo(dT) chains, coated on to magnetic beads (Dynal® Dynabeads mRNA Purification kit), bound the poly A^+ RNA in the presence of a high concentration of salt, and non-poly A^+ species were removed along with the supernatant. Subsequent washes with low salt buffers then allowed for the elution of the poly A^+ mRNA. This magnetic procedure for the isolation of mRNA was much faster and more efficient than other methods that require precipitation steps or the use of organic solvents. Typically, yields of mRNA were expected to be in the region of 1% of the total RNA used, which corresponds to the concentration of mRNA in the total RNA pool. Concentrations of approximately $1\mu g.\mu l^{-1}$ mRNA are not readily detectable via agarose gel electrophoresis unless multiple samples (10µl each) are pooled, and fluorometric and spectrophotometric methods of quantification were also not suitable. The concentration of $1\mu g.\mu l^{-1}$ was thus assumed for each mRNA sample. Precautions for the prevention of RNA degradation were also strictly followed to ensure that mRNA degradation was eliminated, a crucial requirement for the synthesis of full-length cDNA and for the construction of representative cDNA libraries.

4.3.2 Conversion of mRNA into complementary DNA (cDNA)

The validity of any result obtained downstream from a cDNA library is dependent on the original quality of the library itself. Consequently, utmost care was taken to ensure that a representative library for each treatment (Al-exposure and control) was obtained. Fluorometric quantification of initial cDNA synthesis products revealed that yields were $1 - 3ng.\mu l^{-1}$. As such yields are considered low, the maximum volume of cDNA permitted was used in subsequent reactions. Digestion of the cDNA using restriction enzymes *Alu* I and *Rsa* I resulted in the reduction of the average size of the cDNA within each of the populations. This eliminated the preferential PCR amplification of naturally small cDNAs during the subtraction process. Once the digestion of the cDNA and ligation of the a1/a2 and b1/b2 adaptors on to the cDNA was complete (3.3.3), it was possible to amplify the samples via PCR to obtain sufficient product for the subtraction process. This is a major advantage of this PCR-based protocol (Patel and Sive, 1996), in that only very small amounts of starting material are required.

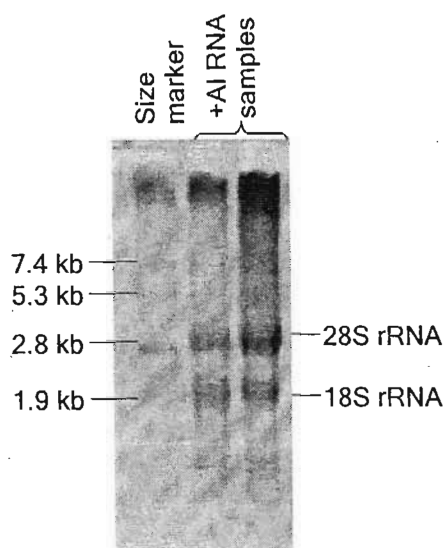

Fig. 7: Fractionation of representative RNA samples via denaturing agarose gel (1.2% [w/v]) electrophoresis, as described by Ingelbrecht *et al.*, 1998, and visualised via ethidium bromide staining and short-wavelength UV radiation. Lane 1: RNA size ladder 1 (Roche); Lane 2 & 3: 10µg RNA samples isolated from root tips exposed to 250µM Al ($AlCl_3$ in 1mM $CaCl_2$) for 24 hours using a hydroponics system.

4.3.3 Isolation of cDNAs associated with the aluminium phytotoxic response

On average, eukaryotic cells contain approximately 1pg of mRNA (equivalent to one million molecules) which has resulted from the transcription of about 15 000 different genes (Sargent, 1987). Of these one million mRNAs, the majority are considered to be of low abundance and present at less than 20 copies per cell. The detection of a rare transcript thus becomes very difficult, unless the effective concentration is further increased via subtractive hybridisation to remove common and abundant sequences. Although some redundancy is still expected in a subtracted library, the process of subtraction significantly reduces the number of clones that need to be analysed for the detection of sequences of interest. This approach has been estimated to improve the sensitivity of screening at least ten-fold (Sargent, 1987). The number of rounds of subtraction that are required to adequately remove common sequences varies from tissue to tissue and cell-type to cell-type, depending largely on the complexity of the initial cDNA populations, that is, the total number of different cDNA sequences present in each population. This can vary anywhere between five and 20 rounds of subtraction, however, the number of handling steps should be restricted as much as possible to minimise handling and the accidental

loss of trace amounts of cDNA. After each round of subtraction, 5µl aliquots of PCR amplified cDNA were fractionated by means of agarose gel electrophoresis, stained with ethidium bromide and viewed under short wavelength UV radiation (Fig. 8). A visible difference in the cDNA smear size and banding profile was observed between the two populations (A and B), as common sequences were removed through streptavidin binding and removal of the biotinylated tracer-driver and driver-driver hybrids. As no change was observed in the cDNA profile between the fourth, fifth and sixth subtractions, six rounds of subtraction were thus deemed sufficient to remove the majority of common and abundant sequences in this study.

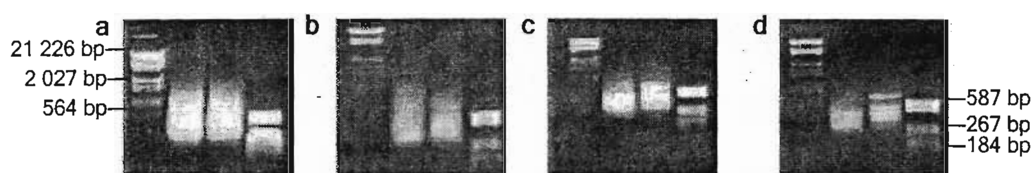

Fig. 8: Progressive removal of common abundant sequences from two populations of cDNA over the first four cycles of hybridisation (a: 1st cycle; b: 2nd cycle; c: 3rd cycle; and d: 4th cycle) via PCR based subtraction. (Lanes 1: Marker 3 [λDNA digested with *Eco*RI and *Hind*III]; Lanes 2: Tracer cDNA A [Al-exposed treatment]; Lanes 3: Tracer cDNA B [control treatment]; Lanes 4: Marker 5 [pBR322 digested with *Hae*III].

4.3.4 Cloning of subtracted cDNAs

The Lambda ZAP® II vector system is a useful combination of the convenience of a plasmid system, with blue-white colour selection, with the high construction and packaging efficiency of a lambda library. The system also allows for the *in vivo* excision of the pBluescript ® phagemid, allowing for characterisation of the insert in a plasmid system.

Once subtraction was completed, and a final round of PCR amplification performed, the two cDNA populations were prepared for cloning into the Lambda ZAP® II Pre-digested *Eco*RI/CIAP-Treated vector (Stratagene). This involved the additional step of ligating *Eco*RI adaptors onto the amplified B cDNA, as no internal *Eco*RI site was present in the b1/b2 adaptor set, only an *Eco*RV site, which was not suitable for cloning into this particular vector. While these new adaptors were pre-digested, the corresponding restriction site in the a1/a2 adaptors on the A cDNA needed to be cleaved with *Eco*RI before ligation into the vector. Once both sets of cDNA had been ligated into the cloning vector, they were termed SubA and SubB (subtractive libraries A and B).

4.4 GENERAL ANALYSIS OF ALUMINIUM-CHALLENGE-ASSOCIATED cDNA LIBRARY

The titre of the libraries was determined via the preparation of serial dilutions, followed by the plating out thereof with appropriate host cell lines, and counting the number of plaques (Table 6). The Sub A primary library titre (8.8×10^5 pfu.ml^{-1}) was approximately 6 fold below the range deemed acceptable by the manufacturers (5×10^6 to 1×10^7 pfu.ml^{-1}, Stratagene). Despite a repetition of the packaging process, the titre still did not improve to within this acceptable range. The titre of the Sub B library (6.5×10^4)was almost 100 fold below this acceptable range, and similarly did not improve after a further attempt at packaging. These libraries were nevertheless used, as increasing the amount of phage stock used during the plating out of the library allowed for sufficient clones to be obtained for further analysis. Furthermore, differential expression results associated with Al stress would be obtained from the Sub A library, the titre of which was within acceptable limits. The primary libraries were unstable and not suitable for long-term storage at 4°C, with a rather dramatic decrease in titre observed after six months, thus necessitating the amplification of the primary libraries to obtain stable high titre stocks for long-term storage.

Table 6: Characteristics of Lambda ZAP® II subtractive libraries prepared from mRNA isolated from N12 root tips. Sub A - Al-exposure treatment (250µM $AlCl_3$ [in 1mM $CaCl_2$] for 24 hours); Sub B - control treatment (1mM $CaCl_2$for 24 hours).

Library	Primary titre (pfu.ml^{-1})	%Non -recombinants	Amplified titre (pfu.ml^{-1})	Average insert size (bp)*
Sub A	8.8×10^5	4%	1.6×10^{10}	485
Sub B	6.5×10^4	28% (very high)	2.2×10^9	305

* Excluding the length contribution made by vector arms (approximately 125 bp)

The average insert size of each library was determined via the sampling of a number of random plaques, followed by PCR amplification of the insert and fractionation by means of agarose gel electrophoresis (Fig. 9, Table 6). The average sizes of the inserts was found to be approximately 485 and 305 bp for the Sub A and Sub B libraries respectively, which compares favourably with the expected size range of between 250 and 500bp (Patel and Sive, 1996). The inclusion of X-Gal and IPTG into the plating medium enabled the identification of non-recombinant clones (containing no insert) and thus the percentage of these clones present in the library. The manufacturers of the cloning kit indicate that between 1 and 10% non-recombinants are to be expected, with the Sub A library falling within these limits with 4% of clones containing no insert. The Sub B library, however, contained approximately 28% non-recombinant clones,

which is considered rather high, almost three times that which is considered acceptable. As with the lower titre, the packaging process was not optimised to attempt to rectify the characteristics of this library to within the recommended limits. Instead, when selecting clones for downstream applications, appropriate care was taken to ensure that non-recombinant clones were not sampled.

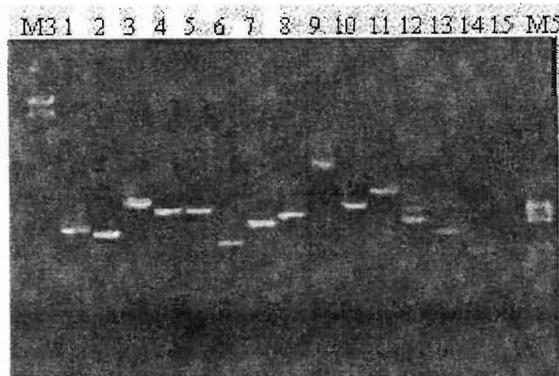

Fig. 9: A subset of PCR amplified plaques from subtractive library A, fractionated via agarose 1.2% (w/v) gel electrophoresis. cDNA library was prepared from mRNA isolated from N12 root tips exposed to 250µM Al for 24 hours in hydroponics. Note multiple banding in lanes 12, 13 and 14. Such samples were discarded.

4.5 EXPRESSION ANALYSIS OF ALUMINIUM-CHALLENGE ASSOCIATED cDNA LIBRARIES

4.5.1 Reverse Northern hybridisation analysis

DNA array technology is currently at the forefront of gene expression research, surpassing differential display as the technique of choice for the isolation of differentially expressed genes. Labelled mRNA transcripts are bound to arrays of cDNA molecules fixed to a solid support, constructed from glass, silicon, nitrocellulose or nylon membranes. Membrane-based arrays have been shown to be extremely useful in the detection of low-abundance messenger RNA sequences, with detection limits estimated to be approximately one mRNA transcript out of a population of ten thousand (Bouchez and Höfte, 1998). With the inclusion of subtractive hybridisation, the detection sensitivity for rarer differentially expressed sequences can be increased even further. One of the main advantages of array technology is that information on hundreds or even thousands of specific genic fragments can be obtained simultaneously, giving a broader view of gene expression changes between samples (Baldwin *et al.*, 1999). This technology was thus employed to identify mRNA sequences that were preferentially expressed under conditions of Al stress.

a. Array querying

The array containing cDNA sequences from the Sub A library was hybridised to radio-labelled target cDNA from the Al-challenge and control treatment. Generally, the signal strengths of individual spots on the array were not very strong, possibly due to a low concentration of probe on the membranes. Membranes were thus left to expose to the phosphor screens for longer periods, sometimes up to one week, to obtain images with sufficient resolution to allow for further analysis (Fig. 10).

b. Array analysis

From the raw data (Fig. 10) it was clear that some clones appeared to be differentially expressed under conditions of Al stress, with very faint or no signal at certain addresses on the array queried with the control total cDNA, and a clear signal obtained with Al-exposed total cDNA. However, the subtractive process is not totally effective at removing all common sequences, and there were many clones that hybridised with equal intensity to both target cDNA populations.

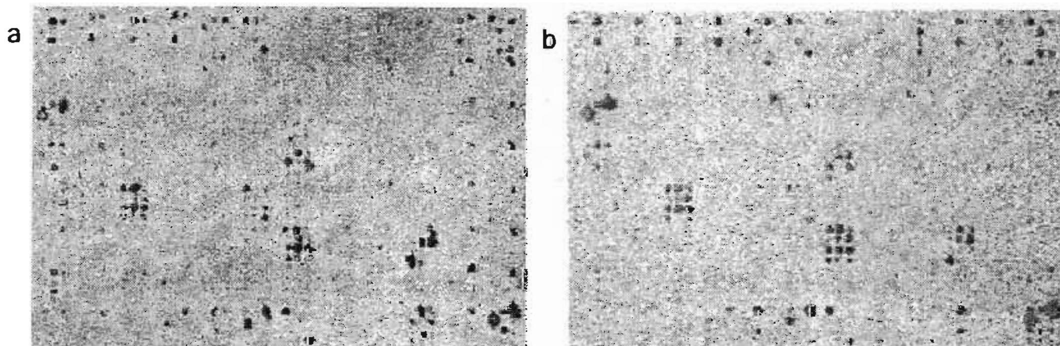

Fig. 10: Probing of total mRNA, isolated from (a) Al-exposed (250µM Al in 1mM $CaCl_2$ for 24 hours) N12 root tips and (b) from control treatment (1mM $CaCl_2$ for 24 hours) root tips, with subtractive library A (control treatment cDNA subtracted from Al challenge cDNA) array.

Array images were further analysed using QuantArray® Microarray Analysis Software (Version 3.0, Packard Bioscience). This software allowed for the superimposition of raw data images (a) and (b) (Fig. 11) and calculated quantitative comparisons of signal intensities for each address on corresponding array images queried with the two different target cDNA populations.

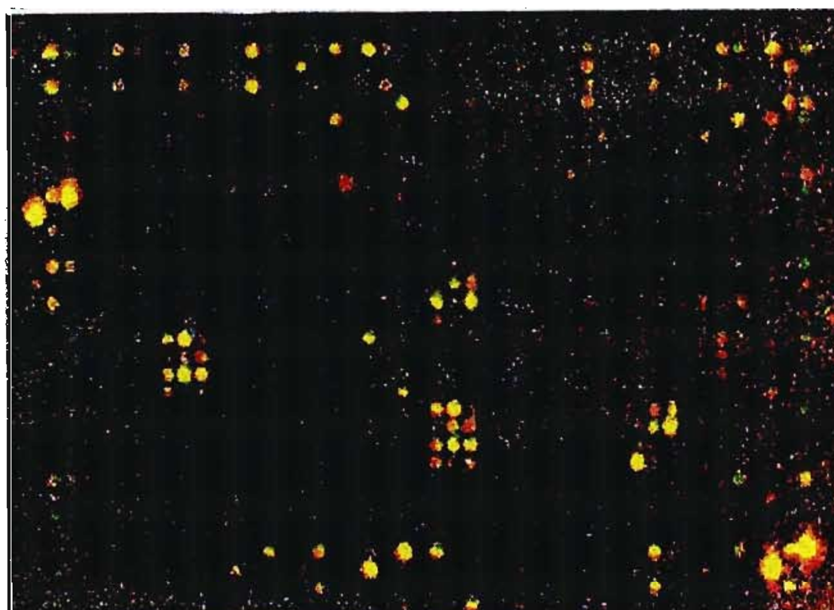

Fig. 11: Superimposition of array image results from the hybridisation of the Sub A (Al-specific) library array to control treatment and to Al-exposure cDNA, using QuantArray® Microarray Analysis Software (version 3.0, Packard Bioscience). Regions of green signal indicate signal from control treatment cDNA hybridisation, regions of red signal indicate signal from Al-exposure cDNA hybridisation, and yellow indicates regions of overlap between the red and green signals.

Relative expression at each address on the array under the two different treatment conditions was also represented in the form of pie-charts (Fig. 12). Due to the faintness of the signal at many locations on the array, the brightness filter for spot intensity brightness was set at 25. Spots with intensities lower than this threshold value were not distinguishable from background signal and thus not considered reliable data points. Expression of a clone under control conditions and Al stress conditions were represented by green and red fractions of the pie respectively. Spots indicating a red fraction of greater than half were regarded as being preferentially expressed under conditions of Al stress and thus of interest to this study.

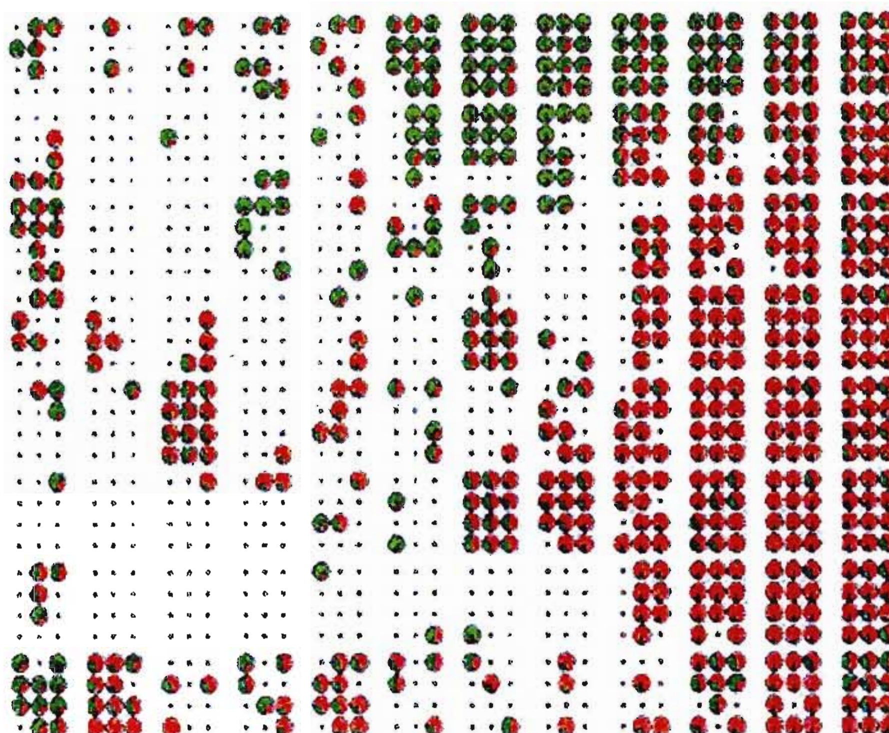

Fig. 12: Pie representation of the relative expression of array clones under conditions of Al-stress (red) and control treatments (green) using QuantArray® Microarray Analysis Software (version 3.0, Packard Bioscience). Smaller pie charts indicate data points below the brightness threshold of 25; larger pie charts indicate data points above the brightness threshold of 25.

The data from figure 12 was also presented in the form of a spreadsheet, using the QuantArray® Microarray Analysis Software, whereby specific values for the relative expression of different clones under the Al challenge and control conditions could be obtained. Clones were grouped into ten categories of 10% intervals according to their expression levels under conditions of Al stress as a percentage of the total expression under both sets of conditions. For example, clones grouped into the 10.1 - 20% category for expression under conditions of Al stress would be expected to have a corresponding expression level under control conditions of between 80.1 – 90%. These categories were represented in the form of a pie-chart (Fig. 13), which gave an indication of how many specifically Al-induced genes were present in the subtracted Al-exposed cDNA library. From this figure, 33% of the clones that were arrayed could be classified as inducible under conditions of Al stress while the remaining 67% were preferentially expressed under control conditions.

4.5.2 Confirmation of differential expression patterns: Northern hybridisation analysis

Clones that displayed preferential hybridisation with labelled cDNA generated from Al-stressed root mRNA were selected for Northern hybridisation analysis. This served as a confirmation of the differential expression of certain mRNA transcripts under Al treatment, as indicated by array data. Eleven different clones were selected for Northern hybridisation analysis and used to probe size-fractionated total RNA from control and Al stress treatments. However, no conclusive results were obtained from these analyses, with no distinct hybridisation signal for the RNA of either treatment (Fig. 14). It is likely that these clones represented very rare transcripts in the mRNA population used to synthesise the labelled total cDNA populations, thus accounting for the inability to obtain a detectable signal.

4.6 CLONE IDENTIFICATION

Twenty-five clones that displayed Al-induced expression patterns, as indicated by array data (Figs 10 to 12), were selected for sequence analysis. The cDNA inserts of these clones were excised from phage particles, converted into plasmid form and subjected to single run partial sequencing. All of the fragments were sufficiently short due to the restriction digestion of cDNA during library construction, allowing for complete sequencing of the clones. Sequences were edited using Sequence Navigator 1.0.1 to remove primer and vector arm sequences and these edited sequences submitted to the NCBI EST and non-redundant protein databases for homology searches using the BLASTn and BLASTx algorithms respectively (Table 7).

A certain degree of redundancy existed in the SubA library, with only 16 different sequences out of a possible total of 25 (36% redundancy). There were also seven ribosomal sequences identified (60S rRNAs, L2, L12 and L34). The EST identities were assigned with greater confidence than the non-redundant protein identities, with the expect (E) values for the former all extremely low, even zero in several cases, and bit scores above 130. The non-redundant protein identities were not as easily assigned, with many sequences displaying relatively low bit scores of less than 80, as well as relatively high E-values. In most instances, the protein identities assigned were based on homologies with proteins of plant origin, including genetically well-characterised species such as *Arabidopsis thaliana, Solanum tuberosum, Oryza sativa,* and *Zea mays.* Two of the proteins were not of plant origin, namely the probable flagellar protein (*Trypanosoma cruzi),* and the UV radiation resistance associated gene *(Homo sapiens).* However, the homologies to these proteins were low, with bit scores of 37 and 32 respectively,

and E-values of 0.034 and 1.0. These identities, therefore, could not be assigned to these clones with any degree of confidence and were thus rejected as putative protein identities. It is likely that these sequences encode very different proteins, the functions of which have yet to be elucidated in plants.

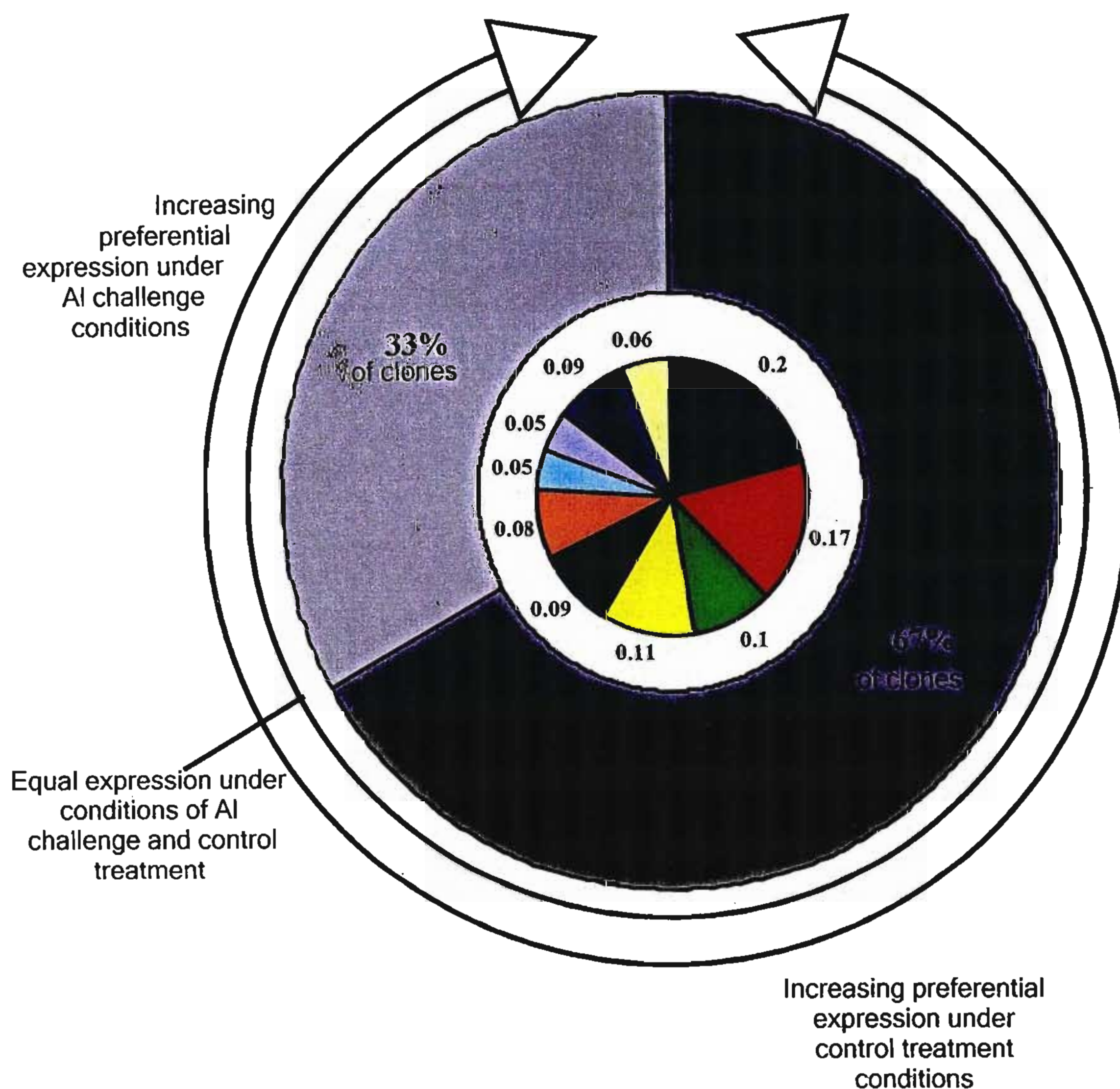

Fig. 13: Proportion of clones preferentially expressed under conditions of Al challenge (250µM Al in 1mM $CaCl_2$ for 24 hours) and under control conditions (1mM $CaCl_2$ for 24 hours). Values allocated to inner pie-segments indicate the proportion each segment represents of combined expression in both challenge and control treatments. Segments represent groupings of clones falling within 10% intervals of percentage expression under Al challenge conditions.

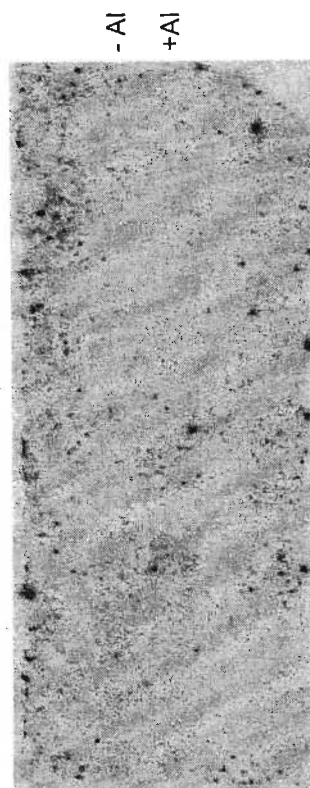

Fig. 14: Representative phosphor-image from Northern hybridisation analyses, using putative Al-induced genic fragment to probe membrane bearing 10µg total RNA isolated from Al-challenged (+Al) (250µM Al in 1mM $CaCl_2$ for 24 hours in hydroponics) N12 root tips and 10µg total RNA from control (-Al) (1mM $CaCl_2$ for 24 hours in hydroponics) root tips.

Another of the protein identities assigned was that of a root specific protein, isolated in rice (*Oryza sativa*). Although this gene may not be relevant to the Al stress response of the plant, it offers the potential for the isolation of root specific promoters for applications in root-targeted gene expression in sugarcane. Consequently, this cDNA fragment was used to probe a membrane bearing RNA isolated from different parts of the sugarcane plant, including the root and the leaf, as well as from an undifferentiated cultured cell mass (callus). The root, leaf and callus material were exposed to Al stress (250µM for 24 hours) prior to RNA isolation to determine the possible effect of Al stress on differential expression of this root specific sequence. Also included in the assay were diamide stressed roots to determine the possible effects of oxidative stress on differential expression. The RNA samples were prepared and fractionated as previously described (section 3.5.2) and transferred to positively charged nylon membrane (Hybond™ N^+, Amersham) (RNA membrane courtesy of Dr Derek Watt). The cDNA probe was radio-labelled and hybridised to the membrane as previously described (section 3.5.2) and exposed to high resolution phosphor screens (Cyclone™ Storage Phosphor

Screen [Packard]). After between 24 and 56 hours of exposure, the images on the phosphor screens were captured and viewed (Fig. 15).

The radio-labelled cDNA probe bound to the RNA from all the root samples, with the exception of the diamide-treated root sample. It is possible that this was the result of a degraded RNA sample, as the ribosomal bands in the corresponding gel image were not as distinct, with some smearing visible. The band corresponded to a size of approximately 600 bp, compared with the 260 bp size of the cDNA fragment, which was cleaved using restriction enzymes during the process of library construction and thus not full length. This band was not visible at all in the leaf and callus samples, indicating that this protein is indeed root specific. The band does not, however, appear to be limited to the samples exposed to Al, which indicates that it is not differentially expressed in response to the metal, as originally indicated by the array data.

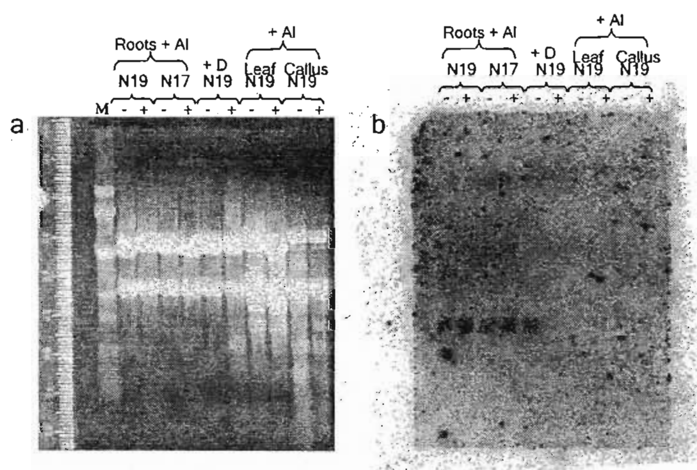

Fig. 15: Differential expression of an N12-derived root specific cDNA fragment in sugarcane cultivars N19 and N17. (a) Fractionated RNA samples, stained with ethidium bromide (b) Northern hybridisation analysis of root, leaf and callus RNA using root specific cDNA fragment as probe. (+Al: Al stress treatment ; +D: Diamide stress treatment; - : Control treatment).

Table 7: Putative identities and characteristics of selected sequences expressed in N12 root tips in response to challenge with 250μM Al for 24 hours in hydroponics.

Clone No.	cDNA size (bp)	Algorithm used to assign putative identities and characteristics against NCBI databases (dbEST and non-redundant protein)							
		BLASTn (dbEST)				BLASTx (nr)			
		Putative EST identity	Accession No.	Bit score	E-value	Putative protein identity	Accession No.	Bit score	E-value
1A.A1	431	Pathogen-induced *(Sorghum bicolor)*	BE599149	666	0.0	Ubiquitin conjugating enzyme *(Zea mays)*	AF034946	246	$2x10^{-65}$
5A.H1	442	Pathogen-induced *(Sorghum bicolor)*	BE599149	642	0.0	Ubiquitin conjugating enzyme *(Zea mays)*	AF034946	243	$3x10^{-64}$
1A.A2	274	Pathogen-infected compatible 1 *(Sorghum bicolor)*	BM330257	345	$1x10^{-92}$	60S Ribosomal protein L2 *(Lycopersicon esculentum)*	P29766	56	$4x10^{-11}$
1AE6	283	Pathogen-infected compatible 1 *(Sorghum bicolor)*	BE597703	371	$1x10^{-100}$	60S Ribosomal protein L2 *(Lycopersicon esculentum)*	P29766	97	$3x10^{-20}$
1A.A3	262	Rhizome 1 *(Sorghum halepense)*	AI723943	442	$1x10^{-122}$	60S Ribosomal protein L34 *(Arabidopsis thaliana)*	AC021046	66	$7x10^{-11}$
1A.A4	260	Dark grown *(Sorghum bicolor)*	BE362328	321	$2x10^{-85}$	Root specific protein (extensin-like)* *(Oryza sativa)*	S53012	121	$2x10^{-27}$
1A.A11	437	Ovary *(Sorghum bicolor)*	BE917673	678	0.0	Peptidyl prolyl *cis-trans* isomerase *(Zea mays)*	P21569	253	$2x10^{-67}$
1A.A12	459	Ovary *(Sorghum bicolor)*	BG412395	361	$4x10^{-97}$	Ubiquinol cytochrome c reductase *(Solanum tuberosum)*	P48502	75	$2x10^{-13}$
1A.D1	170	Ovary *(Sorghum bicolor)*	BG412682	134	$3x10^{-29}$	Ubiquinol cytochrome c reductase *(Solanum tuberosum)*	P48502	38	0.028
6A.E6	410	Ovary *(Sorghum bicolor)*	BG412395	357	$6x10^{-96}$	Ubiquinol cytochrome c reductase *(Solanum tuberosum)*	P48502	75	$1x10^{-13}$
8A.D7	446	Ovary *(Sorghum bicolor)*	BG412395	446	$6x10^{-96}$	Ubiquinol cytochrome c reductase *(Solanum tuberosum)*	P48502	75	$2x10^{-13}$

1A.B11	340	Dark grown (*Sorghum bicolor)*	BE360666	618	1×10^{-175}	Plasma membrane integral protein (aquaporin)* *(Zea mays)*	AF326487	224	1×10^{-58}
1A.C1	386	Embryo (*Sorghum bicolor)*	BG355725	664	0.0	Probable flagellar protein (*Trypanosoma cruzi)*	A61144	37	0.034
1A.C11	429	Rhizome 2 (*Sorghum propinquum)*	BG605767	341	3×10^{-91}	Putative nitrilase-associated protein (*Arabidopsis thaliana)*	AC006836	70	3×10^{-12}
1A.F9	306	Pathogen-infected compatible 1 (*Sorghum bicolor)*	BM327776	335	1×10^{-89}	Putative nitrilase-associated protein (*Arabidopsis thaliana)*	Z6936	70	3×10^{-12}
1A.H10	306	Pathogen-infected compatible 1 (*Sorghum bicolor)*	BM327776	335	1×10^{-89}	Putative nitrilase-associated protein (*Arabidopsis thaliana)*	Z6936	70	3×10^{-12}
1A.C12	282	Endosperm (*Zea mays)*	AI833933	349	9×10^{-94}	Elongation factor 1 alpha (*Cicer arietinum)*	AJ004960	124	2×10^{-28}
1A.D11	383	Juvenile leaf and shoot (*Zea mays)*	BI245224	531	1×10^{-148}	60S ribosomal protein L12 (*Arabidopsis thaliana)*	NP181256	531	1×10^{-148}
1A.D12	353	Juvenile leaf and shoot (*Zea mays)*	BI273486	531	1×10^{-148}	60S ribosomal protein L12 (*Arabidopsis thaliana)*	NP181256	182	4×10^{-46}
4A.G1	383	Juvenile leaf and shoot (*Zea mays)*	BI245224	523	1×10^{-146}	60S ribosomal protein L12 (*Arabidopsis thaliana)*	NP181256	178	7×10^{-45}
1A.E9	262	Rhizome 1 (*Sorghum halepense)*	AI723943	418	1×10^{-114}	60S ribosomal protein L34 (*Arabidopsis thaliana)*	NP177120	69	1×10^{-11}
1A.G11	337	Floral-induced meristem 1 (*Sorghum propinquum)*	BF656109	613	1×10^{-173}	Glyceraldehyde-3-phosphate dehydrogenase (*Zea mays)*	DEZMGC	194	1×10^{-49}
4A.G9	270	Immature panicle 1 (*Sorghum bicolor)*	BI099615	287	2×10^{-75}	UV radiation resistance-associated gene (*Homo sapiens)*	A012958	32	1.0
7A.H10	360	Mature stalk (*Saccharum* sp.)	AI105619	613	1×10^{-173}	Tubulin beta chain (*Hordeum vulgare)*	P93176	224	2×10^{-58}
3A.F10	110	Early embryo (*Zea mays)*	AW331644	167	3×10^{-39}	Putative UDP-glucose dehydrogenase (*Oryza sativa)*	AC079887	57	6×10^{-8}

* Protein identities in parenthesis indicate identities assigned of slightly lower homology than, but closely related to, the main identity

CHAPTER 5
DISCUSSION

Considerable progress has been reported in the pursuit of understanding Al tolerance in other crop species. Aluminium tolerance has been successfully enhanced in several crops, most notably wheat (Carver *et al.*, 1993; Barinaga, 1997; Scott *et al.*, 2001), with such advances encouraging similar research towards improving genetically less-characterised crop species such as sugarcane (Märländer, 2000). This study therefore aimed to analyse the expression of Al stress-induced genes in a specific sugarcane cultivar, N12, indicated from field data as being of a higher tolerance level than several of the other common commercial varieties currently cultivated in South Africa.

5.1 PERFORMANCE OF HYDROPONICALLY-GROWN SUGARCANE UNDER CONDITIONS OF ALUMINIUM STRESS

The primary requirement for such a study was the creation of a suitable system that enabled the controlled exposure of plants to Al. Hydroponics was the system of choice, affording the additional advantage of allowing easy access to the roots for growth and morphological observations and for subsequent harvesting of root material for molecular analysis. There has been little literature published outlining the use of hydroponics systems in sugarcane, particularly in the study of Al exposure related phenomena. Hetherington and coworkers (1986) described a rudimentary hydroponics set-up that was used to analyse Al tolerance ratings in Australian sugarcane cultivars. However, the plants used were newly germinated and thus still very dependent on their setts and sett roots for nutrient requirements, factors that may have impacted the results obtained by that group. Those workers (Hetherington *et al.*, 1986) also reported the absence of visible Al toxicity effects on the roots in their study, whereas brown necrotic regions and mucilage exudation were clearly observed in the Al exposed plant roots in this study. This discrepancy may also be attributed to the presumed use of sett roots by Hetherington's research team, and the inclusion of the nutrient rich setts with the plants during the exposure. These setts supply nutrients to the growing plant, thus lessening the reliance of the plant on nutrients obtained via the Al challenged root system. There is also the potential of interferance with the chemistry of Al ions in the rhizosphere and thus further off-setting the negative effects of Al.

The hydroponics system used in this study ensured that the plants were sufficiently mature to possess well-developed plant roots and thus be independent of the sett. As this entailed the use of approximately five-week old plants, a slightly modified method for supporting the plants within the system was employed, with Neoprene® collars securing the plants in their positions in the lid holes of each vessel. A similar hydroponics system was employed by Cramer and Titus (2001) during their investigation of the effect of dissolved inorganic carbon on Al toxicity in tomato plants. Seedlings were germinated and grown for 3 weeks in solid media prior to transferral to hydroponics, with the hypocotyls of the tomato plants similarly wrapped in foam rubber and inserted through the plastic lids of the nutrient vessels. Although there are some limitations associated with this set-up, such a hydroponics system for sugarcane has the potential for applications in other studies relating to root growth phenomena, such as the study of the effects of flooding, nematode predation, or even ion deprivation.

Traditional hydroponics systems have been successfully used in other crops to study the effects of Al on growth characteristics. Hamel and coworkers (1998) demonstrated that root growth inhibition in hydroponically-grown wheat seedlings was proportional to the dosage of Al supplied. The results obtained in that study showed that the inhibition of root growth in an Al tolerant wheat cultivar reached a plateau at a maximum value of approximately 70% when exposed to concentrations of 250μM Al. Sugarcane cultivar N12, however, displayed a notably more tolerant response to the metal, with only approximately 36% inhibition at 250μM Al. This was consistent with the literature, in which sugarcane is regarded as being more tolerant of Al than crop species such as wheat and maize (Sumner and Meyer, 1971; Hetherington *et al.*, 1986; Nuss, 1987). However, since no Al tolerance-specific breeding programmes have been undertaken in sugarcane as in these other crops, it is possible that this phenomenon has arisen serendipitously as screening for other quality traits has, in some instances, been conducted on acidic soils. Cultivar N12, in particular, is known to perform well on acidic soils, even reacting negatively to efforts to decrease the pH through the application of lime (Turner *et al.*, 1992). It thus follows that the probability of such a cultivar also performing well under conditions of Al stress is rather high, being adapted to cope with and indeed thrive in such a situation.

However, there does remain the possibility that this tolerance may be further enhanced, should a specific mechanism or genetic sequence associated with this trait be isolated, thus increasing the feasibility of Al tolerance-specific breeding and molecular engineering of cultivars.

5.2. EVALUATION OF MOLECULAR STRATEGIES

The fact that this particular sugarcane cultivar appeared to perform well in response to Al at dosage levels normally considered toxic to many other crop species, indicated the strong potential for the existence of a genetic basis responsible for this phenomenon. This study therefore further attempted to isolate any such genic fragments, with the view to the potential isolation of Al tolerance-related gene sequences.

5.2.1. Isolation and manipulation of genetic material

A critical step towards the isolation of putatively Al tolerance-related genes involved the isolation of intact genetic material from Al-exposed root apex cells. The isolation and comparison of RNA populations obtained from challenged and unchallenged root cells allowed for the identification of sequences differentially expressed under conditions of Al stress and thus possibly involved in the tolerance response. Thus, without the successful isolation of undegraded total RNA, this process would have become considerably less informative.

A paper by Espino and coworkers (1998) reported that Al-exposed calli cells yielded lower amounts of RNA than unexposed cells. This was attributed to the probable retardatory effect of Al on DNA synthesis, thus resulting in diminished synthesis of RNA. This could consequently impede the isolation and discovery of a sequence supposedly expressed in response to the very metal which is inhibiting its transcription. However, if such a sequence does confer the tolerant phenotype, then it could possibly be expressed on a constitutive basis, thus allowing the DNA and RNA synthesis systems of the plant to be constantly prepared for and thus unaffected upon exposure to Al. Furthermore, such a non-differentially expressed sequence would not be readily detectable by the comparison of Al-exposed and -unexposed root RNA isolates. However, there have been published reports of Al inducing the expression of certain transcripts (Ezaki *et al.*, 1995; Hamel *et al.*, 1998; Richards *et al.*, 1998), thus one cannot rule that the possibility of Al-induced inhibition of DNA and RNA production excludes the potential for the isolation of Al-induced transcripts. While the total RNA yields obtained in this study did not appear to decrease noticeably following the exposure of roots to Al, the feasibility does remain that Al may have affected the synthesis of DNA and thus certain RNA transcripts.

The conversion of this easily degradable RNA to more robust cDNA allowed for more rigorous analysis of this genetic material via the preparation and screening of subtractive cDNA libraries.

The populations of cDNA inserts within these libraries were enriched for genic fragments whose expression was putatively induced or enhanced by the exposure of the root tips to Al in the hydroponic challenge. The degree of this enrichment was tested via the implementation of the reverse Northern hybridisation technology, whereby aliquots of each library insert were individually spotted onto nylon membranes and hybridised to unsubtracted radio-labelled cDNA. Enrichment, or subtraction efficiency, was not as high as was expected, as evidenced by the high number of ubiquitously expressed ribosomal protein sequences present in the subtracted libraries. It should be noted that these sequences were for ribosomal proteins, the transcription and subsequent translation of such sequences being utterly necessary for the production of ribosomal units and thus equally essential for the continued expression of mRNA in the cell. This did not, therefore, indicate rRNA contamination, which would have suggested a problem with the poly(A)$^+$ RNA isolation protocol. This would have lead to gross under-representation of mRNA sequences and rendered the cDNA libraries largely uninformative. However, the process of cDNA subtraction should have removed the majority of abundant sequences as well as those common to both sets of treatments. This would presumably have included ubiquitous transcripts such as those encoding the ribosomal proteins. From these results, where ribosomal protein sequences constituted a considerable 28% (7 of the 25 sequenced clones), one must assume that either subtraction efficiency was not very high, or that conditions of Al stress did, in fact, significantly enhance the expression of these ribosomal sequences.

Patel and Sive (1996) recommended between five and 20 rounds of subtractive hybridisation for a 20-fold enrichment of cDNA in each population. Sagerström *et al.* (1997) conservatively estimated that a 50- to 100-fold enrichment is to be expected after only the first one or two rounds of subtraction, with more complex and diverse cell populations requiring more rounds to achieve this same level of enrichment. The fractionation profiles of the two cDNA populations in this study appeared to indicate sufficient subtraction had occurred after six rounds of hybridisation, with little observable changes in the profiles after four rounds. However, unsuccessful subtractions are usually attributable to insufficient rounds of subtraction, thus it strongly appears that six rounds of subtraction were not sufficient. Future studies should, therefore, seek to employ further hybridisation steps to ensure more efficient removal of abundant and common sequences to enhance sensitivity in the detection of rarer and differentially expressed genes.

5.2.2 Applicability of array technology to this study

Insufficient subtraction was further evidenced during the hybridisation of arrays representing each library with unsubtracted radio-labelled cDNA populations. For example, Al treatment total cDNA should have displayed considerably less hybridisation with the control treatment array than with the Al treatment array, due to the removal of common sequences. However, this clear distinction between the two membranes was not observed in this study, reinforcing that the perception that the level of subtraction was perhaps not sufficient to remove the majority of common and abundant sequences. A suggestion by Sagerström *et al.* (1997) was the substitution of unsubtracted with subtracted radio-labelled cDNA populations for the analysis of arrays. Not only has this been shown to increase sensitivity ten-fold in the detection of rare clones, but it simultaneously allows for the distinguishing of differentially expressed from those that are merely rare.

Perhaps a further adjustment to improve the likelihood for the detection of differentially expressed sequences would be the inclusion of more clones on the array membrane to increase the representativeness of the cDNA libraries. Increasing the number of clones randomly sampled and screened from each library would, therefore, serve to increase the potential for the isolation of putative Al-induced transcripts.

Although this study was not successful in obtaining conclusive results regarding the differential expression of Al induced transcripts in sugarcane roots, array technology remains a very useful and powerful technology in the search for genes associated with particular responses. With the incorporation of the adjustments mentioned, such an Al stress related sequence may yet be isolated in sugarcane using this approach.

5.2.3 Inconclusive Northern hybridisation analyses

Despite the apparent enhanced expression of some clones under conditions of Al stress, as indicated by array data, such results were not confirmed when subjected to Northern hybridisation analyses. This is of concern, as this confirmatory step is essential if a clone is to be identified as truly differentially expressed. Several of these analyses were performed, with several different clones used as probes, yet no conclusive results were obtained. No specific hybridisation signal was obtained with either the control or experimental RNA. The quantity of total RNA fractionated and transferred to each membrane was then increased from 10 to 15μg to

allow for the possibility that probe sequences were low-level expressors in both treatments. However, even under these conditions, conclusive hybridisation signal was still not obtained.

The Northern hybridisation performed using the root specific sequence as probe did yield a result, confirming the root specificity of the sequence, but not the Al stress-induction of expression. This particular membrane was prepared independently by another researcher (Dr Derek Watt), using independently isolated RNA. Membranes prepared for this study using RNA isolated from this study again did not show any hybridisation with this root-specific probe. It thus appears that the problem lay with the quality of the membranes, and not with the probe or hybridisation protocol. Although the fractionated RNA appeared undegraded, there remains the possibility that some sequences may have undergone some degradation to the extent that hybridisation was affected. Furthermore, the possibility that RNA was not permanently fixing to the membranes was also considered, with the consequent removal of most RNA from the membrane during prehybridisation. This would account for the lack of signal observed. However, the phenomenon of RNA not adhering to the membranes, despite the use of alkaline transfer medium during blotting, could not be readily explained, although preliminary analyses by a colleague indicate that this may indeed be occurring.

One suggestion to combat the problems associated with the Northern hybridisation analyses has been the implementation of virtual Northerns, one of the applications mentioned in the literature accompanying the SMART™ cDNA kit (CLONTECH). This technique involves the conversion of easily degradable total RNA to more stable cDNA, using the SMART™ cDNA synthesis kit, prior to fractionation, thus minimising the loss of transcripts during the preparation of the membrane. This modification shall thus be strongly considered for future studies in this field.

5.3 EFFECT OF ALUMINIUM ON GENE EXPRESSION IN SUGARCANE ROOTS

There have been several successes reported with regards to the isolations of Al-induced gene transcripts in other plant species, some of which have been putatively linked to the tolerant phenotype. In wheat, sequences encoding genes for cysteine-rich proteins and metallothionein-like metal-regulated proteins were isolated by differential screening of cDNA libraries (Snowden and Gardner, 1993). Oxidative stress-related genes have been isolated in response to Al exposure in *Arabidopsis thaliana* (Richards *et al.*, 1998). Some examples include peroxidase, glutathione-s-transferase, superoxide dismutase, Bowman-Birk protease inhibitor and blue

copper-binding protein. An oxidoreductase enzyme, normally induced by pathogen treatment, was also found to be induced under conditions of Al stress.

No gene sequence previously related to proposed mechanisms of Al tolerance in other crop species were immediately apparent from the 25 clones sequenced and identified in this study. These clones all appeared to be involved in general housekeeping functions, such as mitochondrial respiration and the production cytoskeletal elements (see Table 8), with no stress-related themes seeming to emerge. Many of the clone inserts were fairly small in size, ranging from a mere 110bp to 459bp, but this is largely attributable to the restriction digestion step in the subtraction protocol. While this step ensured that preferential amplification of naturally smaller transcripts did not occur, it did result in libraries containing shorter inserts, to which identities are, unfortunately, more difficult to assign. While shorter sequences may have been sufficient to assign a clone to a particular family of proteins, the specific function may yet remain elusive. One suggestion to overcome this obstacle would be the probing of full-length cDNA libraries with these shorter fragments. However, this is a rather time-consuming and labour-intensive route, and was not considered due to the time constraints on this study.

Aluminium has been shown to have deleterious effects on the expression of certain cytoskeletal regulatory genes (Cruz-Ortega *et al.*, 1997). Blancaflor *et al.* (1998) have also shown the positive association of Al toxicity symptoms with changes in the organisation and stability of cytoskeletal elements in maize. Tubulin is regarded as the major constituent of microtubules and thus an integral part of the cell cytoskeletal structure. Another structurally-related protein sequence identified was that of the extensin-like clone. Reported expression in response to wounding in tomato stems has been shown by Showalter *et al.* (1991). More recently, however, the expression of extensin has been demonstrated to be up-regulated in *Pisum sativum* in response to ozone stress, and indeed under conditions of NaCl stress and Al stress. Although the expression of these sequences in sugarcane has not conclusively been shown to be Al-induced, there remains the possibility that the gene expression products of these sequences may somehow be involved in the response of this cultivar to Al exposure.

Also of interest is the reported dark-induction of several oxidative stress-related genes in *A. thaliana* (Richards *et al.*, 1998). Two of the clones isolated from sugarcane in this study displayed EST homology to dark-grown *Sorghum bicolor* sequences, which may be linked to an oxidative stress response. The protein identities assigned to these clones were the root specific

or extensin-like protein and the PM integral protein, the former having been linked to oxidative stress (Sävenstrand *et al.*, 2000).

It has also been shown that expression of elongation factor 1 alpha in plants is influenced by environmental factors, such as wounding (Morelli *et al.*, 1994) and low temperature (Berberich *et al.*, 1995). Strains of yeast that contain mutant alleles of this sequence have displayed several important phenotypes, including altered growth patterns, resistance to antibiotics, and reduced accuracy in translational events. Any changes in the activity of this gene product thus have the potential to have a marked impact on the cell. There is thus the possibility that the enhanced expression of this sequence under conditions of Al exposure may somehow be involved in the response of the plant to the metal, whether it be the toxicity response or the activation of a tolerance mechanism. However, conclusive evidence linking the enhanced expression of this gene with Al has yet to be shown.

Also worth noting is the reported perturbation of ubiquinol cytochrome c reductase activity in rat livers in response to Al-binding (Toninello *et al.*, 2000). This has been proposed to favour the production of reactive oxygen species, resulting in oxidative stress. While nothing conclusive can be drawn regarding the interaction of Al with this enzyme in plant cells, it may provide some clues as to a potential mechanism of or response to Al toxicity in sugarcane.

Several of the other protein identities putatively assigned have also been shown to have some role in certain stress-response pathways in addition to primarily house-keeping functions. Some ubiquitin conjugating enzymes have been shown to be more highly expressed in response to heat-shock and certain heavy metal ions (Shyi-Kae *et al.*, 1999). It is possible that these enzymes function within their known cellular role of targeting malformed proteins for degradation, proteins possibly formed as a result of the applied stress (Clark *et al.*, 1997). The induction of peptidyl prolyl *cis-trans* isomerases under conditions of environmental stress have also been reported. In maize, regulation of a cyclophilin, a particular class of peptidyl prolyl *cis-trans* isomerase, by salicylic acid has also been shown (Marivet *et al.*, 1995), a substance known to be involved in one of the stress response pathways of plants (Durner *et al.*, 1997; Varet *et al.*, 2002) Plasma membrane proteins and, more specifically, aquaporins, are known to be very much under the developmental and hormonal control of the plant, as well as strongly influenced by environmental factors (Maurel, 1997). Due to their role in water transport across the PM, these proteins are thus believed to play a possible role in the regulation of overall water balance in plant cells under conditions of stress (Maurel, 1997). Glyceraldehyde-3-phosphate

dehydrogenase and nitrilase protein families have also been shown to have enhanced expression in response to anaerobiosis (Sivalinganna and Sachs, 1997) and bacterial infection (Pace and Brenner, 2001) respectively. While there has been no evidence directly linking these genes to Al-related phenomena, there does remain the possibility that these sequences may in some way be involved in the general stress responses of sugarcane.

The root specific protein was also potentially interesting in that the response to Al exposure by a plant is primarily a root specific phenomenon, and thus the isolation of such a protein may have relevance to this response. The root specific protein with which significant homology to this clone was obtained was isolated from rice, with a putative function yet to be assigned. The root specificity of this sequence in sugarcane was confirmed through Northern hybridisation analysis, and although this sequence does not appear to be Al-induced, it does indeed appear to be root specific. Such a sequence could have practical applications in the area of genetic modification, where the availability of potentially strong promoters to express useful traits in a root specific manner would be very useful, particularly in the area of resistance to nematodes and other root pathogens (De Pater and Schilperoort, 1992; Bower *et al.*, 2001).

5.4 FUTURE WORK

The production of transgenic crop varieties has been regarded as an important contributor in the pursuit for increased agricultural production (De la Fuente-Martínez and Herrera-Estrella, 1999) and the manipulation of abiotic stress responses in plants has thus been the focus of many research efforts. Tolerances to abiotic stresses are generally complex traits, due to the wide range of morphological and physiological variables involved. These include plant structure, osmotic adjustment, membrane and protein stability, antioxidant capacity, hormonal regulation and root morphology and depth (Ribaut *et al.*, 2002). Tolerances to heat, cold and salinity have been modified in a variety of species, such as *A. thaliana* and millet (*Setaria italica* L.) (Alia *et al.*, 1998; Sreenivasulu *et al.*, 1999). Kasuga and co-workers (1999) succeeded in producing a line of *A. thaliana* modified to contain the gene encoding transcription factor *DREB1A*, responsible for the regulation of several stress tolerance genes. The resulting transgenics were able to tolerate drought, salt and cold significantly better than wild-type individuals. This suggests that many of the stress responses in plants are inducible by a wide range of stresses, and are not specific to one particular set of conditions. The manipulation of one such pathway could thus result in tolerance to many abiotic factors. Some of the genic fragments isolated in

this study may also be related to a general stress response in sugarcane, but should first be further investigated to confirm their involvement in the response of the plant to Al stress.

Nine different genes, previously reported to be expressed under conditions of Al stress in *A. thaliana,* tobacco, yeast and wheat, were expressed in *A. thaliana* by Ezaki and co-workers (2000). The resultant transgenic individuals displayed no apparent deleterious effects on phenotypic expression due to the transgene, with four of the nine genes actually enhancing the Al tolerance of the plant containing the corresponding transformation. Similarly, the expression of a bacterial citrate synthase gene in tobacco and papaya (De la Fuente *et al.*, 1997) has been demonstrated to enhance tolerance to Al, due to the chelatory role of organic acids such as citric acid in the immobilisation of Al in the rhizosphere.

The potential exists for the transformation of crop species with Al tolerance related genes with the view towards improving crop performance on acidic soils. Although the production of transgenic sugarcane plants is based on a seemingly simple concept, in practice there are many biochemical and biological steps involved (Moore, 1999). Fortunately, several characteristics of sugarcane have already been altered genetically, such as the inclusion of the *bar* gene for herbicide resistance (Gallo-Meagher and Irvine, 1996). The technology for the transformation of this crop is thus already in place, should an Al tolerance transformation programme be initiated. There does remain the possibility that the trait of Al tolerance in sugarcane may be multigenic, which would result in the trait being significantly more difficult to manipulate genetically. While in certain wheat cultivars, for example, tolerance has been reported to be under the control of a single gene (Delhaize *et al.*, 1993a), in other cultivars it has been shown to be multigenic (Aniol and Gustafson, 1990). Should such a finding reveal the trait to be multigenic in sugarcane, the genic sequences could still be useful with regards to MAS programmes and early selection of new tolerant cultivars. Thus the successful isolation of an Al stress induced gene sequence is but the first small step towards the production of Al tolerant sugarcane.

Table 8: Possible roles of clones putatively identified through sequence homology with the NCBI non-redundant protein database entries (BLASTx), expressed in N12 roots in response to Al-induced stress.

Protein identity	Acc. no.	Possible role in the cell	Reference
Ubiquitin conjugating enzyme	AF034946	Provides cellular mechanism for the targeting of short-lived and malformed proteins for degradation	Clark *et al.*, 1997; Shyi-Kae *et al.*, 1999
Peptidyl prolyl *cis-trans* isomerase	P12569	Potential molecular chaperone under conditions of environmental stress, mediating and maintaining correct 3-D structures of proteins	Marivet *et al.*, 1995; Kurek *et al.*, 1999
Ubiquinol cytochrome c reductase	P48502	Forms part of Complex III in the mitochondrial respiratory chain, which catalyses electron transfer from succinate and nicotinamide adenine dinucleotide-linked dehydrogenases to cytochrome c	Toninello *et al.*, 2000; De Lonlay, *et al.*, 2001
Root specific protein (extensin-like)*	S53012	Structural protein of the cell wall, involved in cell expansion and required for correct development of root hairs	Showalter *et al.*, 1991; Arsenijevic-Maksimovic *et al.*, 1995; Baumberger *et al.*, 2001
Tubulin β chain	P93176	Major constituent of microtubules, involved in cellular structure	Fuchs *et al.*, 1993
Plasma membrane integral protein (aquaporin)*	AF326487	Facilitates water transport across the PM, ppossibly involved in the regulation of overall plant water balance	Maurel, 1997; Maeshima, 2001
Putative nitrilase-associated protein	ACC0068 36	Family of thiol enzymes, responsible for the conversion of nitriles to carboxylic acid e.g. indole-3-acetonitrile(IAN) to indole-3-acetic acid (IAA, also known as auxin)	Bartel and Fink, 1994; Pace and Brenner, 2001
Elongation factor 1α	AJ004960	Catalyses polypeptide chain elongation, also known to have associations with microtubules and the cytoskeleton	Durso and Cyr, 1994; Morelli *et al.*, 1994; Dinman and Kinzy, 1997; Hashimoto *et al.*, 1999
Glyceraldehyde-3-phosphate dehydrogenase	DEZMGC	Key enzyme catalysing the oxidation and subsequent phosphorylation of aldehydes to acyl phosphates	Sivalinganna *et al.*, 1997
Putative UDP-glucose dehydrogenase	AC079887	Catalyses the irreversible oxidation of UDP-glucose to glucoronic acid (precursor to several hemicellulose cell wall components)	Tenhaken and Thulke, 1996

* identities in parenthesis indicate identities assigned during BLASTx searches of slightly lower homology than, but closely related to, the main identity

5.5 CONCLUSIONS

Of the more than 20 different genes that have previously been identified as inducible by Al stress, most seem to be general stress-related genes, inducible by a wide range of different conditions. These include wounding, pathogen infection and oxidative stress, categories into which many of the clones identified in this study could easily be classified. However, due to the inability to obtain conclusive expression analyses regarding these clones, no definite conclusions can be drawn in linking them to the Al tolerant phenotype.

Although nothing conclusive can be determined from the results obtained in terms of Al-related gene expression, this study has successfully initiated the use of hydroponics systems in the analysis of root-related phenomenon in sugarcane. Many of the problems facing production in the South African sugar industry are effected at root-level, such as nematode predation, mineral deficiencies, drought and, of course, Al toxicity. The heterogenous nature of most soils, coupled with the difficulty in isolating the effects of single factors is a major obstacle in the study of many of these issues, obstacles that can now be overcome via the use of these hydroponics systems.

REFERENCES

Alia, Hayahshi, H., Sakamoto, A., Murata, N. (1998) Enhancement of the tolerance of *Arabidopsis* to high temperatures by genetic engineering of the synthesis of glycinebetaine. *The Plant Journal* **16:** 155 – 161.

Allison, J.D., Brown, D.S. and Novo-Gradac, K.J. (1990) MINTEQA2/PRODEFA2, a geochemical assessment model for environmental systems. Version 3.11 User's Manual. Environmental Research Laboratory, United States Environmental Protection Agency, Athens, Georgia.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215:** 403 – 410.

Andersson, M. (1988) Toxicity and tolerance of aluminium in vascular plants. *Water, Air and Soil Pollution* **39:** 439 – 462.

Aniol, A. (1996) Aluminium uptake by roots of rye seedlings of differing tolerance to aluminium toxicity. *Euphytica* **92:** 155 – 162.

Aniol, A. and Gustafson, J.P. (1990) Genetics of tolerance in agronomic plants. In: A.J. Shaw (Ed.) *Heavy Metal Tolerance in Plants: Evolutionary Aspects.* CRC Press, Inc., Florida. pp 255 – 267.

Anonymous (1999) South African Sugar Association Annual Report 1998/1999, 32p.

Anonymous (2001a) South African Sugar Association Annual Report 2001/2002, 36p.

Anonymous (2001b) South African Sugar Association Industry Directory 2001/2002, 41p.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1999) Differential display of eukaryotic mRNA: meeting the demands of the new millenium? *Journal of Plant Physiology* **154:** 561 – 570.

Arsenijevic-Maksimovic, I., Broughton, W.J. and Krause, A. (1995) Three different cDNAs (Accession No. X86029, X86030 and X86028) encoding extensin-like proteins from *Vigna unguiculata* root hairs. *Plant Physiology* **109:** 338.

Baker, A.J.M. and Walker, P.L. (1990) Ecophysiology of metal uptake by tolerant plants. In: A.J. Shaw (Ed) *Heavy Metal Tolerance in Plants: Evolutionary Aspects.* CRC Press, Inc., Florida, USA. pp 155 – 177.

Baldwin, D., Crane, V. and Rice, D. (1999) A comparison of gel-based, nylon filter and microarray techniques to detect differential RNA expression in plants. *Current Opinion in Plant Biology* **2:** 96 – 103.

Baligar, V.C., Schaffert, R.E., Dos Santos, H.L., Pitta, G.V.E. and Filho, A.F. De C.B. (1993) Soil aluminium effects on uptake, influx and transport of nutrients in sorghum genotypes. *Plant and Soil* **150:** 271 – 277.

Barinaga, M. (1997) Making plants aluminum tolerant. *Science* **276:** 1497.

Barnabas, B., Kovacs, G., Hegedus, A., Erdei, S. and Horvath, G. (2000) Regeneration of doubled haploid plants from *in vitro* selected microspores to improve aluminium tolerance in wheat. *Journal of Plant Physiology* **156:** 217 – 222.

Barnes, J.M. and Botha, F.C. (1998) Progress towards identifying a marker for rust resistance in sugarcane variety NCo376. *Proceedings of the South African Sugar Technologists' Association* **72:** 149 – 151.

Barnes, J.M., Rutherford, R.S. and Botha, F.C. (1997) The identification of potential genetic markers in sugarcane varieties for the prediction of disease and pest resistance ratings. *Proceedings of the South African Sugar Technologists' Association* **71:** 57 - 61.

Bartel, B. and Fink, G.R. (1994) Differential regulation of an auxin-producing nitrilase gene family in *Arabidopsis thaliana*. *Proceedings of the National Academy of Science* **91:** 6649 – 6653.

Basu, A., Basu, U. and Taylor, G.J. (1994a) Induction of microsomal membrane proteins in roots of an aluminum-resistant cultivar of *Triticum aestivum* L. under conditions of aluminum stress. *Plant Physiology* **104:** 1007 – 1013.

Basu, U., Basu, A. and Taylor G.J. (1994b) Differential exudation of polypeptides by roots of aluminum-resistant and aluminum-sensitive cultivars of *Triticum aestivum* L. in response to aluminum stress. *Plant Physiology* **106:** 151 – 158.

Basu, U., Good, A.G., Aung, T., Slaski, J.J., Basu, A., Briggs, K.G. and Taylor, G.J. (1999) A 23-kDa, root exudates polypeptide co-segregates with aluminum-resistance in *Triticum aestivum*. *Physiologia Plantarum* **106:** 53 – 61.

Baumberger, N., Ringli, C. and Keller, B. (2001) The chimeric leucine-rich repeat/extensin cell wall protein LRX1 is required for root hair morphogenesis in *Arabidopsis thaliana*. *Genes & Development* **15:** 1128 – 1139.

Bennet, R.J. (1995) The use of hematoxylin in screening perennial ryegrass (*Lolium perenne*) for aluminium tolerance. *South African Journal of Plant and Soil* **12:** 65 – 67.

Bennet, R.J. and Breen, C.M. (1991) The aluminium signal: New dimensions to mechanisms of aluminium tolerance. *Plant and Soil* **134:** 153 – 166.

Bennet, R.J. and Granger, J.E. (2000) The aluminium response network in *Themeda triandra* Frosk. III: Regulatory mechanisms and aluminium tolerance. *South African Journal of Plant and Soil* **17:** 160 – 169.

Berberich, T., Sugawara, K., Harada, M. and Kusano, T. (1995) Molecular cloning, characterization and expression of an elongation factor 1 alpha gene in maize. *Plant Molecular Biology* **29:** 611 – 615.

Berding, N. and Skinner, J.C. (1987) Traditional breeding methods. In: *Copersucar International Sugarcane Breeding Workshop.* Copersucar Technology Center, Brazil. pp 269 – 320.

Blamey, F.P.C., Edwards, D.G. and Asher, C.J. (1983) Effects of aluminum, OH:Al molar ratios, and ionic strength on soybean root elongation in solution culture. *Soil Science* **136:** 197 – 207.

Blancaflor, E.B., Jones, D.L. and Gilroy, S. (1998) Alterations in the cytoskeleton accompany aluminium-induced growth inhibition and morphological changes in primary roots of maize. *Plant Physiology* **118:** 159 – 172.

Bouchez, D. and Hofte, H. (1998) Functional genomics in plants. *Plant Physiology* **118:** 725 – 732.

Bower, N.I., Casu, R.E., Maclean, D.J. and Manners, J.M. (2001) Identification of markers of the defense response in sugarcane. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 624 – 625.

Braga, D.P.V, Arrigoni, E.D.B., Burnquist, W.L., Silva-Filho, M.C. and Ulian, E.C. (2001) *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 331 – 336.

Budíková, S. (1999) Structural changes and aluminium distribution in maize root tissues. *Biologia Plantarum* **42:** 259 – 266.

Butterfield, M.K. (1995) Plant breeding strategies for the future. *Proceedings of the South African Sugar Technologists' Association* **69:** 219 – 220.

Calba, H. and Jaillard, B. (1997) Effect of aluminium on ion uptake and H^+ release by maize. *New Phytologist* **137:** 607 – 616.

Cambraia, J., Galvani, F.R., Estevão, M.M. and Sant'Anna, R. (1983) Effects of aluminum on organic acid, sugar and amino acid composition of the root system of sorghum (*Sorghum bicolor* L. Moench). *Journal of Plant Nutrition* **6:** 313 – 322.

Carson, D.L. and Botha, F.C. (2000) Preliminary analysis of expressed sequence tags for sugarcane. *Crop Science* **40:** 1769 – 1779.

Carver, B.F., Whitmore, W.E., Smith, E.L. and Bona, L. (1993) Registration of four aluminum-tolerant winter germplasms and two susceptible near-isolines. *Crop Science* **33:** 1113 – 1114.

Cato, S.A., Gardner, R.C., Kent, J. and Richardson, T.E. (2000) A rapid PCR-based method for genetically mapping ESTs. *Theoretical and Applied Genetics* **102:** 296 – 306.

Christiansen-Weniger, C., Groneman, A.F. and van Veen, J.A. (1992) Associative N_2 fixation and root exudation of organic acids from wheat cultivars of different aluminium tolerance. *Plant Soil* **139:** 167 – 174.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proceedings of the National Academy of Science* **81:** 1991 – 1995.

Clark, A., Nomura, A., Mohanty, S. and Firtel, R.A. (1997) A ubiquitin-conjugating enzyme is essential for developmental transitions in *Dictyostelium*. *Molecular Biology of the Cell* **8:** 1989 – 2002.

Cocker, K.M., Evans, D.E. and Hodson, M.J. (1998) The amelioration of aluminium toxicity by silicon in wheat (*Triticum aestivum* L.): malate exudation as evidence for an *in planta* mechanism. *Planta* **204:** 318 – 323.

Conkling, M.A., Cheung, C-L., Yamamoto, Y.T. and Goodman, H.M. (1990) Isolation of transcriptionally regulated root-specific genes from tobacco. *Plant Physiology* **93:** 1203 – 1211.

Cramer, M.D. and Titus, C.H.A. (2001) Elevated root zone dissolved inorganic carbon can ameliorate aluminium toxicity in tomato seedlings. *New Phytologist* **152:** 29 – 39.

Crawford, S.A., Marshall, A.T. and Wilkens, S. (1998) Localisation of aluminium in root apex cells of two Australian perennial grasses by x-ray microanalysis. *Australian Journal of Plant Physiology* **25:** 427 – 435.

Cruz-Ortega, R., Cushman, J.C. and Ownby, J.D. (1997) cDNA clones encoding 1,3-β-glucanase and a fimbrin-like cytoskeletal protein are induced by Al toxicity in wheat roots. *Plant Physiology* **114:** 1453 – 1460.

Cumming, J.R. and Taylor, G.J. (1990) Mechanisms of metal tolerance in plants: Physiological adaptations for exclusion of metal ions from the cytoplasm. In: R.G. Alscher and J.R. Cumming (Eds) *Stress Responses in Plants: Adaptation and Acclimation Mechanisms.* Wiley-Liss, Inc., New York. pp 329 – 356.

Da Silva, J.A.G., Ulian, E.C. and Barsalobres, C.F. (2001) Development of EST-derived RFLP markers for sugarcane breeding. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 318 – 322.

Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) *Basic Methods in Molecular Biology.* Appleton and Lange, Connecticut, USA. pp 129 – 156.

De la Fuente-Martínez, J.M. and Herrera-Estrella, L. (1999) Advances in the understanding of aluminium toxicity and the development of aluminium-tolerant transgenic plants. *Advances in Agronomy* **66:** 103 – 120.

De la Fuente, J.M., Ramírez-Rodríguez, V., Cabrera-Ponce, J.L. and Herrera-Estrella, L. (1997) Aluminum tolerance in transgenic plants by alteration of citrate synthesis. *Science* **276:** 1566 – 1568.

Delhaize, E., Craig, S., Beaton, C.D., Bennet, R.J., Jagadish, V.C. and Randall, P.J. (1993a) Aluminum tolerance in wheat (*Triticum aestivum* L.). I. Uptake and distribution of aluminum in root apices. *Plant Physiology* **103:** 685 – 693.

Delhaize, E., Hebb, D.M., Richards, K.D., Lin, J-M., Ryan, P.R. and Gardner, R.C. (1999) Cloning and expression of a wheat (*Triticum aestivum* L.) phosphatidylserine synthase cDNA. *Journal of Biological Chemistry* **274:** 7082 – 7088.

Delhaize, E. and Ryan, P.R. (1995) Aluminium toxicity and tolerance in plants. *Plant Physiology* **107:** 315 – 321.

Delhaize, E., Ryan, P.R. and Randall, P.J.(1993b) Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiology* **103:** 695 – 702.

De Lima, M.L. and Copeland, L. (1994) The effect of aluminium on respiration of wheat roots. *Physiologia Plantarum* **90:** 51 – 58.

De Lonlay, P., Valnot, I., Barrientos, A., Gorbatyuk, M., Tzagoloff, A., Taanman, J.W., Benayoun, E., Chretien, D., Kadhom, N., Lombes, A., de Baulny, H.O., Niaudet, P., Munnich, A., Rustin, P. and Rotig, A. (2001) A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure. *Nature Genetics* **29:** 57 – 60.

De Pater, B.S. and Schilperoort, R.A. (1992) Structure and expression of a root-specific rice gene. *Plant Molecular Biology* **18:** 161 – 164.

D' Hont, A., Garsmeur, O., Raboin, L.M., Paulet, F., Begum, D., Wing, R. and Glaszmann, J.C. (2001) Chromosome walking towards a major resistance gene for common rust of sugarcane. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 315 – 317.

Didierjean, L., Frendo, P., Nasser, W., Genot, G., Marivet, J. and Burkard, G. (1996) Heavy-metal-responsive genes in maize: identification and comparison of their expression upon various forms of abiotic stress. *Planta* **199:** 1 – 8.

Dinman, J. and Kinzy, T. (1997) Translational misreading: Mutation in translational elongation factor 1α differentially affect programmed ribosomal framshifting and drug sensitivity. *RNA* **3:** 870 – 881.

Driscoll, C.T. and Schecher, W.D. (1990) The chemistry of aluminium in the environment. *Environmental Geochemical Health* **21:** 28 – 49.

Durieux, R.P., Jackson, W.A., Kamprath, E.J. and Moll, R.H. (1993) Inhibition of nitrate uptake by aluminum in maize. *Plant and Soil* **151:** 97 – 104.

Durner, J., Shah, J. and Klessig. D.F. (1997) Salicylic acid and disease resistance in plants. *Trends in Plant Science* **2:** 266 – 275.

Durso, N.A. and Cyr, R.J. (1994) Beyond translation: Elongation factor-1-alpha and the cytoskeleton. *Protoplasma* **180:** 99 – 105.

Espino, F.J., González-Jaen, M.T., Ibáñez, J., Sendino, A.M. and Vázquez, A.M. (1998) Aluminum and low pH effects on translatable RNA population from bean calli. *Protoplasma* **201:** 85 – 91.

Ezaki, B., Gardner, R.C., Ezaki, Y. and Matsumoto, H. (2000) Expression of aluminium-induced genes in transgenic Arabidopsis plants can ameliorate aluminium stress and/or oxidative stress. *Plant Physiology* **122:** 657 – 665.

Ezaki, B., Yamamoto, Y. and Matsumoto, H. (1995) Cloning and sequencing of the cDNAs induced by aluminium treatment and P_i starvation in cultured tobacco cells. *Physiologia Plantarum* **93:** 11- -18.

Foy, C.D. (1983) The physiology of plant adaptation to mineral stress. *Iowa State Journal of Research* **57:** 355 – 391.

Fuchs, U., Moepps, B., Maucher, H.P. and Schraudolf, H. (1993) Isolation, characterization and sequence of a cDNA encoding gamma-tubulin protein from the fern *Anemia phyllitidus* L.SW. *Plant Molecular Biology* **23:** 595 – 603.

Gallo-Meagher, M. and Irvine, J.E. (1996) Herbicide resistant transgenic sugarcane plants containing the *bar* gene. *Crop Science* **36:** 1367 – 1374.

Gallego, F.J. and Benito, C. (1997) Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theoretical and Applied Genetics* **95:** 393 – 399.

Gascho, G.J., Anderson, D.L. and Bowen, J.E. (1993) Sugarcane. In: W.F. Bennet (ed.) *Nutrient Deficiencies & Toxicities in Crop Plants.* The American Phytopathological Society, USA. pp 37 – 42.

Godbold, D.L. and Jentschke, G. (1998) Aluminium accumulation in root cell walls coincides with inhibition of root growth but not with inhibition of magnesium uptake in Norway spruce. *Physiologia Plantarum* **102:** 553 – 560.

Grabski, S. and Schindler, M. (1995) Aluminum induces rigor within the actin network of soybean cells. *Plant Physiology* **108:** 897 – 901.

Habu, Y., Fukada-Tanaka, S., Hisatomi, Y. and Iida, S. (1997) Amplified restriction fragment length polymorphism-based mRNA fingerprinting using a single restriction enzyme that recognizes a 4-bp sequence. *Biochemical and Biophysical Research Communications* **234:** 516 – 521.

Hairiah, K., Van Noordwijk, M., Stulen, I., Meijboom, F.W. and Kuiper, P.J.C. (1993) Phosphate nutrition effects on aluminium avoidance of *Mucuna pruriens* var. *utilis*. *Environmental and Experimental Botany* **33:** 75 – 83.

Hamel, F., Breton, C. and Houde, M. (1998) Isolation and characterization of wheat aluminium-regulated genes: possible involvement of aluminium as a pathogenesis response elicitor. *Planta* **205:** 531 – 538.

Harper, S.M., Edwards, D.G., Kerven, G.L. and Asher, C.J. (1995) Effects of organic acid fractions extracted from *Eucalyptus camaldulensis* leaves on root elongation of maize (*Zea mays*) in the presence and absence of aluminium. *Plant and Soil* **171:** 189 – 192.

Hashimoto, A., Toshiyuki, K., Yamada, S., Kubo, T. and Imaseji, H. (1999) cDNA cloning of translational elongation factor-1 alpha from *Nicotiana paniculata* (Accession No. AB019427). *Plant Physiology* **119:** 364.

Hawes, M.C., Gunawardena, U., Miyasaka, S. and Zhao, X. (2000) The role of root border cells in plant defense. *Trends in Plant Science* **5:** 128 – 133.

Heinz, D.J. (1987) Sugarcane improvement: current productivity and future opportunities. In: *Copersucar International Sugarcane Breeding Workshop.* Copersucar Technology Center, Brazil. pp 55 – 70.

Hetherington, S.J., Asher, C.J. and Blamey, F.P.C. (1986) Tolerance of sugarcane to Al in soil and solution culture. *Proceedings of Australian Society of Sugar Cane Technologists* 63 – 68.

Hewitt E.J. (1966). *Sand and Water Culture Methods used in the Study of Plant Nutrients.* Commonwealth Agricultural Bureaux, Farnham Royal, Buckinghamshire (2nd Edition).

Huckett, B.I. (1995) Biotechnological intervention. *Proceedings of the South African Sugar Technologists' Association* **69:** 221 - 224.

Hue, N.V., Craddock, G.R. and Adams, F. (1986) Effect of organic acids on aluminium toxicity in subsoils. *Soil Science Society of America Journal* **50:** 28 – 34.

Ingelbrecht, I.L., Mandelbaum, C.I. and Mirkov, T.E. (1998) Highly sensitive northern hybridization using a rapid protocol for downward alkaline blotting of RNA. *BioTechniques* **25:** 420 – 425.

Jo, J., Jang, Y-S., Kim, K-Y., Kim, M-H., Kim, I-J. and Chung, W-I. (1997) Isolation of *ALU1-P* gene encoding a protein with aluminum tolerance activity from *Arthrobacter viscosus. Biochemical and Biophysical Research Communications* **239:** 835 – 839.

Jones, D.L., Shaff, J.E. and Kochian, L.V. (1995) Role of calcium and other ions in directing root hair tip growth in *Limnobium stoloniferum.* I. Inhibition of tip growth by aluminum. *Planta* **197:** 672 – 680.

Jorge, R.A. and Arruda, P. (1997) Aluminum-induced organic acids exudation by maize roots of an aluminum-tolerant tropical maize. *Phytochemistry* **45:** 675 – 681.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnology* **17:** 287 – 291.

Keltjens, W.J. (1988) Short-term effects of Al on respiration and nitrate reductase activity of two sorghum genotypes differing in Al-susceptibility. *Communications in Soil Science and Plant Analysis* **19:** 1155 – 1163.

Kidd, P.S. and Proctor, J. (2000) Effects of aluminium on the growth and mineral composition of *Betula pendula* Roth. *Journal of Experimental Botany* **51:** 1057 – 1066.

Kochian, L.V. (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **46:** 237 – 260.

Koyama, H., Takita, E., Kawamura, A., Hara, T. and Shibata, D. (1999) Over expression of mitochondrial citrate synthase gene improves the growth of carrot cells in Al-phosphate medium. *Plant Cell Physiology* **40:** 482 – 488.

Kurek, I., Aviezer, K., Erel, N., Herman, E. and Breiman, A. (1999) The wheat peptidyl prolyl *cis-trans*-isomerase FKBP77 is heat induced and developmentally regulated. *Plant Physiology* **119:** 693 – 703.

Lambert, K.N., Ferrie, B.J., Nombela, G., Brenner, E.D. and Williamson, V.M. (1999) Identification of genes whose transcripts accumulate rapidly in tomato after root-knot nematode infection. *Physiological and Molecular Plant Pathology* **55:** 341 – 348.

Landell, M.G.A. (1989). Comportamento de cana-de-açúcar (*Saccharum* spp.) frente a níveis de alúmino, em solução nutriva. FCAV/UNESP, PhD Thesis, p.117. In: Drummond, R.D.,

Guimarães, C.T. Felix, J., Ninamango-Cárdenas, F.E., Carneiro, N.P., Paiva, E. and Menossi, M. (2001). *Prospecting sugarcane genes involved in aluminium tolerance. Genetic and Molecular Biology* **24:** 221-230.

Larsen, P.B., Degenhardt, J., Tai, C-Y., Stenzler, L.M., Howell, S.H. and Kochian, L.V. (1998) Aluminum-resistant Arabidopsis mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. *Plant Physiology* **117:** 9 – 18.

Lazof, D.B., Rincón, M., Rufty, T.W., Mackown, C.T. and Carter, T.E. (1994) Aluminum accumulation and associated effects on $^{15}NO_3^-$ influx in roots of two soybean genotypes differing in Al tolerance. *Plant and Soil* **164:** 291 – 297.

Legendre, B.L. and Burner, D.M. (1997) New opportunities in the development of sugarcane cultivars. Augmenting traditional approaches with molecular techniques. *Sugar Cane* **5:** 4 – 9.

Liu, Q.Y., van der Meer, J.P. and Reith, M.E. (1994) Isolation and characterization of phase-specific complementary DNAs from sporophytes and gametophytes of *Porphyra purpurea* (Rhodophyta) using subtracted complementary DNA libraries. *Journal of Phycology* **30:** 513 – 520.

Loper, M., Brauer, D., Patterson, D. and Tu, S-I. (1993) Aluminum inhibition of NADH-linked electron transfer by corn root plasma membranes. *Journal of Plant Nutrition* **16:** 507 – 514.

Maeshima, M. (2001) Tonoplast transporters: Organization and function. *Annual Review of Plant Physiology and Plant Molecular Biology* **52:** 469 – 497.

Marienfeld, S. and Stelzer, R. (1993) X-ray microanalysis in roots of Al-treated *Avena sativa* plants. *Journal of Plant Nutrition* **141:** 569 – 573.

Marivet, J., Frendo, P. and Burkard, G. (1995) DNA sequence analysis of a cyclophilin gene from maize: developmental expression and regulation by salicylic acid. *Molecular and General Genetics* **247:** 222 – 228.

Märländer, B. (2000) Breeding patterns and biotechnological approach in sugarcane and sugarbeet. *Zuckerindiustrie* **125:** 951 – 956.

Marschner, H. (1991) Mechanisms of adaptation of plants to acid soils. *Plant and Soil* **134:** 1 – 20.

Masion, A. and Bertsch, P.M. (1997) Aluminium speciation in the presence of wheat root cell walls: a wet chemical study. *Plant, Cell and Environment* **20:** 504 – 512.

Maurel, C. (1997) Aquaporins and water permeability of plant membranes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48:** 399 – 429.

McIntyre, L., Aitken, K., Berding, N., Casu, R., Drenth, J., Jackson, P., Jordan, D., Piperidis, G., Reffay, N., Smith, G., Tao, Y. and Whan, V. (2001) Identification of DNA markers linked to agronomic traits in sugarcane in Australia. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 560 – 562.

McQualter, R.B., Harding, R.M., Dale, J.L. and Smith, G.R. (2001) Virus derived transgenes confer resistance to Fiji disease in transgenic sugarcane plants. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 584 – 585.

Meyer, J. (1999) Monitoring soil fertility trends. *The Link* **8(1):** 4 - 5. (Published by SASA Experiment Station)

Meyer, J.H., Van Antwerpen, R. (2001) Soil degradation as a factor in yield decline in the South African sugar industry. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 8 – 15.

Meyer, J.H., Van Antwerpen, R. and Meyer, E. (1996) A review of soil degradation and management research under intensive sugarcane cropping. *Proceedings of the South African Sugar Technologists' Association* **70:** 22 – 28.

Miller, T.E., Iqbal, N., Reader, S.M., Mahmood, A., Cant, K.A. and King, I.P. (1997) A cytogenetic approach to the improvement of aluminium tolerance in wheat. *New Phytologist* **137:** 93 – 98.

Mirkov, T.E. (2001) Progress and potential for sugarcane improvement through transgenic plant technologies. *Proceedings of the 24th International Society of Sugar Cane Technologists Congress* **11:** 575 – 577.

Miyasaka, S.C., Buta, J.G., Howell, R.K. and Foy, C.D. (1991) Mechanism of aluminum tolerance in snapbeans. Root exudation of citric acid. *Plant Physiology* **96:** 737 – 743.

Miyasaka, S.C., Kochian, L.V., Shaff, J.E. and Foy, C.D. (1989) Mechanisms of aluminium tolerance in wheat: an investigation of genotypic differences in rhizosphere pH, K^+, and H^+ transport, and root cell membrane potentials. *Plant Physiology* **91:** 1188 – 1196.

Moffat, A.S. (1999) Engineering plants to cope with metals. *Science* **285:** 369 – 370.

Moon, D.H., Ottoboni, L.M.M., Souza, A.P., Sibov, S.T., Gaspar, M. and Arruda, P. (1997) Somaclonal-variation-induced aluminum-sensitive mutant from an aluminum-inbred maize tolerant line. *Plant Cell Reports* **16:** 686 – 691.

Moore, P.H. (1999) Progress and developments in sugarcane biotechnology. *Proceedings of the 23rd International Society of Sugar Cane Technologists Congress* **22:** 241 – 257.

Morelli, J.K., Shewmaker, C.K. and Vayda, M.E. (1994) Biphasic stimulation of translational activity correlates with induction of translation elongation factor 1 subunit α upon wounding in potato tubers. *Plant Physiology* **106:** 897 – 903.

Msomi, N. and Botha, F.C. (1994) Identification of molecular markers linked to fibre using bulk segregant analysis. *Proceedings of the South African Sugar Technologists' Association* **68:** 41 – 45.

Mudge, J., Cregan, P.P., Kenworthy, W.J., Orf, J.H. and Young, N.D. (1997) Two microsatellite markers that flank the major soybean cyst nematode resistance locus. *Crop Science* **37:** 1611 – 1615.

Mugai, E.N., Agong, S.G. and Matsumoto, H. (2000) Aluminium tolerance mechanisms in *Phaseolus vulgaris* L.: Citrate synthase acitivity and TCC reduction are well correlated with citrate secretion. *Soil Science and Plant Nutrition.* **46:** 939 – 950.

Muñoz, F.J., Dopico, B. and Labrador, E. (1998) A cDNA encoding a proline-rich protein from *Cicer arietinum.* Changes in expression during development and abiotic stress. *Physiologia Plantarum* **102:** 582 – 590.

Nuss, K.J. (1987) Breeding sugarcane for poor soils. In: *Copersucar International Sugarcane Breeding Workshop.* Copersucar Technology Center, Brazil. pp 203 – 216.

Ostatek-Boczynski, Z., Kerven, G.L. and Blamey, F.P.C. (1995) Aluminium reactions with polygalacturonate and related organic ligands. *Plant and Soil* **171:** 41 – 45.

Ownby, J.D. (1993) Mechanisms of reaction of hematoxylin with aluminium-treated wheat roots. *Physiologia Plantarum* **87:** 371 – 380.

Pace, H.C. and Brenner, C. (2001) The nitrilase superfamily: classification, structure and function. *Genome Biology* **2:** reviews0001.1 – 0001.9.

Parker, D.R., Norvell, W.A. and Chaney, R.L. (1995) GEOCHEM-PC: Chemical speciation program for IBM and compatible personal computers. In: R.H. Loeppert (Ed.) *Soil Chemical Equilibrium and Reaction Models.* Soil Science Society of America, Madison. pp 253 – 269.

Patel, M. and Sive, H. (1995) PCR-based subtractive cDNA cloning. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (Eds). *Current Protocols in Molecular Biology, Vol. 1.* John Wiley and Sons, Inc., USA.

Pellet, D.M., Papernick, L.A., Jones, D.L., Darrah, P.R., Grunes, D.L. and Kochian, L.V. (1997) Involvement of multiple aluminium exclusion mechanisms in aluminium tolerance in wheat. *Plant and Soil* **192:** 63 – 68.

Pintro, J., Barloy, J. and Fallavier, P. (1997) Effects of low aluminum activity in nutrient solutions on the organic acid concentrations in maize plants. *Journal of Plant Nutrition* **20:** 601 – 611.

Rengel, Z., Piñeros, M. and Tester, M. (1995) Transmembrane fluxes during Al stress. *Plant and Soil* **171:** 125 – 130.

Ribaut, J-M., Banziger, M. and Hoisington, D. (2002) Genetic dissection and plant improvement under abiotic stress conditions: drought tolerance in maize as an example. *JIRCAS Working Report* **(2002):** 85 – 92.

Richards, K.D., Schott, E.J., Sharma, Y.K., Davis, K.R. and Gardner, R.C. (1998) Aluminum induces oxidative stress genes in *Arabidopsis thaliana. Plant Physiology* **116:** 409 – 418.

Roach, B.T. and Daniels, J. (1987) A review of the origin and improvement of sugarcane. In: *Copersucar International Sugarcane Breeding Workshop.* Copersucar Technology Center, Brazil. pp 1 – 32.

Robinson, N.J. (1990) Metal-binding polypeptides in plants. In: A.J. Shaw (Ed) *Heavy Metal Tolerance in Plants: Evolutionary Aspects.* CRC Press, Inc., Florida, USA. pp 195 – 214.

Sabelli, P.A.(1996) Differential Screening. In: G.D. Foster and D. Twell (Eds) *Plant Gene Isolation: Principles and Practice*. John Wiley and Sons Ltd., West Sussex, England. pp 125 – 155.

Sagerström, C.G., Sun, B.I. and Sive, H.L. (1997) Cloning: Past, present and future. *Annual Review of Biochemistry* **66:** 751 – 783.

Sambrook, J. and Russel, D.W. (2001) *Molecular cloning. A laboratory manual.* 3rd Edition. Cold Spring Harbour Laboratory Press, New York.

Sargent, T.D. (1987) Isolation of differentially expressed genes. *Methods in Enzymology* **152:** 423 – 432.

Sasaki, M., Kasai, M., Yamamoto, Y. and Matsumoto, H. (1994) Comparison of the early response to aluminum stress between tolerant and sensitive wheat cultivars: root growth, aluminum content and efflux of K^+. *Journal of Plant Nutrition* **17:** 1275 – 1288.

Sasaki, M., Kasai, M., Yamamoto, Y. and Matsumoto, H. (1995) Involvement of plasma membrane potential in the tolerance mechanism of plant roots to aluminium toxicity. *Plant and Soil* **171:** 119 – 124.

Sävenstrand, H., Brosché, M., Ängenhagen, M. and Strid, Å. (2000) Molecular markers for ozone stress isolated by suppression subtractive hybridisation: specificity of gene expression and identification of a novel stress-regulated gene. *Plant, Cell and Environment* **23:** 689 – 700.

Schroeder, B.L., Robinson, J.B., Wallace, M. and Turner, P. (1994) Soil acidification: occurrence and effects in the South African sugar industry. *Proceedings of the South African Sugar Technologists' Association* **68:** 70 – 74.

Schumann, A. (1998) Soil acidification: Causes and management. *The Link* 7(**4**): 9. (Published by SASA Experiment Station)

Schumann, A. (1999) Soil acidification revisited: Confirmation from a commercial field. *The Link* **8**(**1**): 1. (Published by SASA Experiment Station)

Scott, B.J., Fischer, J.A. and Cullis, B.R. (2001) Aluminium tolerance and lime increase wheat yield on the acidic soils of central and Southern New South Wales. *Australian Journal of Experimental Agriculture* **41:** 523 – 532.

Shann, J.R. and Bertsch, P.M. (1993) Differential cultivar responses to hydroxo-aluminum complexes. *Soil Science Society of America Journal* **57:** 116 – 120.

Showalter, A.M., Zhou, J., Rumeau, D., Worst, S.G. and Varner, J.E. (1991) Tomato extensin and extensin-like cDNAs: structure and expression in response to wounding. *Plant Molecular Biology* **16:** 547 – 565.

Shyi-Kae, Y., Chen, P-E. and Yen, H.E. (1999) Characterization of a gene encoding ubiquitin-conjugating enzyme from *Mesembryanthemum crystallinum* (Accession No. AF176040). *Plant Physiology* **121:** 685.

Sibov, S.T., Gaspar, M., Silva, M.J., Ottoboni, L.M.M., Arruda, P. and Souza, A.P. (1999) Two genes control aluminum tolerance in maize: Genetic and molecular mapping analyses. *Genome* **42:** 475 – 482.

Siegel, N. and Haug, A. (1983) Calmodulin-dependent formation of membrane potential in barley root plasma membrane vesicles. A biochemical model of aluminium toxicity in plants. *Physiologia Plantarum* **59:** 285 – 291.

Silva, I.R., Smyth, T.J., Moxley, D.F., Carter, T.E., Allen, N.S. and Rufty, T.W. (2000) Aluminum accumulation at nuclei of cells in the root tip: fluorescence detection using lumogallion and confocal laser scanning microscopy. *Plant Physiology* **123:** 543 – 552.

Sivaguru, M., Fujiwara, T., Šamaj, J., Baluška, F., Yang, Z., Osawa, H., Maeda, T., Mori, T., Volkmann, D. and Matsumoto, H. (2000) Aluminum-induced 1→3-β-D-glucan inhibits cell-to-cell trafficking of molecules through plasmodesmata. A new mechanism of aluminum toxicity in plants. *Plant Physiology* **124:** 991 – 1005.

Sivaguru, M. and Paliwal, K. (1993) Differential aluminum tolerance in some tropical rice cultivars. II. Mechanism of aluminum tolerance. *Journal of Plant Nutrition* **16:** 1717 – 1732.

Sivalinganna, M. and Sachs, M.M. (1997) Molecular characterization and promoter analysis of the maize cytosolic glyceraldehydes-3-phosphate dehydrogenase gene family and its expression during anoxia. *Plant Molecular Biology* **33:** 97 – 112.

Smith, R.W. (1972) Relations among equilibrium and non-equilibrium aqueous species of aluminium hydroxy complexes. *Advances in Chemistry Series* **106:** 250 – 279.

Snowden, K.C. and Gardner, R.C. (1993) Five genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. *Plant Physiology* **103:** 855 – 861.

Snyman, S.J., Leibbrandt, N.B. and Botha, F.C. (1998) Buster resistant sugarcane. *Proceedings of the South African Sugar Technologists' Association* **72:** 138 – 139.

Sreenivasulu, N., Ramanjulu, S., Ramachandra-Kini, K., Prakash, H.S., Shekar-Shetty, H., Savithri, H.S. and Sudhakar, C. (1999) Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. *Plant Science* **141:** 1 – 9.

Staβ, A. and Horst, W.J. (1995) Effect of aluminium on membrane properties of soybean (*Glycine max*) cells in suspension culture. *Plant and Soil* **171:** 113 – 118.

Stevenson, G.C. (1965) *Genetics and breeding of sugar cane.* Longmans, Green and Co. Ltd, London. 284p.

Suhayda, C.G. and Haug, A. (1986) Organic acids reduce aluminum toxicity in maize root membranes. *Physiologia Plantarum* **68:** 189 – 195.

Sumner, M.E. (1970) Aluminium toxicity – a growth limiting factor in some Natal sands. *Proceedings of the South African Sugar Technologists' Association* **44:** 176 - 182.

Sumner, M.E. and Meyer, J.H. (1971) Incidence of toxic aluminium in sandy soils. *Proceedings of the South African Sugar Technologists' Association* **45:** 212 – 216.

Taylor, G.J. (1988a) Aluminum tolerance is independent of rhizosphere pH in *Triticum aestivum* L. *Communications in Soil Science and Plant Analysis* **19:** 1217 – 1227.

Taylor, G.J. (1988b) Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat). V. Nitrogen nutrition, plant-induced pH, and tolerance to aluminum; correlation without causality. *Canadian Journal of Botany* **66:** 694 – 699.

Taylor, G.J. (1991) Current views of the aluminum stress response; the physiological basis of tolerance. *Current Topics in Plant Biochemistry and Physiology* **10:** 57 – 93.

Taylor, G.J. (1995) Overcoming barriers to understanding the cellular basis of aluminium resistance. *Plant and Soil* **171:** 89 – 103.

Taylor, G.J. and Foy, C.D. (1985a) Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat). I. Differential pH induced by winter cultivars in nutrient solutions. *American Journal of Botany* **72:** 695 – 701.

Taylor, G.J. and Foy, C.D. (1985b) Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat). III. Long-term pH changes induced in nutrient solutions by winter cultivars differing in tolerance to aluminum. *Journal of Plant Nutrition* **8:** 613 – 628.

Taylor, G.J. and Foy, C.D. (1985c) Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat). IV. The role of ammonium and nitrate nutrition. *Canadian Journal of Botany* **63:** 2181 – 2186.

Tenhaken, R. and Thulke, O. (1996) Cloning of an enzyme that synthesizes a key nucleotide-sugar precursor of hemicellulose biosynthesis from soybean: UDP-glucose dehydrogenase. *Plant Physiology* **112:** 1127 – 1134.

Thokoane, L. and Rutherford, R.S. (2001) Differential gene expression in sugarcane in response to challenge by the fungal pathogen *Ustilago scitaminea* (Sydow) (Sugarcane smut). *Proceedings of the International Society of Sugar Cane Technologists* **24:** 609 – 611.

Toninello, A., Clari, G., Mancon, M., Tognon, G. and Zatta, P. (2000) Aluminum as an inducer of the mitochondrial permeability transition. *Journal of Biological Inorganic Chemistry* **5:** 612 – 623.

Turner, P.E.T., Meyer, J.H. and Wood, R.A. (1992) Some results of field experiments to test lime and phosphogypsum for aluminium amelioration. *Proceedings of the South African Sugar Technologists' Association* **66:** 44 – 49.

Van Antwerpen, R. and Meyer, J.H. (1996) Soil degradation under sugarcane cultivation in Northern KwaZulu Natal. *Proceedings of the South African Sugar Technologists' Association* **70:** 29 – 33.

Van der Biezen, E.A., Hedyani, J., Parker, J.E. and Jones, J.D.G. (2000) cDNA-AFLP display for the isolation of *Peronospoa parasitica* genes expressed during infection in *Arabidopsis thaliana. Molecular Plant-Microbe Interactions* **13:** 895 – 898.

Varet, A., Parker, J., Tornero, P., Nass, N., Nürnberger, T., Dangl, J.L., Scheel, D. and Lee, J. (2002) NHL25 and NHL3, two NDR1/HIN1-like genes in *Arabidopsis thaliana* with potential role(s) in plant defense. *Molecular Plant-Microbe Interactions* **15:** 608 – 616.

Verkleij, J.A.C. and Schat, H. (1990) Mechanisms of metal tolerance in higher plants. In: A.J. Shaw (Ed) *Heavy Metal Tolerance in Plants: Evolutionary Aspects*. CRC Press, Inc., Florida, USA. pp 179 – 193.

Vierstra, R. and Haug, A. (1978) The effect of Al^{3+} on the physical properties of membrane lipids in *Thermoplasma acidophilum. Biochemical and Biophysical Research Communications* **84:** 138 – 143.

Viola, R.E., Morrison, J.F. and Cleland, W.W. (1980) Interaction of metal(III)-adenosine 5'-triphosphate complexes with yeast hexokinase. *Biochemistry* **19:** 3131 – 3137.

Wagatsuma, T., Ishikawa, S., Obata, H., Tawaraya, K. and Katohda, S. (1995) Plasma membrane of younger and outer cells is the primary specific site for aluminium toxicity in roots. *Plant and Soil* **171:** 105 – 112.

Watanabe, T., Osaki, M., Yoshihara, T. and Tadano, T. (1998) Distribution and chemical speciation of aluminum in the Al accumulator plant, *Melastoma malabathricum* L. *Plant and Soil* **201:** 165 – 173.

Weig, A., Franz, J., Sauer, N. and Komor, E. (1994) Isolation of a family of cDNA clones from *Ricinus communis* L. with close homology to the hexose carriers. *Journal of Plant Physiology* **143:** 178 – 183.

Wenzl, P., Patino, G.M., Chaves, A.L., Mayer, J.E. and Raol, M. (2001) The high level of aluminum tolerance in signalgrass is not associated with known mechanisms of external aluminum detoxification in root apices. *Plant Physiology* **125:** 1473 – 1484.

Wilkinson, M. (1991) Purification of RNA. In T.A. Brown (Ed) *Essential Molecular. A Practical Approach Vol. 1.*Oxford University Press, New York. pp 69 – 87.

Wilson, M.A., Bird, D. McK. and Van der Knaap, E. (1994) A comprehensive subtractive cDNA cloning approach to identify nematode-induced transcripts in tomato. *Molecular Plant Pathology* **84:** 299 – 303.

Yang, Y-H. and Zhang, H-Y. (1998) Effect of citric acid on aluminum toxicity in the growth of mungbean seedlings. *Journal of Plant Nutrition* **21:** 1037 – 1044.

Yang, Z.M., Sivaguru, M., Horst, W.J. and Matsumoto, H. (2000) Aluminium tolerance is achieved by exudation of citric acid from roots of soybean (*Glycine max*). *Physiologia Plantarum* **110:** 72 – 77.

Young, N.D. (1999) A cautiously optimistic vision for marker-assisted breeding. *Molecular Breeding* **5:** 505 – 510.

Zhang, X. and Jessop, R.S. (1998) Analysis of genetic variability of aluminium tolerance response in triticale. *Euphytica* **102:** 177 – 182.

Zhang, X.G., Jessop, R.S. and Ellison, F. (1999) Combining ability for aluminium tolerance in triticale. *Journal of Agricultural Science, Cambridge* **133:** 371 – 377.

Zheng, S.J., Ma, J.F. and Matsumoto, H. (1998) Continuous secretion of organic acids is related to aluminium stress during relatively long-term exposure to aluminium stress. *Physiologia Plantarum* **103:** 209 – 214.