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DECIPHERING GENETICS OF NITROGEN-FIXING SYMBIOSIS IN LOTUS JAPONICUS

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DECIPHERING GENETICS OF NITROGEN-FIXING SYMBIOSIS IN *LOTUS JAPONICUS*

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ABSTRACT

As the world population approaches seven billion and is predicted to reach nine billion by the year 2040 it is essential to improve our agricultural methods in order to meet the growing demand for food. The challenge is to increase the yield without negatively affecting the environment.

A new approach that would make use of beneficial plant-microbe interactions should be considered. One of these interactions is the establishment of nitrogen-fixing symbiosis between plants and soil bacterium, commonly known as *Rhizobium.* However the ability to interact symbiotically with *Rhizobium* is almost completely restricted to leguminous plants. Therefore, understanding how the legume-rhizobium symbiosis is established might allow us to improve or engineer new N_2 acquiring plant-microbe associations.

In recent years, we witnessed many breakthrough discoveries that improved our understanding of these interactions; however, significant gaps in our knowledge of this important biological process still remain. The research objective of my thesis has been, therefore, to enhance our understanding of the mechanisms governing the development of nitrogen-fixing root nodule symbiosis in a model legume, *Lotus japonicus.*

The key findings of this thesis are as follows: (1) the identification and characterization of many symbiosis-relevant loci in *L. japonicus*; (2) discovery of an alternative mechanism for successful rhizobial colonization of legume roots; (3) the molecular cloning of a gene that is required for root hairs development *in L. japonicus;* this is in relation to the function of root hair as the primary sites for the initial physical contact and entry of the compatible nitrogen-fixing bacteria inside the host-plant root and last; (4) discovery of a key signaling element that is necessary and sufficient for nodule organogenesis. This breakthrough finding demonstrated that perception of the plant hormone cytokinin is crucial for development of the symbiotic root nodule.

Keywords: symbiosis, nitrogen-fixing, legumes, rhizobia, epidermis, root hairs cortex, nodule organogenesis, nodule.

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AM - arbuscular mycorrhiza
AON - autoregulation of nodulation
BA - benzyladenine
bHLH - basic helix-loop-helix
CAPS - cleaved amplified polymorphic sequences
CEM - crack entry mechanism
CGS - common symbiosis genes
CX - cortex
DAI - days after infection
DIC - Differential interference contrast
EMS - Ethyl methanesulfonate
EN - endodermis
EP - epidermis
GFP - green fluorescence protein
GRAS - GAI, RGA, SCR
HAR1 - hypernodulation aberrant root formation
HK - histidine Kinase
IC - infected cells
IT - infection thread
LCO - lipo-chitooligosaccharides
LTR - long terminal repeat
LRR - leucine-rich repeat
MMt - million metric tones
N - Nitrogen
NAA - Naphthylacetic acid
NC - nodule cortex
NF - nod factors
Nod - nodulating
NP - nodule primordial

NFS - nitrogen fixing symbiosis

ORF - open reading frame

P - Phosphorous

PBS - primer binding site

PPF - photosynthetic photon flux

PPT - polypuring track

RACE - rapid amplification of DNA ends

RHD - root hair-dependent

RNS - root nodule symbiosis

RT-PCR - reverse transcription-polymeryse chain rection

SEM - scanning electron microscopy

SSR - simple sequence repeat

TILLING - targeted induced local lesions in genomes

TRIM - terminal-repeat retrotransposons in miniature

UBI - ubiquitin

UTR - untranslated region

VB - vascular bundle

CHAPTER 1
GENERAL INTRODUCTION

1.1 From an agricultural point of view.

The intensive farming brought about by the Green Revolution resulted in many positive changes which allowed the level of food production to keep pace with the growing human population. While providing an unprecedented level of national food security in some countries and lifting large numbers of people out of poverty and hunger, the Green Revolution had and still has negative effects. Excessive use of expensive fertilizers and pesticides, new irrigation practices and heavy dependence on a few major cereal varieties are among those factors cited as the "curse" of the Green Revolution (Hazel Peter, 2003).

The environmental damage and loss of biodiversity associated with the Green Revolution still remain to be adequately addressed. In this context, one of the most important factors to consider is the environmental destruction associated with the intensive use of industrial nitrogen (N) and phosphorous (P) fertilizers.

It is ironic that, in spite of being one of the most abundant elements on Earth, nitrogen is also one of the most growth-limiting factors, which is primarily because of its inaccessibility to plants. Historically, the production of industrial N fertilizers has followed the trend of human population. The annual amount of N fertilizer applied to soil has increased from 1.3 million metric tones (MMt) in 1930 to 90 MMt currently being added to agricultural lands (Good *et al.,* 2004).

The reduction of atmospheric di-nitrogen (N_2) through the Haber-Bosch process to ammonium, which is then used to produce N fertilizers, requires large amounts of fossil fuels as an energy source (Galloway *et al.*, 1995). In fact, industrial N_2 fixation (i.e. Haber-Bosch process) accounts for approximately 50% of fossil fuel usage in agriculture, which contributes significantly to increased emissions of greenhouse gases while, at the same time, depletes non-renewable resources.

As only approximately 30% of anthropogenic N is absorbed by plants, a significant amount of N leaches to ground water and the atmosphere (Figure 1), where it is responsible for a variety of negative effects. Some of these harmful effects include the eutrophication and hypoxia of aquatic ecosystems, as well as the build up of N_2O, a greenhouse gas which is approximately 300 times more potent than CO_2 (Forster, 2007).

Finally, an excess of N in drinking water has been linked with met-hemoglobin anemia and other serious illnesses in infants, young children and adults (Galloway *et al.*, 1995).

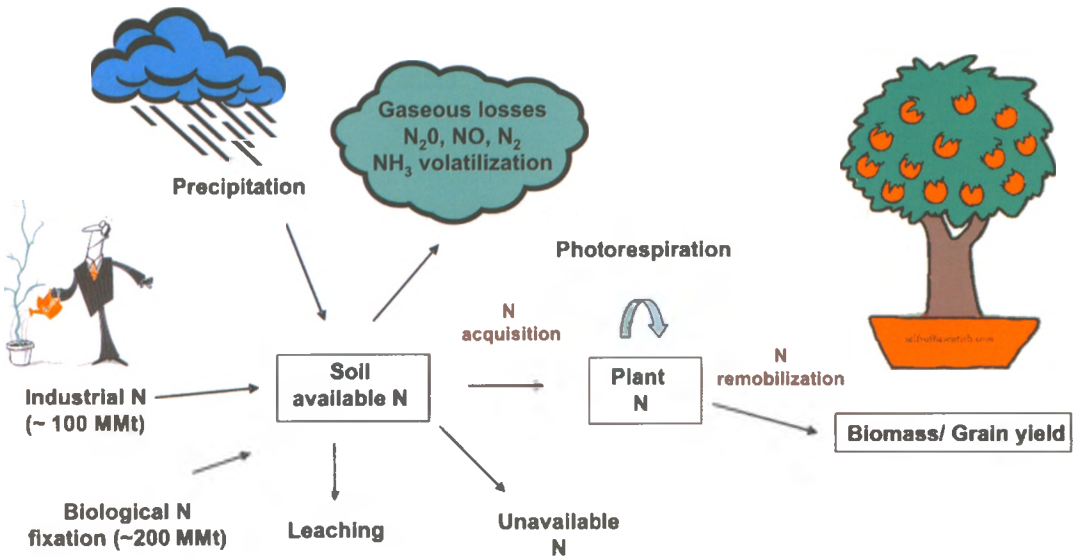

Figure 1. Fates of nitrogen in the environment. Nitrogen available in soils can come from biological N fixation, natural sources such as lightning, volcano eruption, or be supplied in the form of artificial fertilizer. Some of this nitrogen will be lost by leaching to lakes and rivers, converted to unavailable forms or released in gaseous forms. The rest of N can be acquired by plants and is used for biomass production. Figure modified from Good, *et al.* (2004).

Phosphorous fertilizers are produced from rock phosphate deposits. Like N fertilizers, the mining and use of industrial P fertilizers have a negative impact on the environment. Importantly, the available estimates suggest that the relatively inexpensive rock phosphate reserves can be depleted in as little as 50 years (Runge-Metzger, 1995).

In the long term, the negative impact of industrial fertilizers might outweigh the benefits. Instead of sacrificing the ecological health of farmlands, where heavy application of fertilizers is required to boost production levels, we could try to learn from some 475

million years of terrestrial plant evolution, for which a variety of "biofertilizer" strategies that allow the plant to cope with the nutrient limitations, have already been invented (see below). These natural phenomena need to be further explored and understood on the fundamental level. This, in turn, should facilitate the emergence of knowledge that will be required for the establishment of new sustainable plant production systems, where minimum input, limited use of non-renewable resources and preservation of the environment are the guiding principles.

1.2 Legumes, the "holy grail" of sustainable growth.

The legume family is one of the most important groups of plants worldwide. Legumes are not only an important source of proteins and edible oil in human diet but also an excellent basis of fodder and forage crops for animals. Many species of the family are also valued for their medicinal and ornamental properties and some are being used in the production of dyes and timber (Graham *et al.*, 2003).

Legumes are also excellent natural fertilizers. Their remarkable ability to sustain growth under limited soil nutrient conditions and propensity to reinvigorate other plants has been recognized from the earliest historic times. These useful properties reflect a range of evolutionary adaptations (Table 1) which account for the ability of legumes to grow under low N and P conditions.

Of particular importance in this context are the symbiotic interactions of legumes with beneficial soil microorganisms, which provide legumes with nitrogen and also phosphorus and other soil nutrients. Legumes, such as soybean, pea, and alfalfa develop root nodules in which nitrogen fixing bacteria, commonly known as rhizobia, reside and fix atmospheric N_2, thus limiting the need for artificial fertilizer. These mutualistic interactions constitute a major driving force behind the host plant's nitrogen-rich lifestyle. In addition to its positive impact on overall yield and grain composition, the ability of legumes to acquire atmospheric N_2 through root nodule symbiosis significantly improves sustainability under field conditions (Vance *et al.*, 2001)

Table 1. Plant adaptations to low N and P

Strategy	Adaptation
Enhanced acquisition or uptake	Expanded root surface area (more roots and root hairs, cluster roots, and longer roots) Enhanced expression of NO_3^-, PO_4^+, NH_4^+ transporters, aquaporins, and phosphatases Increased organic acid synthesis and exudation Mycorrhizal symbiosis Rhizobial symbiosis
Conservation of use	Internal remobilization Decreased growth rate More growth per unit N or P (nutrient use efficiency) Modified carbon and nitrogen metabolism

Adopted from Vance, C., Plant Physiology, 127, 390 (2001)

It was the propensity for nitrogen fixing root symbiosis (NFS), which is restricted to members of only 10 out of approximately 380 angiosperm families and which is not present in *Arabidopsis thaliana*, that captivated the imagination of plant scientists and prompted vigorous research into the underlying mechanisms (Szczyglowski and Stougaard, 2008).

Recent rapid progress in deciphering the molecular basis of NFS, as fueled by the availability of model legume organisms (Cook, 1999; Handberg and Stougarrd, 1992) and associated genomic resources (Sato *et al.* 2008), has reinvigorated a long-standing interest in improving and/or transferring this ability to plants that are currently unable to do so. Gaining basic knowledge of the mechanisms involved and the realization of the extent to which the orthologous functions operate in important non-legume crop species is the first necessary step in the serious consideration of such a defined goal. **Therefore, the overall objective of this thesis was to perform a research that contributes to the development of the comprehensive molecular picture of NFS in legumes.**

1.3 Root colonization by bacteria.

There are several known ways by which bacteria colonize plant roots. During extracellular root colonization, several rhizospheric bacteria proceed as far as to occupy intercellular space (i.e. residing in between root epidermis and/or cortical cells, as in the case of bacterial endophytes), but they are never internalized within the plant cells. This type of interaction, which was postulated to represent the default bacterial root colonization mode (Sprent and James, 2007; Figure 2), likely pertains to a majority of, if not all plant species but the mechanism(s) that selects for meaningful growth-promoting associations are largely unknown.

The presumed transition from the default, extracellular, root colonization by bacteria to intracellular accommodation, such as the one associated with the NFS, is alleged to have occurred some 55 mya, concomitant with the radiation of a hypothetical progenitor of extant legumes (Kistner and Parniske, 2002). This resulted in two major modes by which extant plants accommodate bacteria within the intracellular compartment (Figure 2).

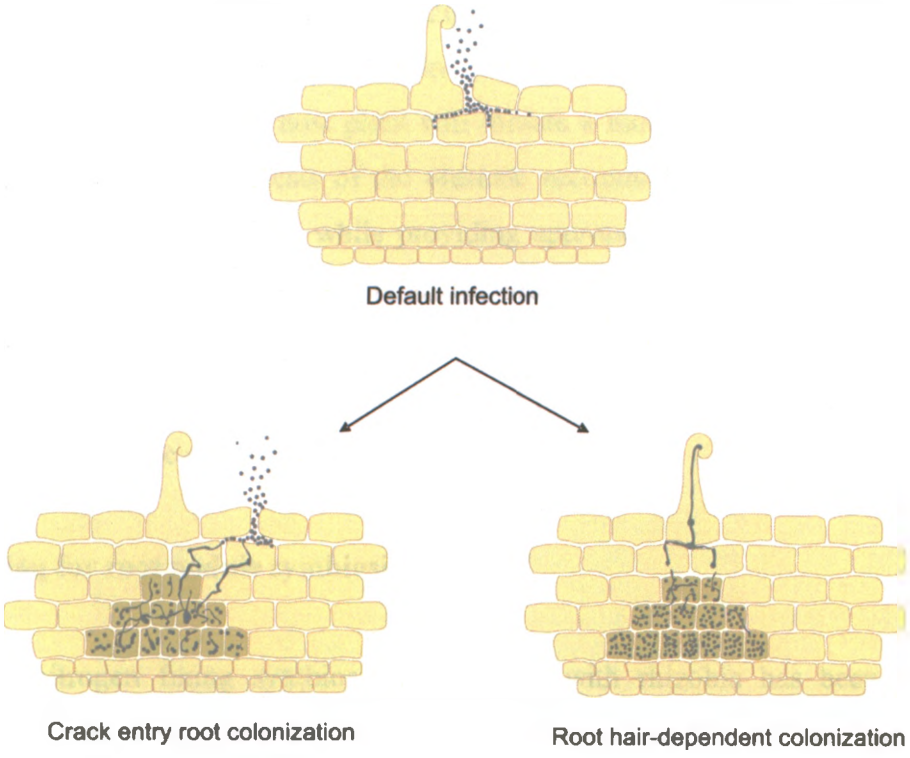

Figure 2. Modes of root colonization by symbiotic bacteria

In many legumes, the internalization of bacteria within plant cells occurs at the root epidermis, where bacteria first enter inside root hairs (i.e. root hair-dependent colonization, RHD) and subsequently proceed to colonize the intracellular space of the root cortex (Guinel *et al.* 2002). Alternatively, bacteria can enter roots through exposed root cortex, either due to mechanical damage or in association with emerging lateral root primordia. This root-hair independent mode of colonization is often referred to as the crack entry mechanism (CEM). The intercellular infection pockets are formed at, or directly beneath, root cracks, from which intracellular colonization of root cortex is initiated. Which root invasion path will be used is dictated by the host plant; although, one interesting example, has also been reported where specific environmental conditions alternate the mode of root colonization by bacteria, (Goormachtig *et al.*, 2004).

In a tropical legume, *Sesbania rostrata*, both RHD and CEM is used, dependent on environmental conditions. In well-aerated soils the RHD mode of colonization is supported. However, when *Sesbania* is grown under limited oxygen availability, such as periodic flood conditions which prevent root hair formation due to accumulation of ethylene, the CEM will be used to assure successful progression of the root colonization by symbiotic bacteria (Goormachtig *et al.*, 2004).

Concomitant with bacterial infection at the root surface and regardless of the mode of root colonization used, the host plant will initiate a morphogenic process, which will culminate with the organogenesis of the ultimate accommodation structures, called root nodules. Nodules house bacteria while providing appropriate physiological conditions in support of symbiotic nitrogen fixation (Oldroyd and Downie, 2008).

In chapters 3 and 4 of this thesis, a genetic modification of root architecture in the model legume *L. japonicus* is described, which led to the discovery of yet another mode of root colonization by the symbiotic bacteria.

1.4 Signaling for root nodule symbiosis.

The nitrogen fixing symbiosis of legumes with rhizobia has been intensively studied. The current knowledge of molecular mechanisms that mediate bacterial entry and the organogenesis of root nodules is predominantly based on research with two model

legumes, *Lotus japonicus* and *Medicago truncatula* (Handberg and Stougaard, 1992; Cook *et al.*, 1999). These plants accommodate their respective microsymbionts, *Mezorhizobium loti* and *Sinorhizobium melliloti,* by the RHD mechanism but differ with regard to the final nodule structure. *L. japonicus* forms determinate nodules, which lack a persistent meristem and, therefore, have a spherical appearance. In contrast, *M. truncatula* develops indeterminate nodules that are characterized by an elongated shape, due to the presence of persistent meristem activity. Regardless of these differences, many mechanisms that have been uncovered thus far are shared by both symbiotic systems. For simplicity, these will be outlined based on the *L. japonicus-M. loti* symbiosis.

The symbiotic partnership between the host plant and symbiotic bacterium is initiated by sophisticated chemical communication. Specific components of root exudates, mostly flavones or isoflavonoids, serve to inform symbiotic bacterium about the presence of the host plant. These direct bacteria to the root surface and also initiate transcriptional reprogramming in the genome of the compatible bacterial symbiont (Denarie *et al.* 1996).

Firstly, the bacterial NodD transcription factor is activated which results, among other, in the synthesis of morphogenic lipochito-oligosaccharide signaling molecules, known as nodulation or Nod factors (NF), Figure 3.

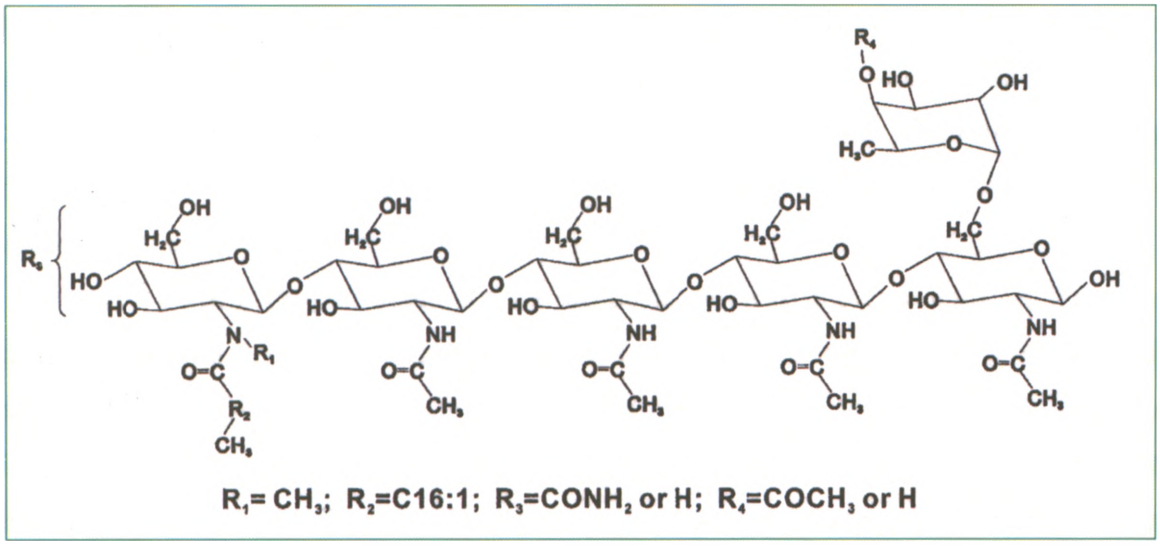

Figure 3. Structure of *M. loti* Nod factor. Figure adopted from Niwa *et al.*, 2001).

The major Nod factor produced by *M. loti*, the natural microsymbiont of *L. japonicus*, is an N-acetylglucosamine pentasaccharide, in which the non-reducing residue is N-acylated with a C18:1 acyl moiety, N-methylated, and carries a carbamoyl group, while the reducing end is substituted with 4-*O*-acetylfucose (Niwa *et al.,* 2001). Ectopic application of this Nod factor on *L. japonicus* roots incites various cellular and molecular responses that are reminiscent of the early responses to Nod factors and/or rhizobial infection in other legume species (Lopez-Lara *et al.*, 1995; Niwa *et al.*, 2001).

NFs act as determinants of host specificity and inducers of various morphogenic processes in the host root (Denarie *et al.*, 1996; Riely *et al.*, 2004). As a result, two interlinked developmental programs are initiated at the root epidermis and within the root cortex (Figure 4).

The epidermal program functions to mediate internalization of bacteria within the host plant root. The initial response of root hairs to the NF-producing rhizobia involves the establishment of *de novo* polar root hair tip-growth and curling, which leads to the formation of typical "shepherd's crook" structures (Esseling *et al.*, 2003). These structures entrap the bacteria and serve as a starting point for the initiation of the infection process. The intracellular infection proceeds via a local invagination of the plant plasma membrane and its subsequent extension, which leads to the formation of growing infection structures, the infection threads (ITs). The intracellular progression of the IT through the root hair and within the root cortex occurs via a tip-growth-like mechanism. This is guided by a specific arrangement of the cytoplasm in the underlying cortical cells, which form so called cytoplasmic bridges. These cytoplasmic bridges are used for ITs to reach the subtending nodule primordium, which has been established within the cortex as the result of the NF-induced root cortical program (van Brussel *et al.*, 1992; van Spronsen *et al.*, 2001).

The cortical program begins with the polarization of the cytoplasm of the outer cortical cells and de-differentiation of the inner cortical cells, which is followed by cell divisions. The third cortical cell layer is proposed to give rise to the first cell divisions leading to the formation of nodule primordia in inoculated *L. japonicus* roots (van Spronsen *et al.*, 2001). These cells undergo successive divisions, which during further development spread to surrounding cell layers, and eventually, give rise to a clearly defined nodule primordium. Once the IT reaches nodule primorida, rhizobia are released

via an endocytic-like process into the plant cell cytosol. They remain separated from the host cytoplasm by the plant–derived peribacteroid membrane to form new intracellular organ-like structures called symbiosomes. Inside these symbiosomes rhizobia differentiate into nitrogen-fixing bacteroids. Thus, interlinked progression of both cortical and epidermal programs gives rise to fully developed nitrogen fixing root nodules (Figure 4)

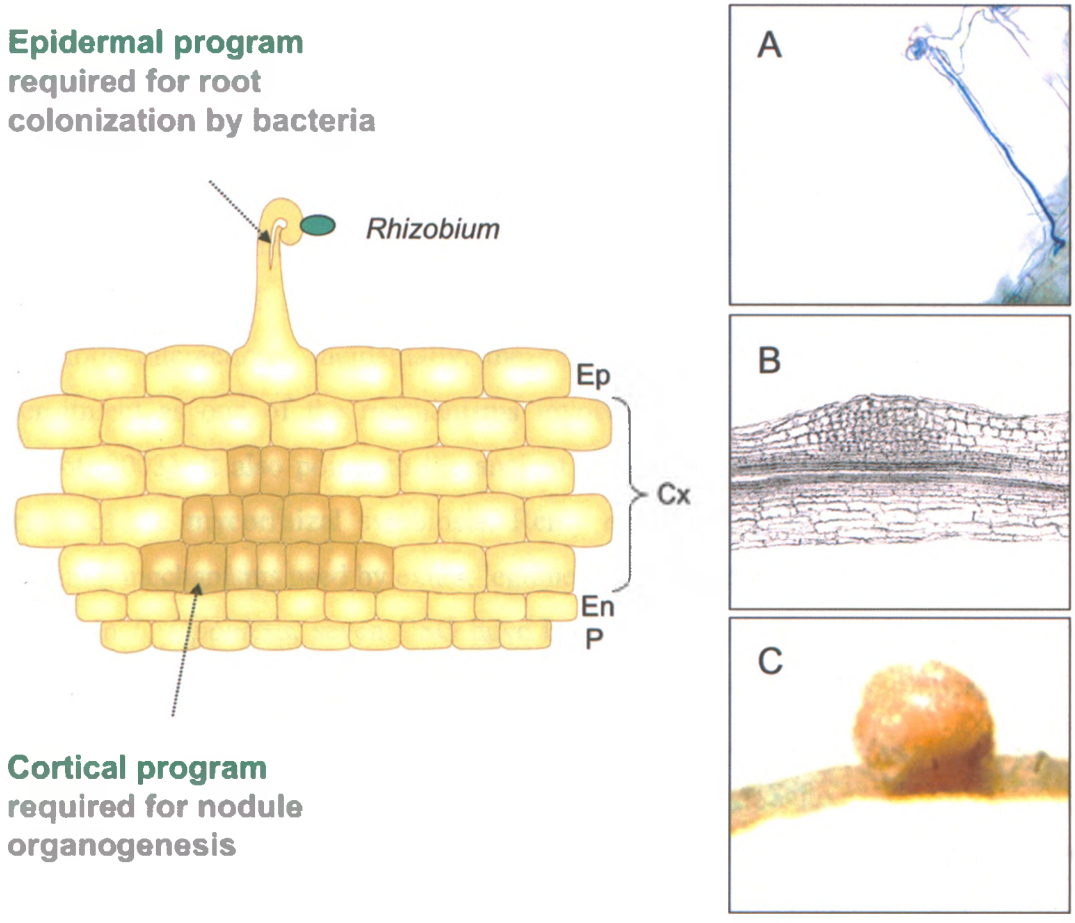

Figure 4. Epidermal and cortical programs required for development of nitrogen-fixing nodules. inset Panel A shows *M. loti* (visualized by blue color) that is migrating inside growing IT, B) NF-dependent activation of cortical cell divisions that resulted in the development of a nodule primordium, C) *L. japonicus* nitrogen-fixing nodule. Ep – epidermis, Cx – cortex, En – endodermis, P - pericicle.

1.5 Nod factor signalling pathway.

Several key perception/transduction elements that mediate host root responses to NF signaling have been identified. In *L. japonicus*, NF is recognized by a complex of two LysM-receptor kinases, called Nod factor receptor 1 and 5 (NFR1 and NFR5; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). Downstream from this perception complex, a set of seven so called common symbiosis genes (CSG) operate to mediate early root responses to symbiotic signaling, including rapid oscillation of cellular calcium concentration, termed Ca^{2+} spiking (Oldroyed and Downie, 2008).

The CSG have been defined based on their key role, not only during the NFS, but also during the formation of phosphate-acquiring symbiosis of plants and arbuscular mycorrhiza (AM) fungi. Deleterious mutations in any of these genes abolish both symbiotic interactions. Incidentally, the existence of CSG supports the notion of gene recruitment as one of the mechanisms involved in NFS evolution (Szczyglowski and Amyot, 2003). It also points to the ancient origin (~ 450 mya based upon the predicted origin of the mycorrhizal symbiosis; Remy *et al.*, 1994) of at least a portion of the signaling mechanisms used by extant legumes to sustain NFS.

In *L. japonicus*, seven CSG have been discovered. Five of these genes, encoding leucine-rich repeats (LRR) symbiosis receptor-like kinase (SYMRK; Stracke *et al.* 2002), the CASTOR and POLLUX ion channels (Ane *et al.*, 2002; Imaizumi-Anraku *et al.*, 2005), and two nuclear pore proteins NUP85 and NUP133 (Saito *et al.*, 2007; Kanamori *et al.*, 2006) were shown to be required for generation of the Ca^{2+} spiking response. Two additional genes, which encode calcium and calmodulin-dependant kinase CCAMK (Levy *et al.*, 2004; Mitra *et al.*, 2004; Tirichine *et al.*, 2006) and its phosphorylation target, the nuclear-localized CYCLOPS protein (Yano *et al.*, 2008) were presumed to be necessary for the interpretation of specific Ca^{2+} signatures (i.e. frequency and amplitude of Ca^{2+} spikes). This, in turn, is considered as required to link the environmental root response pathway to NF, as mediated by NFR1/5 complex and CSG, with the inherent plant developmental mechanisms that mediate both infection and nodule organogenesis.

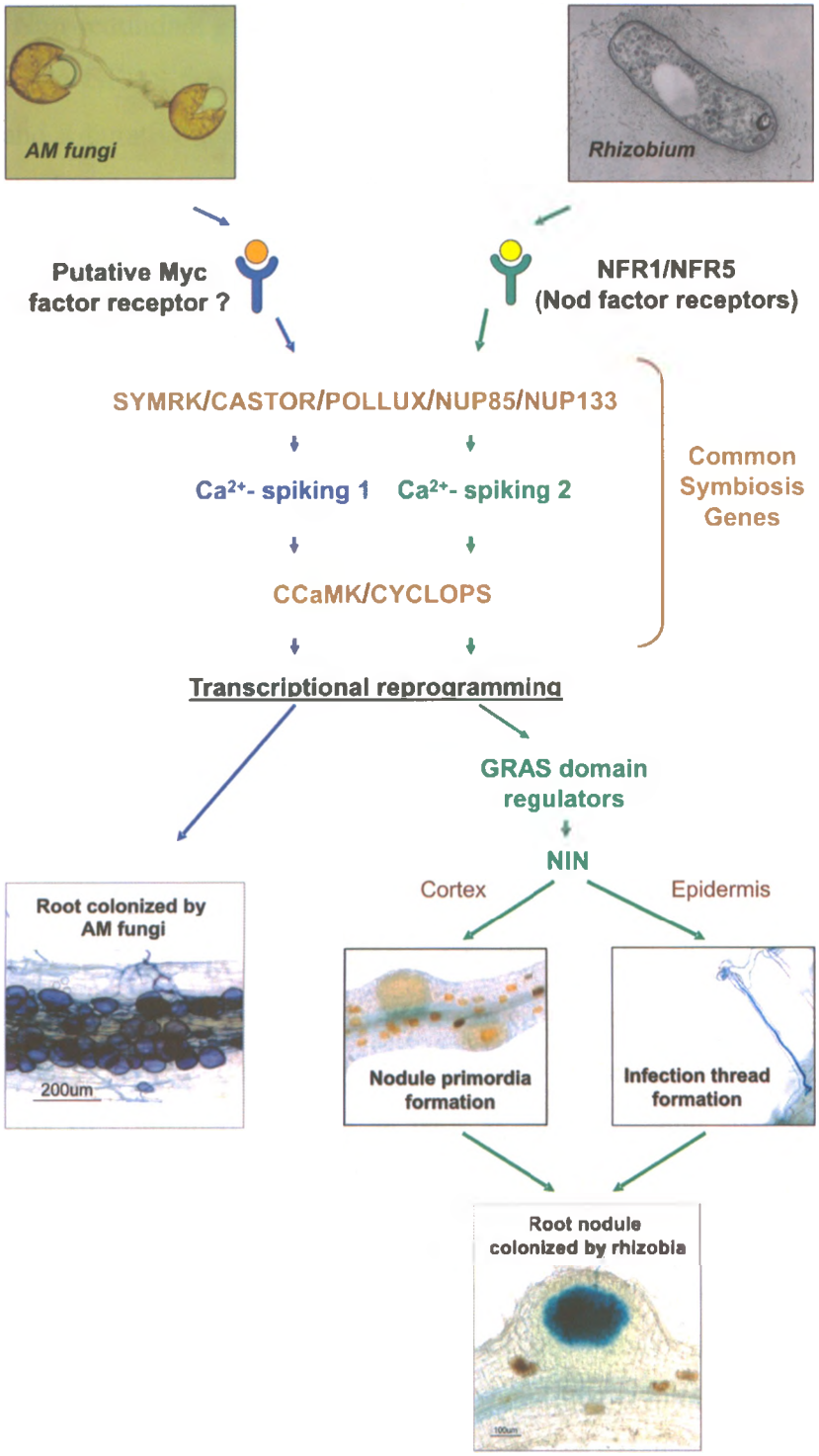

Figure 5. The symbiosis pathway for nodule nitrogen fixing and arbuscular mycorrhiza interactions. Only selected signaling elements are shown. Note that two different Ca^{2+} spiking, Ca^{2+} spiking 1 and Ca^{2+} spiking 2, are generated during AM and NFS, respectively.

Non-redundant plant function that act downstream from the NF receptors and CSG, such as two GRAS-family transcriptional regulators, NSP1 and NSP2 (Heckmann *et al.*, 2006) and a putative transcription regulator NIN (Schauser *et al.*, 1999) have also been shown to be required for IT formation and nodule primordia initiation. However, they were found to be dispensable for AM symbiosis.

Rapid progress in sequencing the *L. japonicus* genome (Sato *et al.*, 2008) constitutes a major driving force behind these increasingly successful experiments, based on which the molecular skeleton of the signal transduction pathway(s) that underlie the mechanisms of micro-symbiont accommodation and the organogenesis of symbiont-specific structures begins to emerge (Figure 5).

In chapter 5 of this thesis, a set of experiments is described which led to a major breakthrough discovery by linking the NF-dependent root response to cytokinin signaling, while defining the latter as necessary and sufficient for nodule organogenesis in *L. japonicus*.

1.6 Homeostasis of root nodule symbiosis.

Nodule organogenesis is initiated by NF but the extent of nodulation is strictly controlled by the host plant. It has been shown that the host plant will restrict or allow nodule development depending on various environmental factors, including the availability of combined nitrogen as well as developmental cues associated with plant growth (Caetano-Anolles and Gresshoff, 1991; Francisco and Harper, 1995; Nutman, 1952; Pearsons *et al.*, 1993).

There are at least two mechanisms that are responsible for controlling nodule number. The first one operates locally to restrict the number of successful infection events within the root susceptible zone, which is located just behind the growing root tip (Vasse *et al.*, 1993; Penmetsa and Cook, 1997). The second mechanism, called autoregulation of nodulation (AON), involves systemic regulation, wherein nodule formation on younger root tissues is inhibited by prior nodulation events (Caetano-Anolles and Gresshoff, 1991). This feed-back regulation involves root to shoot and shoot to root signaling events that assure the homeostasis of the symbiotic interaction (Figure 6)

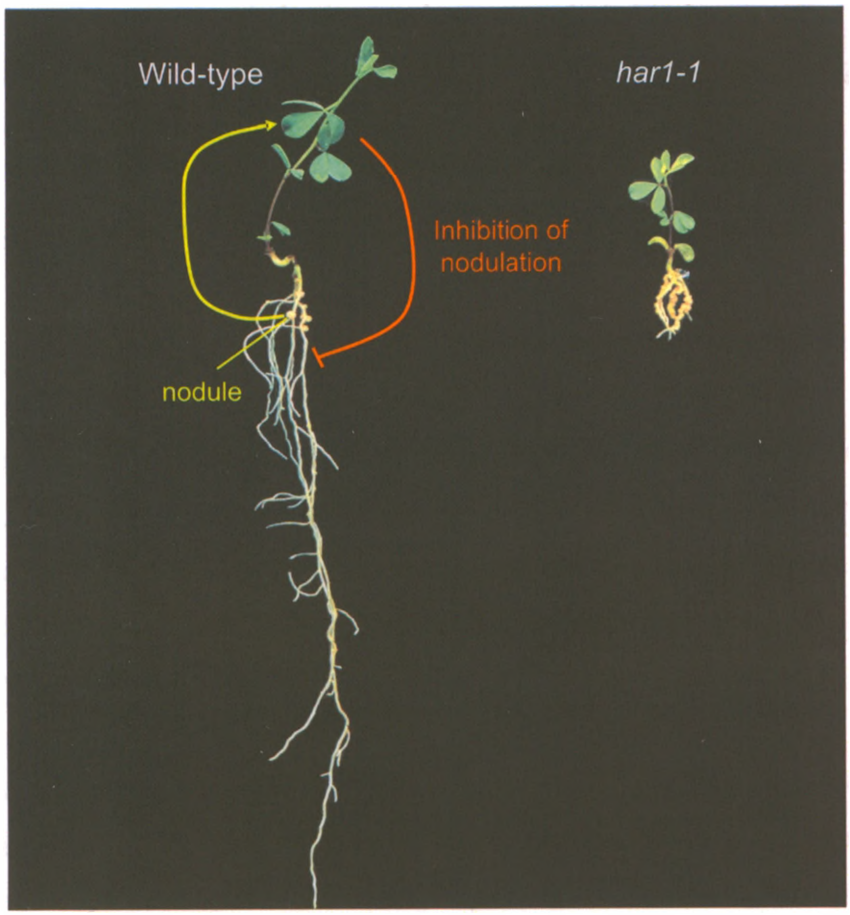

Figure 6. AON operates via root-shoot-root derived feedback signaling to inhibit nodulation on younger root tissues in wild type plants. AON is impaired in *L. japonicus har1-1* mutant resulting in the hypernodulating phenotype.

In *L. japonicus*, the Har1 receptor kinase (Wopereis *et al.*, 2000; Krusell *et al.* 2002) was defined as essential elements of AON. Mutations in these locus impair the ability of the plant to systemically regulate nodule development, such that a significantly increased number of nodules are formed (Figure 6). To learn more about AON, an approach has been designed to identify genetic suppressors of the *L. japonicus har1-1* hypernodulation phenotype. Two main goals for this screen were put forward. First, since the *har1-1* mutation impairs the systemic mechanism of nodule auto-regulation, a screen for suppressor mutations could potentially identify additional components of this interesting regulatory pathway. Secondly, given the hypermorph nature (hypernodulation)

of the *har1-1* mutation, a variety of symbiotic phenotypes, including those difficult to select for in the wild-type genetic background, were predicted to be identified. In chapter 2, of this thesis, results of such a genetic screen are described along with the characterization of several novel symbiosis-relevant loci and/or alleles.

1.7 Objectives.

From a plant-biologist perspective, a long-term goal of the research in the area of nitrogen-fixing symbiosis is to uncover and to understand the function of all plant genes that are important for this particular biological process. In order to contribute to such a defined goal, a forward screening aimed at identifying genetic lesions that impair the ability of the host plant to perform the symbiotic interaction was carried out in the biological background of the *L. japonicus har1* mutation. Based on this screen the following specific objectives were proposed:

1) To characterize a collection of 26 Nod- mutant lines. The detailed molecular genetic characterization of these mutants was predicted to reveal novel plant functions involved in the root development and early signaling events during nodule primordia formation (Chapter 2).

2) To perform functional characterization and molecular cloning of the *L. japonicus LjRHL1* locus. To answer the question how bacteria colonize the root of the *Lrhl1-1* mutants in spite of an apparent lack of the epidermal program (Chapter 3 and 4).

3) To map-based clone *LjS32-AA* locus which is required for initiation of cortical program (Chapter 5).

4) To generate a double mutant between *LjS32-AA* and *Ljrhl1*-1 to further understand the relationship between the epidermal and cortical programs during nodule organogenesis (Chapter 5).

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CHAPTER 2

Genetic suppressors of the *Lotus japonicus har1-1* hypernodulation phenotype.

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2.1 Contributions made by Bogumil Karas:

- Screening and isolations of mutant lines: analyzed symbiotic and non-symbiotic phenotypes of all mutant lines that were initially isolated from *har1-1* suppressor screen. Mutant lines were grouped into two categories: the first group exhibited very strong symbiotic phenotypes (no nodules) while the second category included mutants with more subtle phenotypes (Table 1 & 3).

- Mapping of suppressor lines with strong defects: generated all F1 and F2 plants. Together with Dr. Jeremy Murray, technicians and summer students positioned loci for all these mutant lines on *L. japonicus* genetic map (Table 1)

- Establishment of four complementation groups for mutants with defects in root hair development (Table 3)

- Construction of *har1-1* introgression line (Generations 1 & 2)

- Positioning of *LjS32-AA* locus on *L. japonicus* chromosme IV (Figure 5).

2.2 Introduction.

Lotus japonicus har1 mutants respond to inoculation with *Mesorhizobium loti* by forming an excessive number of nodules due to genetic lesions in the *HAR1* autoregulatory receptor kinase gene (Wopereis et al. 200). To expand the repertoire of mutants available for the genetic dissection of the root nodule symbiosis (RNS), a screen for suppressors of the *L. japonicus har1-1* hypernodulation phenotype was performed. Out of 150,000 M2 plants analysed, a total of 61 stable *L. japonicus* double mutant lines were isolated. In the context of the *har1-1* mutation, 26 mutant lines were unable to form RNS, while the remaining 35 mutant lines carried more subtle symbiotic phenotypes forming either white ineffective nodules or showing reduced nodulation capacity. When challenged with *Glomus intraradices*, 18 of the 61 suppressor lines were unable to establish a symbiosis with this arbuscular mycorrhiza (AM) fungus. Using a combined approach of genetic mapping, TILLING, and sequencing, all non-nodulating mutant lines were characterized and were shown to represent new alleles of at least 9 independent symbiotic loci. The class of mutants with reduced nodulation capacity was of particular interest since some of them may specify novel plant functions that regulate nodule development in *L. japonicus*. To facilitate mapping of the latter class of mutants, an introgression line, in which the *har1-1* allele was introduced into a polymorphic background of *L. japonicus* ecotype MG20, was constructed.

Given the hypermorphic nature of the *har1-1* mutation, a variety of symbiotic phenotypes and associated loci, including those difficult to select for in the wild-type genetic background, were identified and characterized.

2.3 Results.

2.3.1 Screening for suppressors of the *har1-1* hypernodulation phenotype.

In the absence of rhizobia, the *L. japonicus har1-1* mutant exhibits altered root architecture, a non-symbiotic root phenotype, which is characterized by the inhibition of root elongation and a concomitant increase in lateral root formation (bushy root phenotype; Wopereis *et al.*, 2000). When challenged by *M. loti*, *har1-1* mutant roots develop the hypernodulation phenotype, where supernumerary nitrogen fixing nodules are

formed. This exaggerated symbiotic development of *har1-1* roots constituted the basis for a screen for genetic suppressors of *har1-1* hypernodulation. Any mutation that counteracted *har1-1*-dependent hypernodulation, including those resulting in non-nodulating (Nod$^-$) phenotypes, has been defined for the purpose of this work as a suppressor mutation.

Of 20,000 *har1-1/har1-1* seeds treated with ethyl methanesulfonate, approximately 10,000 fertile M1 plants were obtained. Three week old M1 plants, grown in the absence of rhizobia, were uprooted and the morphology of their root systems was visually evaluated to identify any modification of root architecture resulting from a dominant mutation. Since all M1 plants displayed the bushy root morphology of the un-inoculated *har1-1* plant, they were replanted and grown to maturity to produce M2 progeny. A total of approximately 150,000 M2 plants, derived from 100 independent pools (see Materials and Methods), were subjected to further phenotypic analysis by scoring their nodulation phenotypes at 21 days after inoculation (dai). The stable mutant lines at the M4/M5 generation level were further analyzed at 10, 21, and 48 dai using *M. loti* strain NZP2235 that carried a constitutive reporter *hemA:lacZ* cassette, which permitted histochemical detection to observe the progression of infection events. Overall, 26 suppressor lines were found to be characterized by easily identifiable mutant phenotypes, showing strong defects in the development of RNS, while the additional 35 mutant lines carried more subtle symbiotic aberrations. The molecular analysis, using a *har1-1* allele-specific cleaved amplified polymorphic sequence (CAPS) marker, confirmed that all selected suppressor lines were homozygous for the *har1-1* mutant allele (data not shown). Consequently, they were assumed to represent double mutants that carry secondary mutations that are responsible for the observed departure of their nodulation phenotype from the hypernodulation of the *har1-1* parental line. This assumption was tested and confirmed through classical genetic and molecular analyses (see below).

2.3.2 Nodulation defective suppressor lines.

When evaluated at 21 dai, 26 *L. japonicus* suppressor lines did not form root nodules (Table 1). The *har1-1* mutant was hypernodulated at this stage, developing on average 41 ± 0.5 (n = 10) pink nodules. Further phenotypic evaluation, at 48 dai, revealed

Table 1. Phenotype, flanking microsatellite markers, and predicted loci for *L. japonicus* suppressor lines with strong defects in RNS.

Name	Segregation ratio	Nodulation		AM	Chr.	Flanking marker		Candidate gene
	wt:*har1*:sm:dm	21dai	48dai	21dai		Upper	Lower	
LjB21-1	198:62:77:20	-	-	+	1,2	tel	TM0324	*NIN*
LjB46-C	60:34:32:9	-	-/+	nd	1,2	tel	TM1659	*NUP133*
LjB62-D	157:40:51:12	-	-/+	-	1,2	TM0324	TM1659	*NUP133*
LjB80-A	150:55:46:16	-	-	-	1,2	tel	TM00512	*NUP133*
LjB83-B	82:24:21:5	-	-/+	-	1,2	tel	TM00512	*NUP133*
LjB88-B2	101:29:26:8	-	-	-	1,2	tel	TM1659	*NUP133*
LjSup12	102:40:32:6	-	-/+	-	1,2	tel	TM1659	*NUP133*
LjB32-A	157:54:34:21	-	-	+	1,2	tel	TM0507	*SYM70*
LjB85-E	179:56:46:18	-	-	+	1,2	TM0370	TM0329	*SYM70*
LjB90-A	232:50:28:19[a]	-	-	+	1,2	TM0370	TM0329	*SYM70*
LjB46-D	106:35:28:7	-	-/+	-	1,2	TM0011	TM0496	*SYM24*
LjB32-BA	135:47:46:11	-	-	+	1	TM0122	tel	*SYM80,CASTOR*
LjS41-1	226:87:81:24	-	-	+	1	TM0295	tel	*SYM80,CASTOR*
LjB5-2	109:32:33:13	-	-	-	1	TM0122	tel	*CASTOR*
LjB68-B	137:38:50:19	-	-	-	1	TM0122	tel	*CASTOR*
LjS46-1	192:59:60:22	-	-	-	1	TM0835	tel	*CASTOR*
LjS50-1	178:51:60:13	-	-	-	2	TM0076	TM0230	*SYM6, SYMRK*
LjB31-E	168:64:64:21	-	-	+	2	TM0257	TM0504	*NFR-5*
LjS4-1	225:65:58:16	-	-	+	2	TM0257	TM0550	*NFR-5*
LjS58-1	158:54:57:15	-	-	+	2	TM0257	TM1037	*NFR-5*
LjB91-B	112:18:26:16	-	-	-	3	TM0155	TM0129	*LjCCaMK*
LjS95-B	109:32:33:13	-	-	-	3	TM0035	TM0142	*LjCCaMK*
LjB12-1A	114:44:42:9	-	-	-	6	TM0336	tel	*POLLUX*
LjB50-C	120:28:41:16	-	-/+	-	6	TM0336	tel	*POLLUX*
LjS49-D	97:30:25:12	-	-	-	6	TM0336	tel	*POLLUX*
LjSup3	141:67:62:7	-	-	-	6	TM0336	tel	*POLLUX*

sm- single mutant; dm- double mutant; nd- not determined; AM – arbuscular mycorrhiza; (+) wild-type mycorrhiza; (-) aborted mycorrhiza; tel- telomere; .Chr.- chromosome; 1,2 – the gene has been positioned within the region that is translocated between Gifu chromosome 1 and MG-20 chromosome 2 (Hayashi et al., 2001); [a]$P < 0.05$.

that six of the 26 lines that showed a Nod$^-$ phenotype at 21 dai developed a few nodules on some plants (Nod$^{-/+}$; Table 1), while the rest remained Nod$^-$. These nodulation patterns clearly contrasted with the hypernodulation phenotype of the *har1-1* parental line, which at 48 dai developed on average 56 ± 4.1 nodules. When grown in the absence of rhizobia all 26 mutant lines displayed a short and bushy root phenotype, characteristic of the *har1-1* parental line.

In order to further classify the above described suppressor lines, two experimental avenues, the assessment of their capacity to form AM symbiosis and the genetic mapping of the underlying mutant loci, were followed. Of the 26 Nod$^-$ mutants tested, 16 failed to establish a structurally intact symbiosis with the AM fungus, *G. intraradices*, as evidenced by the inability of the fungus to effectively enter, and to form arbuscules and vesicles, within the host roots (Table 1). In addition, line LjB13-B, and LjB74-A, which were capable of developing nodules (Table 3), were impaired in their ability to form AM symbiosis, bringing the total number of mycorrhiza-defective plants identified by the suppressor screen to eighteen (Table 1 and 3).

2.3.3 Mapping suppressor lines with strong defects in root symbiosis.

Given a rapidly growing number of characterized symbiotic loci in *L. japonicus* (Sandal *et al.*, 2006), genetic mapping provides a convenient way of categorizing the newly discovered mutants as representing alleles of a previously characterized locus or as new loci. The 26 double mutant lines (Table 1) with strong nodulation and/or AM phenotypes (homozygous for both the *har1-1* allele and the presumed mutations limiting nodulation) were crossed to *L. japonicus* ecotype Miyakojima (MG20) to establish mapping populations and to test segregation against the predicted 9:3:3:1 ratio (wild-type: *har1-1*: single mutant: double mutant). Most symbiotic phenotypes segregated as predicted, while ratios for line LjB90-A deviated from the expected segregation for two independent recessive loci (Table 1).

Loci for the 26 mutant lines were positioned on the *L. japonicus* genetic map within ~10 cM intervals bounded by microsatellite markers (Table 1). All 26 suppressor loci mapped to locations in the *L. japonicus* genome that contained previously characterized symbiotic loci. Prediction of a candidate gene/locus was made based on the

best fit between the map position and the available phenotypic data (Table 1). For example, AM defective lines were likely to represent new alleles for one of the seven *L. japonicus* common symbiosis genes, *SYMRK*, *CASTOR*, *POLLUX*, *NUP133*, *SYM6*, *LjCCaMK*, or *SYM24* (Table 1).The available sequence information for previously characterized *L. japonicus* symbiotic loci was subsequently used for combined TILLING and sequencing analyses of the mutant lines. Of 26 mutants, the genetic lesions for 23 lines were identified on the molecular level, defining new alleles for *L. japonicus NFR5, SYMRK, CASTOR*, *POLLUX*, *LjCCaMK*, *NUP133*, *SYM70/LjNSP2* and *NIN* loci (Table 2). Line LjB83-B mapped to a 1.6 cM interval on chromosome 1,2 that encompasses *L. japonicus NUP133* and *NIN* loci. Given its AM defective phenotype, this line was predicted to carry a mutation in the *NUP133* locus (Table 1). Sequencing of both *NUP133* and *NIN* did not reveal any nucleotide changes, as compared with the wild-type *L. japonicus* Gifu, suggesting that the underlying mutant phenotype of line LjB83-B is determined by a mutation in an independent locus.

2.3.4 Suppressor lines with more subtle defects in root nodule symbiosis.

The 35 suppressor lines belonging to this category had phenotypic variation that ranged from mutant lines which formed a large number of predominantly white nodule primordia to those forming a low number of mainly pink nodules. The lines were grouped into four classes based on their nodulation and associated developmental and/or infection phenotypes (Table 3).

2.3.5 Class 1: white bump mutants.

Nine suppressor lines belonging to the phenotypic class 1 were characterized by predominantly white bumps/white nodule primordia phenotype (Table 3). Closer examination of these lines revealed that the majority of these lines had only a few intact ITs. Instead, frequent instances of root hair cells containing bacteria in the cytoplasm were observed likely reflecting the disintegration of ITs (Table 3). Four lines within this class (S3-BA, B31-C, B32-BB, and S14) had some ITs that were normal in appearance but failed to ramify into the growing nodule primordia. The remaining five lines formed many, often enlarged, micro-colonies.

Table 2. Supressor lines carrying new alleles, as confirmed by sequencing, are listed together with the identified mutations.

Line name	Mutated gene	Nucleotide change[a]	Amino-acid change	Allele designation
LjB21-1	*NIN*	C_{1785} to T	Q_{519} to stop	*nin-8*
LjB46-C	*NUP133*	G_{2353} to A	W_{656} to stop	*nup133-5*
LjB62-D	*NUP133*	G_{1342} to A	W_{352} to stop	*nup133-6*
LjB80-A	*NUP133*	G_{4496} to A	E_{1029} to K	*nup133-7*
LjB88-B2	*NUP133*	C_{2321} to T	Q_{679} to stop	*nup133-8*
LjSup12	*NUP133*	G_{3587} to A	W_{858} to stop	*nup133-9*
LjB85-E	*SYM70/LjNSP2*	C_{1357} to T	Q_{453} to stop	*ljnsp2-6*
LjB90-A	*SYM70/LjNSP2*	C_{869} to T	A_{290} to V	*ljnsp2-7*
LjB32-BA	*CASTOR*	G_{1594} to A	E_{363} to K	*castor-21*
LjS41-1	*CASTOR*	G_{3053} toA	W_{483} to stop	*castor-22*
LjB5-2	*CASTOR*	G_{1655} to A	G_{383} to E	*castor-23*
LjB68-B	*CASTOR*	G_{10353} to A	W_{840} to stop	*castor-24*
LjS46-1	*CASTOR*	G_{1251} to A	W_{328} to stop	*castor-25*
LjS50-1	*SYMRK*	G_{3777} to A	A_{620} to T	*symRK-13*
LjB13-B[b]	*SYMRK*	C_{1965} to T	P_{386} to T	*symRK-14*
LjS4-1	*NFR5*	G_{1415} to A	G_{472} to E	*nfr5-4*[c]
LjS58-1	*NFR5*	G_{1212} to A	W_{404} to stop	*nfr5-5*[c]
LjB31-E	*NFR5*	C_{237} deleted	Frameshift	*nfr5-6*[c]
LjB91-B	*LjCCaMK*	G_{371} to A	G_{124} to D	*ccamk-5*
LjS95-B	*LjCCaMK*	G_{610} to A	G_{204} to R	*ccamk-6*
LjB12-1A	*POLLUX*	G_{1205} to A	W_{320} to stop	*pollux-11*
LjB50-C	*POLLUX*	G_{1644} to A	Splice site	*pollux-12*
LjS49-D	*POLLUX*	G_{5682} to A	Splice site	*pollux-13*
LjSup3	*POLLUX*	C_{4804} to A	P_{719} to L	*pollux-14*

DNA sequences deposited to the Genbank under the following accession numbers were used as *L. japonicus* Gifu reference: AJ238956 for *NIN*, AJ890252 for *NUP133*, AB162016 for *CASTOR*, AP004579 for SYMRK; AJ575254 for *NFR5*, AM230792 for *LjCCaMK*, AB162017 for *POLLUX,* and AB241456/DQ665943 for *SYM70/LjNSP2*, a putative orthologue of *M. truncatula NSP2* (Allan Downie and Shinji Kawasaki, personal communication). [a]Adenine of the ATG initiator codon is designated as position 1; [b] This line is Nod$^+$ (see Table 3). [c] Allele previously reported by Sandal et al. (2006).

Table 3. Nodulation phenotypes of *L. japonicus* suppressor lines with more subtle defects in RNS.

Line name	Nodule count at 21dai	Nodule primordia/white bumps	Comments:
Gifu	9 ± 1.6	3 ± 1.5	Wild type
har1-1	41 ± 0.5	6 ± 1.4	hypernodulated, parental line
Class 1 (white bumps)			
LjS28-2B[a]	3 ± 1.3	41 ± 5.2	many microcolonies, few ITs, many disintegrated ITs,
LjS57	1 ± 0.7	73 ± 11.7	many microcolonies, few ITs, many disintegrated ITs
LjS29-1	5 ± 2.0	41 ± 6.8	many microcolonies, few ITs, some disintegrated ITs
LjS90-D	3 ± 1.1	58 ± 12.9	many microcolonies, few ITs, many disintegrated ITs
LjS51-1	1 ± 0.8	45 ± 8.1	few microcolonies, few ITs, many disintegrated ITs
LjS3-BA	1 ± 0.7	51 ± 16.4	many ITs, failure to ramify
LjB31-C	2 ± 1.3	63 ± 7.3	many ITs, many disintegrated ITs, failure to ramify
LjB32-BB	4 ± 2.2	70 ± 11.4	ITs present, many disintegrated ITs, failure to ramify
LjS14	4 ± 1.2	37 ± 7.9	ITs present, many disintegrated ITs, failure to ramify
Class 2 (root hair development)			
LjS16-2[b]	1 ± 0.7	37 ± 4.5	root hair mutant, *Ljrhl1-1*
LjB13-C[b]	nd.[c]	nd.[c]	root hair mutant, *Ljrhl1-2*
LjS3-1[b]	nd.[c]	nd.[c]	root hair mutant, *Ljrhl1-3*
LjS24-B[b]	7.2 ± 1.7	19 ± 3.7	root hair mutant, *Ljprh1-1*
LjS67-B[b]	nd.[c]	nd.[c]	root hair mutant, *Ljprh1-2*
LjS88-5A[b]	21 ± 5.0	31 ± 5.5	root hair mutant, *Ljsrh1*
LjB69-A[b]	14 ± 1.9	1 ± 0.5	root hair mutant, *Ljvrh1-1*
LjB12-1B[b]	nd.[c]	nd.[c]	root hair mutant, *Ljvrh1-2*
LjS49-AA[b]	nd.[c]	nd.[c]	root hair mutant, *Ljvrh1-3*
LjS36-1	nd.[c]	nd.[c]	root hair mutant, *Ljvrh1-4*
Class 3 (Nod+, hyperinfected)			
LjS32-AA[d]	5 ± 2.8	0	hyperinfected, deformed nodules, delayed ccd
LjS30- AA[d]	nd.[c]	nd.[c]	hyperinfected, deformed nodules, delayed ccd
LjS56-HA[d]	nd.[c]	nd.[c]	hyperinfected, deformed nodules, delayed ccd
LjS47-A	9 ± 2.8	27 ± 5.6	hyperinfected, delayed ccd
LjS95-E	63. ± 12.8	44 ± 10.3	many IT, delayed ccd
Class 4 (Nod $^+$)			
LjSup11	2 ± 1.3	11 ± 6.7	many IT, exaggerated root hair response
LjB74-A	3 ± 1.4	2 ± 0.7	few ITs, AM$^-$
LjB13-B[e]	6 ± 0.7	16 ± 2.8	exaggerated root hair response, AM$^-$, *symRK-14*
LjS97-CA	10 ± 2.6	32 ± 4.7	
LjS7-3[f]	8, 28	1, 3	only 2 plants examined at 21 dai
LjS13-AB	22 ± 3.3	4 ± 1.9	few IT
LjB88-A	25 ± 5.1	6 ± 2.5	many IT, exaggerated root hair response
LjS8-1A	30 ± 6.1	25 ± 5.5	many IT
LjS24-1B[g]	nd.[c]	nd.[c]	candidate *ALB1* allele
LjS12-5	11 ± 1.9	7 ± 2.4	less bushy root
LjS51-3	nd.[c]	nd.[c]	, supernumerary short lateral roots, fertility problem

[a]locus positioned between markers TM0095-TM0909, chromosome 5 (*SYM7* candidate); [b] Karas *et al.*, 2005; [c]nd.= not determined; [d] these lines form a complementation group; ccd – cortical cell divisions; [e]*SYMRK* allele, see Table 2; [f]actual numbers of nodules and nodule primordia are given for n=2; [g]locus positioned between markers TM0002-TM0036 on chromosome 1,2.

This latter group included line LjS28-2B, which was mapped to a location already occupied by the SYM7 locus (Sandal et al. 2006; Table 3). Since LjS28-2B and *SYM7* (Schauser et al. 1998) both formed white nodule primordia and exhibit a wild type like AM phenotype they were predicted to be allelic. Further phenotypic analysis of LjS28-2B showed that it was capable of forming numerous infection pockets, with *M. loti* entrapped within curled root hairs, but failed, to a large extent, to support the formation of ITs (Fig 1). This greatly limited the entry of *M. loti* into the roots of the mutant plant.

In addition, several instances, where the cytosol of curled root roots of the mutant plant. In addition, several instances, where the cytosol of curled root hair cells contained dispersed bacteria were observed, suggesting that occasionally the structural integrity of the initiated ITs was compromised in this mutant resulting in the release of IT contents into the epidermal cell. We named this locus *Epidermal Entry Defective* (*EED*), to reflect on the presumed function of the underlying gene in mediating the entry of *M. loti* into the *L. japonicus* root system.

2.3.6 Class 2: root hair development mutants.

This class was represented by ten independent suppressor mutant lines, reflecting four distinct *L. japonicus* loci, where defects in root hair development lead to an altered nodulation pattern of the mutant roots (Table 3). A detailed phenotypic and genetic characterization of these lines was reported earlier (Karas *et al.*, 2005).

2.3.7 Class 3: Nod$^+$ hyperinfected mutants.

The group of five mutants belonging to this phenotypic class formed normal ITs and several pink nodules. A subset of these plants was hyperinfected, and three of these lines (S30-AA, S32-AA, and S56-HA) had a low number of often misshapen and pink nodules that were flattened in appearance (Fig 2); no nodule primordia were evident, but the roots had a swollen appearance. The latter three mutants were shown to represent an allelic series by complementation crosses.

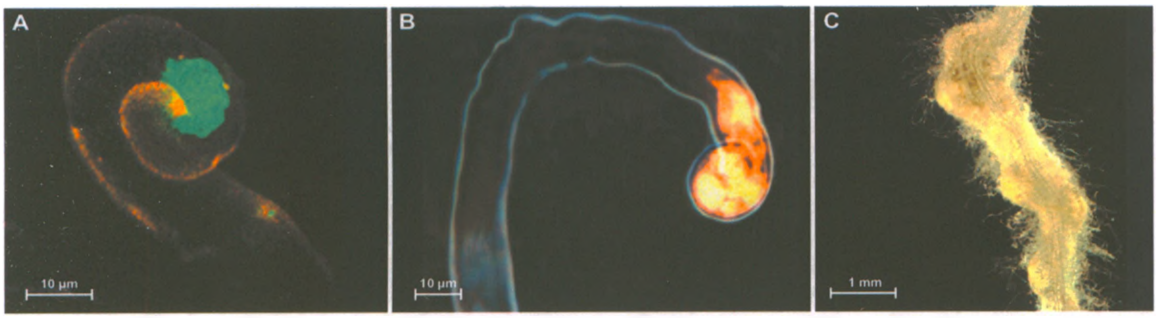

Figure 1. Symbiotic phenotype of the LjS28-2B suppressor line. (A) Confocal image of a curled root hair encapsulating an enlarged colony of the *lacZ:GFP* tagged *M. loti* strain NZP2235 (green). (B) Negative of light microscopy image of a curled root hair containing *lacZ:GUS* tagged *M. loti* (blue GUS staining appears red on the image). (C) White bumps/nodule primordia on Lj28-2B root 21dai.

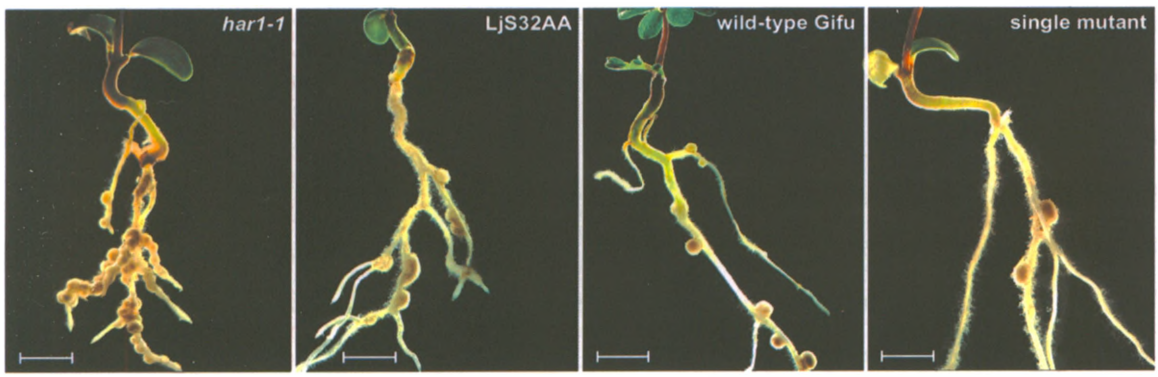

Figure 2. Root nodule phenotypes of *har1-1*, LjS32AA double mutant, wild-type Gifu, and a representative LjS32-AA single mutant, 21 dai. The single mutant shown was selected from the F2 progeny derived from the cross between LjS32-AA double mutant and wild-type Gifu and re-confirmed by progeny testing. Bar scale = 2mm.

The remaining class 3 mutant suppressor lines, S47-A and S95, had many infection threads that managed to descend and partially ramify within underdeveloped nodule structures. Interestingly, when analysed 10 dai, line LjS95 showed only very limited number of nodule formation events in spite of the ongoing abundant infection events at the root epidermis (data not shown). This line, however, became super-nodulated at 21 dai.

2.3.8 Class 4: Nod $^{+}$mutants.

The eleven mutants of class 4 formed intact ITs and pink nodules that were fewer in number in comparison with the *har1-1* parental line. This class of mutants were generally larger and healthier than Nod^{-} plants when grown in the presence of 0.5 mM KNO_3, indicating that at least some of their nodules were able to fix nitrogen. Interestingly, two of these lines, LjS51-3 and LjS12-5, in addition to the low nodulation phenotype, showed altered root architecture. While LjS51-3 had a pleiotropic phenotype including prolific development of very short lateral roots, the root system of LjS12-5A was more elongated and less bushy in comparison to the *har1-1* parental line (Fig. 3).

Notably, this class included two lines, LjB13-B and LjB74-A, that were AM^{-} (Table 3). The LjB13-B line was mapped and confirmed as an unusual Nod^{+} allele of *SYMRK* (Table. 3).

2.3.9 Mapping suppressor lines with subtle phenotypes.

An unambiguously scoreable phenotype is essential to identify homozygous mutant individuals in a mapping population, an essential step in a map based cloning project. All low nodulating suppressor lines were clearly identifiable in the context of the *har1-1* mutation. However, with the exception of very few lines, such as LjS28-2B (see above), these phenotypes turned out to be difficult to select for in either of the wild-type backgrounds (Gifu or MG20). For example, when back-crossed to wild-type Gifu, the low nodulation phenotype of LjS32-AA segregated, although it was difficult to distinguish between the single mutant plants and occasional less-well nodulated wild type plants. As a result, it was necessary to confirm all presumed homozygous single mutant plants by progeny testing to verify the initial selection.

Figure 3. Root nodule phenotypes of LjB51-3 and LjS12-5A double mutant lines in comparison to the *har1-1* parental line, 48 dai. Note proliferating short lateral roots in line LjB51-3. Line LjS12-5A showed a less bushy root phenotype than *har1-1*, with 1-8 nodules per plant. Bar scale = 2mm.

Only those plants for which the corresponding progeny showed a fully elongated root system and the low nodulation phenotype (typically 1-4 nodules per plant), with the concomitant presence of enlarged nodules, were scored as single mutants (Fig. 2). Based on this rather lengthy selection scheme the predicted 9:3:3:1 ratio (86 wild-type: 27 *har1-1* phenotype: 24 suppressed *har1-1* phenotype: 10 double mutant LjS32-AA, $\chi^2 = 0.68$; Fig. 2) was recovered, supporting a recessive mode of inheritance for this mutation.

The difficulty in the unambiguous selection of single mutants was enhanced in the background of the relatively low nodulating *L. japonicus* ecotype MG20. This feature extended the overlap between distributions of the nodule numbers for wild-type and mutant populations, further confounding the selection of the homozygous single mutants. When the LjS32-AA single mutant, which was selected from the cross to the Gifu background, was hybridized with *L. japonicus* MG20, only 1/10, instead of the expected 1/4, of the M2 plants could be unambiguously selected as homozygous LjS32-AA mutants. Using 19 homozygous individuals, the underlying locus has been preliminarily positioned to the lower half of *L. japonicus* chromosome IV (see below).

2.3.10 Construction of the MG20_har1-1 introgression line.

Similar difficulties in the unambiguous selection of homozygous mutants, as described above for line LjS32-AA, have been encountered with numerous suppressor lines with more subtle symbiotic phenotypes, thus hampering the mapping effort. To overcome this obstacle and to make mapping and subsequent cloning procedures more efficient, two approaches were considered. First, mapping based on the selection of double mutants only, which could be clearly identified among segregating phenotypes after the cross with MG20, was envisaged. Following this scenario, only 1/16 of plants from the segregating F2 population were predicted to be informative. Alternatively, the construction of an introgression line, where the *har1-1* allele ("Gifu" background) has been moved into the polymorphic background of the ecotype MG20 (a mapping partner) via marker-assisted backcrossing was considered. The latter scenario would allow for the efficient scoring and mapping of new mutant phenotypes in the uniform background of the *har1-1* mutation (i.e. as double mutants).

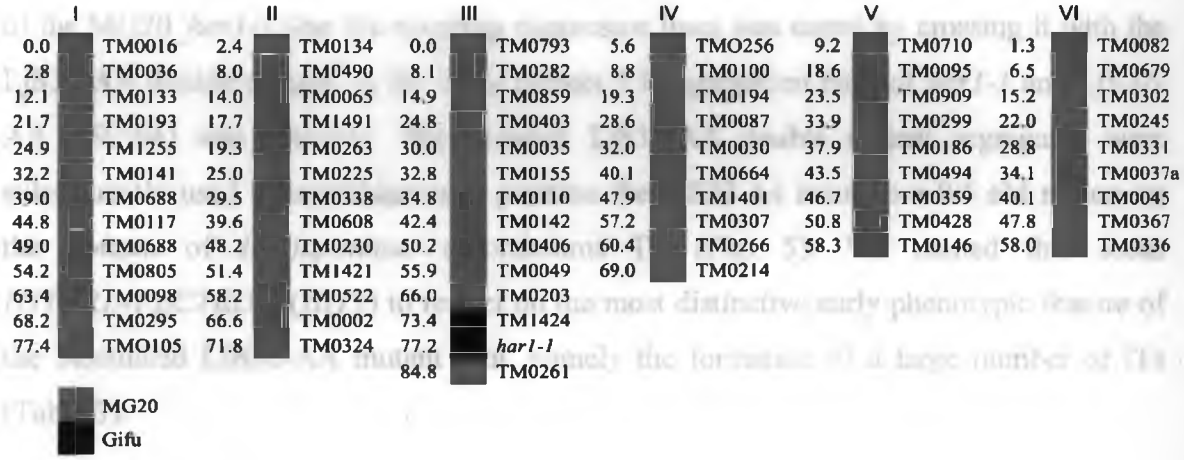

Figure 4. Genetic map of MG20_*har1-1* introgression line. The microsatellite markers used for marker-assisted selection are shown. Numbers indicate a genetic position in centiMorgans (cM) along six *L. japonicus* chromosomes.

Given the relatively large number of suppressor lines which remain to be analysed, the latter has been chosen, since it offers a larger proportion of informative individuals (predicted 1/4 of the segregating population), which should facilitate a robust mapping approach.

The homozygous *L. japonicus* Gifu *har1-1* line was hybridized with the ecotype MG20. After performing four consecutive backcrosses aided by marker-assisted selection, an individual introgression line (called MG20_*har1-1*) carrying MG20 markers throughout the entire genome, except for a ~ 6 cM region on the bottom of chromosome 3 which contained Gifu DNA encompassing the *har1-1* locus, was selected (Fig. 4). The efficacy of the MG20_*har1-1* line for mapping suppressor lines was tested by crossing it with the LjS32-AA double mutant. In the F2, a perfect 3:1 segregation ratio of *har1-1* and LjS32-AA (192:64) was obtained. The selected LjS32-AA double mutant segregants were subsequently used to unambiguously position the *LjS32-AA* locus to a 0.4 cM region on the bottom of *L. japonicus* chromosome IV (Fig. 5). We named this locus *HYPERINFECTED* 1 (*HIT1*) to reflect on the most distinctive early phenotypic feature of the inoculated LjB32-AA mutant root, namely the formation of a large number of ITs (Table 3).

2.4 Discussion.

The analysis of genetic suppressors is a powerful tool for the identification of genes that may directly or indirectly interact with the original mutant locus (for recent examples, see Kwon *et al.*, 2004; Xiao *et al.*, 2004; Yu *et al.*, 2000). We mutagenized the *L. japonicus har1-1* mutant and screened for genetic suppressors of its hypernodulation and root developmental phenotype. The large number of mutants and variety of phenotypes recovered from this screening procedure indicates that many independent symbiotic loci were successfully targeted.

Two classes of mutants were expected; one class that reduces nodulation by interfering directly with the ability of the plant to form nodules. The vast majority of the mutants recovered in this screen belong to this category, including mutants in the common

symbiosis pathway and mutants that are impaired in root nodule development as a pleiotropic secondary effect of their defective root hair development. Another predicted class was expected to restore the wild-type nodulation behaviour by directly interfering with the autoregulatory mechanism of nodulation.

Although at the present stage of the phenotypic analysis it is difficult to categorize all of the obtained mutants unambiguously to either of the two categories, several lines with significantly decreased nodule number were discovered by the suppressor screening. It is possible that at least some of these mutant lines correspond to elements and/or targets of the HAR1 receptor-kinase autoregulatory pathway. On the other hand, their low nodulation phenotypes may be reflective of lesions in genes that regulate nodule number and/or nodule organogenesis via developmental processes that are independent of HAR1. In this context, it is interesting to note that the *HIT1* locus described in this work was mapped within the genetic interval in the *L. japonicus* genome that encompasses the recently discovered *SPONTANEOUS NODULE FORMATION 2* (*SNF2*) locus (Tirichine *et al.*, 2006b). The relationship, if any, between *SNF2*, *HIT1*, and the HAR1-receptor kinase dependent autoregulatory pathway constitutes an interesting subject of the ongoing investigation.

In theory, at least two types of downstream effectors for the HAR1-dependent pathway could be envisaged. First, if HAR1 kinase-dependent signalling targets a positive regulator of root susceptibility to rhizobial signalling, a lesion in such an element would be predicted to prevent nodulation. Consequently, the non-nodulating class of mutant could include lines carrying lesions in the elements/targets of the HAR1 kinase signalling pathway, but would also incorporate defects in any gene required for root susceptibility that may not necessarily be a direct downstream target of HAR1 mediated signalling. It remains, therefore, uncertain if any of the elements of the early root perception/signalling apparatus discovered so far, including two positive regulators of root susceptibility, NFR1 and NFR5, constitute a direct downstream target for HAR1-kinase-dependent signalling.

An alternative scenario, where the HAR1 kinase-dependent pathway activates a negative regulator of root susceptibility and/or nodule organogenesis was also envisaged. Under such a formulated hypothesis, one could expect to recover a gain-of-function or partial gain of function mutation that would result in restriction of nodulation in a HAR1-

independent manner. No dominant gain-of-function mutation was identified in this screen, although a number of suppressor lines remain to be analysed.

Despite screening more than 150,000 M2 individuals, not a single line could be recovered that fully restored the wild-type nodulation and root developmental phenotype of the *har1-1* mutant. Only, line LjS12-5A, which carries a presumed secondary mutation that counteracts the effect of the primary *har1-1* mutation with respect to both the root and nodulation phenotypes, came close to the predicted, complete suppressor phenotype. However, since LjS12-5A also showed a strong defect in shoot elongation (data not shown), further detailed analysis of its complex phenotype in both the single and double mutant background will be necessary to understand the underlying nature of its suppressor effect. Aside from this line and line LjS51-3, which represents a hypermorph with respect to lateral root development, all remaining mutant lines, although affected in nodulation, showed the typical bushy root phenotype of the parental *har1-1* mutant line.

Identification of new alleles for seven known symbiotic genes should contribute to the functional characterization of their products. For example, the phenotypic variation for nodulation ability that was observed between alleles for *NUP133*, *CASTOR*, *POLLUX* and *SYMRK* might give valuable insights into the relevance of certain amino-acid residues for nodulation (Tables 1-3). In particular, the contrasting nodulation phenotypes of *symRK-13* (Nod^-) and *symRK-14* (Nod^+) may help to elucidate the role of different SYMRK receptor kinase domains in signalling events during RNS and AM. One line, LjB83-B, is presumed to carry a mutation in a novel common symbiosis locus in *L. japonicus*. This notion, however, needs to be confirmed through complementation analysis and by map-based cloning of the underlying locus.

The major obstacle encountered while attempting to analyse the suppressor lines characterized by more subtle symbiotic phenotypes was the inability to reliably select for the homozygous single mutant individuals. The successful development of the MG20_*har1-1* introgression line is now facilitating the rapid mapping of more difficult to select phenotypes. Indeed, the *har1-1/har1-1* background may prove to be essential for mapping and characterization of certain suppressor lines due to gene interactions. For example, mutant alleles of *POLTERGEIST* act as suppressors of *clv1* phenotypes but *pol* mutants are virtually indistinguishable from wild type plants (Yu *et al.*, 2000). The use of

the MG20_*har1-1* introgression line should allow cloning and characterization of genes underlying more subtle mutant symbiotic phenotypes identified by the suppressor screen. This will likely clarify their role, if any, in the autoregulatory pathway that limits nodulation in *L. japonicus*.

2.5 Material and methods.

2.5.1 *har1-1* supressor screen.

Twenty thousand homozygous *har1-1/har1-1 L. japonicus* Gifu mutant seed were treated with 0.4% EMS for 3 hours following the previously described protocol (Szczyglowski et al. 1998). Germinated seedlings (M1 plants) were transferred to pots (~30 plants per pot) and were grown for three weeks, upon which, the surviving plants (~10,000) were transferred to new pots (4 plants /pot; 25 pots/flat). Each flat was considered a single pool. Approximately 1500 M2 seeds per single pool (~ 150,000 in totals) were analyzed by scoring their symbiotic phenotype three weeks after inoculation with *M. loti* NZP2235. All putative mutant lines, in which the nodulation characteristics differed from the *har1-1* hypernodulation phenotype, were transferred to new pots and grown to maturity. The symbiotic and root architecture phenotypes of the lines identified in the M2 generation were re-evaluated in the M3 and M4 generations using histochemical staining of a *hemA*:*lacZ* tagged strain of *M. loti*. Selected stable mutant lines from the M4 generation were used in subsequent genetic/phenotypic analyses.

2.5.2 Plant Growth Conditions.

Plants were surface sterilized and germinated as previously described (Szczyglowski *et al.* 1998). F2 plants were transferred to pots containing a 6:1 mixture of vermiculite and sand soaked with B&D nutrient solution (Broughton and Dilworth, 1971), and containing a low concentration (0.5mM) of KNO_3. The plants were maintained in a growth room under a 16/8 hours day/night regime, 200-250 $\mu E\ sec^{-1}\ m^{-2}$ light intensity, at 22°C. After performing selection, the homozygous single mutant plants were transplanted into 4:4:1 mixture of Promix XP (Premier Horticulture, Quakertown, PA), medium size vermiculite (Therm-O-Rock East Inc. New Eagle Pennsylvania), and perlite (Therm-O-

Rock East Inc. New Eagle Pennsylvania), respectively, and were watered with Hoagland's nutrient solution (Hoagland and Arnon 1950) or water as needed.

2.5.3 Mapping of Mutant Nodulation Loci.

All lines that were confirmed as having no nodules or selected lines forming a few nodules were crossed to the wild-type Gifu and allowed to self-pollinate to generate F2 progeny. These plants were phenotyped to determine segregation ratios (Table 1). Homozygous single mutants were selected from the F2 segregating populations based on the nodulation and/or AM phenotype and the absence of the *har1-1* allele. The latter was evaluated using a *har1-1* specific CAPS marker, as described previously (Karas *et al.* 2005). A second cross involving *L. japonicus* ecotype 'Miyakojima' MG20, a mapping partner, was also performed. The resulting F2 plants were evaluated 3-6 weeks after inoculation and scored as wild type, *har1-1*, or nodulation and/or AM defective (Table 1). Typically, 16 homozygous single mutant F2 plants were selected and re-potted. One week later, leaves were harvested from each plant for DNA isolation (Karas et al. 2005). SSR markers were chosen from the Kasuza map (http://www.kazusa.or.jp/lotus/index.html) based on the positions of known symbiotic loci (Sandal et al. 2006) and the AM phenotype of the line being tested. Once tentative linkage was detected about 8-12 additional markers in the area were tested to confirm the linkage and establish the map interval. Candidate mutant loci were subjected to sequencing.

2.5.4 TILLING.

In parallel to the mapping approach, individual DNA preparations of each of the suppressor lines were included in an array that was subjected to TILLING of all sequence-identified genes required for nodulation (forward TILLING population; Perry *et al.*, 2003). In cases where putative mutations were identified through the mismatch mediated cleavage system the corresponding DNA fragment was amplified from the mutant to determine the precise mutation.

2.5.5 Sequencing.

The entire candidate locus was amplified from the corresponding suppressor line. The PCR products were sequenced directly on both strands. The DNA sequence of the entire locus was reconstituted using SeqMan 6.1 sequence analysis software (DNASTAR Inc. USA) and compared to the corresponding wild-type sequences.

2.5.6 Evaluation of Root Nodule Phenotypes.

Plants were inoculated two weeks after germination (1 week after transfer to soil) with *Mesorhizobium loti* strain NZP2235 carrying either the *hemA:LacZ* reporter gene fusion or constitutively expressing GFP. Roots were fixed, stained for β-galactosidase activity, and cleared as previously described (Wopereis *et al.*, 2000). Plants were evaluated using brightfield light microscopy (Nikon SMZ1500) 10, 21 and 42 dai. Nodule counts were performed on at least 15-20 independent individuals for each line and were expressed as a mean nodule number per plant ± 95% confidence interval. For GFP detection, root samples were prepared and analysed as described (Karas *et al.*, 2005).

2.5.7 Culture of Arbuscular Mycorrhizae.

The arbuscular mycorrhizal fungus *Glomus intraradices* (Schenck and Smith) was cultured for at least 6 months on *Zea mays* L. planted in 1:1 sand/Turface. Pot cultures were watered as needed with de-ionized water and were given a low-phosphate nutrient solution approximately once per month; the final concentrations of the following nutrients were used: 0.4 mM KH_2PO_4, 2.5 mM $Ca(NO_3)$, 2 mM K_2SO_4, 1 mM $MgSO_4$, 0.2 mM $FeCl_3$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.5 μM $CuSO_4$, and 0.5μM Na_2MoO_4.

2.5.8 Evaluation of AM Phenotypes.

Seven day old seedlings of *Lotus japonicus* were transplanted (one per pot) into small containers (Stuewe & Sons, Inc. Corvallis, Oregon) filled with substrate. The substrate consisted of 1 part inoculum (substrate from *Z. mays* pot-culture containing desiccated colonized roots, spores, and hyphae) and six parts autoclaved sand and Turface (mixed 1:1) watered to field capacity. Containers were covered with plastic wrap for the

first two weeks of seedling growth after which plants were watered sparingly with de-ionized water; those grown for six weeks were given low-phosphate nutrient solution at three weeks after transplanting. Containers were randomized into racks and plants were kept in a growth room at 24°C/16h days and 20°C/8h nights. During the day, seedlings received approximately 180 μmol m^{-2} s^{-1} PPF (photosynthetic photon flux) from both cool white fluorescent and incandescent bulbs. Eight replicates of each line for each harvest time-point were prepared.

2.5.9 Microscopy of AM.

Upon harvest, the entire root system of each seedling was excised and placed in a separate vial containing 50% ethanol for fixation (1-2 h at room temperature). Roots were then transferred to 10% KOH and cleared for 40 min. in a water bath at 75°C followed by a rinse in tap water and staining for 1h at room temperature. The stain consisted of 5% Shaffer black ink in 5% acetic acid (v/v) (Vierheilig *et al.*, 1998). After staining, roots were placed in 2 ml of water with a drop or two of 5% acetic acid until mounting on slides in 50% aqueous glycerol and then examined under a cover-slip at 100X total magnification. Between five and eight surviving replicates were evaluated per line.

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CHAPTER 3

Invasion of *Lotus japonicus root hairless 1* by *Mesorhizobium loti* involves the Nod factor dependent induction of root hairs

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3.1 Contributions made by Bogumil Karas.

- Performed genetic and phenotypic analyses of all root hair defective mutant lines (Figures 4, 6, 8, 10, 12).

- Generated the material for both light and scanning electron microscopy (Figures 7, 8, 9, 11)

- Positioned the genetic lesions responsible for defects in root hair development on *L. japonicus* genetic map (Figure 5)

- Established four complementation groups for mutants with defects in root hair development (Table 1)

In this study, the analysis of several novel root hair mutants of L. japonicus and the consequences of their associated developmental abnormalities on the outcome of the symbiotic interaction are described. These mutants were identified from a collection of new L. japonicus germlines derived from a screening for genetic suppressors of the L. japonicus har1-1 hypernodulation phenotype (see Chapter 2: 2.2.6).

3.2 Introduction

Root hairs of higher plants represent an important extension of the root epidermal surface with functions in the exploitation of various biotic and abiotic resources of the rhizosphere. In addition to their commonly recognized role in water and nutrient uptake from soil, root hairs of legumes also facilitate symbiosis with the beneficial, nitrogen fixing, soil bacteria commonly known as rhizobia. This symbiosis leads to the formation of new root-derived organs, nitrogen fixing nodules (Szczyglowski and Amyot, 2003).

Initiation of nodule organogenesis involves a highly specific molecular dialogue between the rhizobial microsymbiont and the appropriate host plant. As a result of this dialogue the morphogenic lipochito-oligosaccharide signalling molecules, known as nodulation or Nod factors (NFs), are secreted by the symbiotic bacteria. Acting as determinants of host specificity (Denarie et al., 1996; Riely et al., 2004), NFs are sensed by the root perception apparatus of the compatible host plant. Recent cloning experiments in the model legumes, *L. japonicus* and *M. truncatula*, revealed the involvement of a family of related LysM receptor-like kinases in the NF-dependent perception mechanism(s) that initiates the intracellular colonization of the root by the symbiotic bacteria and the morphogenesis of nodule primordia (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003).

The initial response of root hairs to the NF-producing, compatible strain of rhizobia involves the establishment of *de novo* polar root hair tip-growth and curling, which leads to the formation of typical "shepherd's crook" structures (Lhuissier et al., 2001). These structures entrap the bacteria and serve as a starting point for the initiation of the infection process, which occurs through a local invagination of the plasma membrane and establishment of a growing infection structure, the infection thread (IT). The intercellular

progression of the IT through the root hair towards the underlying nodule primordium occurs via a tip-growth-like mechanism and is guided by a specific arrangement of polarized cytoplasm in the underlying cortical cells (van Brussel et al., 1992; van Spronsen et al., 2001).

Only root hairs localized to the susceptible zone of the root and at a particular developmental stage appear to be fully receptive to NFs (Bhuvaneswari et al., 1981). Although the molecular determinates of root hair susceptibility are not well understood, root hairs which have almost reached their mature size, have been shown to be the most receptive (Gage, 2004 and references therein). Based on the structure-functional analysis of *Sinorhizobium meliloti* NFs, the presence of at least two NF receptors or two NF-dependent signaling mechanisms at the root hair surface was postulated (Ardourel et al., 1994).

In many legumes, including the model legumes *L. japonicus and M. truncatula*, root hairs are the principal sites for attachment and intracellular entry of rhizobia. However, the mechanisms by which rhizobia colonize roots vary significantly among different legume species and various root-hair independent mechanisms of root colonization by rhizobia, including cortical intercellular invasion at lateral root bases, have been described (Boogerd and van Rossum, 1997; Guinel and Geil, 2002 and references therein). Interestingly, in the tropical legume, *Sesbania rostrata*, both intracellular and intercellular modes of root invasion by *Azorhizobium caulinodans* occur. Which invasion pathway is used depends on the particular growth conditions, which determine the availability of susceptible root hairs, and has been shown to be modulated by the plant hormone ethylene (Goormachtig et al., 2004a).

The initial responses of root epidermal and cortical cells to signaling from the NF producing bacteria set off a cascade of signaling events which restrict the extent of successful infections at the root epidermis and nodule organogenesis in the root cortex (van Brussel et al., 2002; Nutman, 1952). Thus, the susceptibility of roots remains under the strict but flexible control of the host plant, which ensures the homeostasis of the plant-microbe symbiosis. Multiple levels of regulation, including local and systemic signaling events have been implicated in this process and have been shown to involve the plant hormone ethylene and Har1 receptor kinase-dependent signaling (Penments and Cook,

1997; Wopereis et al., 2000; Krussel et al., 2002; Nishimura et al., 2002). *L. japonicus* mutants carrying a mutation in the *Har1* gene fail to autoregulate nodule formation, resulting in the formation of an excessive number of nodules (hypernodulation phenotype; Wopereis et al., 2000).

In this study, we describe the analysis of several novel root hair mutants of *L. japonicus* and the consequences of their associated developmental abnormalities on the outcome of the symbiotic interaction. The mutants were identified from a collection of new *L. japonicus* germlines derived from a screening for genetic suppressors of the *L. japonicus har1-1* hypernodulation phenotype. We show that although the various aberrations in root hair development significantly diminish the effectiveness of the mutant roots to support an efficient colonization by the symbiotic bacteria, the progression of the cortical program for nodule primordium organogenesis remains, to a large extent, unaltered and provides an alternative mechanism for successful colonization of the root and the development of nitrogen fixing nodules.

3.3 Results.

3.3.1 *L. japonicus* Wild-Type Root Hair Phenotype.

The root hair phenotype of *L. japonicus* Gifu plants grown on vertical agar plates (see Material and Methods) has been evaluated in terms of zone distribution, site of origin, patterning, and length. transition from the root-hair emergence zone to the root-hair elongation zone has been assigned arbitrarily and is indicated by a dotted line. In wild-type *L. japonicus* Gifu, root hairs begin to emerge from epidermal cells located approximately 1.0 to 1.5 mm behind the root tip (Fig. 1). There is no specific patterning of root hairs within or between different epidermal cell files in *L. japonicus*, since every epidermal cell appears to have the potential to produce a root hair. In fact, a vast majority of epidermal cells form root hairs, and only very sporadically root-hairless cells are observed. The rare root-hairless cells are randomly distributed either as single cells or as short stretches of a few cells within a single epidermal cell file (data not shown).

The initiation of root hairs in the *L. japonicus* root-hair emergence zone begins as a polarized outgrowth from the middle of the outer periclinal cell wall of the epidermal cell

(Fig. 2A). The emergence zone is followed, without any defined boundary, by the root hair elongation zone, which terminates with fully extended root hairs in a root hair mature zone (Fig.1 and Fig. 2B). The morphometric measurement of root hairs showed that the mean length of a mature root hair in *L. japonicus* is 690 ± 120 μm. *L. japonicus* interacts with its natural micro-symbiont *M. loti* to form nitrogen-fixing nodules in a root hair-dependent manner (Szczyglowski et al., 1998). On the cytological level, the major initial steps in this interaction comprise modification of root hair growth and initiation of the infection process which culminates in the development of an IT. The IT traverses the infected root hair on its way to the root cortex (Fig. 2C).

3.3.2 *L. japonicus* Root Hair Mutants.

By performing a screen for genetic supressors of the *L. japonicus har1-1* hypernodulation mutant phenotype (Wopereis *et al.*, 2000) a group of mutants characterized by various aberrations in root hair development and a concomitant low nodulation phenotype (see below) were identified. This group comprises nine independent double mutant lines, which were further analysed with respect to their non-symbiotic root phenotype and also their ability to interact with nitrogen-fixing bacteria. A detailed description of the genetic screen for suppressors of the *har1-1* mutant phenotype and its overall outcome is reported in chapter 2. The initial microscopy observations lead to categorization of the root hair double mutant lines into four distinct phenotypic classes: (1) root hairless; Ljrhl, (2) petite root hairs; Ljprh, (3) short root hairs; Ljsrh, and (4) variable root hairs; Ljvrh. Images of non- symbiotic live roots of the parental *L. japonicus har1-1* mutant line and representative plants belonging to each mutant phenotypic category are shown in Figures 3A and 3B.

Since all root hair mutant lines were derived from chemically mutagenized *L. japonicus har1-1/har1-1* homozygous mutant seeds and the *har1-1* mutant is characterized by the wild-type root hair phenotype (see Fig. 3A), the observed aberrations in the growth and development of root hairs were presumed to be a result of secondary mutations. The presence of the *har1-1* allele was indicated by the overall short stature and the bushy root of the root-hair mutants, reminiscent of the non-symbiotic *har1-1* root phenotype (Wopereis *et al.*, 2000).

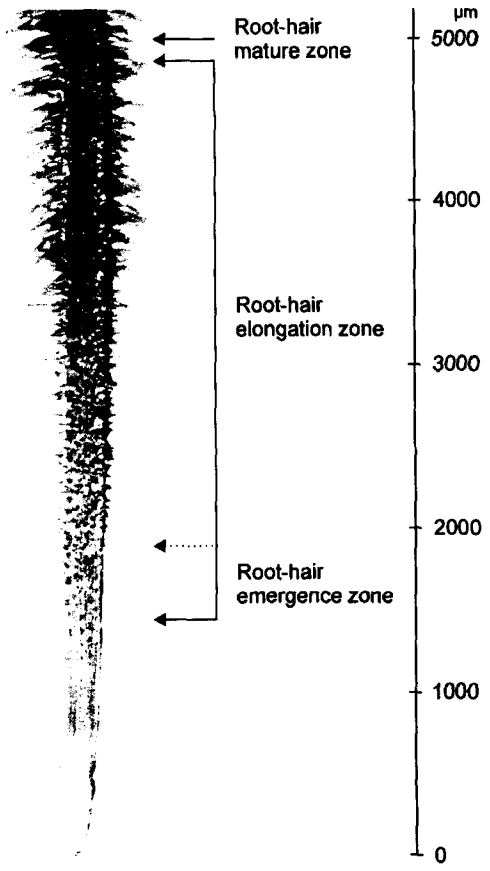

Figure 1. Zones of root hair development in *L. japonicus*. An image of a portion of a *L. japonicus* root is shown and different zones of root hair development are indicated.

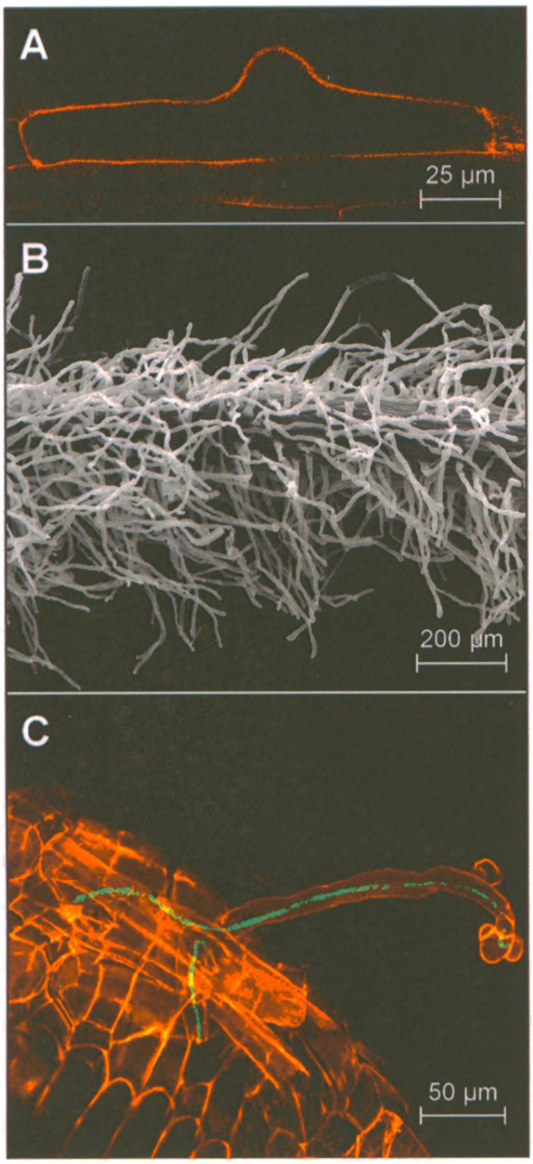

Figure 2. Features of root hairs in wild type *L. japonicus*. (A) A confocal fluorescent image of propidium iodide stained epidermal cell that has initiated root hair growth from the centre of the periclinal cell wall. (B) A scanning electron micrograph of a portion of wild type *L. japonicus* root within the root hair mature zone. (C) A scanning confocal image of an infection thread originating from a microcolony at the curled root hair tip and proceeding down into the base of the epidermal cell. *M. loti* bacteria are tagged with GFP (green fluorescence) and the root tissue has been counterstained with propidium iodide (red fluorescence).

The genotyping analysis, using a *har1-1* allele-specific CAPS marker, confirmed that all nine root-hair mutant lines are homozygous for the *har1-1* mutant allele (data not show). Further genetic analysis (see below) confirms their double mutant genetic background (Table 1). In order to facilitate the distinction between the double vs. single mutant genetic background, the following nomenclature is being used throughout the rest of the text. The double mutants are designated by two corresponding mutant alleles codes (e.g. *Ljrhl1 har1-1*), while a line carrying a mutation at the single locus in otherwise wild-type genetic background is described by the code of the corresponding mutant allele only (e.g. *Ljrhl1*).

3.3.3 Root-Hairless Mutants (class 1).

Three independent double mutant root-hairless *Ljrhl1 har1-1* lines (LjS3-1, LjS13-C, and LjS16-2) were identified. These plants were characterized by an almost complete lack of root hairs (Fig 3A and 3B). When grown on a surface of agarose-solidified medium, sporadic trichoblast cells could be found on the roots of these mutant lines. These, however, were usually localized to the base of the root, near the root-hypocotyl junction, and were found with a frequency of less then 1 root-hair cell per 10 plants. Overall, the root epidermis of the *Ljrhl1 har1-1* double mutants remained virtually hairless (Fig. 3B). Genetic complementation tests revealed that the three independent class 1 mutant lines represent the same genetic locus (Table 1). Therefore, the new mutant alleles were designated as *Ljrhl1-1, Ljrhl1-2*, and *Ljrhl1-3,* and the corresponding wild-type gene as *LjRHL1*. The F1 plants derived from crosses between these three lines and wild-type *L. japonicus* Gifu showed wild-type root hair phenotypes, suggesting a recessive nature of the underlying mutations. This assumption was further confirmed by scoring phenotypic segregations in the resulting F2 populations, which were found to be in good agreement with the predicted Mendelian 9:3:3:1 ratio (wild-type: *har1-1*: root hairless: double mutant) for two independently segregating loci. This confirms that the *LjRHL1* locus is inherited in a recessive fashion and independently of the *HAR1* locus (Table 1).

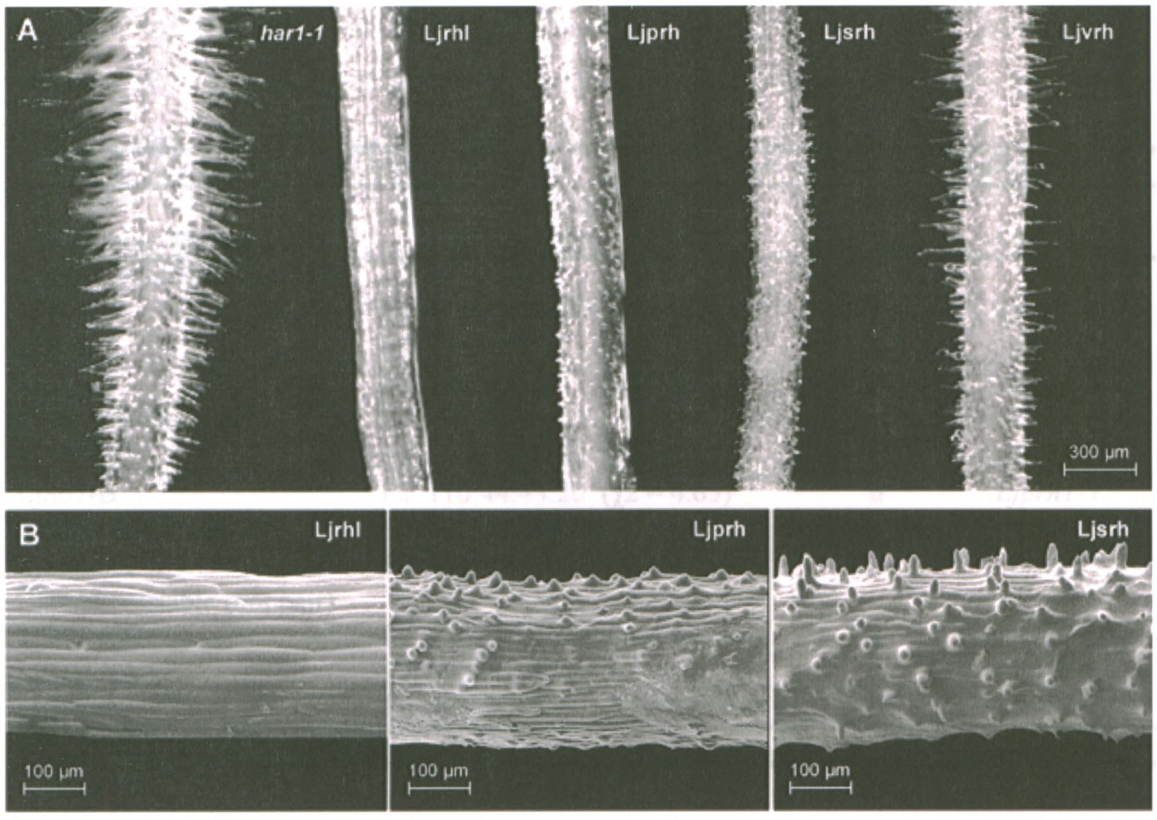

Figure 3. Root hair phenotypes of *L. japonicus* mutant lines. A) The *har1-1* parental line and double mutants *Ljrhl1 har1*, *Ljprh1 har1*, *Ljsrh1 har1*, and *Ljvrh1 har1* are shown. B) Scanning electron micrographs of double mutants *Ljrhl1 har1*, *Ljprh1 har1*, and *Ljsrh1 har1*. Root segments were photographed at approximately 0.3 – 0.5 cm from the root tip.

Table 1. *L. japonicus* root hair mutants. (sm – root hair single mutant, dm –double mutant).

Double Mutant Line	Genetic Control (wt : *har1-1* : sm : dm)		Comp. groups	Allele
Class 1 (root hairless)				
LjS3-1	F2	194:66:78:24 ($\chi2 = 2.10$)	a	*Ljrhl1-1*
LjB13-C	F2	96:30:28:11 ($\chi2 = 0.46$)	a	*Ljrhl1-2*
LjS16-2	F2	75:21:25:8 ($\chi2 = 0.53$)	a	*Ljrhl1-3*
Class 2 (petite root hair)				
LjS24-B	F2	187:69:48:20 ($\chi2 = 3.92$)	b	*Ljprh1-1*
LjS67-B	F2	110:44:43:20 ($\chi2 = 4.65$)	b	*Ljprh1-2*
Class 3 (short root hair)				
LjS88-5A	F2	138:5:3:31 ($\chi2 = 116.26$)*	c	*Ljsrh1*
Class 4 (variable root hair)				
LjB12-IB	F2	137:10:5:37 ($\chi2 = 106.97$)*	d	*Ljvrh1-1*
LjS49-AA	F2	160:6:2:44 ($\chi2 = 149.79$)*	d	*Ljvrh1-1*
LjB69-A	F2	168:8:9:47 ($\chi2 = 139.95$)*	d	*Ljvrh1-1*

* ($P < 0.05$)

3.3.4 Petite Root Hair Mutants (class 2).

Two independent double mutant lines (LjS24-B and LjS67-B) have been identified in the petite root hair phenotypic category (Table 1). In contrast to the root-hairless mutants described above, the epidermal cells of class 2 mutants formed clearly identifiable swellings, indicative of the ongoing initiations of root-hair development (Fig. 3A and 3B). When grown on a surface of agarose-solidified medium these epidermal swellings failed to transit to the tip growth phase, reaching an average length of only 15 ± 5.6 μm (Fig. 3B). However, when grown in soil, slightly longer root hairs were formed, indicating that under the conditions used, a very short period of tip growth phase may have occurred (data not shown). The mutations underlying the phenotype of class 2 mutants were found to be recessive, allelic, and to be inherited independently from the *HAR1* locus (Table 1).

3.3.5 Short Root Hair Mutant (class 3).

A single plant in class 3 (LjS88-5A) was recovered from the mutagenized population and the corresponding new mutant allele was named *Ljsrh1* (Table I). The double mutant *Ljsrh1 har1-1* plants were capable of initiating root hair growth, which transited to the tip growth phase only to be terminated shortly thereafter. When grown on agar plates, the root hairs of the *Ljsrh1 har1-1* mutant reached an average length of 21± 7.9 μm. They were erect and tubular in shape, characteristics which clearly distinguish them from the broad-based epidermal swellings of the petite root hair mutants of class 2 (Fig. 3B). The *Ljsrh1* allele was found to be recessive, as judged based on the recovery of the wild-type root hair phenotype in the F1 generation derived from the cross between *Ljsrh1 har1-1* and wild-type *L. japonicus* Gifu. In the F2 generation, the proportion of plants showing the root hair phenotype deviated significantly from the expected 9:3:3:1 ratio, indicating linkage of *LjSRH1* and *HAR1* on Lotus chromosome three (Table I). However, recombination events between the *LjSRH1* and *HAR1* loci were recovered indicating that the short root hair phenotype was not a result of genetic changes at the *Har1* locus.

3.3.6 Variable Root Hair Mutants (class 4).

Class 4 mutant lines LjB12-IB, LjS49-AA, and LjSB69-A showed a rather complex root hair phenotype. This phenotype was characterized by the presence of variable length root hairs throughout the apical-basal axis of the root. In addition, the overall root hair density was diminished in the double mutant lines as compared with the parental line *har1-1* (Fig. 3A). The lower density of root hairs was found to be a result of a developmental defect(s), which appears to cause some elongating root hairs to collapse (data not shown). The combined genetic/phenotypic analysis revealed the recessive nature of the underlying mutations, while the complementation crosses allowed classification of the three independent double mutant lines into one complementation group (alleles: *Ljvrh1-1*, *Ljvrh1-2*, and *Ljvrh1-3*; Table 1), defining a fourth *L. japonicus* root hair-associated locus, designated as *LjVRH1*. Interestingly, the segregation analysis indicated a genetic linkage between *LjVRH1* and the *HAR1* locus (Table 1), but again, recovery of recombination events between the *LjVRH1* and *HAR1* loci proved that the variable root hair phenotype was not due to genetic changes at the *HAR1* locus.

With regard to all classes of mutants described above, the single mutant lines homozygous for the wild-type *Har1* allele that carry the corresponding homozygous root hair-related mutant alleles have been recovered from the segregating populations (Table I). They were found to have root hair phenotypes identical to their matching parental double mutant lines (see above). However, the phenotypic distinction between single and double mutants was straightforward since, unlike the *har1-1* allele carrying plants, the root-hair single mutants were characterized by a more elongated and less bushy root system, resembling the wild-type root phenotype (data not shown).

Interestingly, when grown on either a surface of agar-solidified medium or in soil, the single *Ljrhl1-1* line had significantly longer roots than wild type *L. japonicus* Gifu (Fig. 4).

3.3.7 Mapping of *L. japonicus* root hair loci.

Analysis of the homozygous root-hair mutants selected from F2 populations derived from crosses of the double mutant lines *Ljrhl1-1 har1-1*, *Ljprh1-1 har1-1*, *Ljsrh1-1 har1-1*, and *Ljvrh1-1 har1-1* to the polymorphic mapping partner, *L. japonicus* ecotype 'MG20', allowed positioning of the underlying loci on the *L. japonicus* genetic map

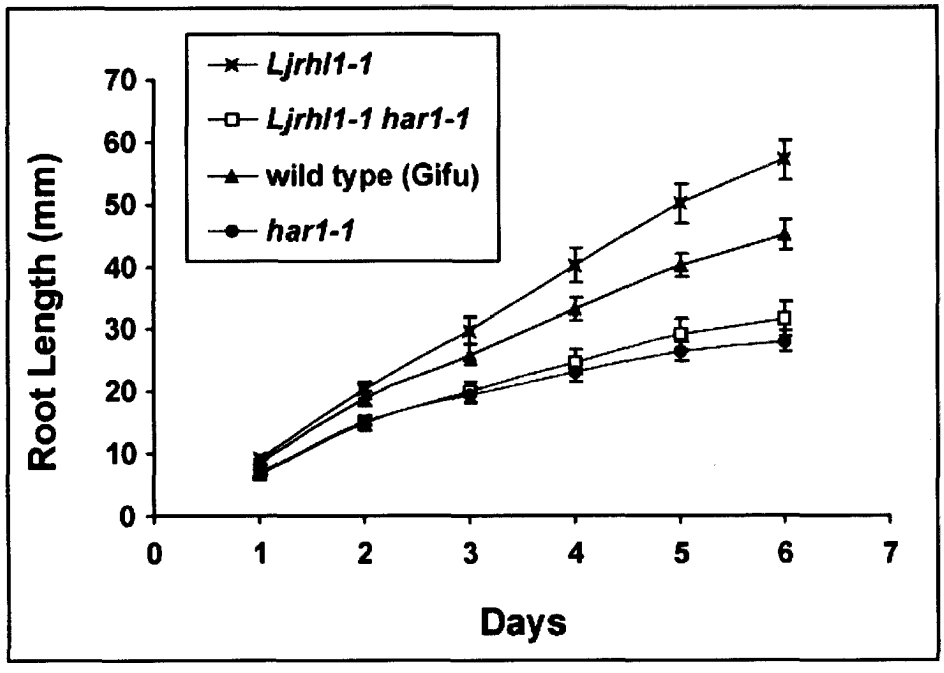

Figure 4. Root elongation rate of *L. japonicus* Gifu and mutant lines. Plants were grown in the dark on vertically positioned agar plates for 6 days.

(Asamizu et al., 2003). Flanking recombinant markers delimit a 0.8 cM interval for the *LjVRH1* and *LjSRH1* loci on chromosome three, a 5.2 cM interval for the *LjPRH1* locus on chromosome five, and a 3.2 cM interval for the *LjRHL1* locus on chromosome six (Fig. 5).

3.3.8 Symbiotic phenotypes of *L. japonicus* root hair mutants.

In the presence of the *har1-1* allele in the double mutant background, all four classes of root hair mutants developed nodules. Initial visual inspection of their nodulation phenotype, in comparison with the parental single mutant *har1-1* line, showed an overall decrease in nodule number, confirming partial *har1-1* suppressor characteristics of the *Ljrhl1-1*, *Ljprh1-1*, *Ljsrh1*, and *Ljvrh1-1* mutant alleles. To examine these novel nodulation phenotypes in more detail, a *M. loti* strain carrying a constitutively expressed *hemA*::*lacZ* reporter gene fusion was used in subsequent experiments, and the nodulation phenotypes of the double mutants were examined 10 and 21 (dai) and compared to the corresponding phenotypes of wild-type Gifu and the *har1-1* parental mutant line (Fig. 6). Ten dai *L. japonicus* Gifu and the *har1-1* single mutant developed wild-type and hyper-nodulation phenotypes, respectively (Fig. 6A). The dark blue colour of the nodules reflects the activity of the bacterially-encoded *lacZ* reporter gene, and is thus indicative of successful root colonization by *M. loti.* Dark blue stained nodules developed on roots of wild-type *L. japonicus* Gifu, *har1-1*, *Ljvrh1-1 har1-1*, and *Ljsrh1 har1-1,* and to a much lesser extent on the roots of *Ljprh1-1 har1-1*, but were absent from the roots of *Ljrhl1-1 har1-1* (Fig. 6A). Instead, when cleared roots were analysed (see Material and Methods), a large number of foci of cortical cell divisions were found throughout the entire root system of the *Ljrhl1-1 har1-1* (Fig 6A). These foci were not found on control un-inoculated *Ljrhl1-1 har1-1* roots, nor were they present after inoculation with *M. loti NodC*::*Tn5* mutant strain that is unable to produce NF, suggesting that they constitute nodule primordia (NP; data not shown). This notion is supported by the results of longitudinal sectioning of segments of the *Ljrhl1-1 har1-1* root, which showed broad-based foci of cortical cell divisions which are indistinguishable from NP (Fig. 6B). Unstained NP were also formed 10 dai on roots of *Ljprh1-1 har1-1* and *Ljsrh1 har1-1*, but were virtually absent from *Ljvrh1-1 har1-1, har1-1,* and wild-type *L. japonicus* Gifu roots (Fig. 6A).

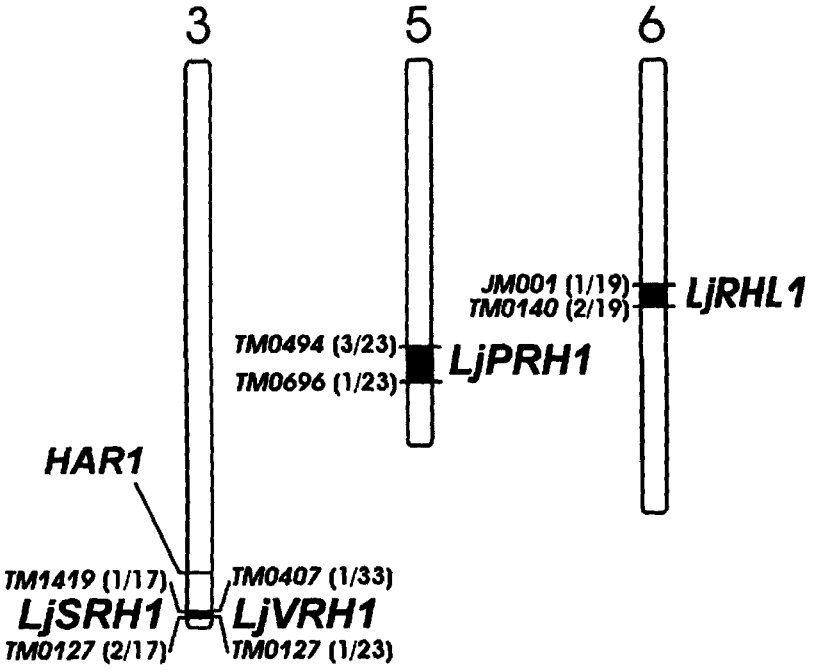

Figure 5. Positions of root hair loci *LjSRH1*, *LjVRH1*, *LjPRH1*, and *LjRHL1* on *L. japonicus* chromosomes 3, 5, and 6. Flanking simple sequence repeat markers are given.

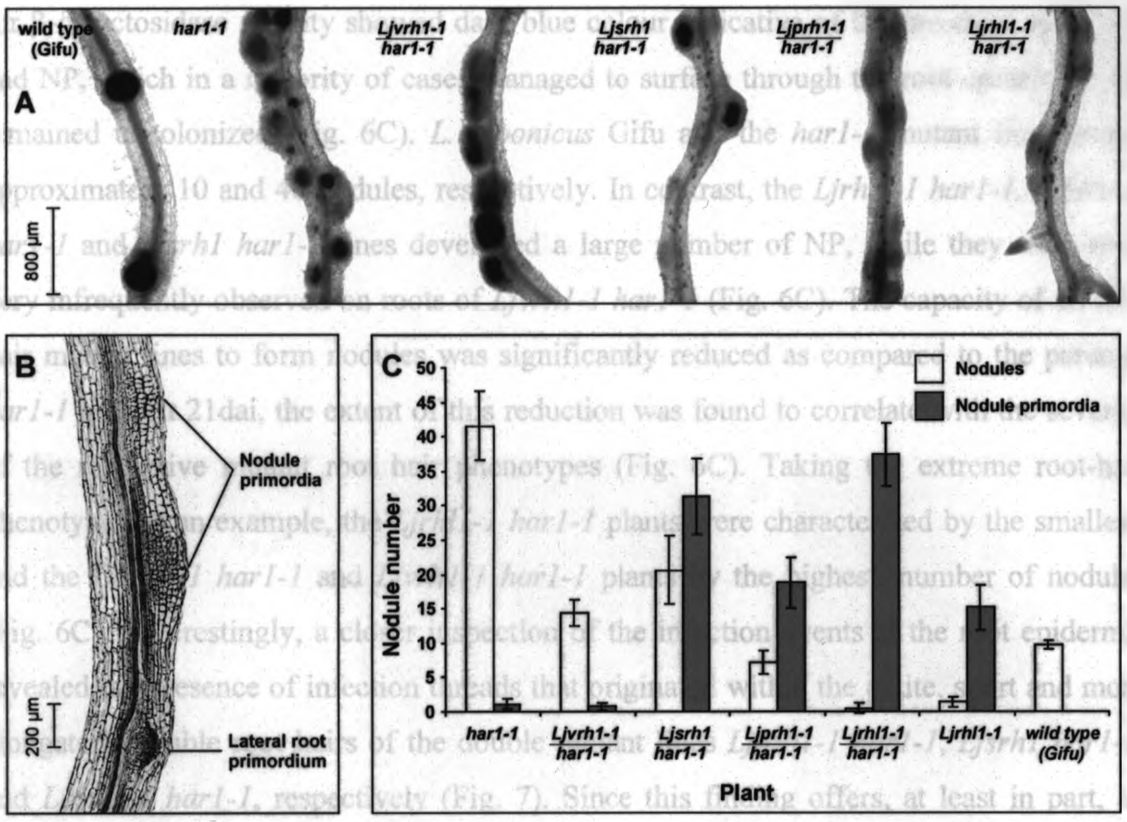

Figure 6. Symbiotic phenotypes of wild type (Gifu) and mutant plants (*har1-1*, and double mutants *Ljrhl1 har1*, *Ljprh1 har1*, *Ljsrh1 har1*, and *Ljvrh1 har1*. (A) Roots of plants 10 dai with *M. loti* strain NZP2235 carrying a *hemA::LacZ* reporter gene construct. Roots of all plants shown were cleared and stained for β-galactosidase activity. B) A longitudinal section (25 µm) of the double mutant *Ljrhl1 har1* 10 dai. NP and emerging lateral root primordia can be easily distinguished. C) Numbers of nodules and NP on the roots of wild type, *har1-1*, and root hair double mutants (*Ljrhl1 har1*, *Ljprh1 har1*, *Ljsrh1 har1*, and *Ljvrh1 har1)* and single mutant (*Ljrhl1-1*) 21 dai with *M. loti* strain NZP2235 (*hemA::LacZ)*. Roots were cleared and stained for β-galactosidase activity before counting. Mean values are shown ± 95% CI (n = 10-15).

When examined 21 dai, roots of all mutant classes, but not wild-type *L. japonicus* Gifu developed two types of symbiotic structures, nodules, which upon a histochemical staining for β-galactosidase activity showed dark blue colour indicative of the presence of *M. loti* and NP, which in a majority of cases managed to surface through the root epidermis, but remained uncolonized (Fig. 6C). *L. japonicus* Gifu and the *har1-1* mutant line formed approximately 10 and 40 nodules, respectively. In contrast, the *Ljrhl1-1 har1-1*, *Ljprh1-1 har1-1* and *Ljsrh1 har1-1* lines developed a large number of NP, while they were only very infrequently observed on roots of *Ljvrh1-1 har1-1* (Fig. 6C). The capacity of all root hair mutant lines to form nodules was significantly reduced as compared to the parental *har1-1* line. At 21dai, the extent of this reduction was found to correlate with the severity of the respective mutant root hair phenotypes (Fig. 6C). Taking the extreme root-hair phenotypes as an example, the *Ljrhl1-1 har1-1* plants were characterized by the smallest, and the *Ljsrh1-1 har1-1* and *Ljvrh1-1 har1-1* plants by the highest, number of nodules (Fig. 6C). Interestingly, a closer inspection of the infection events at the root epidermis revealed the presence of infection threads that originated within the petite, short and more elongated variable root hairs of the double mutant lines *Ljprh1-1 har1-1*, *Ljsrh1 har1-1*, and *Ljvrh1-1 har1-1*, respectively (Fig. 7). Since this finding offers, at least in part, an explanation for the observed nodulation phenotype in these three mutant lines (see Discussion), the further analyses of the nodulation and colonization events have been confined to the most severe root-hair phenotype of the *L. japonicus* mutants of class 1. These analyses were carried out in the context of both double (*Ljrhl1-1 har1-1*) and single (*Ljrhl1-1*) mutant genetic backgrounds. The question we sought to answer was if *M. loti* is capable of colonizing *L. japonicus* roots through a mechanism that does not require root hairs.

3.3.9 Nodulation of *Ljrhl1* in the Double and Single Mutant Backgrounds.

At 21 dai, roots of the single *Ljrhl1-1* mutant developed numerous NP, while nodules could only be observed sporadically (Fig. 6C). The average number of nodules formed by the *Ljrhl1-1* (1.4 ± 0.77) was not significantly different from the *Ljrhl1-1 har1-1* double mutant line (0.5 ± 0.7). However, the plants of both genotypes differ significantly in the overall frequency of NP, with *Ljrhl1-1* forming fewer NP than the corresponding double

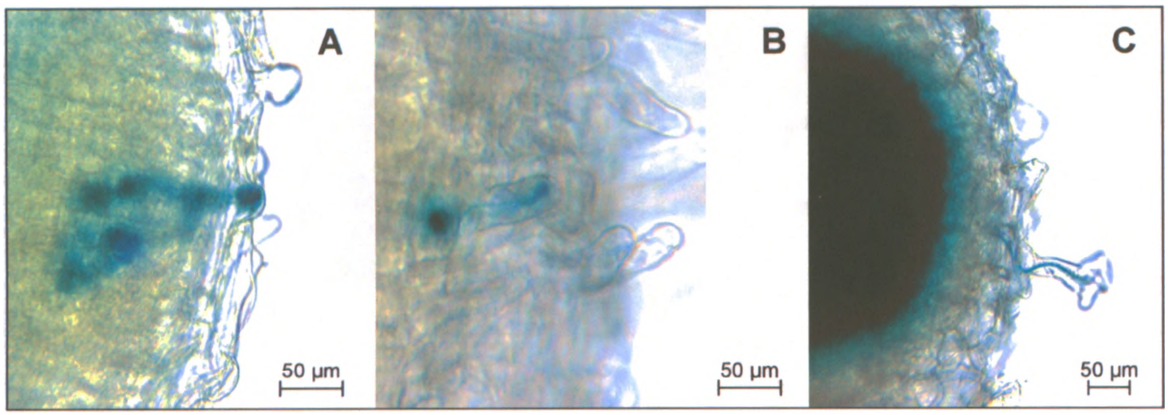

Figure 7. Representative infection events 21dai on double mutants *Ljprh1-1 har1-1*, *Ljsrh1-1 har1*, and *Ljvrh1-1 har1-1*. Roots were cleared and stained for β-galactosidase activity to detect the infecting *M. loti* strain NZP2235 (*hemA::LacZ)*. (A) An abnormally broad IT formed within a mutant root hair of *Ljprh1-1 har1-1*. The IT has ramified within the nodule cortex (B) IT traversing an uncurled root hair on *Ljsrh1-1 har1*. (C) A *Ljvrh1-1 har1-1* root hair exhibiting a curled root hair tip encircling a microcolony from which an infection thread descends.

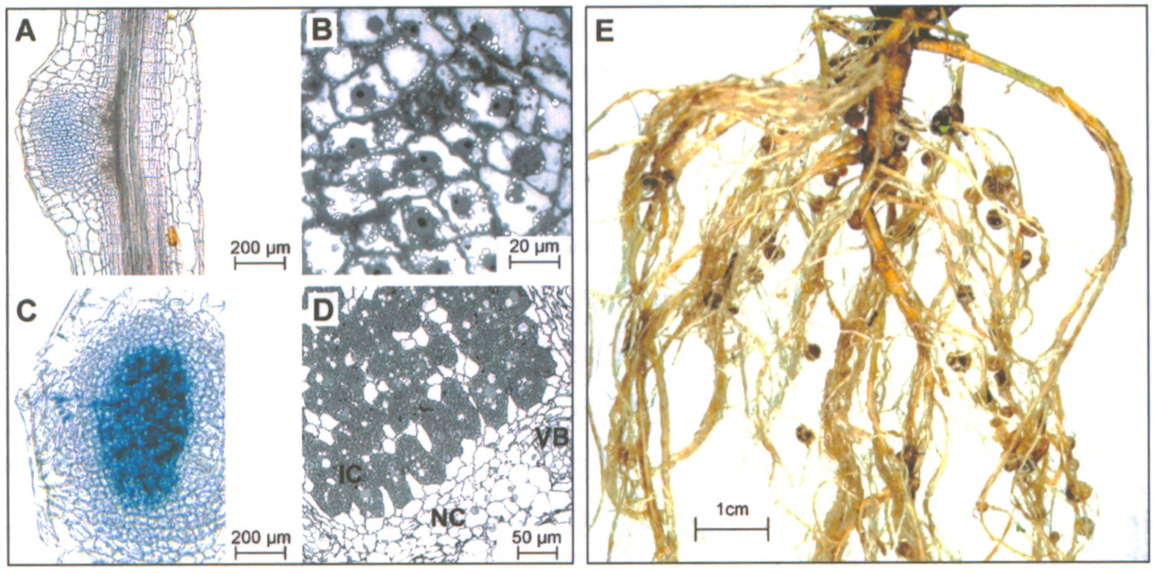

Figure 8. Infection phenotypes of *Ljrhl1-1* (42dai) A, A longitudinal section of a NP stained for β-galactosidase activity. B, A light micrograph of a semithin section of an NP. Note the absence of infected cells. Activated cortical cells with centrally located enlarged nuclei and many starch granules are visible. C, A longitudinal section of an infected nodule stained for β-galactosidase activity showing deep-blue color in the nodule central zone and an IT extending from the nodule apex. D, A light micrograph of a semithin section of an infected nodule. Infected cells (IC) are clearly discernible; NC, nodule cortex, VB, vascular bundle. E, Roots of a 4-month old *M. loti* infected *Ljrhl1-1* plant showing an abundance of nodules.

mutant line (Fig. 6C). At 42 dai, the number of nodules formed by both *Ljrhl1-1 har1-1* and *Ljrhl1-1* increased to a larger extent than the number of NP when compared to plants 21dai, suggesting an improved efficiency with which the roots and/or NP are colonized by *M. loti* (Fig. 8). The microscopic examination of NP revealed structure that is typical of empty nodules, with cortical cells containing enlarged, centrally localized nuclei and numerous starch granules, but deprived of symbiosomes (Fig. 8A and 8B). In contrast, the nodules were characterized by wild-type histology of a determinate type, consisting of a large central area primarily composed of bacteroid-containing cells that is surrounded by concentric layers of uninfected nodule cortex (Fig. 8C and 8D). Examination of histochemically stained sections of these nodules showed the presence of ITs descending from the nodule surface and ramifying within the central region of the nodule infected zone (Fig. 9). A few *Ljrhl1-1* plants were maintained for an extended period of time (4 months, see Material and Methods). When uprooted, these plants showed a fully developed nodulation phenotype, characterized by the presence of 80 to 150 big nodules distributed all over the root system (Fig. 10). A microscopic inspection of younger and older root segments of one individual, which developed approximately 150 nodules, showed that the overall hairlessness was maintained throughout the entire growth period, while sectioning of a few nodules revealed their wild type morphology (data not shown). How does *M. loti* colonize Roots of the *L. japonicus* Root-Hairless Mutant? The ability of *M. loti* to colonize, albeit with a significant delay, roots of the *L. japonicus Ljrhl1-1* mutant could be due to the presence of sporadic root hair cells, an alternative mode(s) of root invasion, or both. The following observations provide support for the existence of at least two novel mechanism of roots invasion by *M. loti*. At 21dai, an occasional association of dark-blue-stained patches of *M. loti* expressing the *hemA*::*lacZ* reporter gene fusion with the cortical surface of the enlarged NP was observed (Fig. 9A). Upon thorough microscopic inspection of many samples no evidence for the formation of ITs that originate at the cortical surface of, and ramify within, the NP could be found. However, sectioning of several samples has uncovered three examples containing clearly identifiable surface bacteria, which appear to penetrate a few cortical cell layers deep inside the NP via a mechanism resembling intercellular invasion (Fig. 9B).

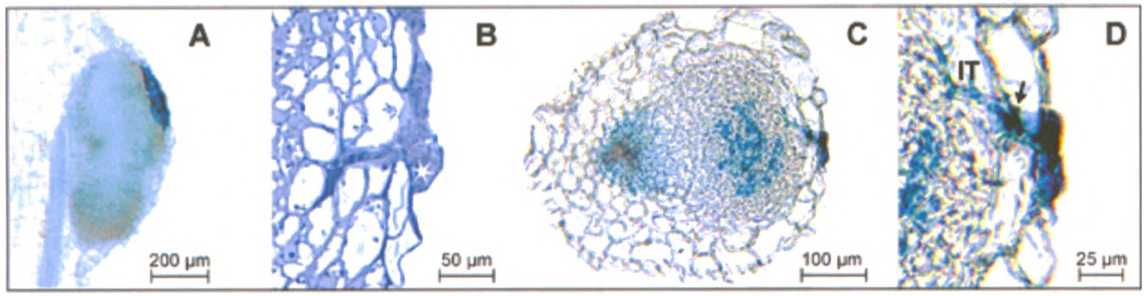

Figure 9. Root hair independent invasion of NP in the *Ljrhl1* mutant by *M. loti* (*hemA::LacZ*) (A) A longitudinal thick section of a NP showing a bacterial patch on the surface visualized by β-galactosidase staining (21 dai). (B) A light micrograph of a toluidine blue stained thin section of a NP showing a surface bacterial patch (*) which progresses via an intercellular route through three layers of cortical cells creating an 'infection pocket' (21 dai). (C) A transverse section (30 µm) of an infected nodule primordium with a surface patch of bacteria leading to the infection pocket and the IT that has ramified within the NP cortex (42 dai). (D) closeup of (C) The bacterial patch is continuous with an infection pocket (arrow) which narrows down into an infection thread (IT).

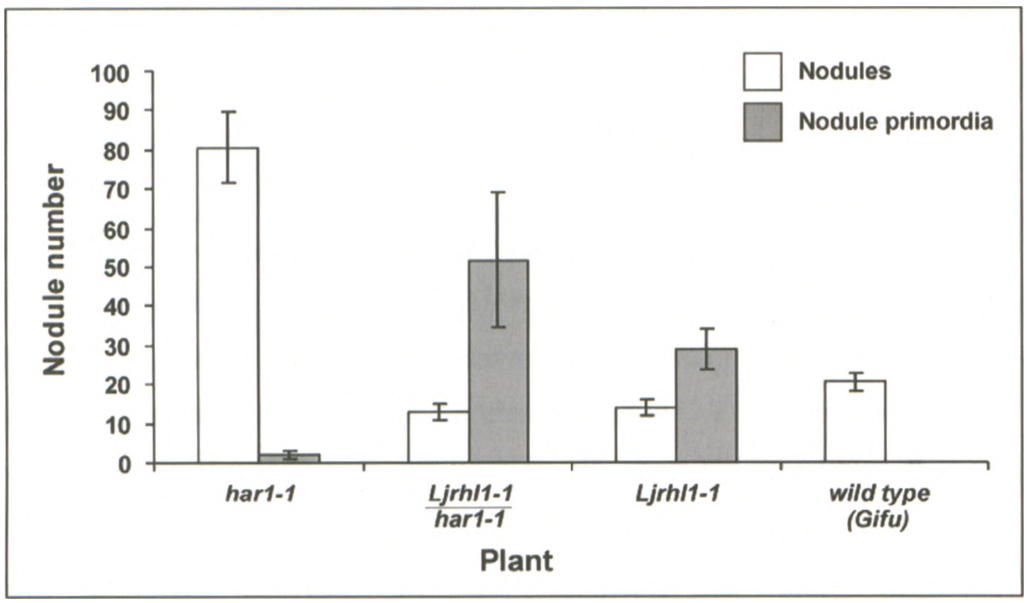

Figure 10. Numbers of nodules and NP on the roots of 49 day old wild type, *har1-1* and root hairless single (*Ljrhl1-1*) and double (*Ljrhl1-1 har1-1*) mutants 42 dai with *M. loti.* The roots were cleared and stained for β-galactosidase activity before counting. Mean values for each genotype represents n = 10 ± 95% CI.

Although infected cells were not found when the consecutive sections derived from these samples were examined (data not show), these initial observations prompted us to look for a similar and/or more advanced invasion events in the older (42 dai) plant material. Sectioning of many nodules, 42 dai, revealed a single example of a successful invasion event that originated from a patch of bacteria localized on the cortical surface of the nodule (Fig. 9C and D). This nodule was section in its entirety and it was clear that the surface patch of bacteria was the only source of infection. The histochemical footprint of *M. loti* expressing the β-galactosidase reporter gene suggests that, in this particular case, the mode of entry inside the nodule involved the formation of a wide intercellular infection pocket that gave rise to a narrower IT, which eventually lead to colonization of the underlying cortical cells of the nodule (Fig, 9C and D).

3.3.10 Nod-factor dependent root hair induction.

While examining roots of soil-grown single and double mutants carrying the Ljrhl1-1 allele, 42 dai, an unexpected discovery of root hairs that were associated with a proportion of the enlarged NP and nodules was made. These root hairs were often observed as groups of two to five, and were usually short but sometimes were very long and deformed (Fig. 11A). In addition, non-nodule associated (epidermal) root hairs, typically singletons, were observed with increased frequency as compared to uninoculated plants. These root hairs were often deformed and/or brunched (Fig. 11B). A closer microscopic examination showed that, in most cases, the nodule-associated hairs originated from cortical cells positioned underneath the fissure in the root epidermis caused by the emerging NP, defining them as the cortical root hairs (Fig. 11C). Importantly, 42 dai, several examples of ITs traversing multiple hairs present on a single nodule or NP, as well as ITs associated with the epidermal root hairs have been found (Fig 11D). Those infection threads that were associated with nodules had typically ramified inside the central zone giving rise to infected cells of the nodule (see Fig. 9C). Since root hairs were only very rarely observed on the un-inoculated single and double mutant lines carrying the *Ljrhl1-1* allele, the augmented presence of root hairs suggested their *de novo* origin upon inoculation. In order to test this hypothesis, the frequency of root hairs on 42 day old roots, which were either un-inoculated or inoculated with wild type or mutant

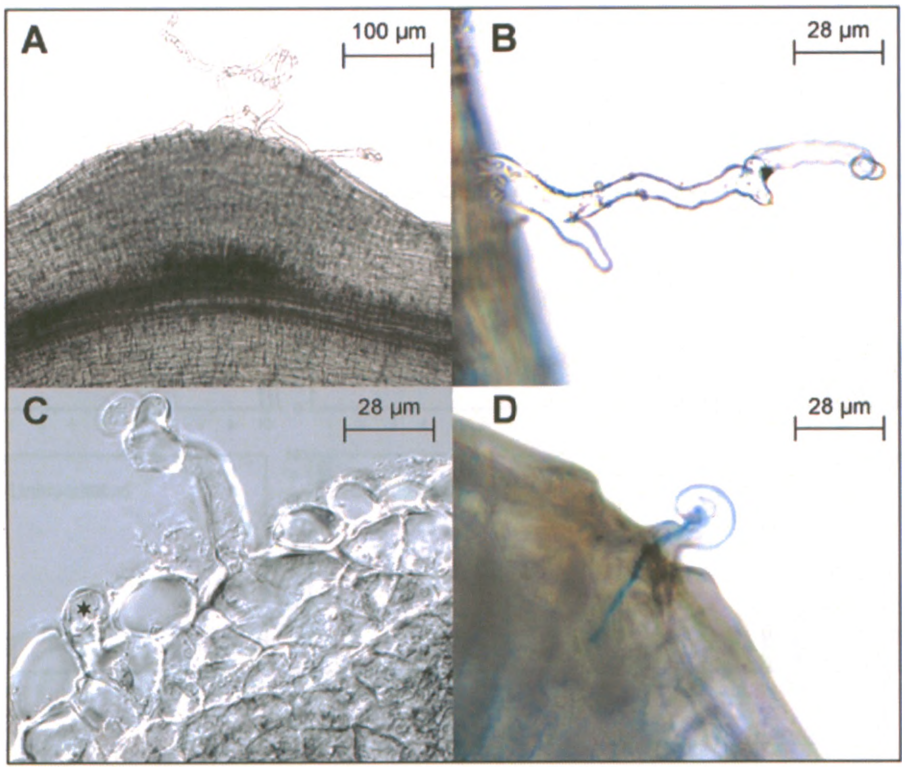

Figure 11. Root hair phenotypes of *Ljrhl1-1* mutant after inoculation with *M. loti.* (A) Three long nodule-associated (cortical) hairs emerging from the apex of a NP. (B) A non-nodule associated (epidermal) root hair with presumed NF induced branching and deformations. (C) A DIC image of an uncolonized NP showing two cortical root hairs, one fully emerged and the second one just emerging (asterisk). (D) IT traversing a root (cortical) hair that has emerged from the site of a fissure in the root epidermis at the apex of an enlarged NP.

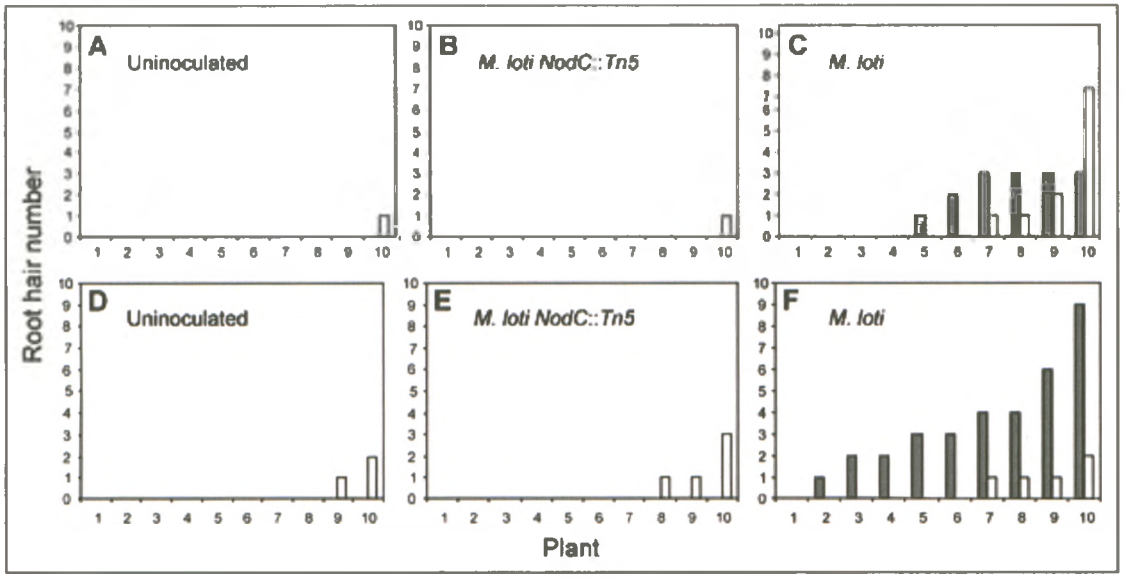

Figure 12. Numbers of nodule-associated (grey bars), and non-nodule associated (white bars) root hairs for individual (1 to 10) plants that were either uninoculated, or were inoculated with the *M. loti* strains as shown. (A-C) single mutant *Ljrhl1-1*; (D-F) double mutant *Ljrhl1-1 har1-1*.

NodC::Tn5 M. loti strains was carefully evaluated. As expected, the uninoculated roots developed hairs only very sporadically (Fig 12A and 12D), while inoculation with *M. loti* significantly increased their frequency (Fig 12C and 12 F). The *M. loti NodC::Tn5* mutant strain was unable to exert a similar effect indicating that the induction of these root hairs is NF dependent (Fig 12 B and 12E). The increase in the frequency of hairs was visible in both genetic backgrounds but was somewhat more pronounced in the *Ljrhl1-1 har1-1* mutant. The majority of hairs formed were found to be associated with nodules and NP (Fig 12F), which are more abundant in the *Ljrhl1-1 har1-1* double mutant as compared to the *Ljrhl1-1* single mutant line. One week later, the inoculated *Ljrhl1-1* developed an average of 38 ± 12 root hairs (66% of which were associated with nodules), while uninoculated control plants of the same age formed only 1.2 ± 2.2 root hairs *per* plant. The presence of ITs, within both the nodule associated and the non-nodule (epidermal) root hairs was clearly observable at this late time-point during symbiotic interaction, suggesting that the both types of root hairs contributed to the colonization of the *Ljrhl1-1* root by *M. loti*.

3.4 Discussion.

Root hair development has been extensively studied in *A. thaliana.* Although a large number of root hair mutants have been identified in this model organism (Grierson et al., 2001), *they* have never been characterized in the context of nitrogen fixing symbiosis, since Arabidopsis is unable to successfully interact with *Rhizobium.* We describe here the wild-type *L. japonicus* root hair phenotype and the isolation and characterization of nine *L. japonicus* root hair mutants carrying lesions in various parts of the root hair developmental pathway(s). These mutant lines, representing four distinct loci, have been identified as suppressors, or partial suppressors, of the *L. japonicus har1-1* hypernodulation phenotype, which by itself provides formal evidence for the essential role of wild-type root hairs during the *L. japonicus – M. loti* interaction. Almost unrestricted nodulation in the genetic background of the *har1-1* autoregulatory mutation offers the ability to observe the otherwise wild-type nodulation events in much greater numbers in comparison to wild-type plants (Wopereis *et al.*, 2000). This feature has guided us through the evaluation of

the nodulation phenotypes of the *L. japonicus* root hair mutants, and was especially useful during the analysis of the *Ljroothairless 1* mutant, where the initial root invasion events are rare. On the cellular level, the various developmental changes in both the root epidermis (the epidermal program) and cortex (the cortical program) are among the earliest observable responses of the host plant towards the compatible NF-producing *Rhizobium* (D'Haeze and Marcelle, 2002; Spaink, 1996). Our results show that the presence of root hairs is not required for the activation of the cortical program in *L. japonicus,* while it is essential for the efficient progression of the epidermal program.

The cortical program, defined by the initiation of root cortical cell divisions and subsequent organogenesis of NP (Guinel and Geil, 2001), is induced efficiently by a NF-producing strain of *M. loti* on roots of *L. japonicus* double mutant lines belonging to all four root hair mutant classes. With one notable exception, namely *Ljvrh1-1 har1-1* (see below), abundant organogenesis of NP occur within the first few days after inoculation with *M. loti.* Over a period of 3-6 weeks, the majority of these NP enlarged in size but remained un-colonized, indicative of a defect in the progression of bacterial invasion at the root epidermis. The observation that the overall number of NP is decreased in the *Ljrhl1-1* single mutant line carrying a functional *HAR1* receptor kinase gene, as compared to the corresponding double mutant *Ljrhl1-1 har1-1*, suggests that, similar to wild-type plants, the organogenesis of NP is subjected to autoregulation in the *Ljrhl1-1* mutant background. Thus, nodule primordia formation and their autoregulation in *L. japonicus* do not require root-hairs. It is, however, intriguing that the number of developing NP is limited in the double mutant genetic background of *Ljvrh1-1 har1-1*. Unlike the other *L. japonicus* root hair mutants described above, the *Ljvrh1-1 har1-1* develops significantly fewer NP that quickly become colonized by *M. loti.* At 21dai, the nodulation phenotype of *Ljvrh1-1 har1-1* resembles the corresponding wild-type phenotype of *L. japonicus* Gifu. Follow up experiments will be needed to fully understand the nature of the mechanism(s) that limits nodulation events in this particular mutant background. In contrast to the normal progression of NP organogenesis, the events at the root epidermis were found to be significantly affected in the root hair mutants. This is reflected in a diminished ability of all classes of *L. japonicus* root hair mutants to develop nodules as compared to the *har1-1* parental mutant line (nodule number, see Fig. 6C). Nevertheless, the root hairs of *petite,*

short, and *variable* root hair mutants support, to some extent, the infection process. At 21dai, the root colonization in these mutant lines proceeded via ITs which originated at the developmentally hindered root hairs. Although growth–terminating root hairs have been shown to be particularly susceptible to NF deformation activity (Esseling *et al.*, 2003, Esseling and Emons, 2004), young growing root hairs were found to entrap the bacteria under soil-growth conditions (Kijne et al., 1992). Root hairs of *petite, short*, and *variable* root hair mutants proceed through different durations of the tip-growth phase, which is destined for premature termination. With the exception of *Ljvrhl-1 harl-1* (see above), longer tip growth phase correlates with greater numbers of infected nodules. It is possible that the duration of the tip-growth phase, and thus the particular physiological status of the growing root hair (Esseling and Emons, 2004), may be one defining factor of the mutant root hair susceptibility to *M. loti* infection. Other developmental factors, such as the ability to curl and entrap symbiotic bacteria, and the propensity to initiate and sustain IT growth, are also important determinants of root hair susceptibility. The detailed evaluation of these processes in the above described *L. japonicus* root hair mutant lines is required in order to fully correlate the various root hair phenotypes with the outcome of the symbiotic interactions. For many legume species, including *L. japonicus*, root hairs are considered to be essential structures for the establishment of successful plant-microbe symbioses. The involvement of root hairs in the entrapment of bacteria, the subsequent initiation of root invasion (which commences as invagination of the root-hair plasma membrane to give rise to an IT), and eventual colonization of nodule structure, have been well documented (Gage, 2004). The nodulation phenotypes of the *L. japonicus* root hair mutants described above provide additional support for the significance of root hairs in bacterial invasion of the root tissue. However, Kawaguchi et al. (2002) reported the identification of the *L. japonicus SLIPPERY* mutant, which in spite of having very few root hairs was able to develop a low number of colonized nodules. Although not investigated further, this observation lead the authors to suggest that an alternative entry mechanism(s) into the *L. japonicus* root may have accounted for the nodulation phenotype of the *SLIPPERY* mutant (Kawaguchi *et al.*, 2002). We set out to test this hypothesis by performing a detailed analysis of the nodulation phenotype of the independently isolated *L. japonicus* root hairless *Ljrhl1-1* mutant. We show that, under the growth conditions used, *Ljrhl1-1* is

capable of interacting with *M. loti* and, given an extended period of time, is able to develop a large number of nitrogen fixing nodules. Evidence is provided for the ability of *M. loti* to enter the nodule structure via a crack entry mechanism through the cortical surface of the NP. Furthermore, we show that in a NF-dependent manner, a partial complementation of the root hairless phenotype occurs. These *de novo* induced root hairs restore the root hair-dependent mode of infection, which proceeds through IT formation and leads to the colonization of the *Ljrhl1-1* root by *M. loti*.

There exist several examples of alternate mechanisms of infection that do not require root hairs. Crack entry mechanisms that rely on intercellular breaching of the epidermal cell layer have been reported in several legume species (Boogerd and van Rossum, 1997; de Faria *et al.* 1988; Subba-Rao *et al.* 1995) as well as in the non-legume *Parasponia* (Lancelle and Torrey, 1984). For example, in the semi-aquatic *S. rostrata,* a close relative of *L. japonicus*, a crack entry mechanism at the sites of lateral root emergence is used as the principal mode of bacterial entry for nodule colonization under hydroponic conditions (Goormachtig et al., 2004a and 2004b). This mechanism manifests itself as the formation of pockets of bacteria embedded in the intercellular matrix that narrow down to intracellular infection threads prior to entering the plant cells (Sprent and Raven, 1992; Ndoye *et al.*, 1994; Subba-Rao *et al.* 1995). Interestingly, in *Chamaecytisus proliferus* (tagasaste) – *Bradyrhizobium* sp. symbiosis, the initially developed infection threads abort prematurely within the root hairs. A crack entry through the intercellular space of the outer layers of the emerging nodule is used instead for colonization, without further participation of ITs (Vega-Hernandez *et al.* 2001).

The single example of root-hair independent invasion described in this work, where *M. loti* colonized cracks at the surface of the emerged nodule, and proceeded through intercellular infection and IT formation to enter the nodule structure, appears to combine features of both the *Azorhizobium caulinodans-S. rostrata* and the bradyrhizobia-tagasaste symbioses. This type of root invasion by *M. loti* appears to be rare, even on plants lacking epidermal root hairs and in spite of the frequent occurrence of bacterial colonies on the nodule surface. The importance of this phenomenon in Lotus is unknown and needs further study. One possibility is that this mode of infection is important under conditions that inhibit root hair growth or susceptibility, such as is the case in *S. rostrata*

and *N. plena* (Goormachtig *et al.*, 2004a, b). Interestingly, it is possible that symbionts that are unable to initiate the normal epidermal infection events, but are capable of inducing NP formation, may be capable of infecting *L. japonicus* through this route. Both *Stylosanthes* and *Arachis*, which are infected via crack entry, exhibit less stringent requirements towards their micro-symbiotic partners (Dart, 1977). In addition, Goormachtig et al. (2004a) report that while mutant bacteria producing un-substituted NFs fail to form functional nodules though root hair-dependent infections, they are able to colonize nodules via intercellular entry at nearly wild type levels. It appears, nevertheless, that the successful colonization of *Ljrhl1-1* roots proceeds mainly through NF-dependent *de novo* root hair formation. Nod factors and related LCOs are known for their strong plant growth promoting effects (Dyachok *et al.*, 2002, Souleimanov *et al.*, 2002, Prithiviraj et al., 2003), and have been shown to cause root hair induction (Hai) on vetch (*Vicia sativa* ssp. *nigra*; Roche *et al.*, 1991; Tak et al., 2004; Zaat *et al.*, 1987, 1989), *S. rostrata* (Mergaert *et al.*, 1993), and *L. japonicus* (van Spronsen *et al.*, 2001). Van Brussel *et al.* (1992) observed the induction of cortical root hair formation upon application of mitogenic Nod factors in the absence of bacteria. It was speculated that NF signalling causes localized weakening of the cell wall, which directs IT formation in the presence of bacteria or root hair formation in their absence (van Brussel *et al.*, 1992, van Spronsen et al., 1994). In our experiments nodule associated (cortical) root hairs, and also nodule independent (epidermal) root hairs, evolved with bacteria present but in the absence of pre-existing root hairs suggesting that the context of the bacteria (*ie.* whether or not it can interact with root hairs) may be important. This notion is supported by the observation that the *nodF nodL* double mutant of *S. meliloti,* which produces altered NF and is unable to induce formation of sheperd's crooks or to initiate ITs when inoculated onto alfalfa seedlings, exerts abnormal activity in terms of inducing tip growth on trichoblasts and non-haired epidermal cells (Ardourel *et al.*, 1994).

In vetch non-mitogenic NFs are stronger inducers of Hai than are mitogenic NFs (Roche *et al.*, 1991; Tak *et al.*, 2004). Furthermore, mitogenic NFs have inhibitory effects on Hai in vetch (Tak *et al.*, 2004), but seem not to inhibit (cortical) root hair formation on NP. The separate ontogeny and differential response to NFs of nodule-associated (cortical) hairs and epidermal root hairs prescribe two distinct classes of root hairs in legumes. Both

cortical and epidermal derived root hairs in *L. japonicus* are NF inducible, a developmental process which does not require the *LjROOTHAIRLESS 1* gene product. One intriguing possibility is that *M. loti*-produced NFs complement, at least partially, the lack of *Ljrhl1-1* gene function. If this is indeed the case, the molecular cloning of *Ljrhl* gene, which has became increasingly possible through rapidly advancing *L. japonicus* whole genome sequencing project (http://www.kazusa.or.jp/lotus), should provide important insight into the role NFs play in the initiation of root hairs.

3.5 Material and methods.

3.5.1 Plant Material and Growth Conditions.

The *L. japonicus* root hair mutants were generated by chemical ethylmethanesulfonate (EMS) mutagenesis of *L. japonicus* ecotype Gifu *har1-1* homozygous seeds (Wopereis et al., 2000) following the previously detailed procedure (Szczyglowski et al., 1998). Seeds were surface sterilized and germinated as described previously (Szczyglowski et al., 1998). All plants (unless otherwise stated) were maintained in a growth room under the 16/8 hours day/night regime, an irradiance of 200-250 umol sec^{-1} m^{-2} (PPF) light intensity, at 22°C, and were occasionally watered with B&D nutrient solution (Broughton and Dilworth, 1971) containing a low concentration (0.5mM) of KNO_3.

3.5.2 Evaluation of Root Length and Root Hair Phenotypes.

The root length phenotypes were evaluated by examining plants grown on vertically positioned agar plates as previously described (Wopereis et al., 2000). The plates were incubated in the dark at 22°C for 6 days. Root length measurements were taken every 24 hours during this period.

For root hair length measurements the agar plate condition were used as described for Arabidopsis (Muller and Schmidt, 2004), except that the subsequent incubation was as in Wopereis et al. (2000). Unstained root images of 5 day old plants were captured with a DMX1200 digital camera (Nikon) connected to a SMZ 1500 microscope (Nikon) and root hair lengths were measured manually, using a standard ruler, directly from enlarged

printed images. For each genotype, a total of 80 root hairs were measured from eight plants. The average root hair length is expressed as a mean value ± 95% confidence interval.

Root hair number was evaluated using 42 (10 plants per each genotype) and 49 (five plants per each genotype) day old plants, which were either uninoculated or inoculated (seven days after transfer to soil) with the appropriate *M. loti* strain (see figure legend). All plants were grown in pots containing a 6:1 mixture of vermiculite and sand. Plants were carefully uprooted, washed and the roots were cut into approximately 5 cm long sections. The root sections were then mounted on slides and examined under 200X magnification using a ZEISS Axioskop 2 plus microscope. For the 49 days old plants, roots were fixed, stained for β-galactosidase reporter gene activity, and cleared as previously described (Wopereis at al. 2000) before root hair counts were made.

3.5.3 Evaluation of Symbiotic Phenotypes.

L. japonicus seedlings were transferred from germination plates to pots containing a 6:1 mixture of vermiculite and sand and allowed to grow for an additional seven days at which point they were inoculated with one of the following *M. loti* strains: NZP2235, NZP2235 carrying a *hemA::LacZ* reporter gene fusion, R7A, and R7A *nodC*::*Tn5* mutant incapable of producing Nod factors (kindly provided by C. Ronson and J. Sullivan, University of Otago, Australia) .

Plants inoculated with *M. loti* strains R7A and R7A *nodC* were examined 28 dai using brightfield light microscopy (Nikon SMZ 1500).

For the histochemical analysis of β-galactosidase reporter gene activity soil grown plants were analyzed 10, 21 and 42 dai. The number of nodule primordia and nodules were scored at 21 and 42 dai (10-15 plants per each genotype). Root sections were processed as described above for root hair counts of 49 day old plants. The cleared specimens were first examined by brightfield light microscopy (ZEISS Axioskop 2 plus). In addition, the microscopic sections were generated by embedding specimens in 3% agarose and sectioning them to 25-35 μm using a VT1000S vibrating microtome (Leica). The images were taken using a DMX1200 digital camera (Nikon) attached to an inverted microscope (Leitz) and processed into montages using Adobe Photoshop 7.0 software.

2002), which destroys an *Mva*1 restiction site present in the wild type allele. Briefly, primers (har1-1Forward: 5'-gattgtgattggaattgcact-3', har-1Reverse: 5'-cgcaatcttatacctcatctcc-3') were used to amplify a 463 bp fragment of the *HAR1* gene, 10 µl of which was subsequently digested with 5 units of *MvaI* enzyme for 1 hour at 37°C. *Mva*1 digested products amplified from wild-type Gifu alleles yielding 369 and 94 bp restriction fragments; those amplified from the *har1-1* mutant allele fail to digest.

Crossing the *L. japonicus* homozygous mutant lines with the wild-type Gifu parental line was performed using a manual emasculation and pollination procedure as described previously (Jiang and Gresshoff, 1997). Root hair and symbiotic characteristics of the resulting F1 and F2 generations were scored and used to determine the genetic basis of the observed phenotypes.

In order to examine allelism, complementation analyses were conducted by cross-pollinating independent double homozygous mutant lines that display either identical or similar phenotypes, and by evaluating their respective F1 hybrid phenotypes. The progeny from two to four independent crosses were evaluated.

3.5.6 Mapping of *Ljrhl1, Ljprh1, Ljsrh1, and Ljvrh1.*

To map the genes underlying the various root hair phenotypes, the double mutants (*Ljrhl1-1 har1, Ljprh1-1 har1, Ljsrh1-1 har1, Ljvrh1-1 har1*) were crossed to the polymorphic mapping partner, *L. japonicus* ecotype 'MG20'. DNA derived from F2 homozygous mutants showing the variable root hair (33 individuals), short root hair (17 individuals), petite root hair (30 individuals), and root hairless (19 individuals) phenotypes was prepared as follows. Approximately 25 mg of leaf tissue was pulverized in a 1.5 ml micro-tube using a hand drill and a plastic pestle in 2X CTAB extraction buffer [2% hexadecyltrimethylammonium bromide (w/v), 100 mM Tris (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone (w/v)] and then centrifuged for 1 minute at 14000 rpm. The supernatant was extracted once with an equal volume of chloroform and the DNA precipitated with 2/3 volume of isopropanol and briefly dried before being re-suspended in 0.25 ml of 10 mM-Tris-HCl (pH 8.0). The resulting DNA samples were analyzed using a selection of available simple sequence repeat markers (Sato et al., 2001; Nakamura et al., 2002; Kaneko et al., 2003; Asamizu et al., 2003, and unpublished data). In addition, the

following simple sequence repeat markers were used: TM0407, TM1419, TM0494, TM0696, TM0140, and JM001. The corresponding primer sequences are as follows: TM0407-F: 5'-aagctattgcatccactggg-3', TM0407-R: 5'-actagggttcattctgtggc-3'; TM1419-F: 5'-gtctaatgatgtgggttggc-3', TM1419-R: 5'-ggagctcaaatattcattacac-3'; TM0494-F: 5'-catagctgcaattccaagag-3', TM0494-R: 5'-tttcgcttgagtcaatgtag-3'; TM0696-F: 5'-ttgaccctaacatgggaatc-3', TM0696-R: 5'-tggacaaatgcatgacacac-3'; TM0140-F: 5'-ggaaatcaatttcgggaggc-3', TM0140-R: 5'-tggacagtaataaatacattcg-3', JM001-F: 5'-aaggaggaagggttttgcac-3', and JM001-R: 5'-caggcctcaagctaaggaca-3'. All marker amplification reactions were carried out in a total volume of 50 μl containing: 20 mM Tris-HCl, pH 8.4, 50 mM KCl and 1.5 mM $MgCl_2$, 1 μM each primer, 200 μM of each dCTP, dATP , dCTP and dGTP, 1 unit of Taq DNA polymerase (Invitrogen) and 5 μl of template DNA. PCR reactions were performed using a GeneAmp PCR System 9700 machine (PE Applied Biosystems) with a single 4 minute denaturation cycle at 94°C followed by 35 cycles (94°C, 30 s; 55°C, 30 s; and 72°C, 1 min). PCR products were separated on 4% agarose gels in 0.5X TBE buffer.

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CHAPTER 4

Conservation of Lotus and Arabidopsis basic helix-loop-helix proteins reveals new players in root hair development.

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4.1 Contributions made by Bogumil Karas:

- Analysis of symbiotic and non-symbiotic phenotypes of *Ljrhl1-1* and *Ljrhl1-2* mutant lines (Figures 1, 2).
- Map based cloning of *LjRHL1* locus. (Figure 3)
- All RT-PCR experiments (Figures 4, 10, 13, 15)
- Construction of all vectors and performance of all hairy root transformation experiments (Figure 5, 11, 12)
- Generation of material and perforance of light microscopy (Figures 7, 8, 9, 11)
- Isolation of Arbidopsis TDNA knockout lines and development of double and triple mutants (Figure 14, Table 1).

In this study, the molecular cloning of the L. japonicus ROOTHAIRLESS locus (see Chapter 3) is described and comparative analyses with Arabidopis, the most advanced model for root hair development research, is presented.

4.2 Introduction.

Root hairs constitute tubular extensions of epidermal cells. Formed by the majority of angiosperm plants, they function to increase root surface area thus facilitating physical anchorage to a substrate while providing a large interface through which nutrients and water are absorbed. Root hairs are also important for plant-microbe interactions, highlighting the key role of these tip growing cells in biotic and abiotic interactions of the root.

Features of root hair development, such as their exterior position, species-specific patterning, and polarized growth, have made them an attractive model for studying various aspects of cell differentiation and growth in the context of root developmental plasticity. Both endogenous and environmental cues have been implicated in the specification of root hair development and various proteins and protein complexes have been characterized as positive or negative regulators of root hair differentiation (Ishida *et al.*, 2008).

In Arabidopsis roots, where files of hair cells alternate with files of non-hair cells, genetic analysis has revealed intricate mechanisms of positional information, epigenetic regulation, and non-cell autonomous signalling in the specification of epidermal cell fate (Guimil and Dunand, 2006). A protein complex containing the transcriptional regulators WEREWOLF (WER) (Lee and Schiefelbein 1999), GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Bernhardt *et al.*, 2003, Payne *et al.*, 2000, Zhang *et al.*, 2003), and TRANSPARENT TESTA GLABRA (TTG1) (Galway *et al.*, 1994, Walker *et al.*, 1999), was shown to promote expression of the *GLABRA2* (*GL2*) gene (Masucci *et al.* 1996), encoding an HD-ZIP transcription factor, to regulate hairless cell differentiation. Conversely, MYB proteins CAPRICE (CPC) (Wada *et al.* 1997), TRIPTYCHON (TRY) (Schellmann *et al.* 2002), ENHANCER of TRY and CPC1 (ETC1) (Kirik *et al.* 2004), and CAPRICE-LIKE MYB3 (CLP3) (Tominaga *et al.*, 2008) were identified as positive regulators of hair cell identity. In addition, various networks of transcription factors and other regulatory elements, encompassing different cellular functions, including chromatin

remodelling, hormonal signalling, ion fluxes, cell cycle progression, and cytoskeleton rearrangement, were shown to be important for initiation and maintenance of polar root hair growth (Guimil and Dunand 2006). The availability of a large collection of root hair mutants in Arabidopsis continues to fuel rapid dissection of genetic networks and associated cellular events governing patterning and growth of root hair cells.

Root hair mutants have been also identified from genetic screens for which the primary goal has been the characterization of loci that support the development of root nodule symbiosis in legume plants (Karas *et al.*, 2005, Kawaguchi *et al.*, 2002, Murray *et al.*, 2006) In many legumes root hairs mediate the initial contact between the legume host and nitrogen fixing soil bacteria, commonly known as *Rhizobium*. They actively participate in the recognition of bacterially-encoded lipochitin-oligosaccharide signalling molecules known as nodulation or Nod factors and, subsequently, in the colonization of the root by bacteria (Karas *et al.*, 2005), processes which are unknown to Arabidopsis. Prior to or concomitant with the initiation of the infection process at the surface of root hair tips, legume roots respond to bacterial signalling by the initiation of cell divisions in the root cortex. This leads to the formation of new lateral organs, root nodules, which eventually host the symbiotic bacteria, providing the appropriate conditions for symbiotic nitrogen fixation to occur (Oldroyd and Downie 2008).

Impairment of root hair development results in defective symbiotic interaction. In the model legume *Lotus japonicus*, deleterious mutations in the *ROOTHAIRLESS1* (*LjRHL1*) locus prevent root hair formation (Karas *et al.*, 2005, Kawaguchi, *et al.*, 2002). Inoculation of the corresponding *Ljrhl1-1* mutant with symbiotic bacteria leads to the initial formation of empty nodule structures, thus uncoupling bacterial colonization of the root from nodule organogenesis (Karas, *et al.* 2005). These features of the *Ljrhl1-1* mutant genetic background have been useful in the functional analysis of root nodule organogenesis (Karas *et al.* 2005, Murray *et al.* 2007) and therefore, we set out to characterize the underlying *LjRHL1* locus at the molecular level.

We show here that the deleterious mutations in the *LjRHL1* gene, encoding a bHLH transcription factor, were responsible for lack of root hairs in the *L. japonicus* *Ljrhl1-1* mutant. Among the 162 affiliates of the Arabidopsis bHLH protein family, LjRHL1 showed the highest homology with the members of group XI, that comprises five

predicted bHLH transcription factors (Heim *et al.* 2003). By performing cross species complementation experiments, we demonstrate functional conservation of *L. japonicus* LjRHL1 and Arabidopsis At2g24260, At4g30980, and At5g58010 but not At1g03040 and At4g02590 proteins. We demonstrate the partially redundant function of At2g24260, At4g30980, and At5g58010, which provides a plausible explanation of why mutations in these loci do not generate a root hair phenotype in Arabidopsis.

4.3 Results.

4.3.1 *Lotus japonicus root hairless* mutants.

In two independent genetic screens, we have identified a class of ethyl-methanesulfonate-induced *L. japonicus* symbiotic mutant lines, where defective interaction with a natural microsymbiont of *L. japonicus*, *Mezorhizobium loti,* was linked with the impairment of root hair development (Karas *et al.,* 2005, Kawaguchi *et al.,* 2002, Murray, *et al.* 2006). Among these mutants, four root hairless lines were found and the detailed analysis of their root and symbiotic phenotypes was described (Karas *et al.,* 2005, Kawaguchi *et al.,* 2002). Three of these lines were put into a single complementation group and assigned an allelic designation, *Ljrhl1-1*, *Ljrhl1-2*, and *Ljrhl1-3* (Karas *et al.,* 2005). However, further analysis of these lines defined them as siblings, carrying the same mutant allele, *Ljrhl1-1* (see below). The fourth line, *slippery* (*slp*), was derived from a separate screen (Kawaguchi *et al.,* 2002) and its relation to *Ljrhl1-1* was unresolved (Figure 1). Based on the similarity of root hair and symbiotic phenotypes in *Ljrhl-1* and *slp*, we predicted that these lines might carry allelic mutations. The roots of F1 plants, derived from a complementation cross between homozygous *Ljrhl1-1* and *slp*, showed the root hairless phenotype thus supporting this prediction (see below). Consequently, we renamed *slp* as *Ljrhl1-2*.

L. japonicus roots have type 1 hair cell patterning; all or almost all root epidermal cells produce hairs (Karas *et al.,* 2005). The *Ljrhl1-1* line remained almost totally hairless when cultivated under various growth conditions (Karas *et al.,* 2005). Root hair formation in *Ljrhl1-2* was very low in comparison with the wild-type control, but its frequency varied depending on growth conditions used. When cultivated in soil, the frequency of root

hair formation in *Ljrhl1-2* was 62 ± 15 per plant (n = 30), while no root hairs were detected in *Ljrhl1-1* under the same growth conditions.

The observed differences in root hair phenotypes between *Ljrhl1-1* and *Ljrhl1-2* correlated, to some extent, with the severity of their corresponding mutant symbiotic phenotypes. When analysed in parallel 21 days after inoculation (dai) with *M. loti* carrying the constitutively expressed GUS reporter gene, the *Ljrhl1-1* line developed mostly uncolonized nodule primordia, and only a few small nodules colonized by rhizobia were formed, thus confirming our previous data (Karas *et al.*, 2005). In contrast, *Ljrhl1-2* formed a limited number of big colonized nodules, although small nodules and uncolonized nodule primordia were as abundant in *Ljrhl1-2* as in *Ljrhl1-1* (Figure 2). The presence of big nodules at 21 dai suggested that the root hairs formed by *Ljrhl1-2* were able to support limited root colonization by the bacteria in a root-hair-dependent manner, similar to the wild-type interaction (Karas *et al.*, 2005).

The initial growth of both mutant lines was as vigorous as that of wild-type plants and no significant differences, aside from root hair formation and nodulation pattern, were observed. However, unlike *Ljrhl1-2*, the *Ljrhl1-1* line showed increased sensitivity to growth conditions (e.g. watering) and grew less vigorously later during development, which was also reflected in the smaller pod size of this line (data not shown). When grown vertically on the surface of agar plates, both mutants had increased root elongation, compared to wild type plants (Figure 1, see also Karas *et al.*, 2005).

4.3.2 Map-based cloning of *LjRHL1/SLIPPERY* locus.

The initial mapping experiments positioned the *Ljrhl1-1* mutation within a 3.2 cM interval on the long arm of *L. japonicus* chromosome 6, between the microsatellite (SSR) markers JM001 and TM0140 (Karas, *et al.* 2005). Fine mapping and subsequent genotyping of 760 *Ljrhl1-1* mutants, derived from an F2 segregating population of a cross between the *Ljrhl1-1* homozygous line (ecotype Gifu) and a polymorphic wild-type *L. japonicus* MG-20, enabled us to narrow down this region to 48.8 kb (Figure 3). This region is flanked by recombination points, as defined by BK001 and BK003 molecular markers, which are located on two overlapping TAC clones, BM2019 and TM1904, respectively (Figure 3a, b). Four genes, encoding a putative zinc finger protein,

Figure 1. Root hair phenotypes of *L. japonicus* wild-type-Gifu (**left**), *Ljrhl1-1*(**center**), and *Ljrhl1-2* (**right**) mutant lines. Six day old seedlings are shown; note the emerging difference in root length between wild type and mutant plants.

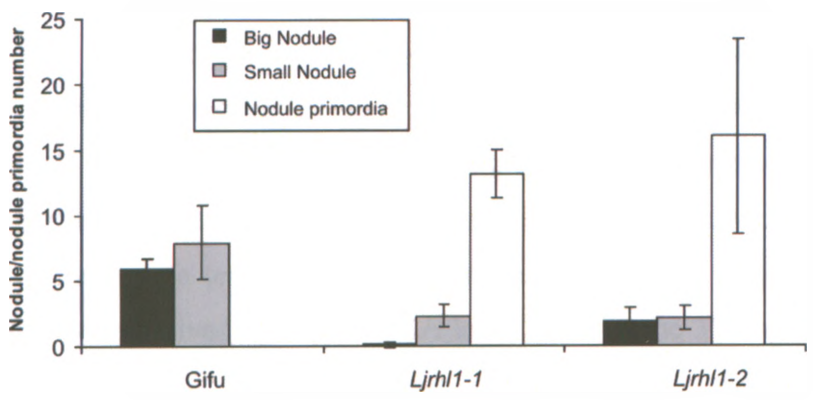

Figure 2. Number of nodulation events in wild type *L. japonicus* Gifu, and *Ljrhl1-1* and *Ljrhl1-2* mutant plants, 21 dai with *M. loti*. The nodules colonized by *M. loti* were categorized as wild type-like big and small nodules. The nodule primordia category encompasses all remaining nodulation events that occurred without successful *M. loti* colonization. For each genotype at least 15 plants were scored.

an aldehyde dehydrogenase, an unknown protein, and a basic helix-loop-helix (bHLH) transcriptional factor, were predicted in this region by a blast search of the Arabidopsis genome (Figure 3C). The PCR amplification and sequencing of all four genes from the *Ljrhl1-1* and *Ljrhl1-2* mutants and the corresponding wild-type Gifu, revealed the presence of mutations in the gene encoding the bHLH transcriptional regulator (Accession. no. FJ375304), but not in the other three genes. The *Ljrhl1-2* line carried a 371 bp insertion in the predicted exon 2 of this gene, while the three *Ljrhl1* lines had an identical single base pair substitution of G_{997} to A in predicted exon 3 and were, therefore, considered to be siblings (Figure 3D). A pair of gene specific primers that were localized in the first and the fourth exon of the predicted *bHLH* gene were used in genomic PCR amplification (Figure 4). For both wild-type and *Ljrhl1-1,* the expected 967 base-pair (bp) genomic fragment was amplified. In contrast, a 1338 bp fragment was generated when the *Ljrhl1-2* genomic DNA was used as a template. Sequencing of all genomic fragments obtained confirmed that they were derived from the *bHLH* locus and that the 371 bp insertion was indeed present in the *Ljrhl1-2* allele. The same primer pair was used in an RT-PCR (reverse transcription polymerize chanin reaction) approach with total RNA derived from uninoculated roots of all three genotypes (Figure 4). A 447 bp cDNA fragment was amplified from both wild-type and *Ljrhl1-1*, while several weak bands of higher molecular mass were obtained for the *Ljrhl1-2* genetic background. The latter result indicated that alternative forms of the *bHLH* mRNA were produced due to the presence of the insertion (see below). Together, these results defined the *bHLH* gene as a viable candidate for the *LjRHL1* locus.

To further test this prediction, a full copy cDNA was obtained that corresponded to the *bHLH* mRNA (see below) and *in planta* complementation experiments were performed. A binary vector, containing the cauliflower mosaic virus (CaMV) 35S promoter driving expression of a C-terminal translational fusion between the green fluorescent protein (GFP) and the *bHLH* cDNAs was introduced into *L. japonicus* roots by *Agrobacterium rhizogenes*-mediated transformation (Murray, *et al.* 2007). The resulting transgenic hairy roots, which emerged from points of inoculation on the hypocotyl of *Ljrhl1-1* and *Ljrhl1-2* mutant plants, formed root hairs with efficiency comparable to wild-type plants (Figure 5).

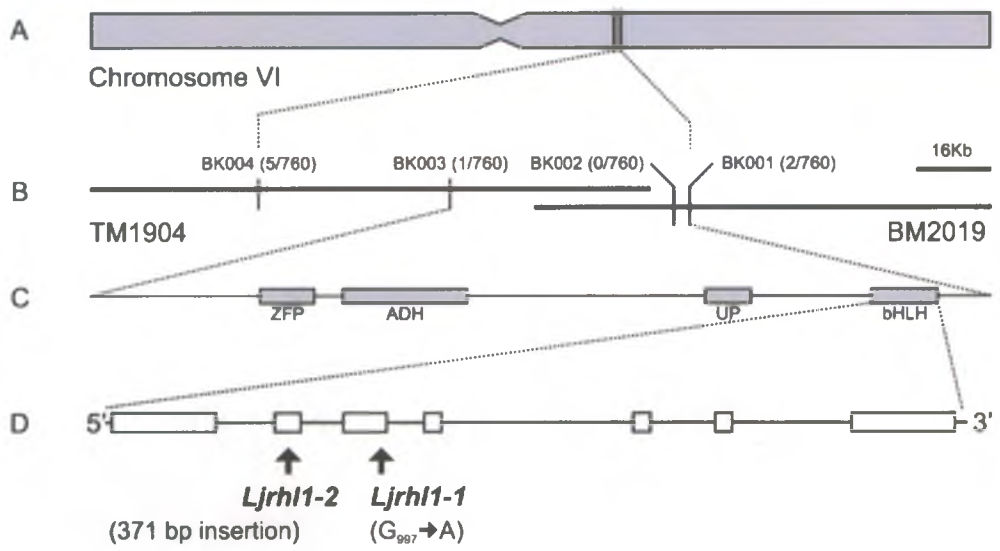

Figure 3. Map-based cloning of the *LjRHL1* locus. (**A**) A schematic of *L. japonicus* chromosome VI. (**B**) Two overlapping TAC clones, TM1904 and BM2019, with genetic markers linked to the *LjRHL1* locus are indicated. Number of recombinants versus total number of homozygous mutant individuals tested for a given marker is shown in parenthesis. (**C**) The region delimited by BK003 and BK001 genetic markers was predicted to contain four genes encoding a presumed zinc finger protein (ZFP), an alcohol dehydrogenase (ADH), unknown protein (UP), and a bHLH domain-containing protein (bHLH). (**D**) Exon-intron structure of the *LjRHL1* gene. The positions of genetic lesions in *Ljrhl1-1* and *Ljrhl1-2* mutant alleles are indicated.

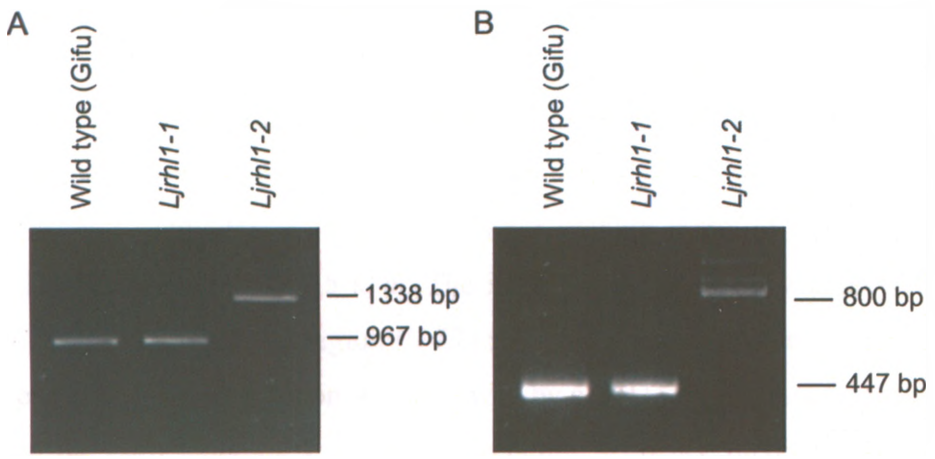

Figure 4. Results of the genomic and mRNA amplification experiments in wild type *L. japonicus* Gifu, and *Ljrhl1-1* and *Ljrhl1-2* mutant plants (**A**) *LjRHL1-1* gene specific primers were used to amplify a genomic region positioned between introns 1 and 4 of the *LjRHL1-1* locus (see Material and Methods). Note a higher molecular mass of the *Ljrhl1-2*-derived product, which resulted from the insertion of the *LjTRIM1* transposable element. (**B**) RT-PCR products using the same primer pair as in **A**. Multiple bands corresponding to several alternative forms of the *LjRHL1-1* transcript were found in roots of the *Ljrhl1-2* mutant.

However, instances where transgenic roots showed patchy root hair formation were also observed. We have attributed this phenotype to the sporadicallychimeric nature of hairy root tissues. When transformed with the control vector, containing the CaMV 35S promoter and GFP but lacking the *bHLH* cDNA, the hairy roots that formed remained hairless, thus recapitulating the phenotype of the original mutant plants (Figure 5). Cumulatively, based on the data obtained, we concluded that the *bHLH* gene identified through a map-based cloning approach indeed corresponded to the *LjRHL1* locus.

4.3.3 *LjRHL1* encodes a bHLH transcriptional factor.

Using a combined approach of reverse transcription-polymerase chain reaction (RT-PCR) and 5'- and 3'- rapid amplification of cDNA ends (RACE), a full copy cDNA of 1444 nucleotides (nts) in length, corresponding to the *LjRHL1* mRNA, was reassembled (see Material and Methods). It contained an open reading frame of 1161 nts encoding a predicted LjRHL1 protein of 40.5 kDa. The initiation ATG codon was preceded by 229 nucleotide long 5' untranslated region (UTR) that contained an in frame TGA stop codon. At the 3' end, the TGA stop codon was followed by a 3' UTR of 55 nts. Alignment of the mRNA and the corresponding *L. japonicus* genomic sequence predicted an *LjRHL* gene structure of 7 exons and 6 introns (Figure 3d).

A search for conserved protein domains using BLAST and Pfam algorithms identified the presence of a bHLH motif in the predicted LjRHL1 protein, which was localized between amino-acid residues 181 and 236 (Figure 6). A comparison with a consensuses sequence for plant bHLH domains (Heim, *et al.* 2003) showed that key amino acid residues were present at the conserved positions within the bHLH of LjRHL1 (Figure 6). Thus, the most critical His-Glu-Arg (H_E_R) residues for amino acid contact with nucleotide bases were present at the conserved 5, 9, and 13 amino acid positions, respectively, within the predicted bHLH domain. Furthermore, the hydrophobic residues, presumed to be important for bHLH dimerization and the stability of the resulting DNA-protein dimers (Ferre-D'Amare *et al.* 1993), such as Leu23, were also found at the equivalent positions in the bHLH domain of LjRHL1 (Figure 6).

A search for homologous proteins in Arabidopsis identified several significant hits to the predicted bHLH proteins, with the top five encompassing all members of the

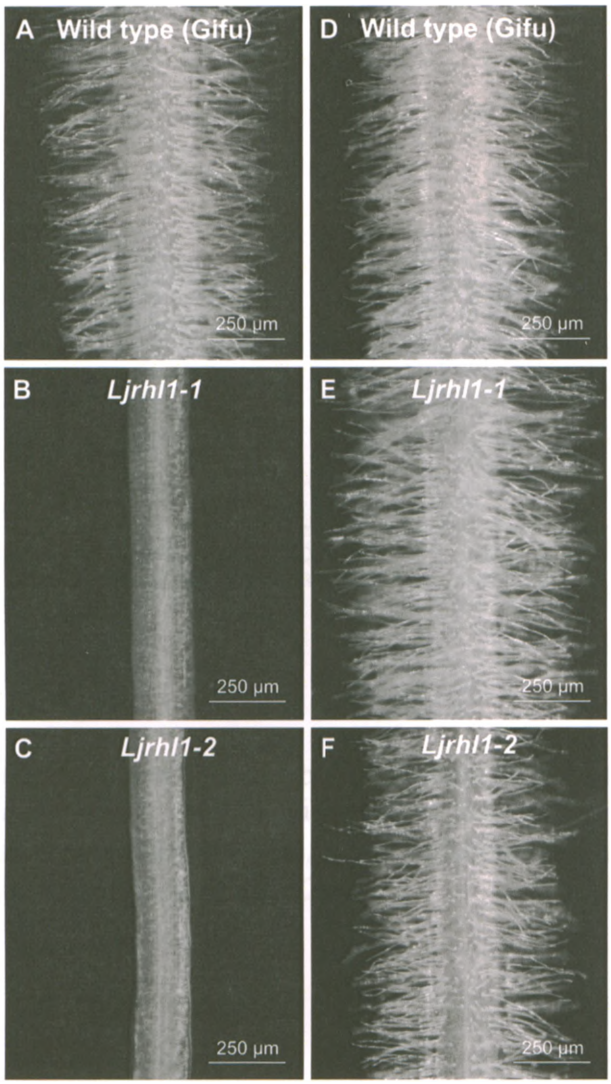

Figure 5. Expression of *LjRHL1,* driven by a constitutive 35S CaMV promoter, complements the root-hairless phenotype of the *Ljrhl1-1* and *Ljrhl1-2* allelic mutants in transgenic hairy roots. (**A, B**, and **C**) Transgenic hairy roots carrying the *CaMV* 35S promoter only. (**D, E**, and **F**) Transgenic hairy roots expressing *LjRHL1-1* mRNA.

Figure 6. Alignment of the predicted LjRHL1 bHLH domain with a consensus sequence for plant bHLH (Heim *et al.,* 2003). Amino acid residues that have been predicted to be important for contact with a nucleotide base (*), DNA backbone (+), and protein-protein interactions (●) are indicated, as in Heim *et al.,* (2003).

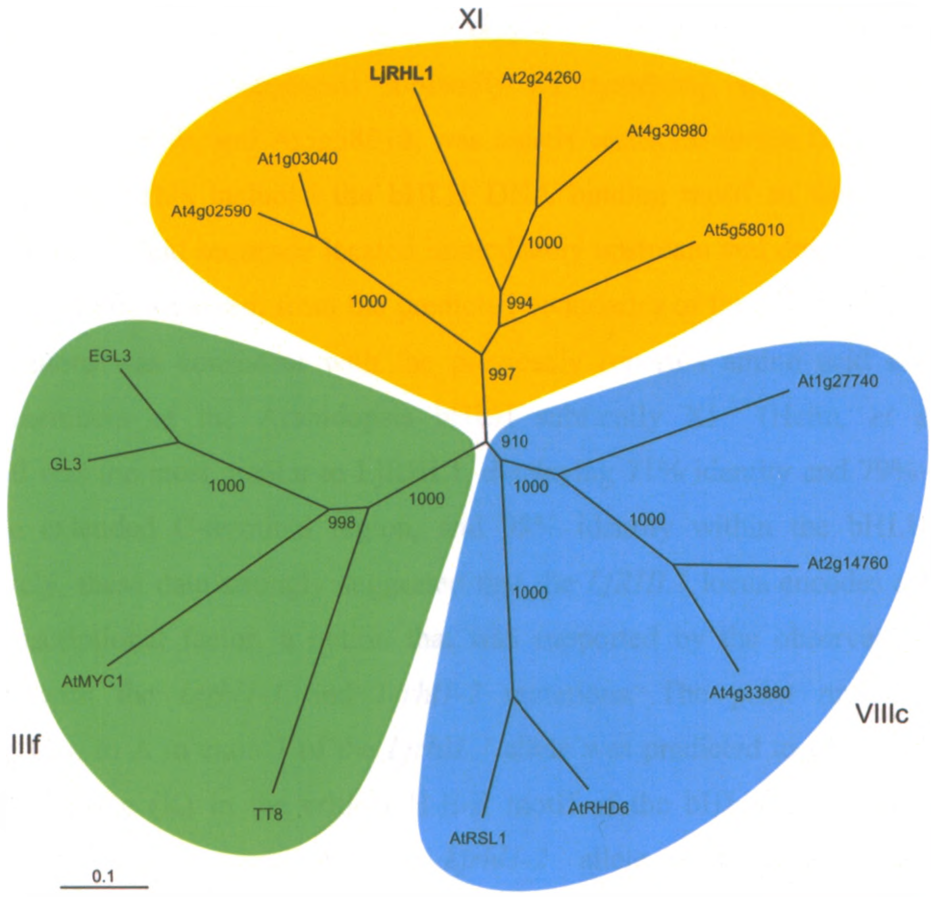

Figure 7. Phylogenetic analysis of the LjRHL1 protein. Unrooted tree based on an amino acid alignment of full length sequences of *L. japonicus* LjRHL1-1 and members of the *A. thaliana* bHLH-domain protein family belonging to group XI (**top**), and subgroups IIIf (**bottom left**), and VIIIc (**bottom right**). EGL3 and GL3 (subgroup IIIf), and AtRSL1 and AtRHD6 (subgroup VIIIc) were previously shown to be involved in specification of root epidermal cell fate in *A. thaliana* (Bernhardt *et al.*, 2003, Heim *et al.*, 2003, Menand *et al,.* 2007).

Arabidopsis bHLH protein subfamily XI (Figure 7). The amino-acid conservation between LjRHL1 and Arabidopsis subfamily XI members, At1g03040, At2g24260, At4g02590, At4g30980, and At5g58010, was mostly restricted to the C-terminal portion of these proteins. This included the bHLH DNA binding motif as well as additional stretches of amino acid sequence located immediately upstream and downstream and also at some distance downstream from the predicted boundaries of the bHLH domain (Figure 8). This pattern was consistent with the previously reported amino acid conservation between members of the Arabidopsis bHLH subfamily XI (Heim, *et al.* 2003). At2g24260 was the most similar to LjRHL1, displaying 71% identity and 79% similarity within the extended C-terminal region, and 98% identity within the bHLH domain. Cumulatively, these data strongly suggested that the *LjRHL1* locus encodes a functional bHLH transcriptional factor, a notion that was supported by the observed phenotypes associated with the *Ljrhl1-1* and *Ljrhl1-2* mutations. The point mutation altering nucleotide G_{997} to A in exon 3 of the *Ljrhl1-1* allele was predicted to replace the amino-acid E_{243} to lysine (K) in the critical H-E-R motif of the bHLH domain (Figure 3d). Analysis of the 371 bp insertion in the *Ljrhl1-2* allele identified this sequence as a member of a group of long terminal repeat (LTR) retrotransposons defined as terminal-repeat retrotransposons in miniature or TRIM (Witte *et al.* 2001). We named this retrotransposon *LjTRIM1*.

LjTRIM1 has all sequence features characteristic for this class of retrotransposons (Figure 9) including short overall length, 139 bp long terminal direct repeats (TDRs), and an internal domain of 88 bp containing a primer binding sequence (PBS) and a polypurine track (PPT). Its insertion at the bottom strand of the *LjRHL1* locus lead to the typical 5 bp target site direct duplication and resulted in the generation of at least five different species of *LjRHL1* mRNA (Figure 5B). These mRNAs (I to V) retained the original *LjTRIM1* insertion but differed in splicing patterns of the adjacent introns, 1, 2, and 3. While these introns were correctly spliced in mRNA I, introns 1 and 2 were retained within mRNA II and III, respectively. mRNA IV retained both introns 1 and 2, while intron 3 was spliced normally. Finally, mRNA V retained all three introns. Regardless of the splicing pattern, the premature termination of translation likely occurred in all these mRNA species due to the presence of a predicted in frame translation terminating codon in *LjTRIM1*.

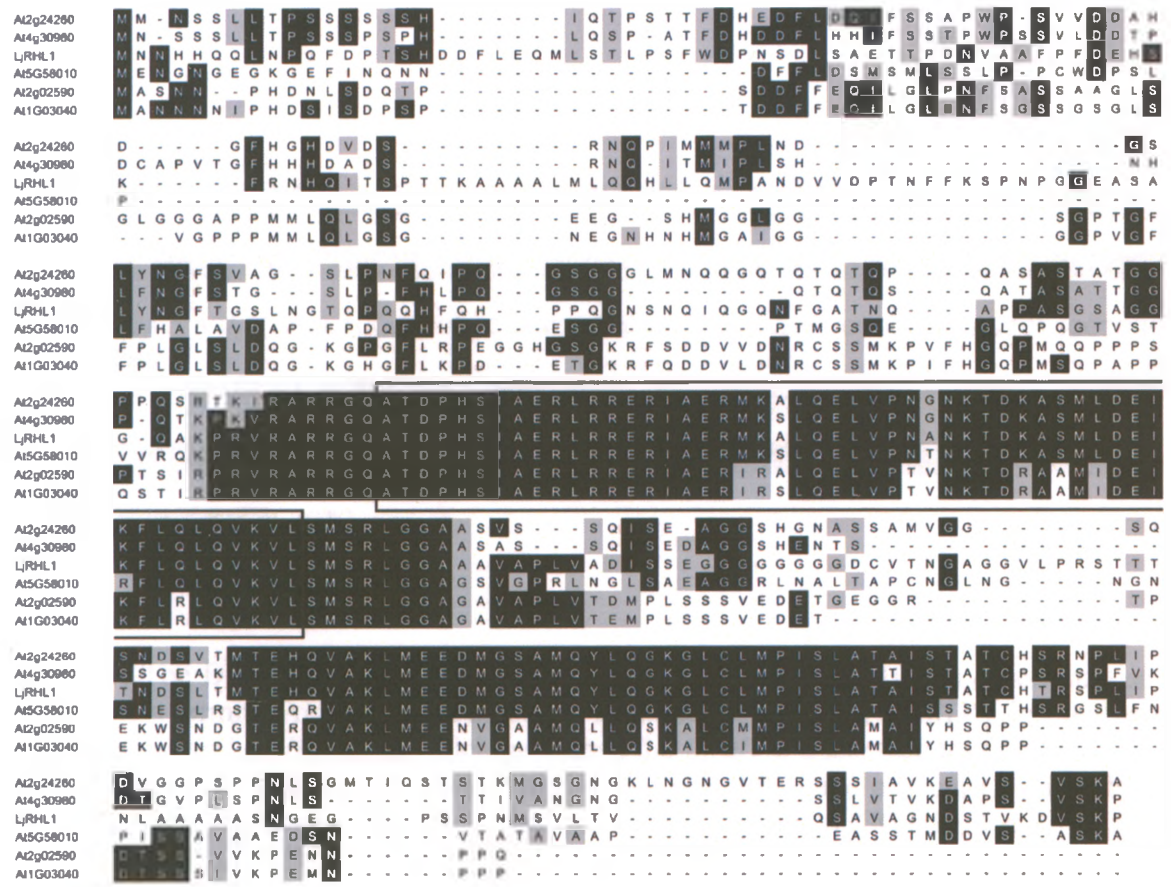

Figure 8. Sequence alignment of the predicted LjRHL1 protein and members of the *A. thaliana* bHLH domain proteins from group XI (Heim, *et al.* 2003). The conserved amino acid residues are highlighted, while the predicted bHLH domain is boxed.

Figure 9. The DNA sequence of *LjTRIM1*, a long terminal repeat (LTR) retrotransposon defined as a terminal-repeat retrotransposon in miniature (TRIM). *LjTRIM1* inserted into the *L. japonicus LjRHL1* locus which generated the *Ljrhl1-2* allele (see text for additional details). LTRs are highlighted; the primer binding site (PBS) and polypurine track (PPT) are underlined. Note also the five base pair direct repeats (GGGTG) that have been generated at the *LjTRIM1* insertion site within the *LjRHL1* locus.

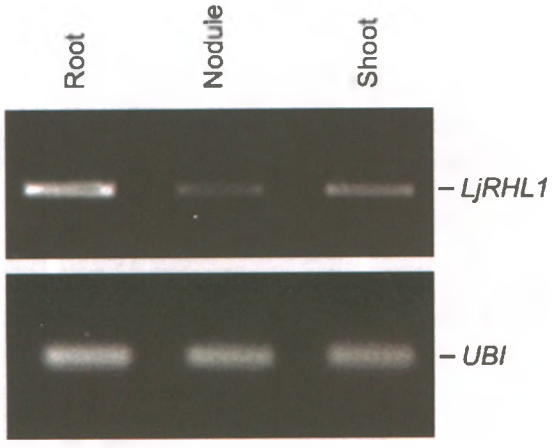

Figure 10. RT-PCR results (30 cycles) showing the steady-state levels of *LjRHL1* and the ubiquitin (UBI) mRNAs in *L. japonicus* roots, nodules, and shoots.

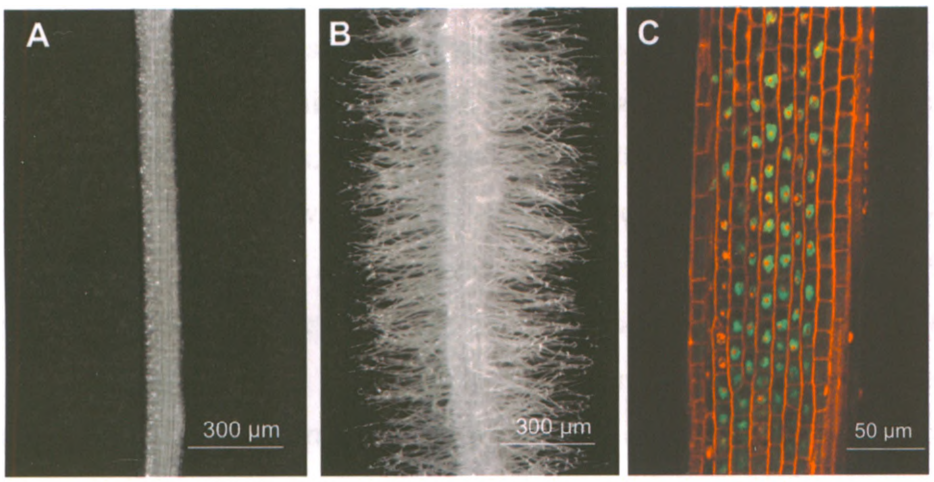

Figure 11. Complementation of the *Ljrhl1-2* root hairless phenotype and localization of the LjRHL1 protein. The *Ljrhl1-2* mutant was inoculated with the *A. rhizogenes* strain AR10 and the resulting transgenic hairy roots carrying either a *LjRHL1* promoter-*GFP* control construct (**A**), or a chimeric *GFP-LjRHL1* gene construct driven by the *LjRHL1* promoter (**B, C**), are shown. The equivalent mature root regions, where fully developed root hairs are normally present, are shown in panels A and B. (**C**) The meristematic/elongation zone of the transgenic hairy root showing expression of GFP-LjRHL1 in the nuclei of epidermal cells.

Expression analysis using RT-PCR demonstrated that *LjRHL1* mRNA was detectable in all *L. japonicus* tissues tested (Figure 10). When the GFP-LjRHL1 protein fusion was expressed in transgenic hairy roots under the control of the *LjRHL11* promoter, root hair growth in the *Ljrhl1-1* and *Ljrhl1-2* was restored, and the protein was localized in the nucleus of cells in the cell elongation zone of the root (Figure 11).

4.3.4 Functional conservation of *L. japonicus* LjRHL1 and Arabidopsis bHLH proteins.

The amino-acid sequence conservation between Arabidopsis bHLH proteins of subfamily XI and LjRHL1 prompted us to perform cross-species complementation experiments (Figure 12). Expression of *At1g03040* and *At4g02590* under the control of the CaMV 35S promoter in transgenic hairy roots did not rescue the root hairless phenotype of *Ljrhl1-1* and *Ljrhl1-2* mutants. In contrast, *At2g24260*, *At4g30980*, and *At5g58010* complemented the root hair developmental defect of both *Ljrh1-1* and *Ljrhl1-2* alleleic mutants (Figure 12). Based on the protein structure similarities and functional complementation results, we renamed the three Arabidopsis genes as *LjRHL1-LIKE1* (*AtLRL1*), *AtLRL2,* and *AtLRL3,* respectively. Since our data suggested that these genes might regulate root hair development in Arabidopsis, we set out to test this prediction.

4.3.5 Insertion mutants of *AtLRL1, AtLRL2, and AtLRL3.*

The available T-DNA insertion lines corresponding to the three Arabidopsis genes were identified from the Arabidopsis stock center at TAIR. Two of these lines, SALK_061956 and SALK_006430, carried T-DNA insertions in introns 3 and 5 of *AtLRL1,* respectively. We refer to these lines hereafter as *Atlrl1-1*and *Atlrl1-2*, respectively.

cDNA products corresponding to the *AtLRL1* locus could be amplified from the *Atlrl1-1* line (Fig 13). However, sequencing of the RT-PCR product revealed the presence of only one alternatively spliced form. In this cDNA, exon 2 was joined with exon 4 such that the original open reading frame (ORF) for the remaining portion of the *AtLRL1* cDNA was correctly reconstructed. The resulting loss of exon 3 lead to a 22 amino acid deletion (LRRERIAERMKALQELVPNGNK), which removed three amino acid from the basic

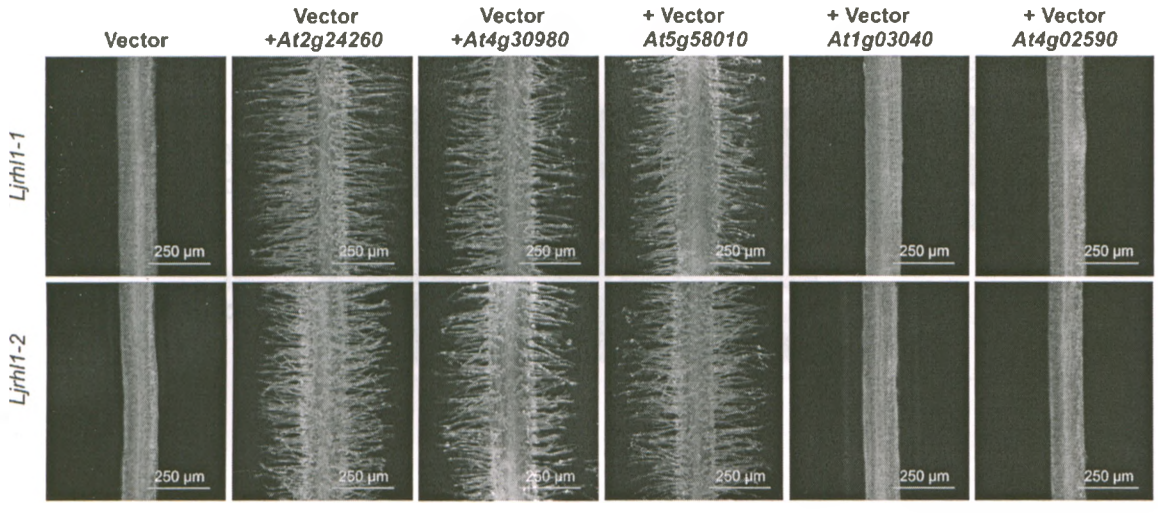

Figure 12. Cross-species complementation experiments. Under the control of *CaMV* 35S promoter, the expression of *At2g24260*, *At4g30980*, and *At5g5810* but not *At1g03040* and *At4g02590*, complements the root hairless phenotype of *Ljrhl1-1* (**top row**) and *Ljrhl1-2* (**bottom row**) allelic mutant lines in transgenic hairy roots.

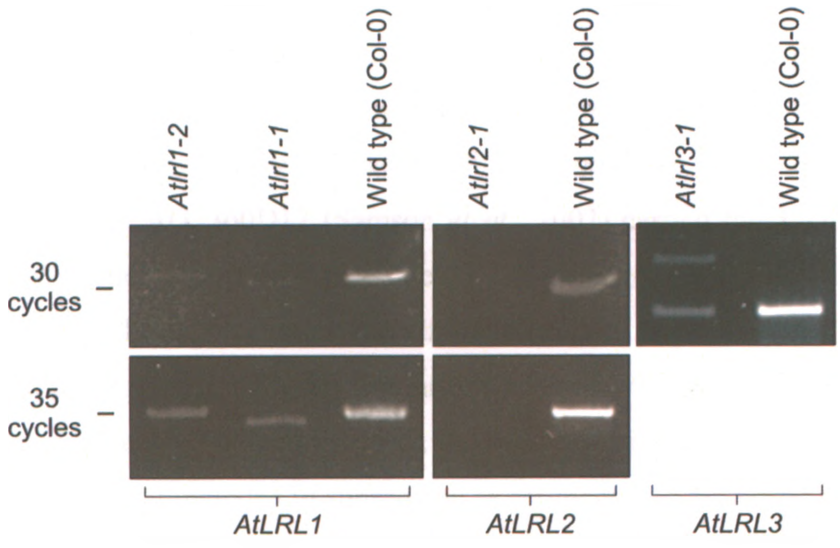

Figure 13. RT-PCR results showing the steady state levels of *AtLRL1*, *AtLRL2, and AtLRL3* mRNAs in *A. thaliana* wild-type (Col-0) and the corresponding homozygous T-DNA insertion lines. Note the lower size of the *AtLRL1* mRNA derived from the *Atlrl1-1* line and lack of detectable *AtLRL2* product in *Atlrl2-1*.

portion, the entire first helical region, and 4 amino acids of the loop region from the bHLH domain of the predicted Atlrl1 protein.

In contrast, the *AtLRL1* cDNA product derived from *Atlrl1-2* was wild-type, indicating correct splicing of intron 5, irrespective of the presence of the T-DNA insertion. However, the level of the *AtLRL1* mRNA in *Atlrl1-2* was very strongly diminished in comparison to wild-type plants (Figure 13). Therefore, both *Atlrl1-1* and *Atlrl1-2* were used in subsequent genetic experiments. In line SALK_011010 (*Atlrl2-1*) the T-DNA was integrated within intron 2 of the *AtLRL2* locus. The *AtLRL2* mRNA could not be amplified from *Atlrl2-1*, although the corresponding cDNA could be obtained from wild-type plants (Figure 13). Therefore, we considered this line as carrying a complete loss-of-function allele of the *AtLRL2* gene (*Atlrl2-1* allele). Three additional lines, SALK_128814, SALK_015021, and FLAG_360D12 (Samson *et al.*, 2002) carried the T-DNA insertion within the *AtLRL3* gene. In the two SALK lines, T-DNA integrated 356 and 136 base pairs upstream from the predicted ATG translation initiation codon, respectively.
Since these lines showed only a slightly diminished level of *AtLRL3* mRNA in comparison with the wild-type plants, as judged based on the semi-quantitative RT-PCR results (data not shown), they were not included in subsequent experiments.

The FLAG_360D12 (*Atlrl3-1*), carried a T-DNA insertion in the last exon, within the predicted 3' UTR of the *AtLRL3* mRNA. Two cDNA species that corresponded to *AtLRL3* mRNA could be amplified from *Atlrl3-1*(Figure 13). While one of these cDNAs was identical to the predicted wild-type mRNA, the second cDNA product was of higher size due to the retention of all introns. Since three independent control RT-PCR reactions, without reverse transcriptase, did not show any discernible products, we presumed that this cDNA corresponded to an unspliced *AtLRL3* mRNA, which likely led to a non-functional mRNA due to the presence of a premature termination codon in the first intron.

4.3.6 *AtLRL1*, *AtLRL2 and AtLRL3* genes regulate root hair development in Arabidopsis.

All single locus homozygous insertion lines described above showed the wild-type root hair phenotype (Figure 14). Therefore, genetic crosses were performed to construct the corresponding double mutants. For *Atlrl1-1* x *Atlrl2-1* and *Atlrl1-2* x *Atlrl2-1* the

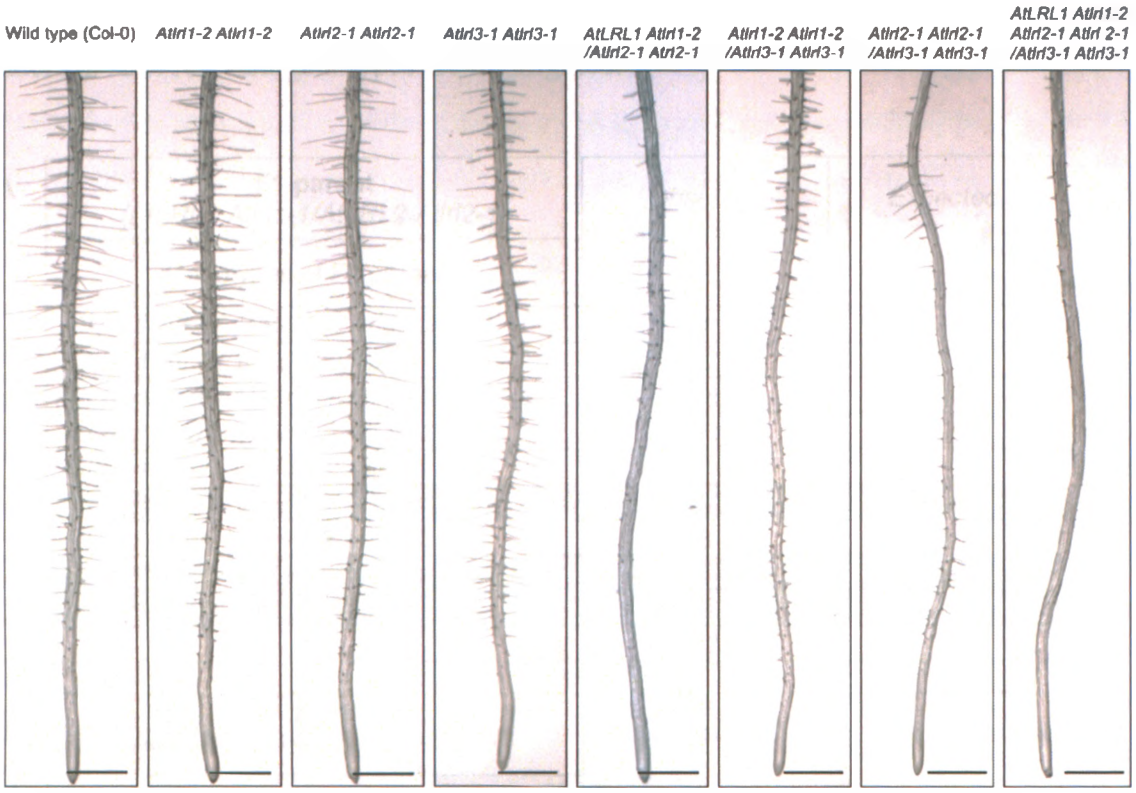

Figure 14. *AtLRL1, AtLRL2, and AtLRL3* act redundantly to positively regulate root hair development in *A. thaliana*. (**A**) Roots of 5-day-old homozygous single and double mutant lines are shown in comparison with the wild-type (Col-0). The corresponding genotypes are indicated. Note that for *Atlrl1-1* x *Atlrl2-1* and *Atlrl1-2* x *Atlrl2-1* crosses the phenotype of only one representative progeny, *AtLRL1 Atlrl1-2 / Atlrl2-1 Atlrl2-1*, is shown. Bar = 500 μm.

Table 1. The results of genotyping and scoring F2 and F3 progenies of the indicated F1 and F2 parental individuals, respectively, are shown. The F1 parents were derived from *Atlrl1-1 Atlrl1-1* x *Atlrl2-1 Atlrl2-1* (**A**) and *Atlrl1-2 Atlrl1-2* x *Atlrl2-1 Atlrl2-1* (**B**) genetic crosses. The F2 parent (**C**) was selected from the segregating population listed under **B**. The relevant genotypes are highlighted.

A

F1 parent *(AtLRL1 Atlrl1-1/AtLRL2 Atlrl2-1)*	*Observed*	*Expected*
Genotypes of F2 progeny		
AtLRL1 AtLRL1/AtLRL2 AtLRL2	4	4
AtLRL1 AtLRL1/AtLRL2 Atlrl2-1	12	9
AtLRL1 AtLRL1/Atlrl2-1 Atlrl2-1	14	4
AtLRL1 Atlrl1-1/AtLRL2 AtLRL2	13	9
AtLRL1 Atlrl1-1/AtLRL2 Atlrl2-1	21	18
AtLRL1 Atlrl1-1/Atlrl2-1 Atlrl2-1	2	9
Atlrl1-1 Atlrl1-1/AtLRL2 AtLRL2	3	4
Atlrl1-1 Atlrl1-1/AtLRL2 Atlrl2-1	1	9
Atlrl1-1 Atlrl1-1/Atlrl2-1 Atlrl2-1	0	4
Total	70	70

B

F1 parent *(AtLRL1 Atlrl1-2/AtLRL2 Atlrl2-1)*	*Observed*	*Expected*
Genotypes of F2 progeny		
AtLRL1 AtLRL1/AtLRL2 AtLRL2	6	6
AtLRL1 AtLRL1/AtLRL2 Atlrl2-1	13	11
AtLRL1 AtLRL1/Atlrl2-1 Atlrl2-1	14	6
AtLRL1 Atlrl1-2/AtLRL2 AtLRL2	10	11
AtLRL1 Atlrl1-2/AtLRL2 Atlrl2-1	21	22
AtLRL1 Atlrl1-2/Atlrl2-1 Atlrl2-1	8	11
Atlrl1-2 Atlrl1-2/AtLRL2 AtLRL2	8	6
Atlrl1-2 Atlrl1-2/AtLRL2 Atlrl2-1	9	11
Atlrl1-2 Atlrl1-2/Atlrl2-1 Atlrl2-1	0	6
Total	89	89

C

F2 parent *(AtLRL1 Atlrl1-2/Atlrl2-1 Atlrl2-1)*	*Observed*	*Expected*
Genotypes of F3 progeny		
AtLRL1 AtLRL1/Atlrl2-1 Atlrl2-1	35	19
AtLRL1 Atlrl1-2/Atlrl2-1 Atlrl2-1	39	37
Atlrl1-2 Atlrl1-2/Atlrl2-1 Atlrl2-1	0	19

double homozygous genotype was not recovered from among all F2 individuals analysed (see below). However, 20 plants in total that showed significantly diminished root hair development were selected from both segregating populations (Figure 14). The initiation of root hairs appeared not significantly affected in these plants, as evidenced by the formation of bulges. However, subsequent root hair elongation was severely reduced and occurred only sporadically, giving rise to a patchy short root hair phenotype (Figure 14). All of these individuals were homozygous for either *Atlrl1* or *Atlrl2-1*, while they remained heterozygous for the second locus (see Table 1).

Out of 70 F2 segregants of *Atlrl1-1* x *Atlrl2-1* only two and one individuals for each of the *AtLRL1 Atlrh1-1/Atlrl2-1 Atlrl2-1* and *Atlrl1-1 Atlrh1-1/AtLRL2 Atlrl2*-1 genotype categories were recovered, respectively, and they showed a decreased root hair phenotype. Since in total ~18 such individuals were expected, this suggested that the combination of mutant alleles was detrimental (see Table 1). The second segregating population where 89 F2 segregants of *Atlrl1-2* x *Atlrl2-1* were analysed, 17 showed a decreased root hair phenotype (Figure 14), which approximated the expected number of 22 plants with *AtLRL1 Atlrh1-2/Atlrl2-1 Atlrl2-1*and *Atlrl1-2 Atlrh1-2/AtLRL2 Atlrl2-1* genotypes (see Table 1). Cumulatively, these results suggested that the *Atlrl1-1* allele was more harmful than *Atlrl1-2* in the presence of *Atlrl2-1*.

When the progeny of the selfed *AtLRL1 Atlrh1-2/Atlrl2-1 Atlrl2-1* plant were analyzed, again no double homozygote insertion line was recovered, further confirming that such a genotype was not viable (see Table 1).

Double homozygous lines of *Atlrl1-2 Atlrl1-2/Atlrl3-1 Atlrl3-1* and *Atlrl2-1 Atlrl2-1/Atlrl3-1Atlrl3-1* genotypes could be recovered and were characterized as having a defective root hair phenotype (Figure 14). Although initiation appeared not affected, the subsequent elongation of root hairs was severely diminished in these lines, resulting in a predominantly spiky, short root hair phenotype.

4.3.7 Expression of *AtLRL3* requires *CPC*, *CPC*-like MYB, and *LjRHD6* gene functions.

Having established that the *AtLRL1*, *AtLRL2*, and *AtLRL3* genes, positively affect root hair development, we analyzed their expression in roots of three different mutant

Arabidopsis genetic backgrounds: *cpc1* (Wada et al., 1997), *cpc2 try-29760 etc1 cpl3-1* quadruple mutant (Tominaga *et al.*, 2008), and *Atrhd6-3* (*root hair defective 6*) *Atrsl1-1* (*rhd six-like1*) double mutant (Menand *et al.*, 2007). Since *AtRHD6* was shown to be positively regulated by *CPC* in Arabidopsis (Menand *et al.*, 2007), the analysis of the steady state level of its corresponding mRNA in the mutant backgrounds listed above was performed in parallel. The *AtLRL1* and *AtLRL2* mRNAs were detected in all mutant backgrounds tested, although a splice-variant for *AtLRL1* mRNA that retained exon 2 of the corresponding gene was identified in the *cpc-1* genetic background (Figure 15). In contrast, the *AtLRL3* mRNA was present in *cpc1* but was absent from both the quadruple and the double mutant lines.

4.4 Discussion.

In this study, we investigated the molecular basis of root hair formation in *L. japonicus* by analysing two mutant lines carrying deleterious mutations in the *LjRHL1/SLIPPERY* locus. The main premise of this research was to identify and characterize the underlying defective gene on the molecular level in order to facilitate a more informed use of *Ljrhl1-1* and *Ljrhl1-2* in experiments that aim at functional dissection of the mechanisms by which legumes accommodate symbiotic bacteria. However, the prospect of performing a comparative analysis between *L. japonicus* and Arabidopsis was also considered. Unlike Arabidopsis, all or almost all of the *L. japonicus* root epidermal cells produce hairs (Karas *et al.*, 2005), thus no pattern of root hair and root hairless cells is formed (Dolan and Costa, 2001).

We showed that the *LjRHL1* locus encodes a predicted bHLH transcription factor which is indispensable for root hair formation in *L. japonicus*. While the presence of *LjRHL1* mRNA in all *L. japonicus* tissues tested suggests that this locus might play a role in other regulatory processes in addition to root hair development, the only consistently discernible pleiotropic effect of *Ljrhl1-1* and *Ljrhl1-2* allelic mutations observed was a slightly increased rate of root elongation in comparison with the wild-type genetic background. Lack of a trichome phenotype indicates that *LjRHL1* is either not involved or acts redundantly to regulate the development of epidermal cells in aerial tissues.

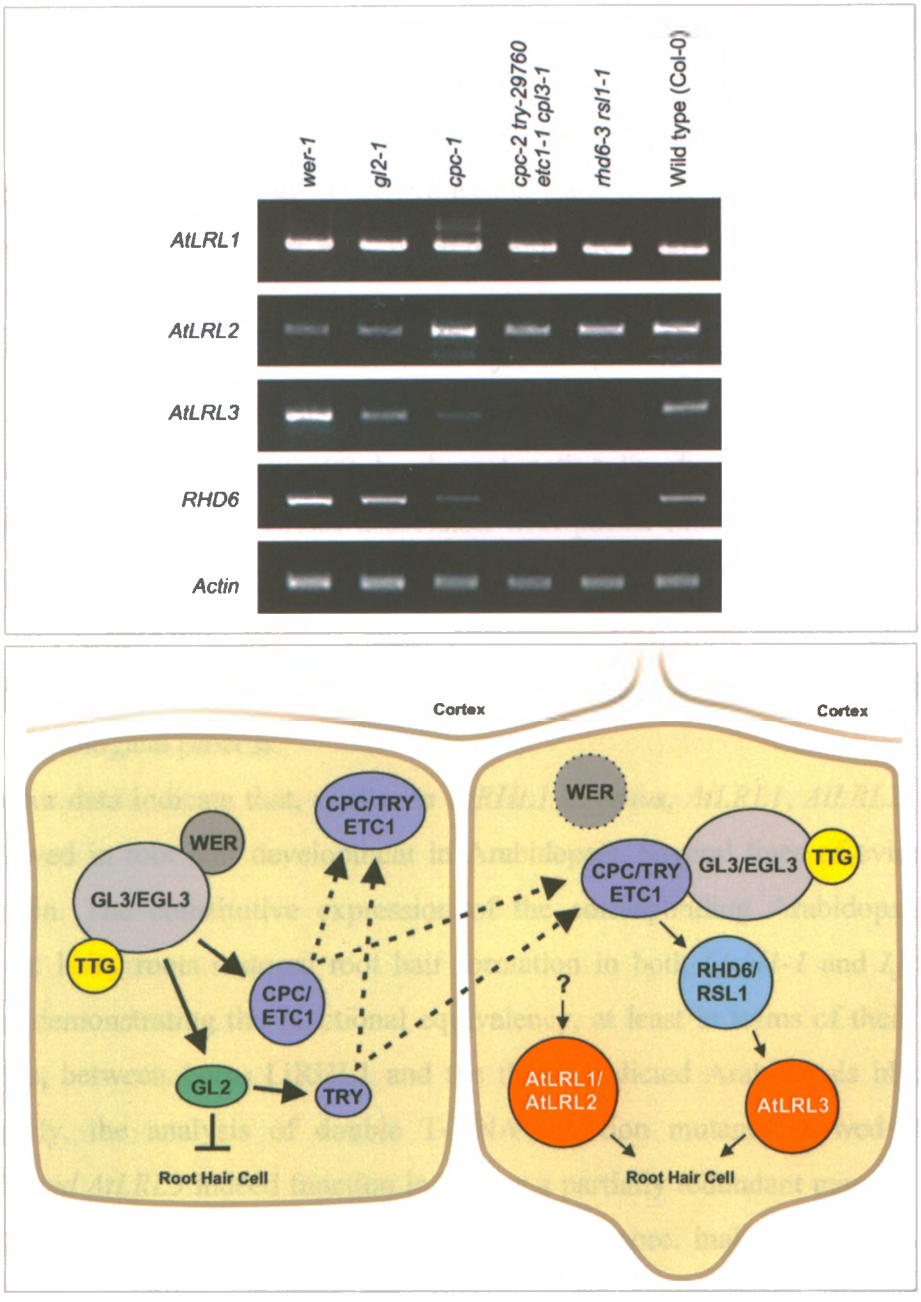

Figure 15. Upper panel: RT-PCR expression analysis of AtLRL1, AtLRL2, AtLRL3 and RHD6 in roots of *A. thaliana* mutant and wild-type (Col-0) lines (see text for further details). Actin serves as a control. Note that a higher molecular weight AtLRL1 cDNA in cpc-1 represents an alternatively spliced product that retained intron 2. Also, lack of the RHD6 cDNA product in rhd6-3 rsl1-1 is caused by the T-DNA insertion in the RHD6 locus. Lower panel: A model which explains cell-type patterning in the *Arabidopsis* root epidermis (modified from Simon et al 2007). AtLRL3 acts downstream from patterning genes (CPC/TRY/ETC1 and GL3/EGL3). AtLRL1/2 are required for root hair intiation/elongation; however, they are not regulated at transcriptional level by the patterning genes.

Localization of the GFP-LjRHL1 chimeric protein in the nuclei of root cells encompassing the root elongation and not the root differentiation zone suggested that LjRHL1 functions early in the signalling mechanism(s) that specifies root hair development in *L. japonicus*. This expression pattern might also be pertinent to root elongation, since cell growth and differentiation are interlinked and tightly controlled (Guimil and Dunand, 2007).

The homology search clustered the LjRHL1 protein together with the members of subgroup XI of the Arabidopsis bHLH protein family. Only one out of five members of this subgroup, namely At4g02590, has been functionally classified. This protein is likely involved in regulatory processes associated with pollen tube guidance and/or reception (Pagnussat et al., 2005). Although possible functional redundancy between *At1g03040*, *At4g02590*, *At2g24260* (*AtLRL1*), and *At4g30980* (*AtLRL2*) has been suggested based on their structural similarities (Heim et al., 2003), this was neither proven nor linked to any particular biological process.

Our data indicate that, similar to *LjRHL1* in *Lotus*, *AtLRL1*, *AtLRL2*, and *AtLRL3* are involved in root hair development in Arabidopsis. Several lines of evidence support this notion. The constitutive expression of the corresponding Arabidopsis cDNAs in transgenic hairy roots restored root hair formation in both *Ljrhl1-1* and *Ljrhl1-2* allelic mutants, demonstrating the functional equivalency, at least in terms of their biochemical properties, between *Lotus* LjRHL1 and the three predicted Arabidopsis bHLH proteins. Importantly, the analysis of double T-DNA insertion mutants showed that *AtLRL1*, *AtLRL2, and AtLRL3* indeed function in at least a partially redundant manner to positively regulate root hair formation in Arabidopsis. Furthermore, inability to recover a double homozygote insertion genotype for *Atlrl1/2* allele combinations suggested that the presence of one of these bHLH proteins is required for viability. Thus, in addition to their role in the root epidermis, the *AtLRL1*and *AtLRL2* genes likely function in other key developmental mechanisms in Arabidopsis. Whether like *At4g02590*, they act to regulate pathways relevant to Arabidopsis gametophytic development remains unresolved.

In spite of the apparent defect in root hair development in the double insertion line, our data show that accumulation of *AtLRL1*and *AtLRL2* transcripts in Arabidopsis roots was not strictly dependent on known positive regulators of root hair cell

differentiation/patterning, including CPC, TRY, ETC1, and CLP3. Furthermore, the *AtRDH6* and *AtRSL1-1* genes, which mediate the development of Arabidopsis root hair cells by acting downstream from the regulatory complexes that specify pattern formation in the epidermis (Menand et al., 2007), were also not required for accumulation of these transcripts. Thus, *AtLRL1*and *AtLRL2* might be constitutively transcribed in the root epidermis. Intriguingly, however, the presence of an alternatively spliced form of the *AtLRL1* mRNA in *cpc-1* might indicate the involvement of a mechanism of post-transcriptional regulation; this, however, remains to be further investigated. Finally, we can not entirely rule out the possibility that *AtLRL1*and *AtLRL2* function in a parallel regulatory pathway(s), independent from the epidermis patterning genes, contributing to root hair initiation and/or being pertinent to root hair elongation in Arabidopsis.

In contrast to *AtLRL1* and *AtLRL2,* the *AtLRL3* transcript did not accumulate to any detectable level in either the quadruple or the double *Atrhd6-3 Atrsl1-1* mutant backgrounds. Thus, *AtLRL3* participates in the positive regulation of root hair development in Arabidopsis by acting downstream of the epidermal pattern formation genes and the *AtRHD6* and/or *AtRSL1* genes.

The presence of networks of redundantly acting bHLH proteins in Arabidopsis likely underscores evolutionary events that led to the expansion of this gene family to its current size of 162 members (Heim et al., 2003; Zhang et al., 2003; Menand et al., 2007). Interestingly, a comparative analysis of Arabidopsis and rice (*Oriza sativa*) predicted the presence of at least 66 bHLH genes in the genome of the presumed most recent common ancestor of monocots and eudicots (Li et al., 2006). The *L. japonicus* genome has 118 predicted bHLH domain–encoding genes (Sato et al., 2008), and this number is close to the presumed 91 bHLH transcription factors represented on the *Medicago truncatula* Gene Chip (Udvardi *et al.*, 2007; Benedito *et al.*, 2008). As the sequenced portion of the *L. japonicus* genome is postulated to account for approximately 91.3% of the plant gene space (Sato *et al.*, 2008; Szczyglowski and Stougaard, 2008), the total number of bHLH-domain encoding genes (~ 130 genes) could be lower in *L. japonicus* than in Arabidopsis and rice. We showed here that at least one member of the *L. japonicus* bHLH-domain protein family, *LjRHL1*, works in a non-redundant manner to specify root hair development, which significantly contrasts with an apparent redundancy of similar

functions in Arabidopsis. Whether this is reflective of differences in the expansion of certain subsets of bHLH genes in *Lotus* and Arabidopsis genomes in association with the species-specific inventions, such as a particular type of root hair patterning, remains an interesting subject for future investigations.

4.5 Material and methods.

4.5.1 Plant material and growth conditions.

L. japonicus root hair mutants were identified from a screen for genetic suppressors of the *L. japonicus* Gifu *har1-1* hypernodulation phenotype, as described (Karas, *et al.* 2005, Murray, *et al.* 2006). For *Arabidopsis thaliana* studies, ecotype Columbia 0 (Col-0) was used as the wild type reference. The Arabidopsis T-DNA insertion lines were selected from the Arabidopsis Biological Resource Center at The Ohio State University (Alonso *et al.* 2003).

All plants were maintained in a growth room under a 16/8 h day/night regime unless otherwise stated. *L. japonicus* plants were subjected to an irradiance of 250 $\mu mol\ s^{-1}\ m^{-2}$ at 22°C, and were occasionally watered with B&D nutrient solution (Broughton and Dilworth 1971) containing 0.5 mm KNO_3. *A. thaliana* plants were subjected to 140-180 $\mu E\ s^{-1}\ m^{-2}$ light intensity at 22°C.

4.5.2 Evaluation of root hair and symbiotic phenotypes.

L. japonicus seeds were germinated as previously described (Karas, *et al.* 2005) and seedlings were transferred to pots containing a 6:1 mixture of vermiculite and sand and grown for an additional 10 days. The roots of *Ljrhl1-1* and *Ljrhl1-2* mutants were scored for the presence of root hairs (n = 30), as described in (Karas, *et al.* 2005).

To evaluate the root hair phenotype of *A. thaliana*, plants were germinated and grown on the surface of vertically positioned agar plates, containing 1 x MS medium, pH 5.8, 1% sucrose, and 1% phytagel for 4 days. The roots were inspected and root hairs that formed were evaluated with respect to their position and were counted within a 1 cm long region, starting from the root tip (n = 10 for Col-0, *Atlrl1-2 Atlrl1-2*, and *Atlrl2-1 Atlrl2-1*; n = 23 for *Atlrl1-2 Atlrl1-2/Atlrl2-1 Atlrl2-1*).

After growing for the first 7 days under sterile conditions, *L. japonicus* seedlings were inoculated with the *M. loti* strain *MAFF303099* carrying the constitutively expressed *β-glucuronidase* (*gusA*) reporter gene (kindly provided by Kazuhiko Saeki, Osaka University, Japan) (Okazaki *et al.* 2007). Histochemical analysis of the reporter gene activity was performed 21 days after inoculation (dai). For each genotype, at least 15 roots were stained as in (Okazaki, *et al.* 2007) and the number of nodules and nodule primorida was scored as described in (Karas, *et al.* 2005).The observations were made using a Nikon SMZ 1500 microscope and images were captured with a Nikon DMX1200 digital camera.

4.5.3 Identification of full length mRNA and coding regions.

The *LjRHL1* cDNA was amplified by RT-PCR of total RNA derived from *L. japonicus* roots using the coding region specific primers (HLH_pcDNA_F, and HLH_pcDNA_R; see list for all primer sequences below). Rapid amplification of the 5' and 3' cDNA ends (RACE) was subsequently carried out by using the First Choice RLM-RACE kit from Ambion (Texas, US), according to the manufacturer's instructions. The full copy *LjRHL1* cDNA was reconstituted based on the obtained sequences. An additional set of two *LjRHL1* mRNA-specific primers, which were positioned at the extreme 5' and 3' ends of the predicted full copy cDNA, was designed (HLH_cDNA_F, HLH_cDNA-R). Using total RNA from *L. japonicus* roots, RT-PCR was again performed and the resulting product was entirely sequenced.

The cDNAs corresponding to the coding regions of the Arabidopsis *bHLHs* genes were amplified by RT-PCR (primers used: AT1G03040_F, AT1G03040_R, AT2G24260_F, AT2G24260_R, AT4G30980_F, AT4G30980_R, AT4G02590_F, AT4G02590_R, AT5G58010_F, AT5G58010_R) and were entirely sequenced for confirmation. Subsequently, these cDNAs were cloned into the pEGAD vector and used in transgenic hairy root experiments (see below).

4.5.4 Expression analysis.

Total RNA from Arabidopsis roots and *L. japonicus* tissues was isolated and converted into 1st strand cDNA as described (Murray, *et al.*, 2007).

To evaluate steady-state levels of the *LjRHL1* mRNA in different *L. japonicus* tissues, the following PCR conditions were used: 5 min at 94°C, 35 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec, followed by 7 min at 72°C (primers: RHL_E1-4_F, RHL_E1-4_R); the ubiquitin cDNA was amplified (primers: Ubi-F, Ubi-R; see primer list below) using similar PCR conditions except that only 30 cycles were performed.

To perform expression analyses of *At2g24260*, *At4g30980*, *At5g58010*, in roots of T-DNA insertion mutants and wild type Arabidopsis lines, the following PCR conditions were used: 5 min at 94°C, 30 or 35 cycles of 94°C for 30 sec, 62°C for 1 min, and 72°C for 30 sec, followed by 7 min at 72°C (primers: AT2G24260_expF, AT2G24260_expF, AT4G30980_expF, AT4G30980_expR, AT5G58010_expF, AT5G58010_expR.); for actin *mRNA*: 5 min at 94°C, 25 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by 7 min at 72°C (primers: Actin-F, Actin-R; see primer list below).

4.5.5 Transgenic hairy roots.

The *LjRHL1* and *Arbidopsis bHLH* cDNAs were fused in frame to the C-terminal end of the *GFP* in the pEGAD vector containing the *CaMV* 35S promoter (Cutler *et al.* 2000). For the LjRHL1 protein localization/complementation study, the 35S promoter was replaced by the cognate *LjRHL1* promoter (nucleotide position -3131 to -223, counting from the predicted *LjRHL1* translation initiation site (primers: RHL_PROMTR_F, RHL_PROMTR_R).

The resulting constructs were transferred into the *Agrobacterium rhizogenes* AR10 strain and *L. japonicus* plants were inoculated using the established protocol (Diaz *et al.* 2005). At least 30 independent plants were transformed for each genotype analysed. The resulting hairy roots were visually evaluated for the presence of root hairs. For the GFP-LjRHL1 localization study, transgenic hairy roots were counter-stained with propidium iodide and GFP fluorescence was visualized and captured using a Leica scanning confocal microscope TCS SP2 (Leica Microsystem).

4.5.6 List of primers used in this study.

Underline sequence denotes restriction site sequence included in the primer. All sequences are presented in a 5' to 3' orientation.

Map-based cloning.

BK001_F	AAATGGGTGCATGTAAAACACA
BK001_R	TCCGAAATAGACCCCAATGA
BK002_F	CTGCTGAGTTCCCCATCAAT
BK002_R	TGGTTAAGGACAAGGGTCAG
BK003_F	TCAAGTCTTTCAACCTAAGTCGTG
BK003_R	ATTGCCATGGATTGTGATTG
BK004_F	GGATTGTAAGGCGAAGTGGA
BK004_R	CTCATCATTTGTTGGAAGATGG

Amplification of LjRHL1 cDNA.

HLH_pcDNA_F	CCACCCAGATCCAAAATTCA
HLH_pcDNA_R	CACCCAAACGCTACGTCAT
HLH_CDNA_F	ATGCAACCCTGTAGCAGAGAA
HLH_CDNA_R	TACGTCATCACGGTTTCGAC

RACE

5'outer race	TGGTGGTTACGGAACTTGGA
5'inner race	TGAGTTGTTGATGATGGTTG
3'outer race	AGCCAAGCTAATGGAAGAAGA
3'inner race	TCCAAACAATCTCGCCAACCT

Amplification of AT1G03040, AT2G24260, AT4G30980, AT4G02590 and AT5G58010 cDNAs.

AT1G03040_F	GAATTCGAATTCTCCTTCATGGCTAATAACAACAACA
AT1G03040_R	AAGCTTAAGCTTAAAATCTACGGTGGAGGATTCA
AT2G24260_F	GAATTCGAATTCAAGAAACCCATCATCATCA

AT2G24260_R	AAGCTTAAGCTTGCCTAACCCCAAAAGTAAACG
AT4G30980_F	GAATTCGAATTCGAAGCCATGAACTCCTCGTC
AT4G30980_R	AAGCTTAAGCTTGTTATCACGGCTTGGAAACG
AT4G02590_F	GAATTCGAATTCGCTCAAACCATGGCTAGTAACA
AT4G02590_R	AAGCTTAAGCTTCTACTGTGGAGGATTGTTCTCAGG
AT5G58010_F	CCCGGGCCCGGGATGGAAAATGGAAATGGAGAAGG
AT5G58010_R	AAGCTTAAGCTTAGGGAGATTAGCGTTTGACTT

Evaluation of LjRHL1 in L. japonicus tissues and confirmation of Ljrhl1-2 mutation.

RHL_E1-4_F	CCGTAACCACCAGATCACCT
RHL_E1-4_R	TTAGCATTGGGGACCAGTTC
Ubi-F	TTCACCTTGTGCTCCGTCTTC
Ubi-R	AACAACAGAACACACAGACAATCC

Evaluation of At2g24260, At4g30980, and At5g58010 in roots of A. thaliana wild type and mutant lines.

AT2G24260_expF	AGCAGAAGAAACCCATCATCA
AT2G24260_expF	AGAAGACGACCTCTCGGTCA
AT4G30980_expF	TTCTCACTCCCCACCAAAAA
AT4G30980_expR	CGTATCCGAAAACACGACCT
AT5G58010_expF	TGTTGGGATCCATCTCTTCC
AT5G58010_expR	TGCTACAGCACTGGAGATGG
RHD6_EXP_F1	CATGAGCTACGGCTTCACAA
RHD6_EXP_R1	TAAAGATTCCATCCCCGTGT
Actin-F	CCTTACAGAGAGAGGTTACATG
Actin-R	GACCTTAATCTTCATGCTGCTTGG

Construction of LjRHL1p::GFP:LjRHL1 plasmids.

RHL_PROMTR_F	TTGATTTGGGTGATCGGATT
RHL_PROMTR_R	ACCGGTACCGGTATTGATGGGGAACTCAGCAG

4.6 Literature cited.

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CHAPTER 5

A Cytokinin Perception Mutant Colonized by *Rhizobium* in the Absence of Nodule Organogenesis

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5.1 Contributions made by Bogumil Karas:

- Establishment of allelic series including three mutant lines: *LjS32-AA*, *LjS30-AA* and *LjS56-HA*
- Map-based positioning of *HIT* locus (using *LjS32-AA* line).
- Isolation of single mutants.
- Development of double mutant line *Ljhit1-1/Ljhit1-1 Ljrhl1-1/Ljrhl1-1* and evaluation of the resulting symbiotic phenotype (Figure 5)
- Analysis of *Nin* mRNA expression in wild type and *hit1-1* roots (Figure 10)

In this chapter, molecular and functional characterization of the L. japonicus LHK1 cytokinin receptor gene, which was identified through a screen for genetic suppressors of the har1-1 hypernodulation phenotype (see Chapter 2: 2.3.7) is described.

5.2 Introduction.

The development of nitrogen fixing nodules on roots of leguminous plants commences with a molecular dialogue between the host plant and a compatible strain of rhizobia leading to the synthesis of bacterially-encoded lipochito-oligosaccharide signalling molecules, the Nod factors (NFs). Plant plasma membrane-derived structures called infection threads (ITs), which originate within root hairs of the host root in a NF dependent manner, act as conduits for rhizobia to enter the root tissues and to progress towards the root cortex where a nodule primordium (NP) has been initiated. The release of bacteria from ITs into the cytosol of a subset of NP cells and subsequent cellular specialization of both symbionts leads to the formation of fully functional nitrogen fixing organs, the root nodules (*1*).

The initiation of cell divisions for NP organogenesis is presumed to arise from the relay of a signal from the epidermally perceived NFs to the root cortex. Several genes in the NF dependent signalling pathway have been identified, including putative NF receptors (*2-4*) and a number of downstream elements (*5-12*). Deleterious mutations in any of these genes abolish bacterial entry into the root and NP development, indicating a crucial role for NF signalling in both processes.

The identification of plants that spontaneously form nodules (*13-15*) and the observation that ectopic application of cytokinins (*16*) or auxin transport inhibitors (*17*) to the root surface leads to the development of nodule-like structures, together demonstrate that the machinery required for NP development is intrinsic to the plant. Consequently, the NF pathway is presumed to trigger nodule organogenesis by regulating the endogenous plant mechanism; however, the nature of the cell division stimulus that brings about nodule primordia inception in the root cortex remains obscure. Here, we show that *Lotus japonicus* plants homozygous for a mutation in the *HYPERINFECTED 1* (*HIT1)* locus show abundant infection thread formation but fail to initiate timely cortical cell divisions

in response to rhizobial signalling. Using a combination of various functional tests and different mutant backgrounds, including *L. japonicus roothairless* mutant (see Chapters 3 and 4) , we demonstrate that the corresponding gene encodes a cytokinin receptor that is required for the activation of the nodule inception regulator *Nin* and nodule organogenesis.

5.3 Results/Discussion.

We performed a screen for genetic suppressors of the *Lotus japonicus har1-1* hypernodulation phenotype that identified three allelic suppressor lines characterized by a low nodulation phenotype and an excessive formation of ITs. The corresponding locus was named *HYPERINFECTED 1* (*HIT1*; *18*). Further detailed phenotypic analysis, performed in both double (*hit1 har1-1*) and single (*hit1*) mutant backgrounds, showed that the three suppressor lines had indistinguishable mutant phenotypes, with the bacterial root invasion via ITs intact and the timely onset of associated cortical cell divisions for NP organogenesis aborted (see below). The *hit1-1 har1-1* and *hit1-1* mutants were chosen as reference lines. When analyzed 10 days after inoculation (dai) with a *Mesorhizobium loti* strain carrying a constitutive *hemA::lacZ* reporter gene fusion, the most noticeable feature of *hit1-1 har1-1* roots was hyper-infection (Figs. 1A and 2A). The large number of ITs that formed in the *hit1-1 har1-1* mutant roots originated within curled root hairs but their progression towards the root cortex was blocked at the epidermis/cortex interface (Fig. 1B). Infrequent ITs that escaped this early blockage and managed to penetrate within the *hit1-1 har1-1* root cortex looped frequently, suggesting they were misguided (Fig. 1C). In spite of abundant infection events at the root epidermis, the root cortex of the *hit1-1 har1-1* mutant failed to initiate NP (Fig. 1C).By 14 dai, many ITs overcame the initial blockage and progressed deeper into the mutant root (Figs. 1D). Cortical cell divisions were initiated coincident with the accumulation of ITs within the root cortex but NP did not develop (Figs. 1D and 1E). *hit1-1* displayed the same mutant phenotypic features as *hit1-1 har1-1*, although the overall number of symbiotic events observed was reduced, likely reflecting the presence of the functional HAR1 autoregulatory receptor kinase (*20*). In *hit1-1*, an initial lack of NP formation in response to rhizobial infection (Fig. 2B) was accompanied by the early onset of hyper-infection (Fig. 2A)

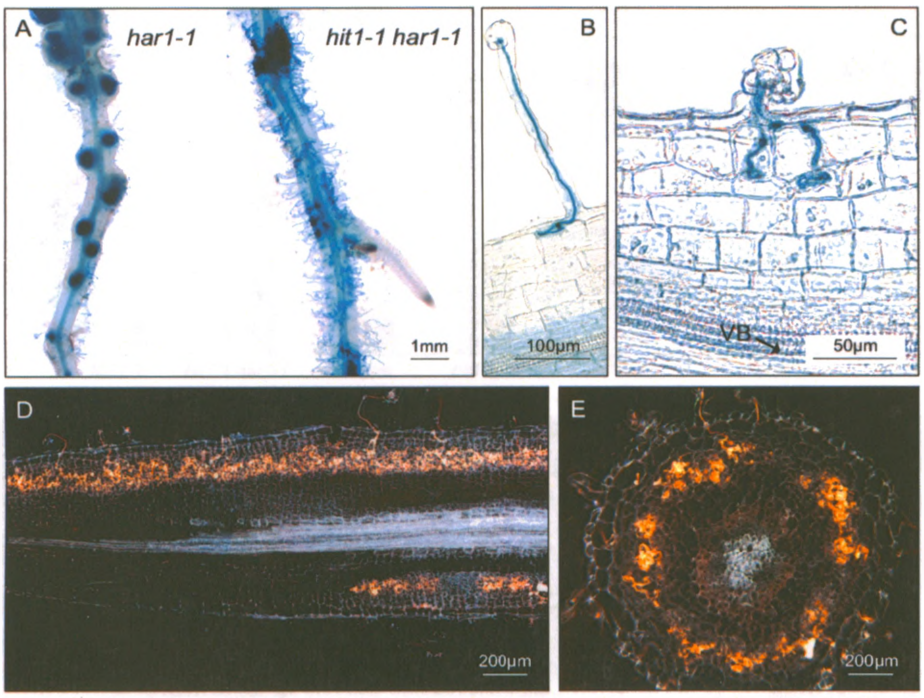


Figure 1. (A-E) Root segments stained for β-galactosidase (*LacZ*) activity 10 (A-C) and 14 (D and E) dai with *M. loti* (A) A large number of ITs gave a blue appearance to the *hit1-1 har1-1* root. (B) An IT (blue) traversed a root hair but became blocked at the epidermis/cortex interface. (C) Misguided ITs looping within the root cortex. Note the absence of subtended cell divisions that would normally be associated with a subepidermal infection (19). Negative images of: (D) a longitudinal section through *hit1-1 har1-1* root showing large number of ITs (red) within the root cortex; (E) Cross-section of the *hit1-1 har1-1* root showing accumulation of ITs around the entire mid-cortex perimeter of the section plane.

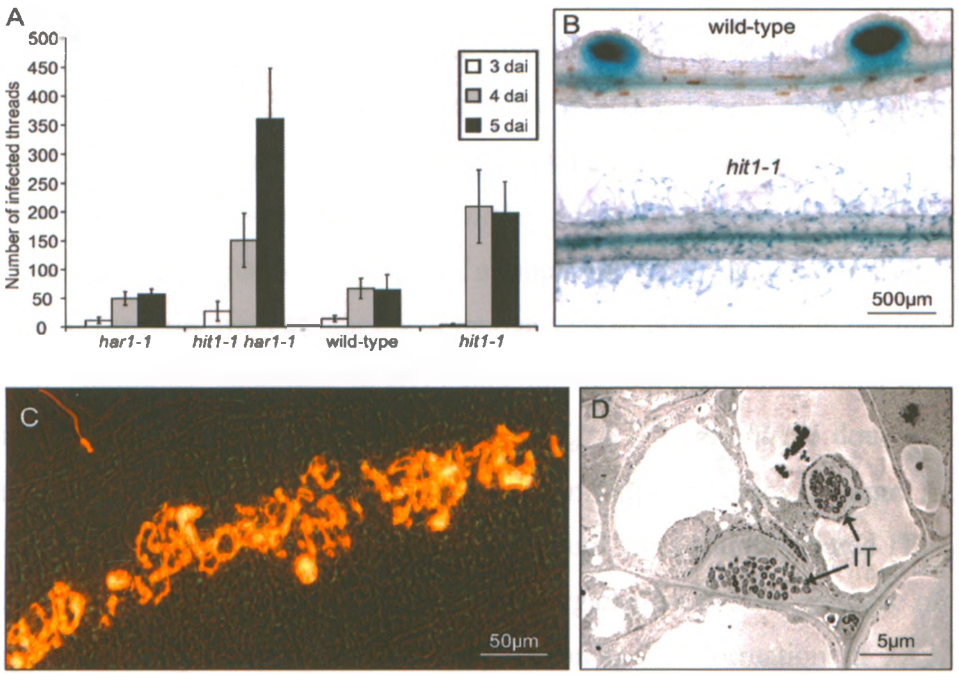


Figure 2. Infection and nodulation events in wild-type and mutant plants. (A) Infection threads were counted at 3, 4 and 5 dai with *M. loti* carrying a *hemA*::*lacZ* reporter gene fusion. Upon histochemical staining, the plants were examined at 20X magnification. All infection threads visible from a single perspective were scored. Means represent 10 plants ± 95% CI. (B) The root phenotypes of wild-type (Gifu) and *hit1-1* roots 7 dai with *M. loti*. Gifu develops fully colonized spherical nodules. *hit1-1* is hyperinfected but nodule development is prevented. (C) A longitudinal section of the *hit1-1* single mutant root segment 10 dai showing a close-up of subepidermal cortical cell layers accumulating a large number of intertwined ITs. (D) TEM image of a 2 μm thin section of *hit1-1* root showing a large pocket of *M. loti* that are confined to ITs. VB: vascular bundle.

with a large number of ITs located within the root cortex (Fig. 2C). Most root cortical cells associated with ITs in *hit1-1* and *hit1-1 har1-1* remained small and un-colonized, with *M. loti* being confined to ITs (Fig. 2D). Occasionally, a local release of bacteria from clustered ITs resulted in enlarged and often flattened nodules (Figs. 3A and 3B), giving rise to the low nodulation phenotype (Fig. 3C).

Intermittently, the development of wild-type-like nodules in both *hit1-1* and *hit1-1-har1-1* was also observed (Fig. 3B). We further investigated a role for the *HIT1* locus in NP organogenesis by studying *Early Nodulin 40* (*ENOD40*) and *Nin* expression, two markers for NP initiation (*12, 21*). Quantitative qRT-PCR analysis showed that steady state levels of the corresponding transcripts in the inoculated roots were strongly reduced in the *hit1-1 har1-1* compared to the *har1-1* parental line, although this difference was not statistically significant between inoculated *hit1-1* and wild type roots (Figs. 4A and 4B). We concluded therefore that the presence of a functional *HIT1* locus is required for normal *ENOD40* and *Nin* expression during the organogenesis of NPs, at least in the *har1-1 hit1-1* background. Additional validation of *HIT1* function in nodule organogenesis was provided by the analysis of the *L. japonicus root hairless* (*Ljrhl1-1*) and *hit1-1* double mutant. In response to inoculation with *M. loti, Ljrhl1-1* initially develops a large number of uncolonized NP, a consequence of the absence of root hairs and associated ITs (*22*). We reasoned that if *HIT1* mediates nodule primordia organogenesis, the presence of a mutant *hit1-1* allele should prevent or significantly attenuate NP formation in *Ljrhl1-1*. The analysis of the *Ljrhl1-1 hit1-1* mutant showed that the development of NP was almost entirely aborted, providing strong evidence for the indispensable role of *HIT1* in nodule organogenesis (Fig. 5). The *hit1-1* phenotype resembled the infection thread "symbiosis" proposed to have been a precursor to nodulation (*23*). Could *HIT1* be the missing evolutionary link?

To begin addressing this question we set out to map-base clone the *HIT1* locus. While this work was in progress Jens Stougaard's group at Aarhus University (Denmark) cloned the *L. japonicus Spontaneous nodule formation 2* locus (13), renamed as *Lotus histidine kinase 1* (*Lhk1*) (24), which is localized to the same genetic interval on chromosome IV as *HIT1*. Given the opposing nodulation phenotypes of *snf2* and *hit1-1*, we tested the hypothesis that *hit1* mutants represent loss of function alleles of *Lhk1*.

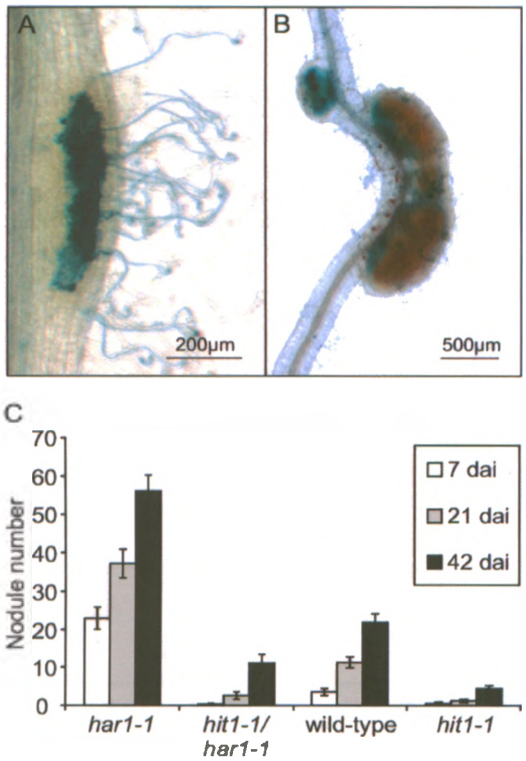


Figure 3. Nodulation phenotype of *hit1-1* single mutant. (A) Abundant infection events 14 dai. Note a large number of IT descending simultaneously toward the root cortex. (B) Rare successful infection events (14 dai) often lead to the formation of grossly enlarged and deformed nodules. (C) Number of nodules formed on the root of *har1-1, hit1-1 har1-1*, wild-type and *hit1-1*, 7, 21, and 42 dai with *M. loti*

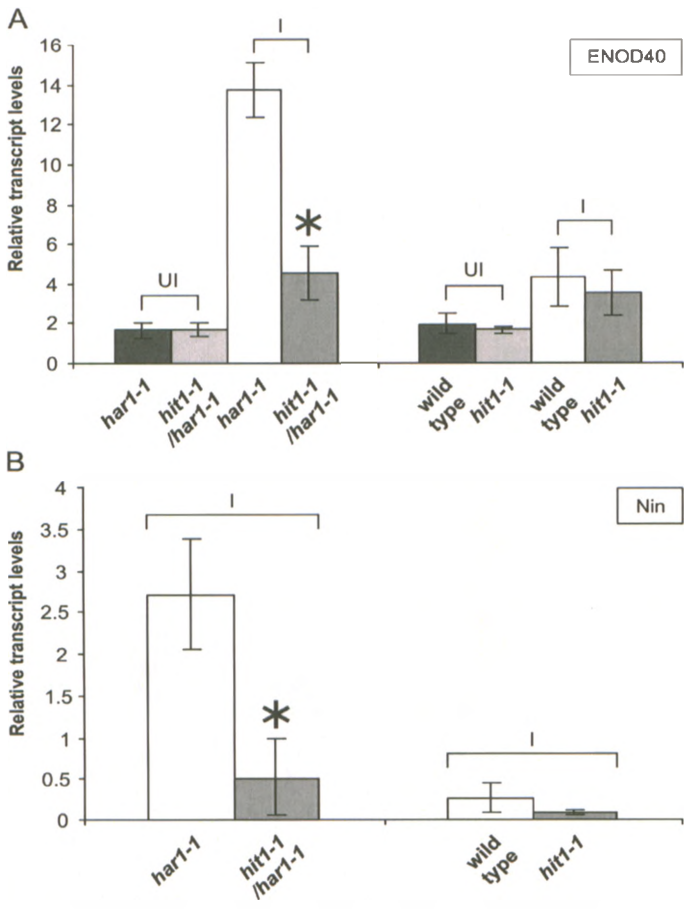


Figure 4. Quantitative RT-PCR analysis of *ENOD40* (A) and *Nin* (B) transcripts in uninoculated roots (UI) and roots inoculated with *M. loti* (I). For *Nin,* data from the inoculated samples only are presented, due to very low transcript levels which resulted in the inconsistent amplification of the corresponding transcript from the uninoculated roots. * indicates $p<0.05$ (t-test) for parental vs. corresponding mutant lines within treatments.

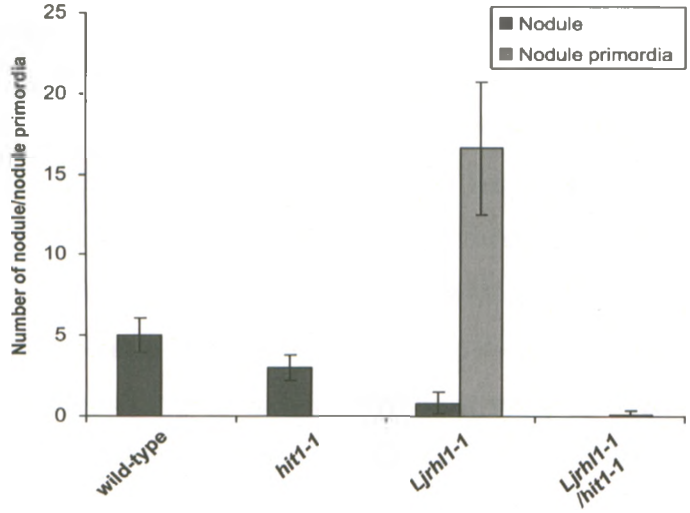


Figure 5. Numbers of nodules and nodule primordia (10 dai) on wild type, *hit1-1, Ljrhl1-1*, and *Ljrhl1-1 hit1-1* mutants. (mean ± 95% CI; n=20).

Lhk1 specific primers were used to amplify the analogous genomic region from the three *hit1* lines. In all three lines mutations were found that were predicted to result in premature stop codons (Fig. 6A). This finding, along with the ability of a wild-type *Lhk1* gene to complement the *hit1-1 har1-1* and *hit1-1* phenotypes in transgenic *L. japonicus* hairy root experiments (Fig. 7) confirmed the identity of the underlying gene as *Lhk1*. Since the *hit1-1* allele carried a G_{1695} to A nucleotide substitution in the splice donor site of intron four, oligonucleotide primers flanking this site were used to amplify the corresponding cDNA. Seven aberrant *hit1-1* splice variants were identified (Fig. 6B). In addition, a polymorphic species was found among the PCR products. A search for the corresponding *L. japonicus* genomic sequence identified a novel gene, here named *Lhk2* (*Lotus histidine kinase 2*), of which the predicted product showed 85% identity at the amino-acid level with LHK1. While *Lhk1* transcripts were present in roots, nodules and shoots (Fig. 6C), the *Lhk2* mRNA was detectable only in roots.

Analysis of the full length *Lhk1* cDNA revealed a 2979-bp open reading frame encoding a predicted protein of 993 amino acids (Fig. 6A). The LHK1 protein had 64% identity with the *Arabidopsis* cytokinin histidine kinase receptor AHK4, and 49 and 45% identity with AHK2, and AHK3, respectively. Like LHK1, LHK2 was more closely related to AHK4 than other *Arabidopsis* cytokinin receptors (Fig. 8).

When expressed in the *sln1*Δ yeast strain carrying a lethal mutation in SLN1 histidine kinase (*25*), *Lhk1* rescued the growth of the yeast strain in a cytokinin dependent manner, demonstrating that LHK1 is a cytokinin receptor (Fig. 6D). In agreement with this notion, roots of *hit1-1* mutants exhibited strong insensitivity to exogenously applied cytokinin (Fig. 10A). A similar cytokinin-insensitive root phenotype was observed in all three *hit1-1 har1-1* double mutant lines(Fig. 11).

Since the accumulation of *Nin* and *ENOD40* transcripts was significantly attenuated in the *hit1-1 har1-1* mutant we next tested if exogenous application of cytokinin regulates expression of these genes in the wild-type roots. *ENOD40* has been shown to be induced by external application of cytokinin to the roots in several legume species (*26*) and this was also the case in *L. japonicus*, albeit the overall induction of *ENOD40* was rather modest (Fig. 11A). In contrast, 50nM benzyl adenine (BA) increased the steady state level of *Nin* transcript 20 fold (Fig. 11A).

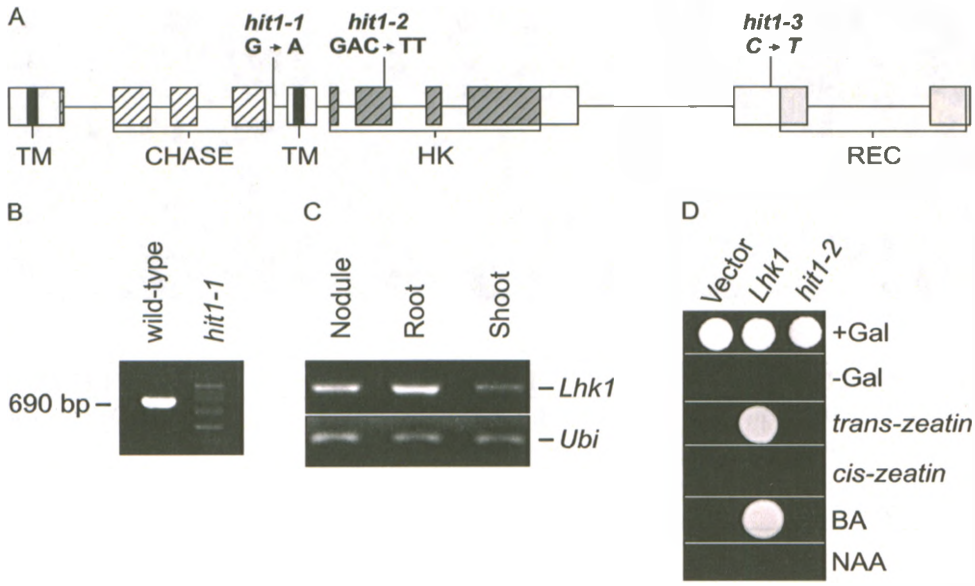
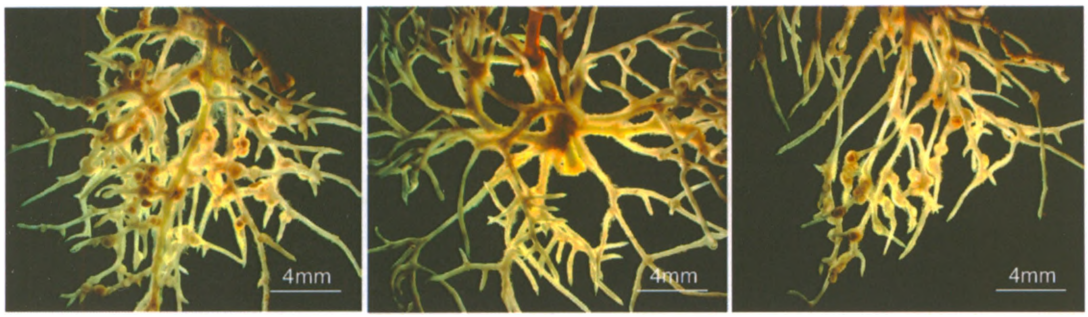


Figure 6. (A) The exon (box)/intron (line) structure of the *Lhk1* gene with positions of the molecular lesions for each of the *hit1* mutant alleles indicated. The corresponding protein domains are indicated: transmembrane domains (TM; black); CHASE domain (white hatched); histidine kinase (HK) domain (grey hatched); receiver (REC) domain (grey). *hit1-1* carries a G_{1695} to A nucleotide substitution that leads to alternatively spliced products, as shown by RT-PCR in panel 3B. *hit1-2* contained two consecutive transversions (GA_{2407-8} to TT) followed by a single base (C_{2409}) deletion. This frame shift results in a premature stop codon 45 bp downstream. *hit1-3* has a C_{4922} to T transition resulting in a premature stop codon before the REC domain. (C) Expression of *Lhk1* in various *L. japonicus* tissues as assayed by RT-PCR. Ubiquitin (*Ubi*) was used as the RNA loading control. (D) *Lhk1* cDNA confers cytokinin responsiveness to yeast cells. TM182 cells were transformed with either p415CYC carrying the *Lhk1* cDNA, the *hit1-2* cDNA or the vector alone. Transformants were plated on a minimal medium with or without galactose (gal), or on a galactose free medium supplemented with the indicated hormone.

A



B

GENOTYPE	AR12	AR12+*LHK1*
Wild-type (Gifu)	28 ± 4.8	20 ± 8.8
hit1-1	0.8 ± 0.54	8.4 ± 2.17
har1-1	154 ± 10	nd
hit1-1 har1-1	0.3 ± 0.3	24.5 ± 6.7

Figure 7. Complementation of *hit1-1 har1-1 and hit1-1* mutant phenotypes with a wild-type copy of *Lhk1 via Agrobacterium rhizogenes* mediated hairy root transformation. (A) Hypernodulated hairy roots of *har1-1* transformed with AR12 control vector and inoculated with *M. loti* (left panel); Hairy roots of *hit1-1 har1-1* transformed with AR12 vector alone, showing almost non-nodulating phenotype (central panel); The AR12 vector containing a full-length copy of the *Lhk1* cDNA (kind gift form Jens Stougaard; Aarhus University, Denmark) was introduced into the *hit1-1 har1-1* double mutant restoring the ability of the chimeric plant to nodulate. (B) Number of nodules formed on hairy roots of different plant genotypes and complementation constructs (Partial restoration of nodule number in hairy roots carrying *Lhk1* complementation construct (i.e. less nodules formed in the *hit1-1* and *hit1-1 har1-1* mutants in comparison to wild-type and *har1-1*, respectively) was not surprising. Positional effects were likely responsible for the observed result. However, we can not entirely rule out the possibility that *Lhk1* has a function in the shoot that is relevant to symbiosis).

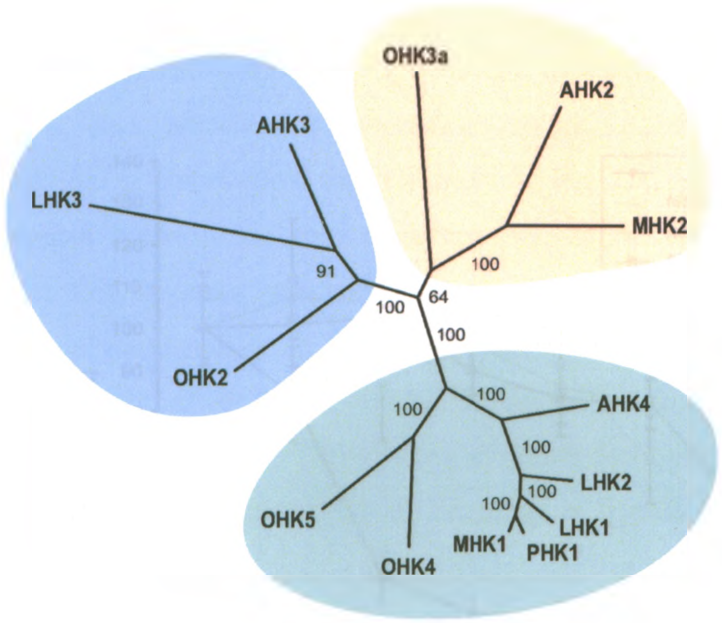


Figure 8. Phylogenetic analysis of LHK1 and LHK1-like proteins. *Lhk1,* a possible ortholog of AHK4, belongs to a clade that includes members from non-legumes and is unlikely to be symbiosis specific. Unrooted tree based on an amino acid alignment of full-length sequences from *L. japonicus (L)*, *Medicago truncatula* (*M*), *Arabidopsis thaliana* (A), *Oryza sativa* (O), and *Pisum sativum* (*P*). Genbank accession numbers: LHK1, DQ848999; LHK2, DQ848998 ; LHK3, AP009230; MHK1, ABE94286; MHK2, CT571263; OHK2, BAB90827; OHK3a, BAF26350; OHK4, XP_469566; OHK5, XP_467688; PHK1, DQ845485. Protein sequences were aligned with CLUSTALW using the default settings. The bootstrapped phylogenetic tree was created using PHYML (http://atgc.lirmm.fr/phyml/) (33). PHK1 sequence was predicted from the provided genomic sequence using Eukaryotic GeneMark.hmm online software (34) (http://exon.gatech.edu/GeneMark/eukhmm.cgi) using the Arabidopsis model, and modified manually. LHK3 protein sequence was predicted from the provided genomic sequence using GenomeScan (35) which is available online (http://genes.mit.edu/genomescan.html).

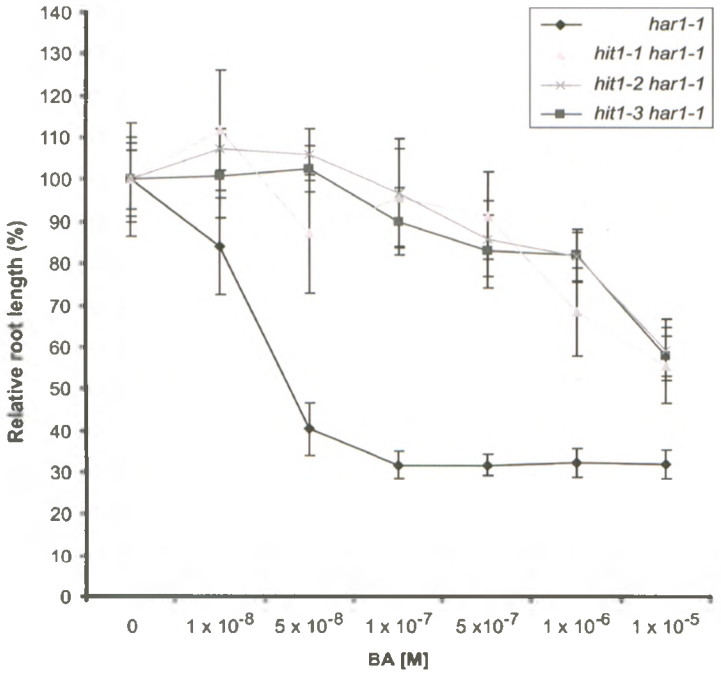


Figure 9 *hit1 har1-1* double mutants showed cytokinin insensitive root phenotype. The double mutant lines were germinated and grown on the surface of agar plates containing different concentration of BA and culture for 7 days. Root length was measured and expressed as described in the Material and Methods section. Each value represents the mean of at least 18 plants ± 95% confidence interval.

This induction required *de novo* protein biosynthesis (Fig. 11B). In *hit1-1* roots, BA-stimulated accumulation of *Nin* transcripts was significantly diminished in comparison with wild-type roots indicating that the high level of *Nin* expression requires the functional *Lhk1* (Fig. 10B).

NF signalling regulates *Nin* expression (3, 15), which is required for the formation of ITs in the root epidermis and initiation of nodule primordia organogenesis in the root cortex (12). Our data indicate that although necessary for *Nin* expression and nodule organogenesis, *Lhk1* is not required for IT formation (Fig. 10C). The reported partitioning of *Nin* expression between the root epidermis and cortex could provide a plausible explanation for this apparent conundrum (3, 15, 27). *Nin* expression, supporting IT formation, may be regulated by an *Lhk1* independent mechanism in the root epidermis, possibly involving another cytokinin receptor. Diminished nodule organogenesis in *hit1-1 har1-1* and *hit1-1* likely restricts local and/or systemic feedback mechanisms that limit root susceptibility to *Rhizobium* infection, resulting in hyperinfection (Fig. 10C). The LHK1 homologues, such as LHK2 and LHK3, are likely to function as cytokinin receptors, which may explain a leaky (formation of some nodules) symbiotic phenotype and lack of more general developmental abnormalities in mutants carrying *hit1* alleles. The *snf2* mutant described in the accompanying manuscript (24) and strongly reduced nodulation in *Medicago truncatula* plants carrying a *MtCREI* silencing construct **(29)** further demonstrate that cytokinin sensing is required to stimulate nodule development. Together, these results specify that the regulators of cytokinin biosynthesis and/or action are crucial downstream targets of NF perception (Fig. 10C) and that recruitment of a cytokinin receptor could have been an essential event during the evolution of nitrogen fixing nodule symbiosis.

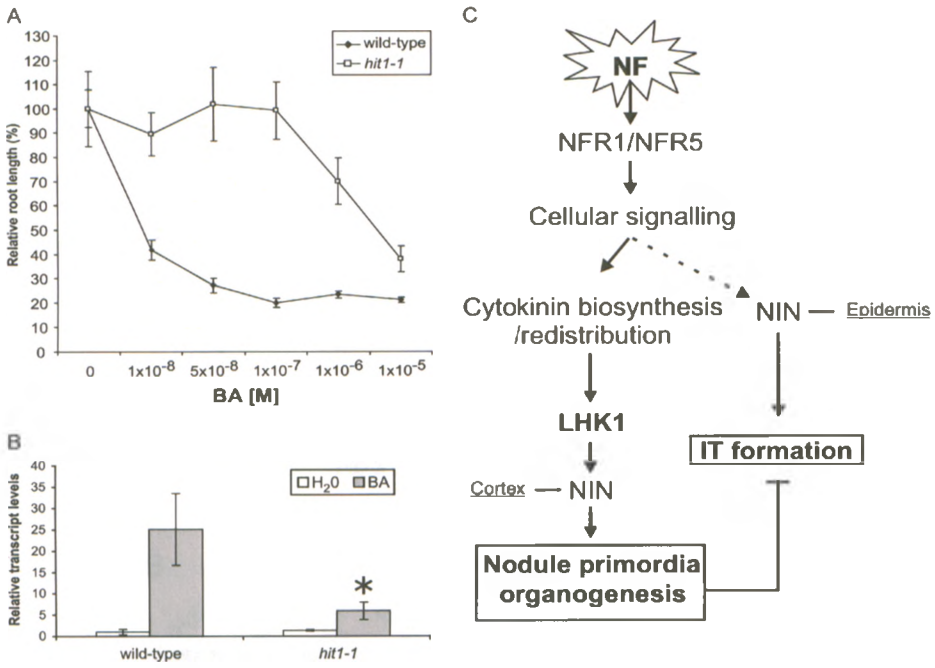


Figure 10. (A) *hit1-1* roots are insensitive to exogenously applied cytokinin. (B) qRT-PCR showing significant attenuation (*$p<0.05$) of *Nin* mRNA in *hit1-1* vs. wild-type roots upon exogenous application of 50 nM BA. Note that BA-stimulated accumulation of *Nin* transcripts in *hit1* roots was significantly higher ($p<0.05$) in comparison to water treatments. (C) The proposed role of LHK1 in NF induced nodule organogenesis. Perception of NF by NFR1/NFR5 stimulates local cytokinin biosynthesis in, or redistribution of cytokinin to, the root epidermis and cortex (28). This is perceived by LHK1, which activates *Nin* expression in the root cortex leading to initiation of nodule organogenesis (for additional discussion, see text).

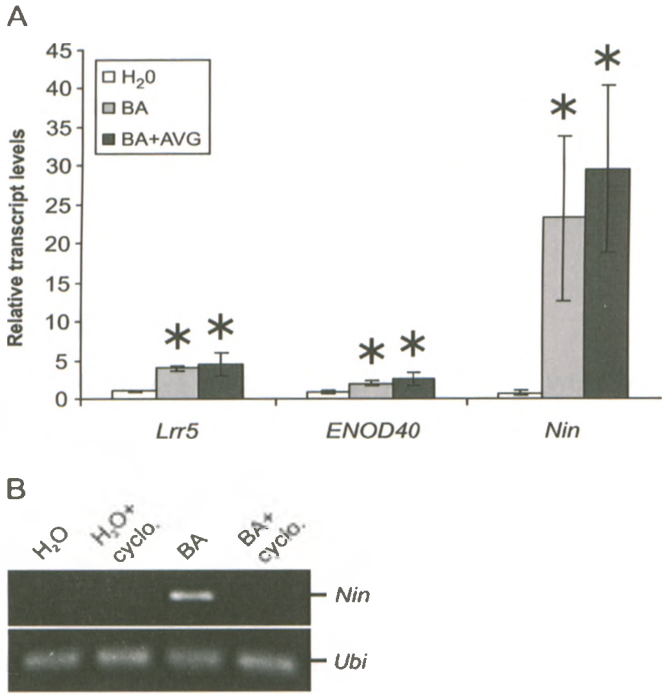


Figure 11. The level of *Lrr5*, *ENOD40*, and *Nin* transcripts increases significantly in wild-type *L. japonicus* roots upon application of 50nM BA, independent of ethylene. (A) Relative transcript levels were determined using qRT-PCR. An expressed sequence tag (EST) for *Lrr5* (Genbank acc. no. CB827384), a homolog of the cytokinin inducible *Arabidopsis Response Regulator 5* (36) identified from the public sequence database was used as a positive control. *indicates $p<0.05$ for water vs. BA and water vs. BA + AVG comparisons for the individual gene targets (B) RT-PCR analysis showing that the BA induction of *Nin* mRNA accumulation in wild-type *L. japonicus* requires *de novo* protein biosynthesis since cyclohexamide, a protein biosynthesis inhibitor, prevented accumulation of *Nin* transcripts.

5.4 Materials and Methods.

5.4.1 Plant Material.

Mutants were generated by chemical EMS mutagenesis of *L. japonicus har1-1/har1-1* (ecotype Gifu) as described previously (18). Genetic complementation tests revealed that the three independent *hit1* lines represent the same locus (18). Seeds were germinated and grown as described (19). Plants were inoculated one week after planting with *M. loti* carrying a *hemA::lacZ* reporter gene and were harvested at 10, 14 and 21 days after inoculation, stained for ß-galactosidase activity and analysed by sectioning and microscopy as previously described (22).

5.4.2 Electron Microscopy.

Root sections from *hit1-1* plants 10 dai with *M. loti* were histochemically stained for ß-galactosidase activity to identify infected regions of the root, which were then fixed and sectioned as described (30). 0.2 μm sections were examined using transmission electron microscopy.

5.4.3 Agrobacterium rhizogenes transformation.

The *A. rhizogenes* AR12 strain carrying the *SNF2* locus was kindly provided by Jens Stougaard (Aarhus University, Denmark). Hairy root transformation was carried out on wild-type, *har1-1*, *hit1-1 har1-1*, and *hit1-1* as described (31), with the following modifications: plants were grown for one week after inoculation with *A. rhizogenes* at which point roots were removed and plants were transferred to liquid culture for 10 days. Shoots with developed hairy roots were transplanted into pots containing a sterilized mixture of vermiculite and sand (6:1), covered with plastic wrap and grown for one week before being inoculated with *M. loti* NZP2235. Three weeks after inoculation, nodules present on all hairy roots which had formed on individual shoots were scored. 15-20 independent shoots per genotype were analyzed.

5.4.4 Characterization of Lhk1 and Lhk2.

Sequence for *Lhk1* was kindly provided by Jens Stougaard (AP009236). Total mRNA was isolated from *L. japonicus* (Gifu) roots and used to synthesize cDNA using the Thermoscript RT-PCR system (Invitrogen). A full length cDNA was generated by 5' and 3' RACE using the FirstChoice RLM-RACE kit (Ambion, Austin TX) and sequenced (Genbank acc. no. DQ848999). A database TBLASTX search using *Lhk2* partial cDNA sequence identified a highly homologous match in the *L. japonicus* draft sequence from the shotgun sequencing database on BAC clone LjB11M03 (BM2030). The BAC was entirely sequenced (Genbank acc. no. AP009229). The following primers based on *Lhk2* sequence were then used to amplify a product from root cDNA which was then sequenced (Genbank acc. no. DQ848998):

HIT2-F, CTTGCTGATGGGTCTTAGCTTGA

HIT2-R, GACGGAGTCATGAGTCAGAGATGGT.

5.4.5 Characterization of pea PHK1.

Primers designed based on *L. japonicus Lhk1* coding sequence (For-GAACGGAACAGCTGGGATAA, Rev- TCCTCTCCAGAAACCCAAGA) were used to amplify a gene fragment from *P. sativum* (Sparkle) DNA (seed kindly provided by Frederique Guinel, Wilfred Laurie University, Canada) using the following conditions: 4-min denaturation cycle at 94°C followed by 35 cycles (94°C, 30 s; 50°C, 30 s; and 72°C, 1 min). The sequence of the resultant product was used to sequentially clone the full length gene using a Universal Genome Walker Kit (BD Biosciences). The following primers were used for long-range PCR using the Expand Long Template PCR System (Roche Diagnostics) to amplify a single product for sequencing the entire gene: PHK-F GGAGCAGATCAAGAAGAAGG, PHK-R AGGGAAGTACTATGGAGATG. *PHK1* locus (Genbank acc. no. DQ845485) was also amplified from two pea symbiotic mutants (*R50* and *sym5*), which show partly similar phenotype to *hit1-1,* but no nucleotide substitutions were found.

5.4.6 Cytokinin treatment of seedlings.

10 day old *L. japonicus* seedlings were transferred to beakers containing either sterile H_20, 50 nM BA, 50 nM BA + 100 nM AVG, or 50 nM BA + 100 μM cyclohexamide, completely shielded from light and incubated at RT overnight with constant aeration, at which point roots were harvested for total RNA isolation and RT-PCR analyses.

5.4.7 Expression Analysis.

Total RNA was extracted and treated with DNAseI using an RNeasy Plant Mini Kit (Qiagen). Oligo-dT primed cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen) in a total reaction volume of 20 μl. Quantitative RT-PCR reactions were performed in triplicate on 2 μl cDNA using the SYBR-GREEN PCR Master Kit (Perkin-Elmer Applied Biosystems) and real time detection was performed on a LightCycler 3.0 (Roche) (2 min soak at 95 °C followed by 50 cycles of 95 °C 0 s, 58° 15 s, 72°C 9 s) and analyzed using LightCycler Software version 3 (Idaho Technology Inc.). Run-to-run variation and primer efficiency differences were corrected using RelQuant version 1 software (Roche). Each mean represents three (Fig. S3) and five (Fig. 4B) biological replicates (± SE). Semi-quantitative PCR reactions were carried out on a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems) (5 min soak at 95 °C, followed by 40 cycles of 94°C 30 s, 60°C 30 s, 68°C 30 s, followed by a 7 min soak at 68°C). All PCR reactions were carried out using High Fidelity Platinum Taq DNA Polymerase (Invitrogen). The resulting cDNAs were sequenced to verify the identity of the PCR products. Primers used:

(Ubiquitin reference gene)	Ubi-F TTCACCTTGTGCTCCGTCTTC,
	Ubi-R AACAACAGAACACACAGACAATCC
(*EIF4A* reference gene)	LjEIF4a-F AGAGGGTTTAAAGATCAAAT
	LjEIF4a-R ATGTCAATTCATCACGTTTT
(*Nin;* Figs. 4B and 11A)	LjNIN-3-F CCACCTTCTCATCTCTTGCTC
	LjNIN-3-R AGACAAGCTCCCATAGGCAGA
(*Nin*; Fig. 10)	LjNIN-F AATGCTCTTGATCAGGCTG
	LjNIN-R AGGAGCCCAAGTGAGTGCTA

(*L. japonicus ARR5*-like EST;	LRR5-F2 CGTCTCACAAAGCGTGTCAT
Lrr5, Acc. no. CB827384)	LRR5-R2 CCATCCAACCCCAAATACTG
(*Enod40-1*; Acc. no. AJ271787)	Enod40-1-F CTGAACCAATCCATCAAATCC
	Enod40-1-R TTGGAGAATGCTCATCTGCT
(*Lhk1*; gene specific primers,	LHK-1F CTGCAGAACAGGTCAACCAA
Fig. 6C)	LHK-1R TCATCATGGATCCTCGCATA
(*Lhk1* primers flanking	LHK-2F GAAGCAACATGGGTGGGTTA
hit1-1 mutation; Fig. 6B)	LHK-2R TCAGCTGCTTCTGCTTTGAC

5.4.8 Root elongation assays.

10-15 seedlings per each hormone concentration were scored as described (32). Root growth was expressed as relative growth (i.e. the ratio of root length in the presence and absence of hormone x 100) In addition to cytokinin (BA), the growth responses of wild-type and *hit1-1* mutant roots to external application of auxin (NAA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed. The *hit1-1* mutant root responses remained unchanged or were slightly elevated (within $5 \times 10^{-8} - 10^{-7}$ M concentration range only) in comparison to wild-type Gifu roots (data not shown). *hit1-1* roots grown on the medium without BA were on average slightly shorter ($\bar{x} = 42 \pm 4.3$ mm; n=35) than wild-type roots ($\bar{x} = 48 \pm 2.95$; n= 38) grown under comparable conditions.

5.4.9 Yeast Complementation.

The entire coding region of *Lhk1* cDNA for wild-type and *hit1-2* were cloned into the yeast expression vector p415CYC under the CYC1 promoter between the *Xba*I and *Xho*I sites. Clones were confirmed by sequencing and then introduced into yeast strain TM182 (*sln1*Δ; kind gift form Tatsuo Kakimoto, Osaka University, Japan) (25). Suspensions of transformants were spotted onto –LEU –URA 2% glucose-containing drop-out media with or without the addition of 2% galactose and containing 10 μM of the following plant hormones (Sigma): BA, *trans*-Zeatin, *cis*-Zeatin, and NAA (1-naphthaleneacetic acid).

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CHAPTER. 6

GENERAL DISCUSSION AND CONCLUSIONS

Although plentiful in the earth's atmosphere, nitrogen (N) is one of the most limiting nutrients for growth and biomass production in all life-supporting environments. To sustain maximal crop yield, modern farming practises rely on industrial fertilizers for enhancement of soil nitrogen content. This comes at an enormous price due to the associated depletion of non-renewable resources, rising production costs and the problem of environmental nitrogen enrichment. Excess nitrogen contributes substantially to such issues as carbon cycle, global warming, water quality, acid rain, biodiversity loss, and air pollution. Nevertheless, all experiments to date indicate that prolonged N supply to the plant will have a positive effect on yield.

Biological nitrogen fixation, provides ~ 175 million metric tons of fixed nitrogen, more than twice the amount of industrially-produced nitrogen, to natural and agricultural ecosystems in an environmentally-friendly and sustainable manner. A significant portion of this biological input is generated as a result of mutualistic associations of nitrogen fixing bacteria with selected groups of plants. Legumes, such as soybean, pea and alfalfa have the ability to engage in this type of beneficial interaction, which permits the host plant to make use of the otherwise inaccessible nitrogen in the air. This interaction enables the plant to develop root nodules in which the symbiotic bacteria reside and fix nitrogen(Szczyglowski and Amyot, 2003). The symbiotically produced combined nitrogen in the form of ammonium (NH_4) fulfills the plant's nitrogen requirements, thus limiting the need for artificial fertilizer (Graham et al. 2003).

Research into the legume-specific functions that mediate efficient sequestration of nitrogen should provide important insight on how to fulfill the increasing need for sustainable plant-based production, while at the same time preserving non-renewable resources and the health of the environment. Symbiotic nitrogen fixation, in addition to its significant agronomic value, also represents the most advanced model for beneficial plant-microbe interactions and developmental signaling in the context of two interacting organisms.

Decades of research into the biology of symbiotic nitrogen fixation have generated a rich intellectual resource, which together with the more recently developed model legumes, *Lotus japonicus* and *Medicago truncatula*, provide exciting new insight into the

molecular basis of this important trait in legumes (Handberg and Stougaard, 1992; Cook et al. 1999).

In the framework of the rapidly progressing *L. japonicus* whole genome sequencing project (Szczyglowski and Stougaard, 2008), a detailed molecular dissection of the host plant elements that underscore the signaling events during symbiotic root development has become possible. Therefore, the overall research objective of my thesis has been to further contribute to our understanding of the mechanisms governing the development of nitrogen-fixing root nodule symbiosis by identifying and characterizing symbiosis-relevant loci in a model legume, *Lotus japonicus*.

Two major research avenues were pursued in this context. First, a forward genetic screen was performed. In contrast to previously reported genetic screens (Buzas et al. 2005; Kawaguchi et al. 2002; Márquez et al. 2005; Perry et al. 2003; Schauser et al. 1998, Szczyglowski et al. 1998; Webb et al. 2000), which have been carried out by chemical or physical mutagenesis of wild-type legume plants, a novel approach was undertaken. The *L. japonicus har1-1* mutant (Wopereis *et al.* 2000) was used to perform a screen for genetic suppressors of its hypernodulation phenotype. This approach resulted in the identification of a large number of symbiosis-defective mutants (see Chapter 2), thus contributing to a resource of genetic variation, which is now being used to interrogate the underlying phenomena. Two categories of novel mutants, where altered phenotypes pointed to presumed defects in either root colonization by symbiotic bacteria or in the organogenesis of nodule structures, were selected for further detailed analyses.

Our genetic screen for suppressors of the *har1-1* phenotype identified a collection of root hair mutants, where monogenic mutations altered the epidermal surface of the root while, at the same time, changed the behavior of the symbiotic partners. In the context of the root colonization events, how do the endophytic or endosymbiotic bacteria enter the root system to promote plant growth and what selective mechanisms determine these processes, are important questions from a biotechnology point of view. Nitrogen fixing bacteria (rhizobia) are known to use a variety of mechanisms to accomplish this task (Guinel and Geil, 2002), but one predominate way is to colonize root hairs in a process which involves the formation of so called infection threads. One class of the mutants selected was characterized by a total lack of root hairs (i.e. *L. japonicus roothairless*

mutants). Since this prevents root hair-dependent infection thread formation, these mutants were used to analyze the symbiotic interaction with bacteria which, were assumed, to rely on root hairs to enter the root. I was able to demonstrate that the symbiotic bacteria were able to enter the mutant root using alternative mechanisms such as crack-entry through breaks in the cortical surface of the nodule or by colonization of cortical root hairs (i.e. protrusions of root cortex that originate as a result of bacterial signaling). The latter mechanism is entirely new and has not been recognized previously (see Chapter 3).

The discovery of *L. japonicus* root hair mutants created new research opportunities to dissect the molecular mechanism(s) underlying alternative modes of root colonization by symbiotic bacteria. Using this newly discovered genetic resource, I demonstrated for the first time how versatile the symbiotic nitrogen fixation bacteria can be in their ability to colonize the root system. The latter is important as it has significant impact on future applications that aim to improve root colonization by beneficial soil microorganisms; contributing, therefore, to the ability of plants to sustain productivity under low nutrient input.

These mutants also constitute invaluable tools in testing emerging models for root colonization by bacteria. Such models are being developed through a system biology approach and their validation will necessitate testing the relevant axioms under perturbed conditions, as exemplified by the well characterized mutant genetic background. Consistent with this assumption, the molecular cloning of the *LjRHL1* locus was performed, revealing a genetic lesion in a presumed transcription regulator belonging to a large plant family of basic helix-loop-helix proteins (see Chapter 4).

Unlike *Arabidopsis*, where a pattern of root hair and root hairless cells is formed, all or almost all of the *L. japonicus* root epidermal cells produce hairs. Therefore, our work provided insight into a mechanism that governs root hair development in a plant species with a random epidermal patterning. This category encompasses the majority of plant species, making this work especially relevant.

Importantly, I was able to show that the *LjRHL1* gene acts in a non-redundant manner to regulate root hair differentiation in *L. japonicus*. Monogenic mutations that completely abolish root hair development are not known in *Arabidopsis,* the model plant that is commonly used to study the mechanisms that govern differentiation of the root

epidermis. By exploring genomic resources available for both *Arabidopsis* and *Lotus*, I have defined three *Arabidopsis* genes as functionally equivalent with *LjRHL1,* and was able to show that they regulate root hair development in at least a partially redundant manner (see Chapter 4).

In more general terms, the identification and molecular characterization of plant genes that control root architecture represents a necessary step in deciphering regulatory circuits that define root plasticity, an important agronomic trait. New opportunities (e.g. linked genetic markers) should emerge from this research, which is predicted to contribute to the future ability to select for appropriate root architecture that meets the desired environmental conditions (e.g. soil type, level of precipitation, etc.) and/or acts to enhance beneficial plant microbe interactions.

In the context of evolutionary events that led to the development of nitrogen fixing symbiosis, the invention of specialized root-derived organs, the nodules, is considered as one of the important steps in enhancing the efficiency of these interactions. SNF has been defined as requiring at least 50 non-redundantly acting plant loci with a concomitant alteration in the expression patterns of more than 1000 genes. This complexity is not surprising, considering that both the selective recognition and development of accommodation organs, the nodules, needs to be accomplished by the host plant. Using an innovative genetics approach and a combination of genomic resources, mutation analysis, and molecular genetics techniques, I was able to contribute to the demonstration that in spite of this complexity, the function of a single gene, *LHK1*, is required and also sufficient to initiate nodule organogenesis (Chapter 5, Figure 1). The corollary of this breakthrough discovery is that the *LHK1* gene was found to encode a cytokinin histidine kinase receptor, a gene not restricted to legumes but present in all plants and known to be involved in the regulation of many fundamental growth and development processes. Further insight into signal transduction elements that operate downstream from *LHK1* might allow a better understanding and subsequent engineering of nodule structures on roots of non-legume plants. An interesting question to address will be whether the formation of empty nodule structures on, for example, rice roots, would aid closer association of this plant with nitrogen fixing bacteria, which in the current rice cultivars are restricted to extracellular space.

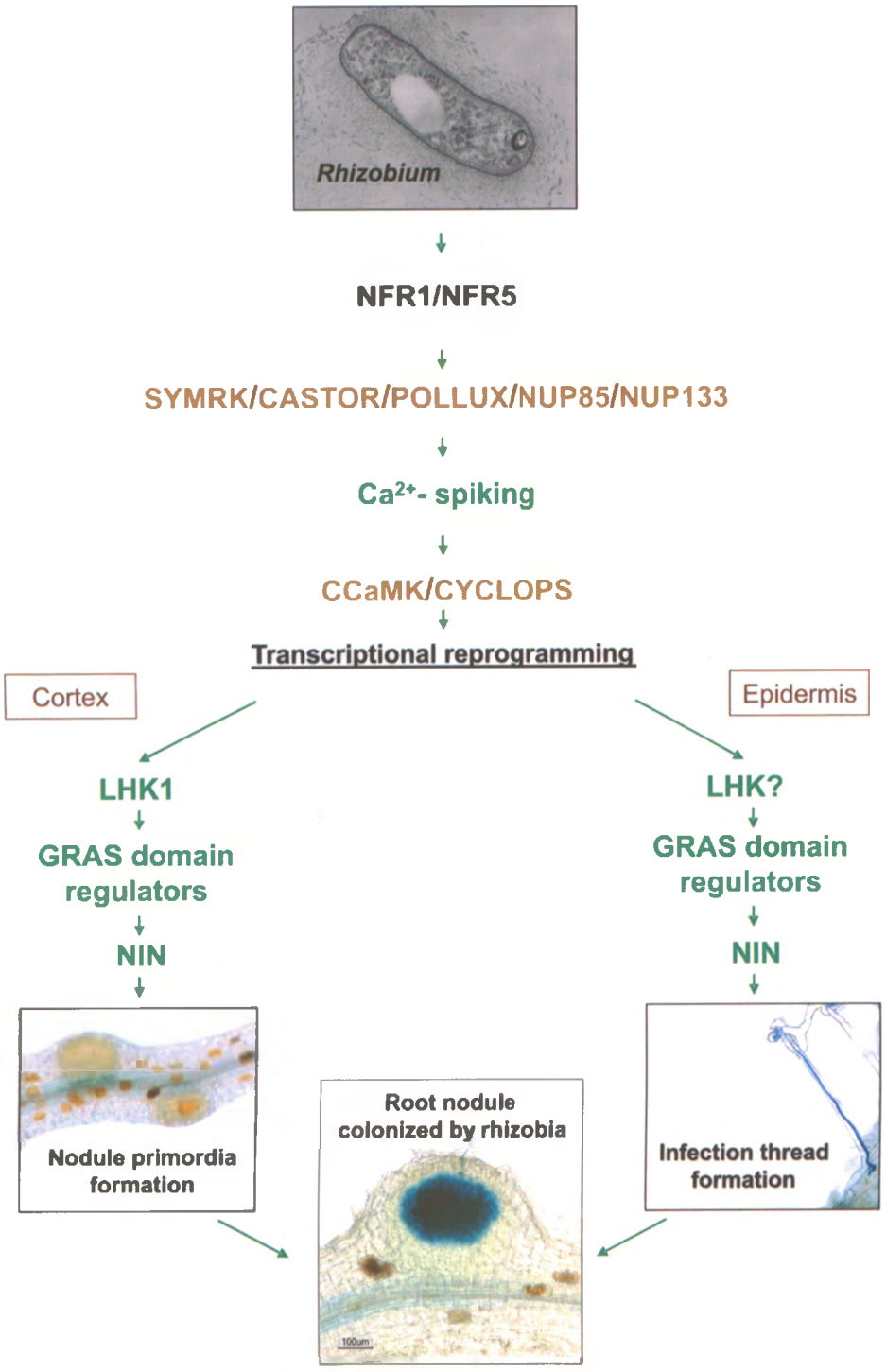

Figure 1. The proposed role of LHK1 in symbiosis pathway. LHK1 is required for activation of cortical program which leads to induction of nodule primordia. Note that a

member of LHK family might be required for activation/progression of the epidermal program.

Research directed towards the enhancement of beneficial nitrogen fixing symbiosis might help in solving another important environmental hurdle associated with the limited accessibility of soil phosphorus to plants. The analysis of the geographical distribution across major sectors of the land biosphere indicates clear prevalence of N_2-fixing plants in phosphorus limited soils of tropical savannas and lowland tropical forest (Houlton et al., 2008). Empirical support was provided for N_2 fixing plants to have an advantage in phosphorus acquisition due to the enhanced production of N-rich extracellular phosphatases, which serve to convert the soil organic P to P-ions that are available for uptake by plant roots (Houlton et al., 2008). Thus, improving or engineering new N_2 acquiring plant-microbe associations can help fix not only the N dilemma but also the P dilemma.

Finally, although the nitrogen fixing nodule symbiosis served as a primary target for this study, the resulting knowledge is expected to be applicable to a broader spectrum of plant-microbe interactions. This, in turn, should facilitate the development of a "biofertilizer" strategy or strategies that also encompass plants outside the legume family. These strategies are envisaged to be rooted in improved capacity of plants to engage in beneficial plant-microbe interactions, which constitutes the long-term goal of this research.

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Appendix I

Permission for inclusion of the Plant Physiology article: "Invasion of Lotus japonicus root hairless 1 by Mesorhizobium loti Involves the Nodulation Factor-Dependent Induction of Root Hairs" as Chapter 3 of this thesis.

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Appendix II

Permission for inclusion of the Science article: "A Cytokinin Perception Mutant Colonized by Rhizobium in the Absence of Nodule Organogenesis" as Chapter 5 of this thesis.

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