Towards a better understanding of the role of reactive oxygen species in legume root nodules

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. Ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 28 sept 2004
des namiddags te vier uur in de Aula

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Thesis Wageningen University, The Netherlands
With references – with summary in Dutch

ISBN 90-8504-092-2

Contents

Outline

Biological N_2 fixation is carried out by prokaryotes, either in the free-living form or in mutualistic symbioses with green algae, legumes and actinorhizal plants. In particular, the rhizobia-legume symbiosis is the major source of fixed N_2 into agricultural systems. Legume root nodules are formed as a result of a complex exchange of molecular signals (flavonoids, Nod factors) between the rhizobia and the plant. During the last few years, the very early stages of nodule formation are being dissected at the molecular level. Evidence has accumulated that reactive oxygen species (ROS) are also required for nodule development. However, many uncertainties still exist. It is not known what specific ROS and at what specific step(s) of nodulation do they participate, how specifically Nod factors can suppress the plant's defensive response or how ROS production escapes control during nodule senescence.

In this thesis we have devised three strategies to dissect nodule formation and senescence at the molecular and cellular levels. For all three strategies, we present optimized metodologies that may be useful for the detailed study of the genes and enzymes associated with ROS production and scavenging.

In **chapter 1** we present an overview of the biochemistry and cellular and molecular biology of the rhizobia-legume symbiosis. The emphasis is also on what is known (little) and needs to be known (much) of ROS metabolism in the symbiosis, from the early stages of the interaction to nodule senescence. From this chapter, it becomes clear that the initial steps of the interaction involves a complex mechanism of molecular signals, which are triggered after recognition of Nod factors by receptors. We are just starting to decipher the signal transduction pathway, in which oscillatory changes in cytoplasmic calcium, ethylene and several protein kinases are key players. It is becoming evident that ROS are also part of the pathway although it is unknown at what specfic stages they, and their associated enzymes, may participate.

In **chapter 2** a novel method is described that allows the isolation of root hairs, with high yield and purity, from the model legume *M. truncatula*. In the course of this work we also found in *M. truncatula* a protein, MtRH2, homologous to pea PsRH2, that is specifically localized in the root epidermis as was further demonstrated by using promoter::GFP fusions. This feature was exploited to monitor the root hair isolation procedure. We propose that this strategy will be useful for the study of ROS involvement in the early stages of symbiosis. Antioxidant enzymes can be assayed in root hair preparations before and after Nod factor perception, or targeted to the root epidermis with constructs involving the *MtRH2* promoter.

In **chapter 3** we show that *A. rhizogenes*-mediated RNAi is a fast and effective tool to study gene function. Using as an example the *KOJAK* gene of *Arabidopsis*, which is involved in root hair development, we show that it is efficiently knocked down. The fluorescent protein DsRED1 was used as nondestructive selectable marker for root co-transformation because it allows to

discriminate between chimaeric and homogeneously transformed roots. The identification of chimaeric roots allowed us to examine the extent of systemic spread of the silencing signal in the composite plants of both *Arabidopsis* and *M. truncatula*. We show that RNA silencing is not spreading to non co-transformed (lateral) roots and only with limited efficiency to the non-transgenic shoot of composite plants. This technology will greatly facilitate also the study of ROS involvement during nodule development and senescence. Constructs of RNAi for key antioxidant genes, like superoxide dismutases (SODs) and peroxidases, can be used to transform model legumes and knock down the genes.

In **chapter 4** we present a third strategy that has been proven to be very helpful to study ROS metabolism. The SOD enzymes catalyze the dismutation of superoxide radicals and thus represent a key defense against the potentially toxic effects of ROS. Transgenic alfalfa plants were designed to overexpress MnSODs in chloroplasts and mitochondria or FeSODs in the chloroplasts. We analyzed the SOD isozyme composition in the transgenic lines and showed that all of them contain low CuZnSOD activities and abundant FeSOD and MnSOD activities in nodules and leaves. We also report a novel compensatory effect in the activities of MnSOD (mitochondrial) and FeSOD (plastidic) in the leaves, which was not caused by changes in the mRNA levels and provide evidence that SOD activity in plants is regulated at least partially at the post-translational level. We also conclude that FeSOD performs important antioxidant functions other than the scavenging of superoxide radicals generated in photosynthesis.

Finally, the objective of **chapter 5** is to provide an overview of the work done and to discuss implications for studies of ROS metabolism during legume nodule formation and senescence.

Chapter 1

General Introduction

Based on:

Matamoros MA, Dalton DA, Ramos J, Clemente MR, Rubio MC, Becana M (2003) Biochemistry and molecular biology of antioxidants in the rhizobia-legume symbiosis. Plant Physiol **133:** 499-509

Ramos J, Bisseling T (2004) Symbiotic nitrogen fixation. *In:* S Amâncio, I Stulen eds, Nitrogen Acquisition and Assimilation in Higher Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 99-132

Dinitrogen (N_2) constitutes 80% of the atmosphere. The quantity of N fixed in nature is about 230 x 10^6 Tm per year. Of this, approximately 87% is the result of biological N_2 fixation, either via symbiotic associations (80%) or by free living organisms (20%) (Gutschick 1980, Vance et al. 1988). In agriculture, nitrogen fertilizers are used in addition to the N_2 fixed by rhizobia-legume symbioses. The current annual world industrial production of ammonia exceeds 130 x 10^6 Tm per year (Bakemeier et al. 1997), most of which is being produced by the costly Haber-Bosch process. Further, the excessive application of fertilizers contributes to environmental hazard by leaching of nitrate into the ground. Thus, biological fixation of N_2 has considerable advantages from an ecological as well as an economical point of view (Sainju et al. 2003).

Rhizobia is the common name given to a group of Gram-negative bacteria that have the ability to infect the roots of legumes and produce nodules (van Berkum and Eardly 1998). Once the nodule is formed, the differentiated bacteria (bacteroids), living in the host plant cells, reduce N_2 to ammonia, which is excreted to the plant cytosol where it is assimilated into organic nitrogen. The plant supplies the bacteroids with carbon skeletons (photosynthates) which are used to provide the energy required for N_2 fixation.

Molecular studies on the symbiosis are greatly facilitated by selecting *Medicago truncatula* and *Lotus japonicus* as model legumes, respectively, for indeterminate or determinate nodulation (Cook 1999, Stougaard 2001). Both legume species have a small diploid genome (approximately 450 Mb/n), are autogamous, have a short generation time and large seed production, and are amenable for transformation. In addition, a large number of mutants and tagged lines have been generated. The chloroplast genome of *L. japonicus* and the genomes of *Sinorhizobium meliloti* and *Mesorhizobium loti* (the bacterial partners) have been entirely sequenced, and the nuclear genomes of *M. truncatula* and *L. japonicus* are being sequenced at a fast pace and are most likely available in 2006 (VandenBosch and Stacey 2003).

Molecular signaling: flavonoids and Nod factors

Colonization of legume roots by rhizobia starts when the bacteria move chemotactically to the roots, where compounds present in the root exudate stimulate bacterial growth. Legume roots secrete flavonoids that serve as chemoattractant to the rhizobia (Caetano-Anolles et al. 1988). This is interesting as these compounds also activate a set of rhizobial genes, the *nod* genes, that are involved in the synthesis of a molecule that sets in motion the process of root nodule formation (for a review see Zuanazzi et al. 1998). The *nod* genes encode several enzymes required for the synthesis of the signal molecules called Nod factors. Rhizobia produce Nod factors with a similar basic structure (Fig. 1).

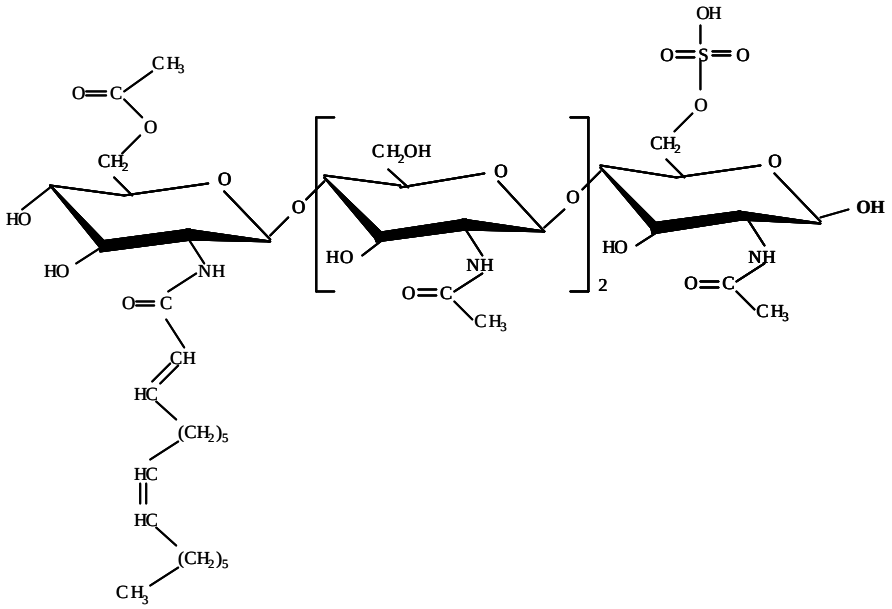

Figure 1. The major Nod factor produced by *Sinorhizobium meliloti*. Its typical characteristics are the sulfate and acetate groups at the terminal sugars as well as the length and number of unsaturated bonds of the acyl group.

This basic structure is made up of a backbone of three to five β−1,4-linked *N*-acetyl-D-glucosamine residues and a fatty acyl chain attached to the non-reducing sugar residue. Because of their structure, the Nod factors are also named lipo-chitooligosaccharides (Dénarié et al. 1996). Legumes are very sensitive to Nod factors as they can recognize very low concentrations, in some cases at the picomolar level. Therefore, it is often suggested that Nod factors are recognized by a high affinity receptor (Dénarié et al. 1996, Heidstra and Bisseling 1996). Further, the amphiphilic nature of Nod factors, with their hydrophobic lipid tail and hydrophilic sugar backbone, suggests that Nod factor receptors are located in the plasma membrane.

In all legume species tested so far, Nod factors are sufficient to induce cortical cell division that develop into primordia. In some species, such as alfalfa, complete nodules are formed (Truchet et al. 1991). Nod factors also induce several responses in the root hairs and other epidermal cells. For example, Nod factors, at picomolar concentrations, are sufficient to induce root hair deformations (Heidstra et al. 1994). Purified Nod factors are in general not sufficient to induce root hair curling or infection thread formation. The latter require the presence of the bacteria (Fig. 2). However, as rhizobial mutants that do not produce Nod factors are unable to induce root hair curling or infection thread formation, it seems likely that Nod factors are essential for the induction of these processes. In addition to root hair deformation, Nod factors induce various other responses in the epidermis. Examples are alkalinization of the medium, calcium influx at the root hair tip, calcium spiking, and membrane depolarization (Ehrhardt et al. 1992, Kurkdijan 1995, Felle et al. 1995, 1996, Shaw and Long 2003). Also, genes are induced in the

root epidermis within a few hours. The best studied of these genes are *ENOD12* and *ENOD11* (Heidstra et al. 1997, Catoira et al. 2000). In cortical cells, purified Nod factors induce cell division and preinfection thread formation, but infection threads are not formed unless rhizobia are present (van Brussel et al. 1992).

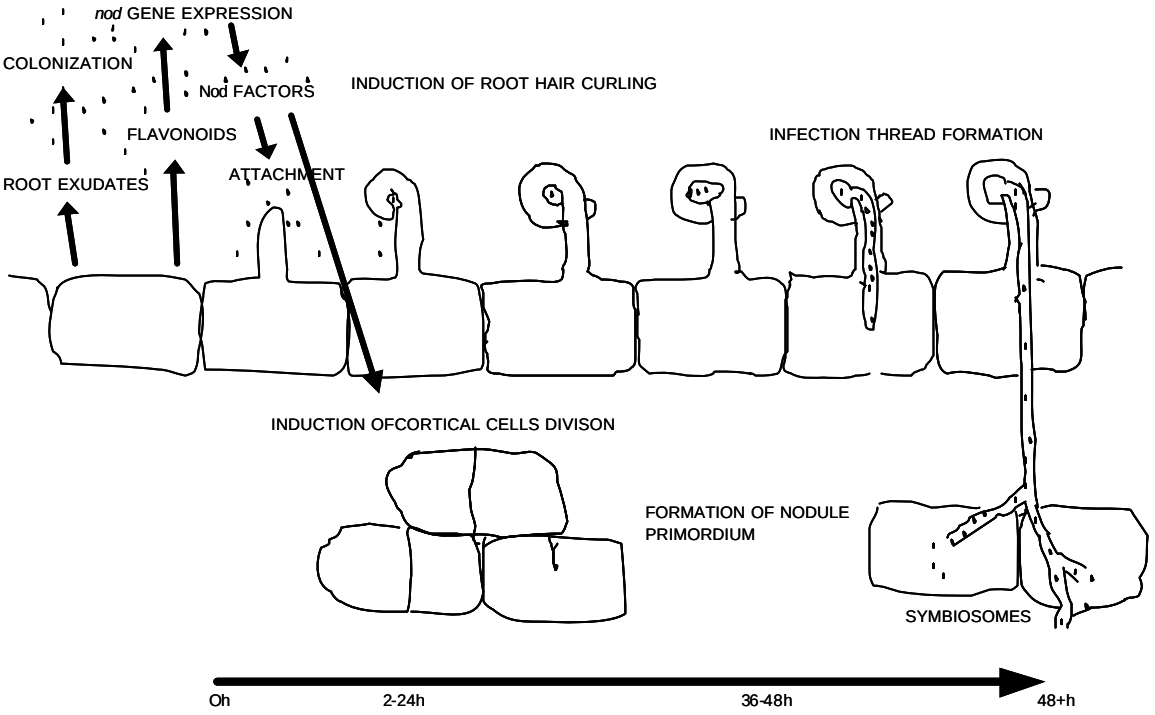

Figure 2. The infection process through the root hairs and the simultaneous formation of the nodule primordium.

Several studies have been initiated aiming to identify the Nod factor receptors. Initially, radiolabeled Nod factors were used to identify binding proteins in *Medicago* spp. Two different Nod factor-binding sites, NFBS1 and NFBS2, have been found (Cullimore et al. 2001). To unravel the mechanisms underlying Nod factor perception and transduction, a genetic approach has recently been applied. In *M. truncatula* (Cook 1999), pea (Borisov et al. 2000), and *L. japonicus* (Stougaard 2001), several genetic loci have been identified that are essential for the early steps in nodulation (Fig. 3). In most cases it has become clear that these are orthologous. A set of mutants (*dmi1*, *dmi2* and *dmi3* in *Medicago*) is blocked in almost all early steps of nodulation. In the *dmi1* and *dmi2* mutants calcium spiking is not induced by Nod factors, whereas this response is induced in the *dmi3* mutant (Wais et al. 2000). This indicates that DMI1 and DMI2 act upstream of DMI3 in the signaling pathway. Nod Factor Perception (NFP) loci have recently been cloned from *Lotus* (*LjSym1* and *LjSym5)* and pea (*Sym10*). These are receptor kinases containing LysM domains (Madsen et al. 2003, Radutoiu et al.

2003) and most likely correspond to Nod factor receptors. Similar Nod factor receptors but specifically involved in infection thread formation were cloned from *M. truncatula* (Limpens et al. 2003). DMI2 and its ortologues from alfalfa, pea, and *Lotus* have been cloned showing that these are receptor-like kinases with a extracellular domain containing three Leu-rich repeats (Endre et al. 2002, Parniske and Downie 2003) Most recently, DMI1 (Ané et al. 2004) and DMI3 (Lévy et al. 2004), and their pea homologues, have been cloned, showing that they are a putative cation channel and a calcium and calmodulin-dependent kinase, respectively.

Nodule formation

Nodule formation requires the triggering of two major processes: cortical cells have to be dedifferentiated, by which a nodule primordium is formed due to cell division, and then the bacteria have to enter the plant. The latter has to be under strict control of the plant. In the best studied infection type, rhizobia invade small emerging root hairs and are able to redirect the growth of a root hair so that a curl with shepherd's crook morphology is formed (Esseling et al. 2003). During the curling process, the bacteria become entrapped in the pocket of the curl. Subsequently, the hydrolysis of the plant cell wall is locally induced and the plasma membrane invaginates (Bauer 1981). New plant material is deposited and a tube-like structure, the so-called infection thread, is produced within the root hair cell (Brewin 1998). The bacteria proliferate in the infection thread, which progresses towards the base of the root hair. The infection thread is external to the cell, as it is surrounded by a plant membrane and contains a matrix of bacterial origin and glycoprotein of plant origin (Brewin 1998). When the infection thread reaches the base of the hair, the bacteria are released in the intercellular space and a new infection thread is formed in a cortical cell. In addition to Nod factors, rhizobial surface polysaccharides are essential factors in the root infection process. Exopolysaccharides contribute to bacteria protection, antigenicity, nutrient gathering, and attachment to surfaces. The ability to establish an effective symbiosis is severely affected in many rhizobial mutants that are deficient in EPS production. In general, they induce nodules devoid of bacteria due to abortion of infection threads or they do not infect at all (Rolfe et al. 1996). Lipopolysaccharides are also often required for proper infection (Perotto et al. 1994).

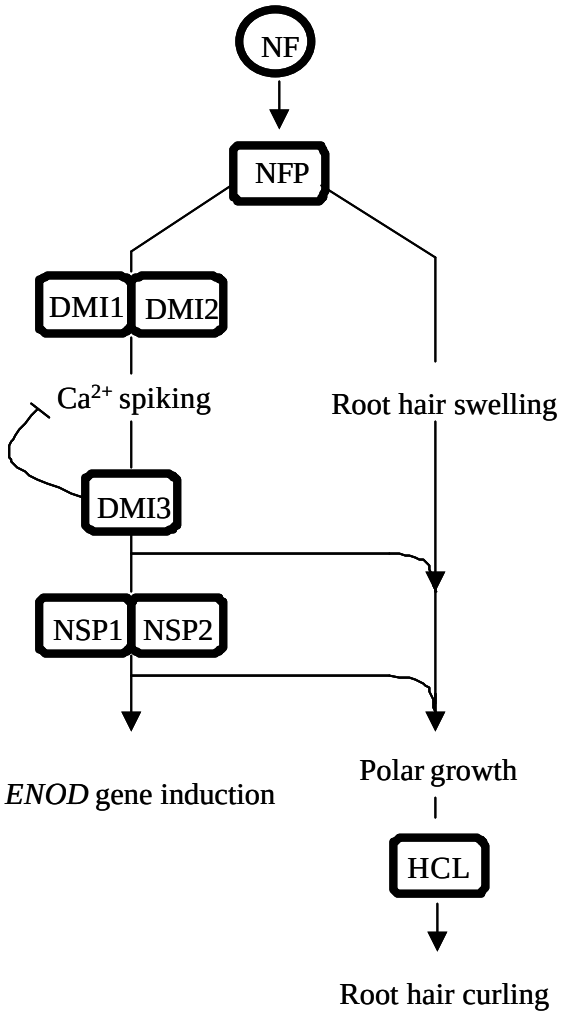

Figure 3. Genetic dissection of the Nod factor signaling pathway. MtNFP is blocked in all Nod factor-induced responses, but the arbuscular-mycorriza symbiosis is not affected. NFP is a good candidate to be directly involved in Nod factor perception. The *Mtdmi1, 2* and *3* mutants still respond to Nod factors with root hair swelling but they fail to reestablish tip growth. Because *Mtdmi1* and *2* mutants cannot respond with Ca spiking, they are placed upstream of *Mtdmi3*. *Mtdmi1, 2* and *3* mutants fail to establish the arbuscular-mycorriza symbiosis and therefore are part of a common signaling pathway activated by both microsymbionts. In the *nsp* mutants, tip growth can be reestablished but in a rather inefficient manner. Furthermore, the induction of gene expression is blocked. *HCL* acts downstream of *nsp* and in this mutant curling and infection are disturbed *(from Limpens and Bisseling 2003)*.

Differentiation into a nodule starts when the infection thread reaches the nodule primordia. Legume nodules can be divided into two types: indeterminate (e.g. pea, alfalfa, vetch) and determinate (e.g. soybean, common bean, *Lotus*) (Fig. 4). The nodule type is a feature of the host plant (Mylona et al. 1995). The overall tissue organization of both forms is similar in having a central tissue, which contain infected cells harboring the bacteroids and uninfected cells that are interspersed between the infected cells. The uninfected or interstitial cells have a specific function in the nitrogen assimilation process. The central tissue is surrounded by the cortex, endodermis, and parenchyma. The nodule parenchyma contains the vascular bundles (van de Wiel et al. 1990). A main difference between the two types of nodules is that indeterminate nodules have a

persistent meristem at their tip, whereas in indeterminate nodules the meristematic activity ceases at an early stage of development. Another major difference concerns their ontogeny, as indeterminate nodules originate from primordia formed in the inner cortex. Primordia formed in the outer cortex are the start of determinate nodule development.

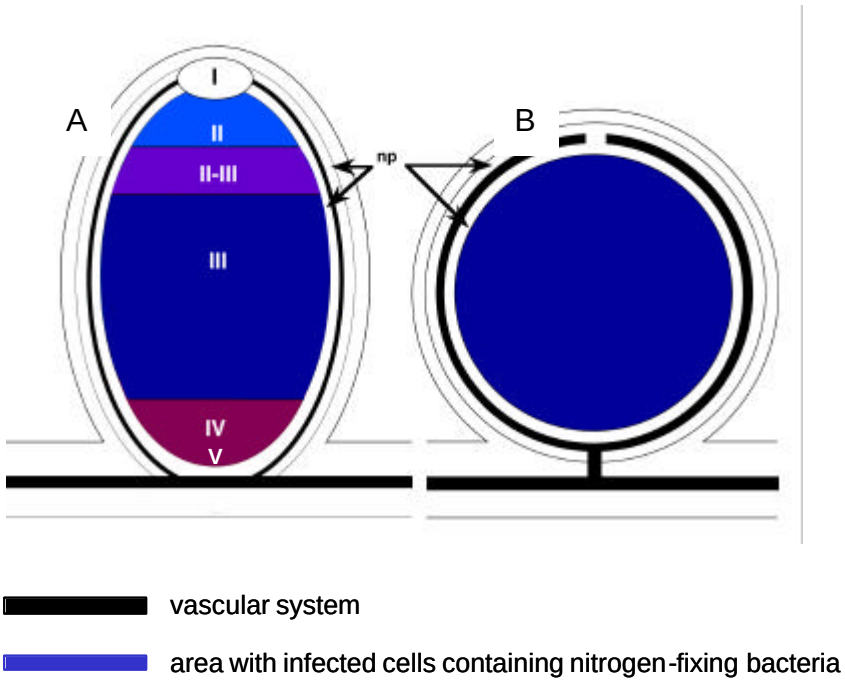

Figure 4. Structure of legume nodules. **A**, Indeterminate nodule. Five zones can be distinguished (Vasse et al. 1990). Directly below the meristem (I), in the prefixation zone (II), cells become infected. Rhizobia are enclosed by the peribacteroid membrane (pbm) and start to differentiate into their symbiotic form, the bacteroids. In the interzone (II-III), bacterial N_2 fixation starts and takes place throughout the N_2 fixation zone (III). In the senescent zone (IV), bacteria are degraded. In zone V, there are undifferentiated rhizobia originating from a late release of bacteria from infection threads lying in the intercellular spaces. The O_2 diffusion barrier is located in the nodule parenchyma (np). **B**, Determinate nodule. The central tissue is surrounded by the np (in which the vascular bundles are located), the endodermis and the cortex. All cells in the central tissue are more or less at the same developmental stage *(modified from Pawlowsky and Bisseling 1996)*.

The persistent meristem in indeterminate nodules produces the continuous addition of new cells at the apex, and therefore the tissues are composed of cells at different stages of development, with the youngest cells near the meristem and the oldest cells near the root attachment point (Fig. 4). The nodule acquires a cylindrical-shape, and it is possible to classify histologically different zones of the central tissue (Vasse et al. 1990, Franssen et al. 1992). The meristem at the nodule apex is called zone I, consisting of non-infected small cytoplasmic rich cells. This is followed by the prefixation (or infection) zone II, where bacteria are released from the infection threads. At the proximal part of this zone, plant cells elongate and bacteria start the differentiation into bacteroids and proliferate. N_2 fixation takes place in zone III. A senescent zone IV is present in old nodules and is located in the most proximal part (the base) of the

nodule. In determinate nodules, cells are at a similar stage of development as their mitotic activity stops soon after infection. The nodule grows then only by cell enlargement due to the absence of an apical meristem. These nodules have a spherical form (Cermola et al. 2000). Recently, a saprophytic zone V (proximal to zone IV) has been described in senescent alfalfa nodules and possibly other indeterminate nodules. In zone V bacteria are released from remaining infection threads, leading to the reinvasion of plant cells that have completely senesced (Timmers et al. 2000).

A nodule host cell may contain several thousand rhizobial symbionts, which are located in a new kind of compartment in the plant cell, the symbiosome. Bacteroids are surrounded by a peribacteroid membrane originating from the plant plasma membrane. However, the peribacteroid or symbiosomal membrane also shares properties with the vacuolar membrane and contains several nodule-specific proteins (nodulins) performing unique functions in the symbiosis. In indeterminate nodules, normally there is a single bacteroid per symbiosome (Vasse et al. 1990). In determinate nodules, the number of bacteroids per symbiosome varies due to merging of the symbiosome membrane rather than by bacterial division (Cermola et al. 2000).

Nitrogen and carbon metabolism in nodules

The reduction of N_2 to NH_3 is catalyzed by the nitrogenase enzyme complex and is a high-energy demanding process (960 kJ per mol of fixed N_2):

$$N_2 + 16\ ATP + 10\ H^+ + 8e^- \longrightarrow 2\ NH_4^+ + H_2 + 16\ ADP + 16\ P_i$$

Nitrogenase has a molecular mass of 220 kD and is composed of two ferrosulfoproteins (dinitrogenase and dinitrogenase reductase) and a cofactor containing Fe and Mo, where the catalytic site is located. The dinitrogenase reductase is reduced *in vivo* by low-potential electron donors, and then electrons are transferred one at a time to the dinitrogenase, with the hydrolysis of two ATP molecules at each electron transfer. All three components of nitrogenase are extremely sensitive to O_2 and become irreversibly inactivated in air.

The high energy costs of N_2 fixation are met by photosynthesis. Thus, photosynthates (mainly sucrose) are transported from the shoot to the nodules via phloem. In nodules, sucrose is hydrolyzed to monosaccharides, which are used for the biosynthesis of organic acids. The high metabolic activity of the nodule is reflected by the abundance of phosphoenolpyruvate carboxylase, which accounts for about 1-2 % of the total soluble protein of nodules (Pathirana et al. 1992). This enzyme catalyzes the formation of oxalacetate, which is subsequently reduced to malate. This dicarboxylic acid is the major energy source for the bacteroids and plant mitochondria, and is used for NH_4^+ assimilation as carbon skeleton in the glutamine

synthase/glutamate synthase pathway (Stitt et al. 2002). The primary product of N_2 fixation is NH_4^+ which is excreted by the bacteroids/symbiosomes. NH_4^+ is poisonous to plant cells and is therefore immediately incorporated into amino acids (Day et al. 2001). Consequently, an essential part of the symbiosis is to make the NH_4^+ produced by the bacteroid accessible to the host. The main pathway for NH_4^+ assimilation involves two enzymes: glutamine synthase, which catalyzes the incorporation of NH_4^+ into glutamate, and NADH-glutamate synthase, which transfers the amide group of glutamine to α−ketoglutarate (Miflin and Habash 2002). Assimilated NH_4^+ is exported from the nodules to the shoot. Legumes with indeterminate nodules transport N in the form of amides, such as glutamine and asparagine, whereas legumes with determinate nodules transport N as ureides, such as allantoin, allantoic acid, and citruline (Smith and Atkins 2002).

Oxygen metabolism in nodules: oxygen diffusion barrier and leghemoglobin

Bacteroids produce ATP by oxidative phosphorylation and consequently have a high O_2 demand. On the other hand, O_2 at high concentrations represses nitrogenase genes and inactivates the enzyme within minutes (Dixon and Wheeler 1986). To solve this dilemma, nodules have mechanisms to provide a low, but steady O_2 supply to the infected cells, while avoiding inactivation of nitrogenase. The main mechanisms include an O_2 diffusion barrier in the mid-inner cortex (nodule parenchyma) and leghemoglobin in the cytosol of infected cells. The occurrence of an O_2 diffusion barrier was first postulated on the basis of direct measurements of O_2 in nodules with selective microelectrodes (Witty et al. 1986). Further experiments revealed that the resistance of the O_2 diffusion barrier has both fixed and variable components. Thus, the barrier adjusts quickly its variable component in response to changes in the external O_2 concentration, so that there is a fine tuning of the O_2 that reaches the central, infected tissue of the nodule. It is generally assumed that the variable component of the barrier is due, at least in part, to changes in the water content of the intercellular spaces of the nodule parenchyma. At least three proteins have been localized specifically to the intercellular spaces of the nodule parenchyma, which suggests that they play a role in the regulation of the O_2 diffusion barrier. These proteins are a glycoprotein (VandenBosch et al. 1989), the hydroxyproline-rich protein ENOD2 (Nap and Bisseling 1990), and a lectin (VandenBosch et al. 1994). However, the molecular mechanism for the operation of the O_2 diffusion barrier still needs to be elucidated.

Once O_2 has reached the infected cells, leghemoglobin, a very abundant (1-5 mM) O_2-carrying hemoprotein, has a critical role in regulating O_2 supply to the bacteroids (Appleby 1992). The very high affinity of leghemoglobin for O_2 (due to a high association rate and a low dissociation rate) is critical to facilitate O_2 delivery to the bacteroids at a very low concentration of free O_2 (10-50 nM) in the cytosol of infected cells (Kuzma et al. 1993). Leghemoglobin needs

to be maintained in the ferrous state to transport and buffer O_2 concentration in the cytosol, but it is very prone to autoxidation, which is favored by the slightly acid pH of the cytosol, and to oxidation by free radicals and other reactive oxygen species (ROS). Leghemoglobin is oxidized to its ferric, inactive form by the superoxide radicals and H_2O_2 (for a review see Becana and Klucas 1992).

Oxygen metabolism in nodules: reactive oxygen species and superoxide dismutases

Although O_2 concentration in the infected region is quite low, several factors are conducive for ROS production in nodules: abundance of leghemoglobin, low redox potential, slightly acid pH (5.5-6.5), and the presence of proteins, such as nitrogenase, hydrogenase, ferredoxin, uricase and flavoenzymes, which can generate ROS (Table 1). Nodules contain an impressive array of antioxidant metabolites and enzymes that scavenge or prevent the formation of the most aggressive ROS, thus protecting N_2 fixation (Dalton 1995). Additionally, antioxidant enzymes control the steady-state levels of the moderately reactive ROS, allowing them to perform important roles at specific sites, environmental conditions, or developmental stages of nodules.

The major antioxidant enzyme systems in nodules and other plant organs are the ascorbate-glutathione pathway and the superoxide dismutase (SOD) enzyme family. Both are essential for N_2 fixation (Table 2). The ascorbate-glutathione pathway involves four enzymes [ascorbate peroxidase (APX), monodehydroascorbate reductase, dehydro- ascorbate reductase, glutathione reductase], which operate in concert to remove H_2O_2 at the expense of the reducing power of NADH or NADPH. Several lines of evidence show that this pathway is critical for nodule functioning (Dalton 1995, Dalton et al. 1998). The activity, protein, and transcript of ascorbate peroxidase, the key enzyme of the pathway, are very abundant in nodules, particularly in the infected and parenchyma cells (Fig. 5A).

In the infected cells, APX protects leghemoglobin and other redox-sensitive proteins from H_2O_2, whereas in the nodule parenchyma (a few cell layers outside the infected zone) the enzyme may participate in the operation of the O_2 diffusion barrier. This barrier has been proposed to be located, for the most part, in the nodule parenchyma and controls O_2 entry into the infected zone. The parenchyma cells have not only high levels of ascorbate peroxidase but also of ascorbate and respiratory dehydrogenases (Fig. 5B).

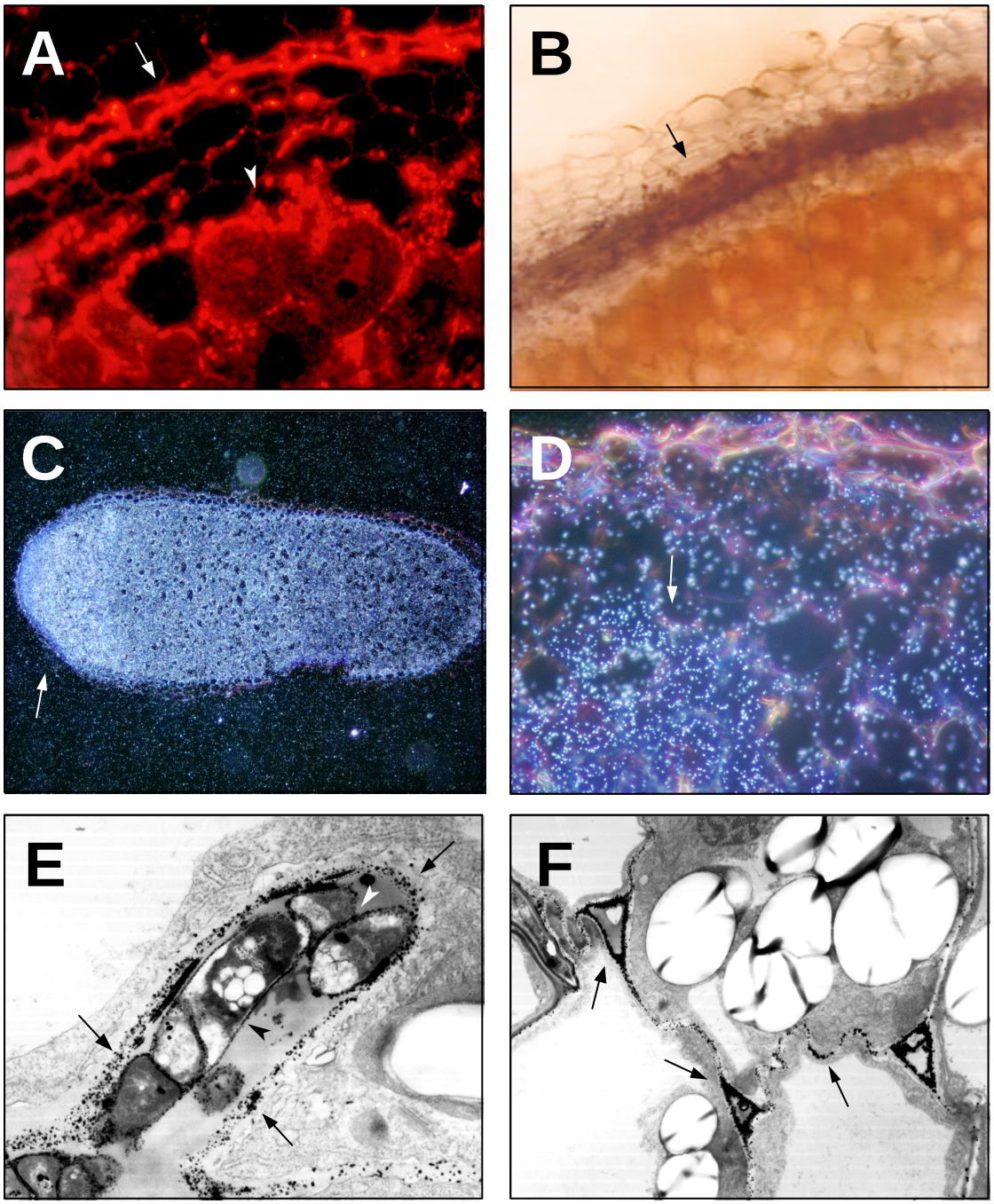

Figure 5. Localization of APX, SODs, and H_2O_2 in alfalfa nodules. **A,** Immunofluorescence localization of cytosolic APX. High levels are evident in the central, infected region (arrowhead) and in a ring of cells in the nodule parenchyma (arrow) *(from Dalton et al. 1998)*. **B,** Tetrazolium staining of respiratory dehydrogenase activity. Activity is enhanced in the nodule parenchyma (arrow), indicating increased respiration associated with the O_2 diffusion barrier and probably with the enhanced level of APX protein shown in A *(from Dalton et al. 1998)*. **C,** *In situ* hybridization of cytosolic CuZnSOD mRNA. Transcript is most abundant in the nodule apex (arrow), which include the meristem and invasion zones *(from Matamoros et al. 2003)*. **D,** *In situ* hybridization of MnSOD mRNA. Transcript is most abundant in the infected region, and especially in the infected cells (arrow) *(from Matamoros et al. 2003)*. **E** and **F,** Localization of H_2O_2. Fresh nodule tissue was perfused with cerium chloride and processed for electron microscopy. The presence of H_2O_2 is marked by the deposition of cerium perhydroxide precipitates, which can be seen in the walls and matrix of infection threads (arrows in E) and in the cell walls and intercellular spaces of the cortex (arrows in F). Note that H_2O_2 can be also observed surrounding the bacteria within the threads (arrowhead in E) *(from Matamoros et al. 2003)*.

Thus, parenchyma cells would regulate O_2 access to the infected region by adjusting their respiratory activity (Dalton et al. 1998). The concentration of the resulting H_2O_2 would be then finely tuned by ascorbate peroxidase, allowing for H_2O_2 to act as a signal molecule for the 'opening' or 'closure' of the O_2 diffusion barrier (Minchin 1997, Dalton et al. 1998).

Table 1. Production of ROS in nodules

ROS	Cellular source
Superoxide radical	Electron transport chains of mitochondria, bacteroids, plastids, endoplasmic reticulum, peroxisomes, and plasma membrane. NADPH oxidase in membranes. Oxidation of leghemoglobin in cytosol. Xanthine oxidase and membrane polypeptides in peroxisomes. Oxidation of nitrogenase and ferredoxin in bacteroids.
H_2O_2	Electron transport chains of mitochondria, bacteroids, plastids, endoplasmic reticulum, and plasma membrane. CuZnSOD in cytosol and plastids, MnSOD in mitochondria and bacteroids, FeSOD in plastids. Fatty acid β–oxidation, urate oxidase, and MnSOD in peroxisomes.
Organic and lipid peroxides	Nonenzymatic lipid peroxidation. Lipoxygenase.
Hydroxyl radical	Reaction of superoxide radical with H_2O_2 catalyzed by trace amounts of Fe or Cu.

SODs are a family of metalloenzymes that catalyze the dismutation of superoxide radicals to O_2 and H_2O_2. Three classes of SODs, differing in their metals at the active site, may coexist in plants and all of them have been found in the nodule plant fraction (Table 2). Recently, the transcripts (and proteins) of cytosolic CuZnSOD and mitochondrial MnSOD have been localized in alfalfa nodules (Fig. 5C, 5D). The CuZnSOD transcript and protein is predominant in the nodule apex, especially in the infection threads, cytosol adjacent to cell walls, and apoplast. In contrast, the MnSOD transcript and protein is abundant in the infected zone, especially in the infected cells. An additional CuZnSOD isozyme, the plastid CuZnSOD, is localized to the amyloplasts, whereas MnSOD is also found in the bacteroids and bacteria within infection threads. The distinct tissue localizations of 'cytosolic' CuZnSOD and MnSOD suggest specific functions for the two enzymes. The CuZnSOD may be associated with cell wall growth in the meristems, infection threads, and apoplast, and with the plant's response to bacterial infection.

The MnSOD would play a role related to the protection and functioning of symbiotic tissue in mature nodules. The FeSODs are the less known SODs in plants but they may perform additional roles to the detoxification of superoxide radicals associated with photosynthesis because the FeSODs are abundant in nodules. In nonphotosynthetic tissue, FeSODs are assumed to be located in the plastids and amyloplasts.

Table 2. Biochemical properties of important antioxidant enzymes of legume nodules

Enzyme	Localization and biochemical properties
CuZnSOD	In cytosol and plastids. Dimer (32 kD, 2 Cu, 2 Zn). Inhibited by KCN and H_2O_2.
MnSOD	In mitochondria and bacteroids. The plant enzyme is a tetramer (82 kD, 4 Mn). The bacterial enzyme is a dimer (43 kD, 2 Mn) and may be cambialistic. Resistant to KCN and H_2O_2.
FeSOD	In plastids and cytosol. Dimer (56-58 kD, 2 Fe). Structurally related to MnSODs. Inhibited by H_2O_2 but resistant to KCN.
Catalase	In peroxisomes and bacteroids. The plant enzyme is a tetramer (220 kD, 4 heme). Inhibited by KCN and aminotriazole. The bacterial enzymes have a subunit size of 63 kD.
Ascorbate peroxidase	Mainly in cytosol (0.9% of total soluble nodule protein). Dimer (subunits of 27 kD, 2 heme). They are inactivated by *p*-chloromercuribenzoate and strongly inhibited by KCN. The cytosolic isoform is distinguished from chloroplastic isoforms by its insensitivity to ascorbate depletion. All isoforms use ascorbate effectively as a reductant, in contrast to classical (guaiacol) peroxidases that do not. K_m = 300 µM for ascorbate and 20 µM for H_2O_2. Membrane-bound isoforms exist in mitochondria and possibly in peroxisomes.
Glutathione reductase	In cytosol, plastids, and mitochondria. Probably, a tetramer (135-190 kD; subunits of 32-60 kD), K_m=23 µM for GSSG and NADPH.

Reactive oxygen species: implication in nodule formation and senescence

Infection of legume roots by rhizobia elicits a hypersensitive reaction. After the first nodule primordia have been formed, an increasing proportion of infection threads abort in a few cortical cells in which both rhizobia and host cells undergo necrosis. The hypersensitive reaction may be part of a mechanism whereby the plant controls infection and thus regulates nodulation (Vasse et

al. 1993). As in the case of attack by pathogens, root cells respond to rhizobial infection with an enhanced production of superoxide and H_2O_2 (Santos et al. 2001, Ramu et al. 2002, D'Haeze et al. 2003). It has not been definitively proven that this is a genuine oxidative burst, but the finding that H_2O_2 accumulation is restricted to the very early stages of nodule formation in *Sesbania* supports this hypothesis. Interestingly, one of the genes more rapidly induced by compatible rhizobia or Nod factors, *rip1*, seems to encode a peroxidase and has *cis*-elements in its promoter region that may be responsive to ROS. Because exogenous H_2O_2 is sufficient to activate *rip1* transcription in the absence of Nod factors, ROS may act downstream in the signal transduction pathway (Ramu et al. 2002). In this respect, both Ramu et al. (2002) and D'Haeze et al. (2003) have concluded that Nod-factor induced nodulation requires H_2O_2.

Most likely, the 'early' production of H_2O_2 is part of an oxidative burst, but, in later stages of nodule formation, H_2O_2 accumulation may be more related to cell wall formation and cross-linking of glycoproteins, both of which are required for succesful infection. An as yet unsolved question is, however, why some rhizobia have success during infection and form functional nodules. It is thought that during infection, rhizobia may escape or inhibit the defensive response. This inhibition has been attributed to the bacterial exopolysaccarides (González et al. 1996). The enzymes responsible for enhanced ROS formation during infection and nodule organogenesis have not been definitively identified. The superoxide radicals are formed in the infection threads (Santos et al. 2001), possibly by a membrane-bound NADPH-oxidase (superoxide synthase), much like the superoxide generation during the oxidative burst in activated neutrophils. Possible sources for H_2O_2 are cell-wall peroxidases, germin-like oxalate oxidases, and diamine oxidases (Wisniewski et al. 2000). We have found that H_2O_2 accumulates in the walls and lumen of infection threads, surrounding bacteria within the threads, and in the apoplast of the nodule cortex. Based mainly on co-localization studies, we propose that CuZnSOD is a source of H_2O_2. This may be important for the cross-linking of cell wall proteins in the apoplast and of the matrix glycoprotein in the infection threads (Wisniewski et al. 2000).

There is a second period in the lifetime of nodules characterized by an enhanced production of ROS. Large amounts of H_2O_2 accumulate in the cells and apoplast in the central zone of senescing soybean nodules (Alesandrini et al. 2003), as well as in surrounding bacteroids in the senescent zone of alfalfa nodules (Fig. 5E, 5F). In the senescing nodule tissue there is a major decrease in antioxidant defenses, oxidative degradation of leghemoglobin to nonfunctional green pigments, and enhanced autolytic processes (Mellor 1989, Matamoros et al. 1999). These are all situations conducive to uncontrolled ROS production. As a consequence, oxidative damage of lipids, proteins, and DNA has been observed in nodules during natural (Evans et al. 1999) and stress-induced (Becana and Klucas 1992, Matamoros et al. 1999) senescence. Similarly, the structural breakdown of organelles, symbiosomes, and bacteroids in the host cells usually accompanies senescence. All these structural and biochemical alterations may be interpreted in

terms of a switch from a reductive to an oxidative state, which may be a general characteristic of plant senescence (Swaraj and Bishnoi 1996).

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Chapter 2

A Method for the Isolation of Root Hairs from the Model Legume Medicago truncatula

Ramos J, Bisseling T (2003) A method for the isolation of root hairs from the model legume *Medicago truncatula.* J Exp Bot **54:** 2245-2250

ABSTRACT

A new method for the isolation of root hairs from the model legume, *Medicago truncatula*, was developed. The procedure involves the propagation of detached roots on agar plates and the collection of root hairs by immersion in liquid nitrogen. Yields of up to 40 µg of root hair protein were obtained from 50-100 root tips grown for 3 weeks on a single plate. The high purity of the root hair fraction was monitored by western blot analysis using an antibody to the pea epidermis specific protein PsRH2. Sequence analyses revealed that the protein homologous to PsRH2 in *M. truncatula*, MtRH2, is identical to the root protein MtPR10-1. The MtRH2 protein proved to be a useful endogenous marker to monitor root hair isolation since it is specifically expressed in the root epidermis and its root hairs.

INTRODUCTION

Leguminous plants are able to establish a symbiosis with soil bacteria collectively known as rhizobia. This interaction culminates in the formation of root nodules and in the differentiation of bacteria into bacteroids, which are capable of reducing atmospheric N_2 into ammonia (for a review see Mylona et al. 1994). Nodule formation involves responses of the host in various root tissues. For example, cell divisions occur in the cortex, whereas deformation and curling of root hairs occur in the epidermis. Both processes are set in motion by signal molecules, the so-called lipochito-oligosaccharides or Nod factors, which are secreted by the bacteria (Dénarié and Cullimore 1993).

Studies on the very early steps of the plant-rhizobial interaction are facilitated if root hairs can be isolated as this would allow biochemical analysis. Root hairs are tubular extensions of epidermal cells that are formed on specialized protoderm cells called trichoblasts (Peterson and Farquhar 1996). Roots are indeterminate growing organs and, as a consequence, root hairs at successive stages of development are lined up along the root. The elongation of root hairs takes places at their tip, and their normal growth as well as Nod factor-induced root hair deformation require a functional actin skeleton (de Ruijter et al. 1999). Root hairs can be classified by their position on the roots, their cytoarchitecture, and their response to Nod factors (Heidstra et al. 1997). Zone I of roots contains growing young root hairs that do not deform upon treatment with Nod factors and that have, at their tip, a relatively large zone devoid of organelles but rich in vesicles. Zone II consists of almost full-grown root hairs, which are able to deform upon application of Nod factors. Zone III contains mature root hairs that lack the vesicle-rich zone and do not deform upon exposure to Nod factors.

Studies on the responses of root hairs to Nod factors have focused on the model legume *Medicago truncatula*. This species has considerable advantages for molecular genetic studies as compared to crop legumes. For example, it has a small diploid genome, self-fertile flowers, and a short generation time (Barker et al. 1990). In addition, powerful genetic tools have been developed for this legume species, including the efficient transformation of several ecotypes by *Agrobacterium tumefaciens* and *A. rhizogenes* (Boisson-Dernier et al. 2001), and the production of BAC libraries, detailed genetic maps, and numerous nodulation and mycorrhizal mutants (Penmetsa and Cook 1997). In this paper, a highly efficient method is described for the isolation of root hairs of *M. truncatula* and the isolation procedure is monitored in detail by using molecular markers.

RESULTS

Root hair isolation procedure

Initially an attempt was made to adapt a method for root hair isolation that was previously used successfully for pea (Röhm and Werner 1987, Gloudemans et al. 1989). However, the root hair fraction obtained with this method was heavily contaminated with other plant material. The roots of *M. truncatula* are considerably thinner than those of pea, and hence it is likely that fragmentation of roots into small pieces occurred before the root hairs were released, resulting in contamination and poor recovery of root hairs. Western blot analysis confirmed that this procedure did not lead to an enrichment of root hair protein (data not shown).

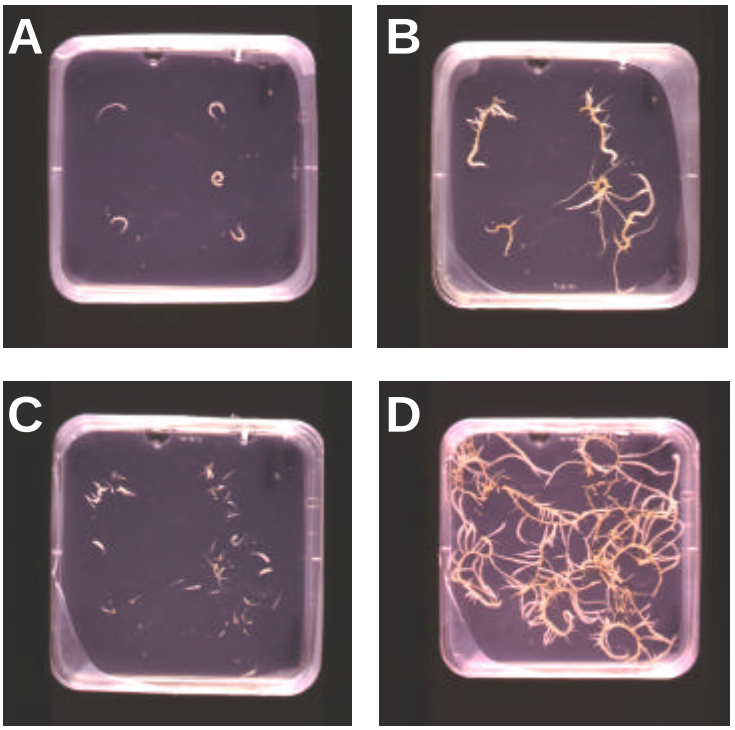

Figure 1. Propagation of detached roots on agar SH medium. The main root of a 3-d-old seedling was detached and allowed to grow for 1 week. Five explants were then taken and placed on a new plate (A). Approximately 1 week later, lateral roots had developed (B). The older tissue was then removed and the root tips were left on the plate (C). After another week, roots had grown profusely and were ready to be used for root hair isolation (D).

To increase root hair yield while minimizing root fragmentation, a procedure was developed to grow roots of *M. truncatula* (R108-1) on the surface of agar plates, so that the root hairs stick out. Root explants were grown on 1.5% agar and 1.5% sucrose in SH medium. The main root of a seedling was detached from the shoot and placed on the agar medium. After 7-10 d, the explant had elongated and developed lateral roots. These lateral roots were subsequently used to

create new explants and were moved onto a new plate (Fig. 1A). A week later the explants had formed lateral roots and covered a larger area of the plate (Fig. 1B). The older parts of the roots were removed and only a 3–6 mm long piece from each lateral root tip was left on the plate (Fig. 1C). If only young root hairs (zones I and II) were to be collected, the plates were used at this time. Otherwise, roots were allowed to grow for another week before collecting the root hairs (Fig. 1D). The roots stayed alive for several months in these conditions, eventually completely covering the plate.

To collect the root hairs, the entire Petri dish was immersed in liquid nitrogen and the surface gently brushed while the plate was submerged in the nitrogen. The root hair suspension was collected and passed through a 400 µm nylon mesh to remove the larger particles. Light microscopy confirmed the high purity of the root hair preparation. However, the weight of the collected root hairs could not be measured, as condensed water would make it unreliable. The amount of total root hair protein produced by the method of Röhm and Werner (1987) from 40 3-d-old seedlings of *M. truncatula* was always <1 µg, whereas, with this method, 25-40 µg of root hair protein was usually obtained from a single 3-week-old plate, containing 50-100 root tips.

The ability of root explants to grow in the absence of hormones was also examined to determine whether the method is specific for *M. truncatula* (R108-1) or can be used for other cultivars (e.g. Jemalong) or species (e.g. *Lotus japonicus* and *M. sativa)*. At the initial stage, root explants of Jemalong behaved like those of line R108-1, growing and developing lateral roots; however, about 2 weeks after detachment from the plant, growth had almost ceased and older tissue was withering and dying. Root tips were able to remain alive on the plates for several weeks, even months, but they did not grow further. However, hairy root explants produced in *M. truncatula* (Jemalong) (Boisson-Dernier et al. 2001, Ramos, unpublished data), *L. corniculatus* (Petit et al. 1987), or *L. japonicus* (Stiller et al. 1997) after infection with *A. rhizogenes* behaved similarly to *M. truncatula* (R108-1); that is, they were able to grow indefinitely. Likewise, detached roots of untransformed *M. sativa* (Aragón) plants were able to propagate *in vitro*.

MtRH2 is a useful marker to monitor root hair isolation

To test the efficiency of the root hair isolation procedure, advantage was taken of the specific expression of *PsRH2* in the root epidermis (Mylona et al. 1995). Constructs were made of the *GFP* gene under the control of the *PsRH2* promoter and these were used to transform *M. truncatula* plants (R108). Fluorescence microscopy showed that GFP expression was confined to the root epidermis and that GFP accumulates in the root hairs of zones I and II (Fig. 2). Thus, in *M. truncatula* transgenic plants, GFP-driven expression by the *PsRH2* promoter is a convenient marker to monitor root hair isolation and to determine the enrichment. Further, roots

of *M. truncatula* plants transformed transiently by *A. rhizogenes* (Boisson-Dernier et al. 2001) harbouring a *PsRH2::GUS* construct confirmed this expression pattern and the lack of expression at the root tip (data not shown).

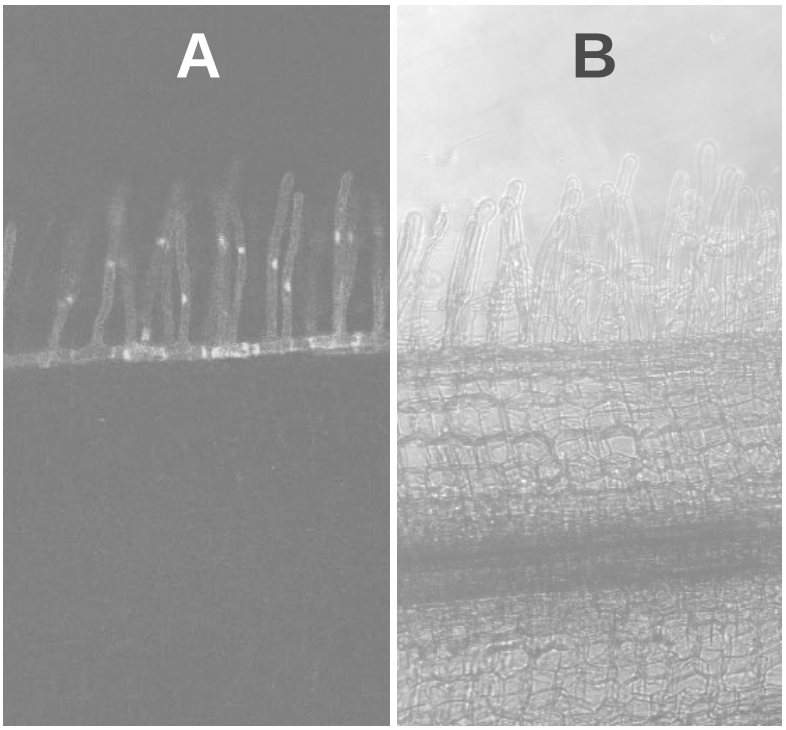

Figure 2. Fluorescence (A) and white light (B) microphotographs of a 3-d-old seedling root. Note that GFP accumulation is localized in the root epidermis.

To find out whether the RH2-like *M. truncatula* protein or mRNA can be detected in root hairs, immunoblots and RT-PCR were used. Protein extracts from roots and root hairs of untransformed plants were analysed by western blotting using the anti-PsRH2 antibody. A single immunoreactive protein band was observed, corresponding to the expected molecular mass (12 kDa) of PsRH2 (Fig. 3). This protein, tentatively identified as MtRH2, was far more abundant in root hairs than in whole root preparations, which provides strong evidence that MtRH2 is localized specifically in the epidermis, as occurs with PsRH2 (Mylona et al. 1995). The expression of the corresponding gene was analysed by RT-PCR with primers based on the *PsRH2* sequence. Figure 4 shows that the transcript was far more abundant in root hairs than in total root RNA preparations. The 253-bp product obtained by RT-PCR was sequenced and its homology at the nucleotide level was found to be 87 % with *PsRH2* and 100 % with *MtPR10-1*, a gene that is constitutively expressed in roots and pathogen-induced in leaves (Gamas et al. 1998). Hence, *MtRH2* is identical to the previously reported *MtPR10-1* gene.

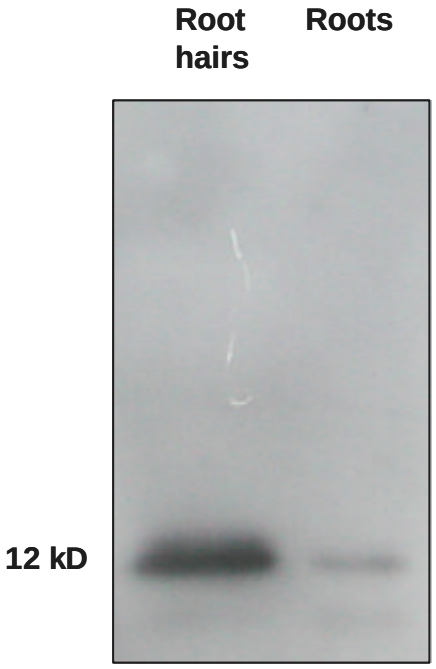

Figure 3. Western blot analysis of proteins from total roots (10 mm including root tips) and root hairs. In both cases, a single immunoreactive protein band was observed, which corresponded to the expected molecular mass (12 kDa) of PsRH2. Lanes were loaded with 5 µg of protein.

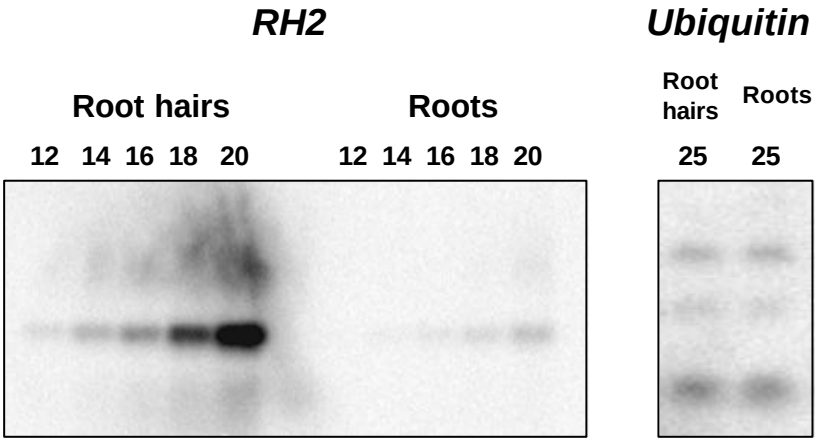

Figure 4. Expression analysis of the *MtRH2* gene by RT-PCR using primers based on the *PsRH2* sequence. The amounts of cDNA template from root hairs or total roots were standardized with ubiquitin. The number of PCR cycles is indicated for each lane to show that the signal is below saturation. The transcript was clearly more abundant in root hair than in total RNA root preparations.

The same primers were used to generate a probe for Southern blot analysis of the *MtRH2 (MtPR10-1)* gene. Root hair cDNA was used as the template and a clone was isolated. Genomic DNA was digested with *Hin*dIII, which cuts once in *MtPR10-1*, outside the probe. A single band was detected (at approximately 6 kb; data not shown), indicating that there is one copy of the *MtRH2* gene in the *M. truncatula* (R108-1) genome. However, the possibility

cannot entirely be ruled out that two copies, in an inverted tandem arrangement, are present.

DISCUSSION

A new method for root hair isolation has been developed, with high yield and purity, in the model legume, *M. truncatula*. The method is especially suitable in those cases where plant material is a serious limiting factor. For instance, transgenic plants can be generated and roots can be used well before seed becomes available, thus providing relatively large amounts of homogeneous material in less than one generation time (approximately in 3 weeks). Another advantage of the method is the possibility to select different root hair populations by simply removing the undesired parts of the root with a scalpel blade before root hair collection. By exploiting the advantages of *A. rhizogenes*-mediated transformation, this method can be expanded to other legume species and allows high amounts of homogeneous root (hair) material to be obtained in a short period. A drawback of the method is, however, that detached roots, whether or not induced by *A. rhizogenes*, do not form nodules.

In this method, use is made of a specific protein marker of root epidermis, RH2, to monitor the root hair isolation process. A construct of the reporter gene *GFP* under the control of the *PsRH2* promoter was made and introduced into *M. truncatula* (R108-1). Transgenic plants expressed GFP specifically in the root epidermis and this property was exploited to verify the high purity and yield of these root hair preparations. In the course of this work, it was also shown that there is a protein (and gene) homologous to PsRH2, MtRH2, in *M. truncatula*. This was proven by analyses of western blots (immunoreactive band with identical molecular mass) and RT-PCR and Southern blots (nucleotide identity and single gene copy). All these analyses are also consistent with a root epidermis-specific expression of MtRH2. Thus, the isolation of root hairs can be readily monitored in untransformed plants by using MtRH2 as a marker. cDNA sequence analysis showed that *MtRH2* is identical to *MtPR10-1*, which is known to encode a root-specific protein (Gamas et al. 1998). Expression of transgenic proteins can, therefore, be specifically targeted to *M. truncatula* root hairs with the use of the endogenous *MtRH2* promoter or homologous promoters such as *PsRH2*. This should be a promising tool for root hair research.

MATERIALS AND METHODS

Plant material

Plants of *Medicago truncatula* Gaertn. R108-1 (c3) were used for these studies because this line is most efficiently transformed (Hoffmann et al. 1997). Seeds, kindly provided by Dr. Trinh (Gif-sur-Yvette, France), were soaked in concentrated H_2SO_4 for 5 min to disrupt the seed coat, rinsed 5 times with water, surface-sterilized for 2 min with a 1:1 mixture (v/v) of 30 %

H_2O_2 and 96 % ethanol, and finally washed 5 times with sterile water. Seeds were then kept in water for 4 h at room temperature and plated on a solid growth medium containing 1 % agar and the following mineral nutrients: 2.72 mM $CaCl_2.2H_2O$, 1.95 mM $MgSO_4.7H_2O$, 2.2 mM KH_2PO_4, 1.26 mM $Na_2HPO_4.12H_2O$ and 80 µM Fe-citrate.$2H_2O$ (Vincent 1970). After incubation at 4°C for 72 h, plates were placed vertically at 15°C in the dark for one day to allow the seeds to germinate.

Growth of explants

A 1.5-cm portion of the main root, including the tip, was cut from the seedling and placed on the surface of SH medium (Schenk and Hildebrandt 1972) containing 1.5 % Phytagar (Life Technologies, Paisley, UK) in a 10 x 10 cm square Petri dish. This high agar concentration prevents growth of the roots under the surface. The plates were covered with aluminium foil to shield them from light and were incubated in a growth chamber at 21°C. After 7-10 d, when the detached root was growing actively and lateral roots had developed, five pieces of 1 cm, containing at least one complete lateral root, were cut from the explant root and placed about 5 cm apart on a new Petri dish. Plates were then incubated under the same conditions as described above. A week later, when the roots had covered the surface of the plate, the lateral roots were sliced 3-6 mm above the tip with a scalpel blade and the older tissue was removed. The roots were allowed to regrow for another week.

Root hair collection

To collect root hairs, the Petri dish (without lid) was immersed in a stainless steel tank (12 x 15 x 4 cm) with liquid nitrogen. When the nitrogen had stopped boiling and the plate was fully frozen, root hairs were gently brushed from the agar surface with a number 2 paintbrush. The brush was kept continuously inside liquid nitrogen, otherwise water condensation may occur resulting in the breakage of the brush hairs. After brushing, the liquid nitrogen containing the root hairs was poured through a 400-µm nylon mesh into a plastic 50-ml tube placed on ice. Liquid nitrogen was allowed to evaporate until 10 ml were left, then the tube was closed with a perforated cap and stored at -80°C.

Reverse transcription-PCR (RT-PCR) of total root and root hair RNA

RNA was isolated using the VS total RNA isolation kit (Promega, Madison, WI). For extraction of root hair RNA, the 50-ml tube (see above) was rinsed with 100 µl of the VS kit extraction buffer to collect the root hairs. For extraction of total root RNA, four 10-mm long lateral roots (including the root tips), grown as described above, were used. A fraction of the extracted root hair RNA (50 %) and total root RNA (5 %) was used for cDNA reverse transcription using Moloney murine leukemia virus reverse transcriptase (Life Technologies) with a poly-A^+ primer.

The amounts of cDNA to be used as templates were standardized with ubiquitin. Primers (forward: GTTGAAGGAAACGGTGG reverse: GTAACCTTCAAGAGCCTTG) were designed based on homologous regions of *MtPR10-1* (Gamas et al. 1998; accession number Y08641) and *PsRH2* (Mylona et al. 1995; accession number S74512). PCR conditions were an initial denaturation step of 5 min at 95°C; 12-20 cycles of 45 s at 95°C, 45 s at 54°C and 45 s at 72°C; and a final elongation step of 10 min at 72°C. PCR products were separated by electrophoresis, transferred to nylon membranes at alkaline pH and hybridized with a ^{32}P-labelled probe generated by PCR using the same primers. Autoradiographs were analysed with a phosphor-imager (Molecular Dynamics, Sunnyvale, CA).

Protein extraction from roots and root hairs

For extraction of hair root protein, the 50-ml tube containing the root hairs (mentioned above) was placed on ice and rinsed with 100 µl of cytoskeleton stabilizing buffer (Morelli et al. 1998) containing 5 mM HEPES, pH 7.5, 10 mM Mg-acetate, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 15 mM β–mercaptoethanol, 0.5 % polyoxyethylene-10-tridecyl ether, and a cocktail of protease inhibitors (Complete; Boehringer-Mannheim, Mannheim, Germany). An aliquot was analysed by microscopy to check whether the root hair preparation was contaminated with other plant material. Samples were then centrifuged at 550 *g* for 30 s and the supernatant was used for analysis.

For extraction of total root protein, four 10-mm long lateral roots (including the tips) were ground in liquid nitrogen. The powder was resuspended in cytoskeleton stabilizing buffer, left to stand at 4°C for 20 min and centrifuged at 15,500 *g* for 1 min. Protein was determined in both supernatants by the Bradford assay using bovine serum albumin as the standard (Bio-Rad, Hercules, CA).

Western blot analysis

Proteins (5 µg) were separated in 12 % (w/v) acrylamide SDS-gels and blotted to polyvinylidene difluoride membranes with a Mini-Trans Blot apparatus (Bio-Rad, Hercules, CA, USA) according to standard protocols. RH2 antibody was prepared in our laboratory. 400 µg of pea root hair protein was separated by 2D gel electrophoresis and the spot representing RH2 was cut from the blot (Mylona et al. 1994). Subsequently, from this material the antibody was raised in a New Zealand rabbit (H. Franssen, unpublished results). The blot was blocked by incubation for 1 h with 5% (w/v) skimmed milk in Tris-buffered saline (10 mM Tris-HCl, pH 7.8; 150 mM NaCl) containing 1 % (v/v) Tween 20. The primary antibody (RH2) was used at a dilution of 1:10,000. After 1-h incubation, the secondary antibody (goat anti-rabbit antibody conjugated to horseradish peroxidase; Boehringer-Mannheim) was added at a dilution of

1:5,000 and incubated further for 1 h. After each incubation, blots were washed three times for 10 min in Tris-buffered saline. Horseradish peroxidase was detected using the ECL luminiscence kit (Amersham Biosciences, Uppsala, Sweden) with X-ray film.

Vectors and plant transformation

The vector used for plant transformation, pBinSL, is a derivative of pBin101 (Clontech). pBinSL contains the *PsRH2* promoter (from the *DRRG49-c* gene; Chiang and Hadwiger 1990), between the restriction sites HindIII and *BamH*I, and the GFP sequence (Davis and Vierstra 1998), between the restriction sites *BamH*I and *Sst*I.

The pBinSL construct was introduced into *A. tumefaciens* strain GV3101 by electroporation. Transgenic plants of *M. truncatula* (R108-1) were obtained essentially as described by Hoffman et al. (1997) and analysed by PCR and Southern blots to determine whether the construct was properly inserted.

Fluorescence microscopy

The roots of transgenic plants transformed with pBinSL were observed and photographed with a confocal microscope, Zeiss LSM510 Axiovert 100, using a plan-neofluar 10x0.3 objective and the LSM-FCS version 2.8 SP1 software of ZEISS.

Southern blot analysis

Genomic DNA was extracted from leaves and flowers of *M. truncatula* following the hexadecyltrimethyl ammonium bromide method (Stewart and Via 1993). DNA (7 µg) was digested with *Hin*d III, electrophoresed, blotted and hybridized as described for RT-PCR. The probe was the same as that used for the detection of *MtRH2* by RT-PCR.

ACKNOWLEDGEMENTS

We gratefully acknowledge Rene Geurts for helping with the confocal picture of GFP and Henk Franssen for providing the RH2 antibody. We are also indebted to Manuel Becana for suggestions and revision of the manuscript.

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Chapter 3

RNA Interference in *Agrobacterium rhizogenes* Transformed Roots of *Arabidopsis* and *Medicago truncatula*

Limpens E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, Bisseling T, Geurts R (2004) RNA interference in *Agrobacterium rhizogenes*-transformed roots of *Arabidopsis* and *Medicago truncatula.* J Exp Bot **55:** 983-992

ABSTRACT

RNA interference (RNAi) is a powerful reverse genetic tool to study gene function. The data presented here show that *Agrobacterium rhizogenes* mediated RNAi is a fast and effective tool to study genes involved in root biology. The *Arabidopsis* gene *KOJAK*, involved in root hair development, was efficiently knocked down. *A. rhizogenes* mediated root transformation is a fast method to generate adventitious, genetically transformed roots. In order to select for co-transformed roots we developed a binary vector that enables selection based on *DsRED1* expression, with the additional benefit that chimaeric roots can be discriminated. The identification of chimaeric roots allowed us to examine the extent of systemic spread of the silencing signal in the composite plants of both *Arabidopsis* and *Medicago truncatula*. It is shown that RNA silencing does not spread systemically to non co-transformed (lateral) roots and only inefficiently to the non-transgenic shoot. Furthermore, evidence is presented which shows that RNAi is cell autonomous in the root epidermis.

INTRODUCTION

In plants several reverse genetic techniques are being used to study gene function, such as co-suppression and antisense suppression. Recent studies have shown that the formation of double stranded RNA (dsRNA) can lead to effective and sequence-specific post-transcriptionally degradation of homologous mRNA. Evidence for the involvement of dsRNA in mediating gene silencing was first discovered in *Caenorhabditis elegans*, and was termed RNA interference (RNAi) (Fire et al. 1998). Since then it has become clear that dsRNA can effectively suppress gene expression in a wide array of organisms, including nematodes, insects, mammals and plants (Tavernarakis et al. 2000, Elbashir et al. 2001). In retrospect, the phenomenon of RNAi was already known in plants as post-transcriptional gene silencing (PTGS), where introduction of transgenes (co-suppression) or infection with manipulated viruses (Virus Induced Gene Silencing or VIGS) resulted in post-transcriptional silencing of homologous endogenous genes (Van der Krol et al. 1990, Ratcliff et al. 2001). RNAi is considered to be an ancient and ubiquitous antiviral system of eukaryotic organisms that has evolved before the divergence of plant and animals (Sharp 2001). The process of RNAi can be divided into a few steps. It is initiated by the production of ds RNA, which is recognized and cleaved by a nuclease, named DICER, to produce 21-25 nucleotide-long small interfering RNAs (siRNAs). In turn these siRNAs are incorporated into a second enzyme complex, called the RNA-induced silencing complex or RISC, which is responsible for the specific degradation of homologous mRNAs in the cytoplasm (Hammond et al. 2001, Hannon 2002). Currently, RNAi is an important tool in the analysis of gene function in both plants and animals. Especially in *C. elegans*, RNAi has been developed to 'genomics' scales (Fraser et al. 2000, Gönczy et al. 2000, Maeda et al. 2001), and also in *Arabidopsis* genome-wide RNAi efforts are underway.

In plants several different approaches are being used to trigger RNAi in living cells. RNAi can be triggered by generating stable transgenic plants that express RNAs capable of forming a double stranded hairpin (Waterhouse et al. 1998, Chuang and Meyerowitz 2000, Wesley et al. 2001). In *Nicotiana benthamiana* RNAi has been applied using *Agrobacterium tumefaciens*-mediated transient expression (Johansen and Carrington 2001) and, in cereals, biolistic delivery of dsRNA to leaf epidermal cells by particle bombardment resulted in interference with the function of endogenous genes at the single cell level (Schweizer et al. 2000). Also, viruses can be manipulated to produce dsRNA intermediates of endogenous genes, which will be targeted for degradation after infection of the plant by the virus (Ratcliff et al. 2001).

Most of these studies have mainly focused on targeting genes in the aerial parts of the plant. Recently it was shown that RNAi can also be used to effectively silence (trans) genes in primary transformed roots of the legumes *Medicago truncatula* (Limpens et al. 2003) and *Lotus japonicus* (Kumagai and Kouchi 2003) by using *Agrobacterium rhizogenes* mediated

transformation. *A. rhizogenes* generates adventitious, genetically transformed roots at the site of inoculation in many dicots and can be manipulated to co-transfer the T-DNA of a binary vector that contains the transgene of interest (Chilton et al. 1982). Upon expression of the *root locus* (*rol)* genes carried on the Ri T-DNA, roots are formed of which a certain number will be co-transformed with the T-DNA of the binary vector (Nilsson and Olsson 1997). *A. rhizogenes*-mediated root transformation has been described for a number of legumes. The transformed roots are morphologically indistinguishable from untransformed roots and, in the case of legumes, can be nodulated by *Rhizobium* and infected by mycorrhizal fungi. *A. rhizogenes*-mediated transformation offers a fast alternative to generate genetically transformed roots, especially in species where generating stable transgenic lines is very time consuming. Furthermore, this method has the advantage that root cultures can be clonally propagated without the requirement of additional plant hormones.

Here we show that *A. rhizogenes* mediated RNAi is a fast and effective tool to study the function of genes involved in root biology, not only in legumes but also in *Arabidopsis*. An *Arabidopsis* gene, *KOJAK (CLSD3)* (Favery et al. 2001, Wang et al. 2001*)*, involved in root hair development was efficiently targeted by *A. rhizogenes* mediated RNAi. As selection marker for co-transformed roots, the gene coding for the fluorescent protein *Ds*RED1 was used as nondestructive selectable marker. This marker offers the additional advantage to discriminate between chimaeric and homogeneously transformed roots. We show that RNA silencing is not spreading to non co-transformed (lateral) roots and only with limited efficiency to the non-transgenic shoot of composite plants. Furthermore, we provide evidence that RNA silencing is cell-autonomously induced in the root epidermis.

RESULTS

A. rhizogenes-mediated root transformation in *Arabidopsis* and *Medicago* using the pRedRoot binary vector

A. rhizogenes-mediated root transformation results in the formation of adventitious roots that are co-transformed with the gene of interest as well as roots lacking this gene. To interpret RNAi experiments it is essential to identify those roots that contain the transgene of interest. Recently it was shown for *Medicago* that selection on co-transformation can be done using kanamycin resistance (Boisson-Dernier et al. 2001). However, in our hands this selection does not effectively discriminate chimaeric roots (data not shown). Therefore the binary vector pRedRoot was developed (Fig. 1A). The pRedRoot vector provides the possiblity to select transgenic roots based on fluorescence since it contains the gene encoding for the red fluorescent protein, *DsRED1* (Matz et al. 1999), under control of the constitutively expressed *UBQ10* promoter of *Arabidopsis* (Norris et al. 1993). In addition to nondestructive identification of co-transformed

roots, this vector also allows the detection of chimaeric roots.

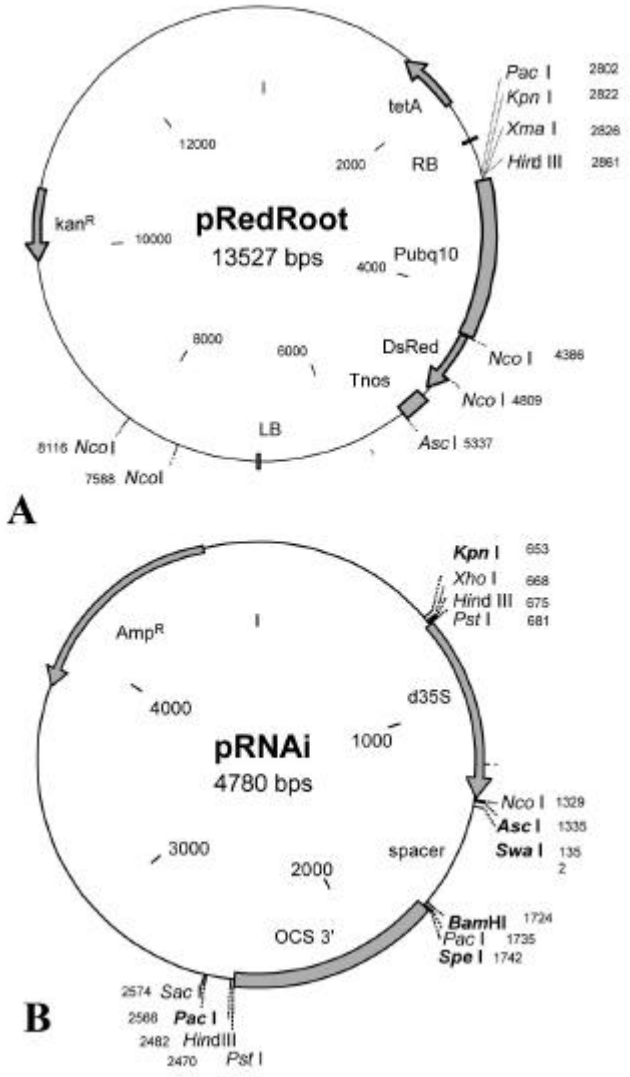

Figure 1. Plasmids used for introduction of RNAi constructs into plants via *A. rhizogenes*-mediated root transformation. (A) pRedRoot binary vector, containing *DsRED1* under the control of the *AtUBQ10* promoter within the T-DNA borders. (B) pRNAi vector used to generate hairpin constructs.

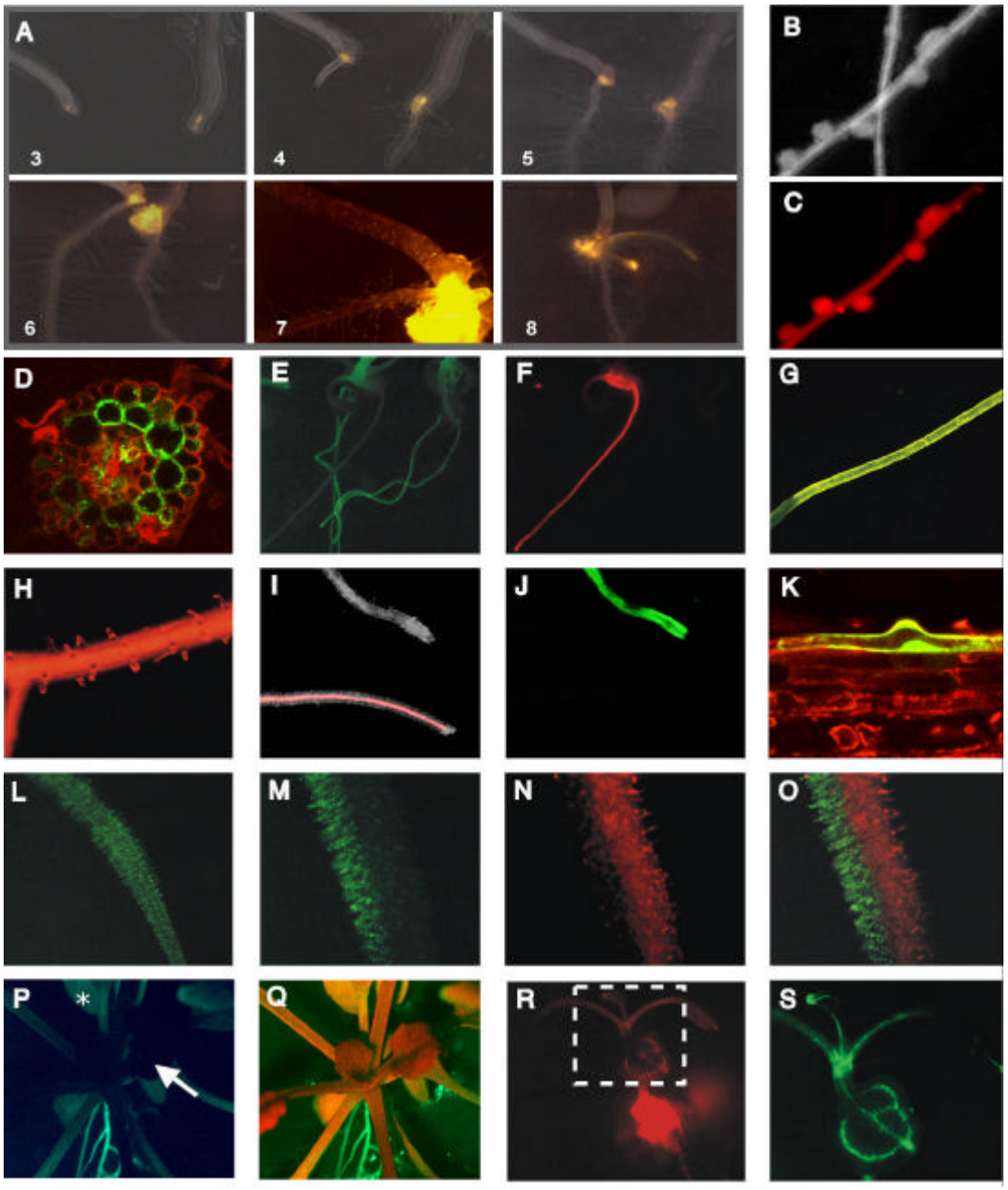

Figure 2. *A. rhizogenes* mediated root transformations in *Medicago* and *Arabidopsis*. (A) Time-laps experiment following DsRED1 expression at one-day intervals in *Arabidopsis*, 3-8 d after infection with *A. rhizogenes* containing pRedRoot. (B) Brightfield picture of *A. rhizogenes* transformed roots of *Medicago*, using the pRedRoot binary vector, 14 d after inoculation with *Sinorhizobium meliloti*, resulting in the formation of root nodules. (C) The same roots as shown in (B) using filter settings to visualize DsRED1 expression (red fluorescence). Only one nodulated root is co-transformed as visualized by red fluorescence. (D) GFP fluorescence in a cross-section of *Arabidopsis* line J0781, showing strong GFP expression in the cortex and stele. Root was counterstained with propidium iodide (0,2 µg/ml). (E) GFP fluorescence in *Arabidopsis* line J0781 transformed with pRR-GFPi, 14 d after transformation. The left plant contains one co-transformed root as visualized by red fluorescence in (F). Only in this co-transformed root GFP is silenced. GFP fluorescence in the non co-transformed roots and the hypocotyl is not affected. (G) Brightfield picture of a 12 d old *A. rhizogenes* transformed root of *Arabidopsis*, co-transformed with pRR-KJKi showing a hair-less root. (H) Segment of an *Arabidopsis* root, co-transformed with pRR-KJKi, showing slightly elongated small root hairs.

(*continued from previous page*) (I) Chimaeric root on J0781, co-transformed with pRR-GFPi in the stele as judged by red fluorescence. An overlay of the DsRED1 channel with the brightfield image is shown. The upper (non-red) root is a lateral root that formed on this chimaeric root. (J) GFP fluorescence in the chimaeric root shown in (I). No GFP fluorescence is visible in the co-transformed chimaeric root, whereas the (non-red) lateral root has regained GFP fluorescence. (K) Confocal picture showing specific GFP fluorescence in the root epidermis of *A. rhizogenes* transformed *Medicago* line R108-RH2::GFP. (L) Fluorescence due to expression of GFP in the root epidermis of the *Medicago* line R108-RH2::GFP. (M-O) Chimaeric root on *Medicago* line R108-RH2::GFP 4 weeks after transformation with pRR-GFPi. GFP expression (M) and DsRED1 expression (N) and a merged picture (O) are shown. GFP expression is only affected in co-transformed root tissue as visualized by red fluorescence. (P) Partial systemic silencing in *Arabidopsis* line 35S::GFP transformed with pRR-GFPi 4 weeks after transformation. Arrow marks a systemically-silenced leaf, asterisk marks a non-silenced leaf. (Q) Brightfield picture (using 525nm long pass filter) of the composite 35S::GFP plant shown in (P). (R) *Arabidopsis* line J0661 transformed with pRR-GFPi 14 d after transformation, showing red fluorescence due to DsRED1 in the callus as well as vascular tissue of the shoot. The boxed area is shown in (S) using filter settings to visualize GFP fluorescence.

To test the pRedRoot vector *Medicago* accessions A17 and R108 as well as *Arabidopsis* accessions Landsberg *erecta*, Columbia and Wassilewskija, were transformed by inoculating freshly cut hypocotyls with the *A. rhizogenes* strain MSU440 (harboring pRiA4) containing pRedRoot. The first red fluorescent *Medicago* roots could be observed approximately 3 weeks after inoculation, whereas in *Arabidopsis* red fluorescent roots were already formed within 8 to 10 d. In Figure 2A a time-laps experiment is shown, following the accumulation of *Ds*RED1 in *Arabidopsis* at one-day intervals. Newly formed roots are visible 4 d after inoculation with *A. rhizogenes*. However, these roots do not originate from cells expressing *DsRED1* and so are not co-transformed. The *A. rhizogenes*-transformed cells form a callus-like structure from which new adventitious roots are induced. To determine whether the observed red fluorescence was the result of *DsRED1* expression, a spectral image of a red fluorescent root versus a control root of *Medicago* was made using fluorescence spectral imaging microscopy (FSPIM). This showed that the spectrum of *Ds*RED1, with an emission peak at 583 nm, could be clearly distinguished from auto-fluorescence present in *Medicago* roots (Fig. 3).

The *A. rhizogenes*-transformed roots of both *Medicago* and *Arabidopsis* have a similar morphology as normal roots. In the case of *Medicago*, they can be nodulated by the symbiotic partner *Sinorhizobium meliloti* (Fig. 2B,C). Co-transformation efficiencies varied between experiments, but on average ~30% of the newly formed roots in *Medicago* were co-transformed (ranging from 1 to 3 co-transformed roots per inoculated seedling), whereas in *Arabidopsis* efficiencies up to 20% were reached. *A. rhizogenes*-mediated transformation results in the generation of homogeneously co-transformed roots as well as chimaeric roots. In general, chimaeric roots were observed in ~50% of the cases in *Medicago*, and only infrequently in *Arabidopsis* (~10%). These results show that pRedRoot is a useful binary vector for *A. rhizogenes*-mediated transformation to identify transgenic roots as well as co-transformed segments in chimaeric roots.

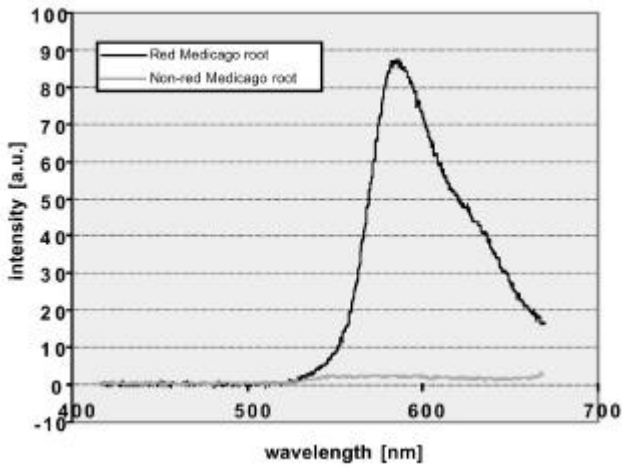

Figure 3. Spectral image of a co-transformed red fluorescent *Medicago* root and a non co-transformed (non-red) root. The *Ds*RED1 protein has a maximum emission at 583 nm.

Silencing of trans-GFP in *Arabidopsis* and *Medicago*

To test the effectiveness of RNAi in *A. rhizogenes* transformed *Arabidopsis* roots, a *GFP* transgene was targeted in order to visualize the silencing effects. Therefore a construct was made containing 584 bp of the coding sequence of *GFP5*, cloned in both the sense and anti-sense direction separated by a 335 bp spacer, under the control of the 35S promoter in pRNAi (Fig. 1B) and subsequently transferred to pRedRoot resulting in the construct pRR-GFPi. RNA transcribed from this construct produces a hairpin structure, resulting in double stranded RNA. The pRedRoot vector enabled the identification of co-transformed roots by red fluorescence of *Ds*RED1, while the efficiency of silencing could be determined by quantification of GFP fluorescence.

In *Arabidopsis* Gal4 enhancer trap line J0781, expressing *GFP* was used (http://www.plantsci.cam.ac.uk/Haseloff/IndexCatalogue.html). J0781 shows strong *GFP* expression in the root cortex and stele and in the hypocotyl (Fig. 2D,E). Transgenic roots were analyzed 12 d after infection with *A. rhizogenes* carrying pRR-GFPi. In 91% (n=98) of the red fluorescent roots no GFP fluorescence could be detected after co-transformation with pRR-GFPi (Fig. 2E,F), whereas roots (n=63) transformed with the pRedRoot control vector all showed bright GFP fluorescence (Table 1). To verify knock down of *GFP* in the *A. rhizogenes*-transformed roots, *GFP* mRNA levels were determined by real-time quantitative RT-PCR (qPCR) (Fig. 4A). Since *Arabidopsis* roots are relatively small, independently transformed roots were clonally propagated in order to isolate sufficient RNA material for the qPCR. The results of the qPCR show that the *GFP* mRNA is substantially reduced, at least 10 times, in the pRR-GFPi transformed roots compared to control roots. Since there was still

residual *GFP* mRNA present in the *GFP*-silenced roots, it was determined whether GFP protein could be detected in these roots. Immunoblotting showed that no GFP protein could be detected in the *GFP*-silenced roots, whereas in the control roots a high level of GFP protein was detected (Fig. 4B). This correlates well with the absence of detectable GFP fluorescence in these roots.

Table 1. Effects of *A. rhizogenes*-mediated RNAi on *Arabidopsis* and *Medicago*

Construct[a]	Transformed Plant	Co-Transformed Roots	Silenced[b]	Not Silenced
pRedRoot	AtJ0781	63	-	63 (100%)
pRR-GFPi	AtJ0781	98	89 (91%)	9 (9%)
pRedRoot	Mt R108-RH2::GFP	12	-	12 (100%)
pRR-GFPi	Mt R108-RH2::GFP	37	32 (86%)	5 (14%)
pRedRoot	*Arabidopsis*	40	-	40 (100%)
pRR-KJKi	*Arabidopsis*	58	53 (91%)	5 (9%)

[a]pRedRoot is an empty plasmid (control). pRR-GFPi and pRR-KJKi are constructs for silencing the *GFP* and *KJK* genes.
[b]Percentages of plants not showing GFP green fluorescence (pRR-GFPi) or of plants showing bulges in root hairs (pRR-KJKi).

Similar results were obtained in *Medicago*, where a stable transformed line R108-*RH2::GFP* was used, which contains the 1.1 kb promoter region of the pea *RH2* gene in front of *GFP* (Ramos and Bisseling 2003). The *RH2* promoter is active in root epidermal cells in the zone of the root starting immediately above the root apical meristem and extending into the region containing mature root hairs (Mylona et al. 1994). In line R108-*RH2::GFP*, GFP fluorescence can be detected in the epidermis of young developing roots (Ramos and Bisseling 2003), which was preserved in *A. rhizogenes* transformed roots (Fig. 2K,L). Four weeks after transformation with pRR-GFPi co-transformed roots were analyzed. In 86% (n=37) of homogeneously transformed roots no GFP fluorescence could be detected (Table 1). In control plants, transformed with the pRedRoot vector, all transgenic roots (n=12) showed clear GFP fluorescence.

Silencing endogenous genes in *Arabidopsis* roots

The potential of *A. rhizogenes*-mediated RNAi with respect to silencing of endogenous genes in *Arabidopsis* was also investigated. For this purpose the gene *KOJAK (KJK/CSLD3)* was selected (Favery et al. 2001, Wang et al. 2001). *KJK* encodes a cellulose synthase-like protein, which is

preferentially expressed in trichoblasts (Favery et al. 2001). Root hair formation is initiated in *kjk* mutants at the correct position but in general stops at the bulge stage, resulting in hair-less roots. A silencing construct for *KJK* was made in the pRedRoot vector and pRR-KJKi was transformed by *A. rhizogenes*-mediated transformation to both *Arabidopsis* accessions Landsberg *erecta* and Wassilewskija. Results are summarized in Table 1. Ninety-one percent (n=58) of the homogeneously transformed roots, as judged by the red fluorescence, showed a root hair phenotype. In most roots (62%), root hairs were initiated but failed to elongate resulting in small bulges (Fig. 2G). But in some roots (29%) root hair cells developed more than a bulge and showed some elongation, resulting in small root hairs (Fig. 2H). Roots transformed with the pRedRoot control vector all contained normal root hairs.

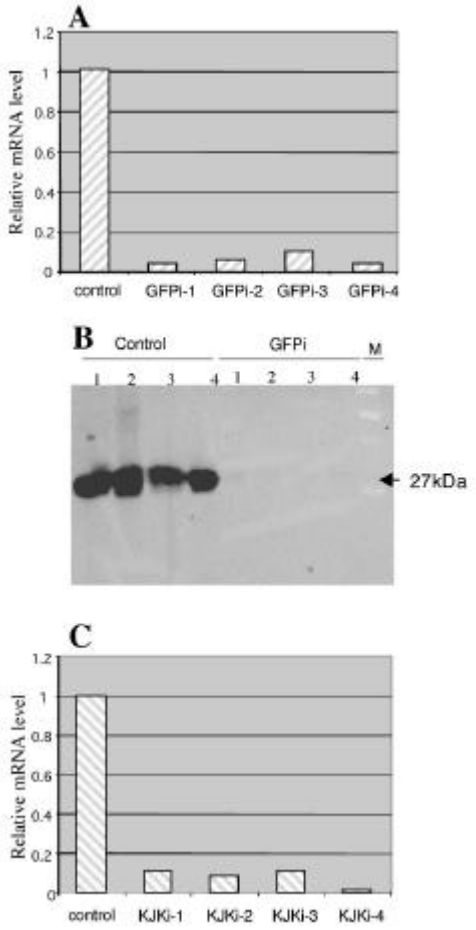

Figure 4. Quantification of mRNA and protein levels in *A. rhizogenes* transformed roots. (A) *GFP* transcript levels of *Arabidopsis* line J0781 co-transformed with pRR-GFPi. RNA was isolated from four 4-week-old independently co-transformed root cultures, and relative transcript levels were determined by qPCR using *ACTIN* as reference. The average of three independent control roots co-transformed with the empty pRedRoot vector is shown. (B) Immunodetection of GFP protein in five 4-week-old independently co-transformed root cultures of *Arabidopsis* line J0781. Control roots are co-transformed with the empty pRedRoot vector. (C) *KJK* transcript levels in four 4-week-old *Arabidopsis* root cultures independently co-transformed with pRR-KJKi. The average of three independent control roots co-transformed with the empty pRedRoot vector is shown.

To verify knock down of *KJK* in the *A. rhizogenes*-transformed roots, the amount of *KJK* mRNA was quantified after clonally propagating the co-transformed roots. qPCR showed that *KJK* mRNA was substantially reduced, at least 8 times, in the pRR-KJKi transformed roots compared to control roots (Fig. 4C). These results show that *A. rhizogenes*-mediated RNAi is an effective and very fast method to silence endogenous genes in *Arabidopsis* roots. Furthermore, a spectrum of phenotypes can be obtained from RNAi.

Systemic spreading of the silencing signal in roots of *Arabidopsis* and *Medicago*

In several plant species it was shown that the induction of RNAi results in systemic spread of the silencing signal (Hamilton et al. 2002). *A. rhizogenes*-mediated root transformation makes it possible to determine whether the interference signal is systemically transported to non-transformed roots or the non-transgenic shoot of the composite plant. To examine the extent of systemic spread of the silencing signal in *A. rhizogenes* transformed roots, the stable *GFP*-expressing *Medicago* (R108-*RH2::GFP*) and *Arabidopsis* (J0781) lines were used. As mentioned above, transformation of these lines with pRR-GFPi efficiently knocked down *GFP* expression. However, no reduction in GFP fluorescence was detected in non co-transformed roots on composite plants that also contained silenced roots (Fig. 2E,F).

The use of *DsRED1* as selectable marker enables the identification of chimaeric roots. This provided the possibility to determine the extent of systemic spread of the silencing signal from co-transformed root tissue to non co-transformed root tissue. Chimaeric roots on *Arabidopsis* J0781 co-transformed with pRR-GFPi showed knock down of *GFP* expression in the entire cortex and stele of those particular roots. Figure 2I shows a chimaeric pRR-GFPi transformed J0781 root that appears to be co-transformed only in (part of) the stele as judged by red fluorescence. However, no GFP fluorescence was detected in both the cortex and the stele of this chimaeric root (Fig. 2J). When such chimaeric pRR-GFPi transformed J0781 roots were clonally propagated, new lateral roots emerged that did not show any *Ds*RED1 fluorescence and regained GFP fluorescence (Fig. 2I,J). This indicates that systemic spread of the silencing signal does take place within the cortex and stele of a single chimaeric *A. rhizogenes* transformed root, but not to non co-transformed lateral roots. Further, the extent of systemic spread of the silencing signal to the epidermis of the root was investigated. Therefore the *Medicago* R108-*RH2::GFP* line was used that expresses *GFP* exclusively in the epidermis (Fig. 2K,L). Upon transformation with pRR-GFPi chimaeric roots were searched for that contained transformed epidermal cell files. Figure 2M,N,O shows such a chimaeric root transformed with pRR-GFPi. Approximately half of the epidermal cell files are co-transformed as visualized by red fluorescence. Strikingly, silencing of *GFP* occurs only in the co-transformed cell files, whereas, in the epidermal cell files lacking *DsRED1* expression, GFP fluorescence intensity is as high as in control roots. Even 6

weeks after transformation, the fluorescence in the non-transformed epidermal cell files was as strong as in control roots, indicating that RNAi is cell autonomous in the root epidermis.

Systemic spreading of the silencing signal to the shoot

RNAi of a trans *35S::gusA* gene in *Lotus japonicus* indicated some systemic spreading of the silencing signal to the non-transgenic shoots of *A. rhizogenes* transformed plants (Kumagai and Kouchi 2003). To investigate the level of systemic spreading of the silencing signal to the shoot two additional transgenic *Arabidopsis* lines were used; one carrying *35S::GFP* and a second Gal4 enhancer trap line, J0661, which shows strong GFP fluorescence in root vascular tissue and in the vascular tissue of the cotyledons and leaves. *A. rhizogenes* mediated RNAi of *GFP* in the *35S::GFP* transgenic line resulted in 20% of the *A. rhizogenes* transformed plants (n=40) showing no, or very low levels, of GFP fluorescence in the shoots four weeks after transformation. In 60% of the plants bright GFP fluorescence was visible in some leaves, whereas other leaves on the same composite plant lacked GFP fluorescence (Fig. 2P,Q), and even variation within one leaf was observed. Twenty percent of the composite plants did not show any sign of silencing in the shoot, while *GFP* expression in the co-transformed roots of these composite plants was efficiently knocked down. In contrast to systemic spreading, albeit inefficiently, in the *35S::GFP* line, no visible reduction of *GFP* expression in the vascular tissue of the leaves of enhancer trap line J0661 was observed 4 weeks after transformation (Fig. 2R,S). Red fluorescence due to the *Ds*RED1 protein could occasionally be observed in the vascular tissue of the shoot (Fig. 2R). Also in the hypocotyl of *A. rhizogenes* transformed enhancer trap line J0781 GFP fluorescence was not reduced (Fig. 2E).

DISCUSSION

Here it is shown, by targeting *KJK/CSLD3*, that endogenous genes can efficiently be silenced in roots of *Arabidopsis* via RNAi by using *A. rhizogenes*-mediated transformation. A high percentage (91% in the case of *KJK*) of the homogeneously transformed roots showed phenotypes identical to the described mutant (Wang et al. 2001). Quantification of mRNA levels by qPCR confirmed the knock down of the corresponding gene. However, residual mRNA could still be detected and also some variation in the level of expression was detected between independently transformed roots. This variation in mRNA levels could be an explanation for the observed variation in phenotypes. In these plants 62% of the homogeneously transformed roots initiated root hairs that stopped at the bulge stage without further elongation, but in 29% of the cases some elongation took place resulting in small root hairs. A similar plasticity in phenotypes has been observed for the *csld3-1* mutant and is thought to be the result of a reduction in the amount of correctly targeted protein to the membrane, resulting in a reduction of delivery of

cellulose polymers to the primary cell wall (Wang et al. 2001). The occurrence of intermediate phenotypes as a result of RNAi has also been reported for stable transformed *Arabidopsis* plants (Chuang and Meyerowitz 2000) and can be an additional tool to gain insight into the function of a gene.

Generally it is thought that a specific mobile silencing signal exists that can travel between cells via plasmodesmata and long distances via phloem (Palauqui et al. 1997, Voinnet et al. 1998, Jorgensen 2002, Mlotshwa et al. 2002). For example, in *Arabidopsis*, biolistic delivery of dsRNA into leaf cells triggered silencing capable of spreading locally and systemically. It was reported that systemic spreading of the silencing signal could be detected two weeks after biolistic delivery starting in the veins of non-bombarded leaves and was clearly evident in non-vascular tissues one month after bombardment (Klahre et al. 2002). Strikingly, *A. rhizogenes* mediated RNAi of trans *GFP* in *Arabidopsis* or *Medicago* roots showed that systemic transport of the silencing signal does not occur to non co-transformed roots. Targeting *GFP* in the Gal4 enhancer trap lines J0661 and J0781 also did not show any systemic spread to the non-transgenic shoot. However, targeting of *GFP* in a *35S::GFP* transgenic line did result in systemic transport of the silencing signal, but the extent of silencing was more limited and greatly variable. Similar results are reported in *L. japonicus* where *A. rhizogenes* mediated silencing of a *35S::gusA* transgene did not spread to non co-transformed roots and was limited and variable in the shoots (Kumagai and Kouchi 2003). The lack of systemic spread of the silencing signal to non co-transformed roots is in agreement with grafting experiments performed in tobacco, which suggested that silencing is unidirectional from the base to the top of the plant (Palauqui et al. 1996, 1997). The observed variation in spatial patterns of silencing in the shoot of the *35S::GFP* line has also been observed in different plant species and for different transgenes under the control of the 35S promoter (Boerjan et al. 1995, Jorgensen et al. 1996, Kunz et al. 1996, Palauqui et al. 1996). The extent of systemic silencing in the shoot could depend on the regulation of the transgene, since no systemic silencing was observed in the shoot of transformed enhancer trap line J0661 and J0781, which express *GFP* under the control of an endogenous enhancer element.

The use of *DsRED1* as selection marker enabled us to select chimaeric roots to examine the extent of systemic spread of the silencing signal within root tissue. The *Arabidopsis* line J0781 shows strong *GFP* expression in the cortex and stele of the root. Chimaeric J0781 roots partly transformed with pRR-GFPi showed silencing of *GFP* in the entire cortex and vascular tissue, indicating that the silencing signal is able to spread systemically in the cortex and stele. Strikingly, lateral roots that formed on these chimaeric roots and were not co-transformed, regained *GFP* expression. This suggests that, within one root system, the silencing signal does not spread to non co-transformed lateral roots. This is most likely a result of the unidirectional movement of the silencing signal. In contrast to systemic spread in the cortex and vascular tissue in *Arabidopsis*,

no cell-to-cell movement of the silencing signal was observed in the epidermis of *Medicago*, demonstrating that silencing in the root epidermis is cell autonomous. One explanation for the fact that spreading of the silencing signal is not observed in the epidermis of *A. rhizogenes* mediated roots could be that epidermal cells become symplastically isolated. By dye-coupling experiments in *Arabidopsis* roots it was shown that cells in the meristem and epidermal cells in the elongation zone are symplastically connected through plasmodesmata, but gradually become symplastically isolated as the epidermal cells differentiate. By the time root hair outgrowth is visible the epidermal cells are symplastically isolated (Duckett et al. 1994). Similarly, it was shown that symplastically isolated stomatal guard cells do not silence systemically (Voinnet et al. 1998). So, the symplastic isolation of cells could cause the immobility of the silencing signal.

RNAi via *A. rhizogenes*-mediated root transformation is a valuable tool to study genes involved in root development and root-microbe interactions. It is a very fast and efficient system to silence genes in roots. In *Arabidopsis*, silenced *A. rhizogenes* transformed roots can already be obtained within 10 d. Especially for plant species with very time consuming regeneration times this methods offers a big advantage. The fact that silencing is triggered cell autonomously in the root epidermis provides the possibility to use inducible and tissue-specific promoters to more specifically regulate RNAi. At the same time it requires a thorough inspection of the chimaeric nature of *A. rhizogenes* transformed roots in order to correctly interpret the observed phenotypes. The pRedRoot vector provides this possibility.

MATERIALS AND METHODS

Plasmids and vectors

To create pRedRoot, the *nptII* gene and NOS terminator of pBINPLUS (Van Engelen et al. 1995) were removed by a Bsu36*I* and Bst98*I* digestion, Klenow treatment and re-ligation. The created plasmid was named pBASIS. The *Arabidopsis* UBQ10 promoter was PCR amplified using genomic DNA of the accession Columbia as template (used primers: 5'-AAGCTTTGTCCCGACGGTGTTGT-3' and 5'-CCATGGCAAAGATCTGCATCTG TTA-3') and subsequently cloned in frame with the ATG start codon of *DsRED1* (Clontech) in a pGEM-T (Promega) derived vector that contained a NOS terminator. A 2.5 kb Hind*III*-Asc*I* fragment containing UBQ10::*DsRED1*-NOS terminator was cloned into pBASIS resulting in pRedRoot (Fig. 1A).

In order to create inverted repeat constructs a 1.3 kb fragment of vector pFGC1008 (described at http://ag.arizona.edu/chromatin/fgc1008.html) containing Sac*I* 2927 bp – Hind*III* 4150 bp (the RNAi box) was PCR amplified and cloned into pBluescriptIISK+ (Stratagene) (primers: 5'-CACTGACGTAAGGGATGA-3' and 5'-ATGAGCTCTTAA TTAAGGATGTGCTGCAAGGCGA-3'). A double CaMV 35S promoter was PCR amplified

from vector pMON999 (primers: 5'-TGATCTCGAGCAAGCTTCTGCAG GTCCAT-3' and 5'-CATGCCATGGAGATCTGCTAGAGTCAGC-3') and cloned (Xho*I*-Nco*I*) in front of this RNAi box to result in the pRNAi vector (Fig. 1B). Inverted repeats were created in pRNAi by two sequential cloning steps. Target regions were first PCR amplified and subsequently cloned (Asc*I*-Swa*I* and BamH*I*-Spe*I*) into the created pRNAi derivative. The resulting inverted repeat construct was inserted (Kpn*I*–Pac*I*) into the pRedRoot binary vector. Target regions were amplified using the following primer combinations:

GFP5 (584 bp) 5'-ATACTAGTGGCGCGCCTTGTTGAATTTAGATGGTGATG-3' 5'-ATGGATCCATTTAAATTTCGAAAGGGCAGATTG-3'

KJK (461 bp) 5'-ATACTAGTGGCGCGCCGTGTGCCAGAAGAAAACC-3' 5'-ATGGATCCATTTAAATGCCAGTAGCCCATCGGAGAAC-3'

The Spe*I*-Asc*I* and BamH*I*-Swa*I* restriction sites included within the primers have been underlined.

Bacterial strains

Agrobacterium strain MSU440 containing the pRi plasmid pRiA4 (Sonti et al. 1995) was used to transform *Medicago* and *Arabidopsis*. The binary vectors were introduced into MSU440 by electrotransformation and grown for 2 d at 28°C under kanamycin selection (50 µg/ml). Integrity of inverted repeat constructs was checked by mini-preps and restriction-digestion with Hind*III*.

Plant material

For *Medicago* the accession Jemalong A17 and the transgenic line R108-*RH2::GFP* were used (Hoffmann et al. 1997, Penmetsa and Cook 1997, Ramos and Bisseling 2003). R108-*RH2::GFP* carries the 1.1 kbp promoter region of the pea *RH2/DRRG* gene (Genbank J03680, Chiang and Hadwiger 1990) in front of *GFP5* (Ramos and Bisseling 2003). For *Arabidopsis* the accessions Landsberg *erecta*, Columbia and Wassilewskija were used. The Gal4 enhancer trap lines J0661 and J0781 are described at http://www.plantsci. cam.ac.uk/ Haseloff/IndexCatalogue.html.

***Agrobacterium rhizogenes*-mediated transformation**

Medicago seeds were surface sterilized by incubating for 10 min in concentrated sulfuric acid, 6x washing in sterile water, 10 min in 4% hypochlorite (commercial bleach) and 7x washing in sterile water, and were subsequently plated on Färhaeus medium (1 mM $MgSO_4.7H_2O$, 0.75 mM KH_2PO_4, 1 mM Na_2HPO_4, 15 µM Fe-citrate, 0.75 mM $Ca(NO_3)_2$, 0.7 mM $CaCl_2$ 0.35 µM $CuSO_4.5H_2O$., 4.69 µM $MnSO_4.7H_2O$, 8.46 µM $ZnSO_4.7H_2O$, 51.3 µM H_3BO_3, 4.11 µM

$Na_2MoO_4.2H_2O$, 0.9 % Daichin agar Brunschwig) containing a filter paper. Seeds were vernalized for 1 d at 4°C and germinated at 25°C for 24 h in darkness (plates upside down). One-day-old seedlings were transferred to new 9 cm-petri dishes containing Färhaeus medium and a half-round filter paper (5 seedlings per plate), and grown at 21°C (16 h light-8 h darkness) after removal of the seed coat. The petri dishes were not completely closed by parafilm to enable aeration. The roots of 5-day-old seedlings were removed at the hypocotyl and the wound surface was inoculated with *Agrobacterium* MSU440 containing the appropriate binary plasmid. The seedlings were co-cultivated with *Agrobacterium* for 5 d at 21°C (16 h light-8 h darkness) and subsequently transferred to "Emergence medium" (3 mM MES pH 5.8 containing 2.5 g/l KNO_3, 0.4 g/l $MgSO_4.7H_2O$, 0.3 g/l $NH_4H_2PO_4$, 0.2 g/l $CaCl_2.2H_2O$, 10 mg/l $MnSO_4.4H_2O$, 5 mg/l H_3BO_3, 1 mg/l $ZnSO_4.7H_2O$, 1 mg/l KI, 0.2 mg/l $CuSO_4.5H_2O$, 0.1 mg/l $NaMoO_4.2H_2O$, 0.1 mg/l $CoCl_2.6H_2O$, 15 mg/l $FeSO_4.7H_2O$, 20 mg/l Na_2EDTA, 100 mg/l myo-inositol, 5 mg/l nicotinic acid, 10 mg/l pyridoxine HCl, 10 mg/l thiamine HCl, 2 mg/l glycine, 1% sucrose, 0.9% Daichin agar containing 300 µg/ml cefotaxime (Duchefa)) and covered by a (half-) filter paper. Plants were grown for 6-18 d on "Emergence medium". In this period new roots are formed that are potentially co-transformed with the T-DNA of the binary vector.

Transformation of *Arabidopsis* was done in a similar way with the following differences: seeds were surface sterilized by incubating for 5 min in 2% hypochlorite (commercial bleach), and 5x washing in sterile water, and were vernalized at 4°C for 3 d. Two-day-old seedlings were used for co-cultivation with the appropriate *Agrobacterium* MSU440 strain (20 plants per plate). Plants were grown on plates with a filter paper, containing 0.5x Murashige and Skoog (MS) salts (Duchefa), 1% sucrose and 0.8% (w/v) Daichin agar, for 3 d (21°C; 16 h photoperiod) and subsequently transferred to 0.5x MS plates containing 300 µg/ml cefotaxime (Duchefa).

Nodulation of *A. rhizogenes* transformed roots

Three weeks after transformation composite *Medicago* plants were starved for nitrate for 3 d (21°C; 16h light-8h darkness) on Färhaeus medium [without $Ca(NO_3)_2$.]. Then plants are transferred to agra-perlite (Maasmond-Westland, The Netherlands) saturated with Färhaeus medium [without $Ca(NO_3)_2$] and inoculated with 1 ml culture of *S. meliloti* 2011 (OD600 = 0.1) per plant and grown for two weeks (21°C; 16 h photoperiod).

Clonally propagating *A. rhizogenes* transformed roots

A. rhizogenes transformed *Arabidopsis* roots were excised (~1 cm above the tip) and transferred to 25 ml ARC medium (Czakó et al. 1993) containing 0.05 mg/l IAA. After 3 d, the root pieces were transferred to new 25 ml ARC medium without IAA and cultured in the dark at

25°C for 4 weeks with gentle shaking (100 rpm).

Microscopy

Imaging of DsRED1 or GFP was done using the Leica MZIII fluorescence stereomicroscope with the appropriate filter settings. Images where processed electronically using Adobe Photoshop 5.5. Spectral imaging of DsRED1 was done according to Gadella et al. (1997). The slit width of the imaging spectrograph was 200 µm and the central wavelength was 550 nm. A 525-nm longpass emission filter was used. Imaging of GFP fluorescence in the J0781 cross-section was performed on a Zeiss LSM 510 confocal laser scanning microsope (Carl-Zeiss) with excitation 488 (GFP) and emission 543 nm (propidium iodide). GFP emission was selectively detected by using a 505-530 nm bandpass filter and propidium iodide emission was detected in another channel using a 560-615 nm bandpass. The root was counter-stained with 0.2 µg/ml propidium iodide.

qPCR

Total RNA was extracted from 4-week-old *Arabidopsis* root cultures according to Pawlowski et al. (1994) followed by DNase I (Promega) treatment. cDNA was made from 1 µg total RNA using the Taqman Gold RT-PCR kit (Perkin-Elmer Applied Biosystems) in a total volume of 50 µl using the supplied hexamer primers. qPCR reactions were performed in triplo on 6.5 µl cDNA using the SYBR-GreenR PCR Master kit (Applied Biosystems) (40 cycles of 95°C for 10 s, 60°C for 1 min) and real-time detection was performed on the ABI 7700 and analyzed using the GeneAmp 5700 SDS software (Applied Biosystems).

Primers used:

AtAct2/8-F:	5’ GGTAACATTGTGCTCAGTGGTGG 3’
AtAct2/8-R:	5’ AACGACCTTAATCTTCATGCTGC 3’
AtKJK-F:	5’ TGAGAGCTCTTGATGGGTTGATGG 3’
AtKJK-R:	5’ TCGGTTTTCTTCTGGCACACGG 3’
GFP4-F:	5’ CTGTCCTTTTACCAGACAACCATTACC 3’
GFP4-R:	5’ CCAGCAGCTGTTACAAACTCAAGAAG 3’

Immunoblotting

Proteins were isolated from 4-week-old *Arabidopsis* J0781 root cultures by grinding 1 g of roots in liquid nitrogen and resuspension in extraction buffer (50 mM Tris-acetate, pH 7.4, 10 mM potassium-acetate, 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF) followed by two subsequent centrifugation steps: 1000 rpm 15 min and 15000 rpm 30 min. The supernatant was used for analysis. Protein concentrations were determined using a protein assay (Bio-Rad).

Proteins (20 µg) were separated on a 12.5% SDS-PAGE gel, blotted onto nitrocellulose paper (Schleicher and Schuell), immunostained with 1:1000 diluted rabbit anti-GFP (Molecular Probes) followed by 1:5000 diluted anti-rabbit-HRP, and detected using the ECL PlusTM Western Blotting Detection Kit for HRP (Amersham Biosciences) on a Storm 840 (Molecular Dynamics). The blot was stained for total protein using Ponceaux S (Sigma Diagnostics).

ACKNOWLEDGEMENTS

The authors thank M. Kwaaitaal (Biochemistry, Wageningen University) and J. Pouwels (Molecular Biology, Wageningen University) for their help with the immunoblotting, Toolbox 21 for their work on *Arabidopsis* line *35S::GFP*, Oscar Vorst and Jos Molthoff (Plant Research International, Wageningen) for their assistance with the qPCR, and J-W Borst (Biochemistry, Wageningen University) for assistance with the FSPIM. The *Arabidopsis* line *35S::GFP* was kindly provided by Renze Heidstra (Molecular Cell Biology, Utrecht University). E.L., T.B. and R.G. are supported by The Netherlands Organization of Scientific Research (NWO) and the E.U. FP5 program QLG-CT2000-00676.

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Chapter 4

Expression Studies of Superoxide Dismutases in Nodules and Leaves of Transgenic Alfalfa Reveal Abundance of Iron-Containing Isozymes, Post-translational Regulation, and Compensation of Isozyme Activities

Rubio MC, Ramos J, Webb KJ, Minchin FR, González E, Arrese-Igor C, Becana M (2001) Expression studies of superoxide dismutases in nodules and leaves of transgenic alfalfa reveal abundance of iron-containing isozymes, post-translational regulation, and compensation of isozyme activities. Mol Plant-Microbe Interact **14:** 1178-1188

ABSTRACT

The composition of antioxidant enzymes, especially superoxide dismutase (SOD), was studied in one nontransgenic and three transgenic lines of nodulated alfalfa plants. Transgenic lines overproduced MnSOD in the mitochondria of nodules and leaves (line 1-10), MnSOD in the chloroplasts (line 4-6), and FeSOD in the chloroplasts (line 10-7). In nodules of line 10-7, the absence of transgene-encoded FeSOD activity was due to lack of mRNA, whereas in nodules of line 4-6 the absence of transgene-encoded MnSOD activity was due to enzyme inactivation or degradation. Transgenic alfalfa showed a novel compensatory effect in the activities of MnSOD (mitochondrial) and FeSOD (plastidic) in the leaves, which was not caused by changes in the mRNA levels. These findings imply that SOD activity in plant tissues and organelles is regulated at least partially at the post-translational level. All four lines had low CuZnSOD activities and an abundant FeSOD isozyme, especially in nodules, indicating that FeSOD performs important antioxidant functions other than the scavenging of superoxide radicals generated in photosynthesis. This was confirmed by the detection of FeSOD cDNAs and proteins in nodules of other legumes such as cowpea, pea, and soybean. The full-length cDNA encoding alfalfa nodule FeSOD was characterized and the deduced protein found to contain a plastid transit peptide. Comparison of sequences and other properties reveals that there are two types of FeSODs in nodules.

INTRODUCTION

The superoxide dismutase (SOD) family of enzymes represents a primary line of defense against the superoxide radical and derived reactive oxygen species in all organisms. Three types of SOD, differing in the metal at the active site, may coexist in plants. The CuZnSOD isozymes are localized in the cytosol, chloroplast stroma, glyoxysomal matrix, apoplast, nucleus, and mitochondrial intermembrane space; the MnSOD isozymes are localized in the mitochondrial and peroxisomal matrices and in the glyoxysomal membrane; and the FeSOD isozymes are primarily localized in the chloroplast stroma (del Río et al. 1992; Bowler et al. 1994; Ogawa et al. 1996; Schinkel et al. 1998). Complete cDNA clones encoding some enzymes of each type have been isolated and used to construct transgenic plants overexpressing SODs in the chloroplasts, mitochondria, and cytosol (Bowler et al. 1991; Scandalios 1993; Van Camp et al. 1996). These studies were performed using model plants for transformation, such as tobacco, but important crop legumes can now be transformed and regenerated with relative ease (Christou 1994). Because legumes are unique amongst crop plants in their ability to fix atmospheric N_2 in symbiosis with rhizobia, transformation of these plants with antioxidant genes could provide additional advantages related to its protective role in nodule activity (Puppo et al. 1982; Dalton et al. 1998; Matamoros et al. 1999).

In this work, we have analyzed the antioxidant composition of nodulated plants of the alfalfa elite genotype N4 and three derived transgenic lines overproducing, respectively, MnSOD in the mitochondria, MnSOD in the chloroplasts, and FeSOD in the chloroplasts. Transgenic alfalfa plants showed a compensatory effect in expression of MnSOD and FeSOD in the leaves. All four lines displayed low CuZnSOD activities and abundant FeSOD and MnSOD activities in nodules and leaves. The full-length cDNA sequence encoding alfalfa nodule FeSOD was characterized and nodules of different legumes were screened for the presence of FeSOD cDNAs and proteins. Results show that the FeSODs of nodules belong to two types of enzymes that can be distinguished biochemically and immunologically and that are probably located in different subcellular compartments.

RESULTS

Composition of SOD isozymes in alfalfa nodules and leaves

SOD activity staining of nondenaturing gels preincubated with potassium phosphate buffer alone or supplemented with either 3 mM KCN or 5 mM H_2O_2 revealed that alfalfa nodules (Fig. 1) and leaves (Fig. 2) contain the three types of SOD that can be found in plants.

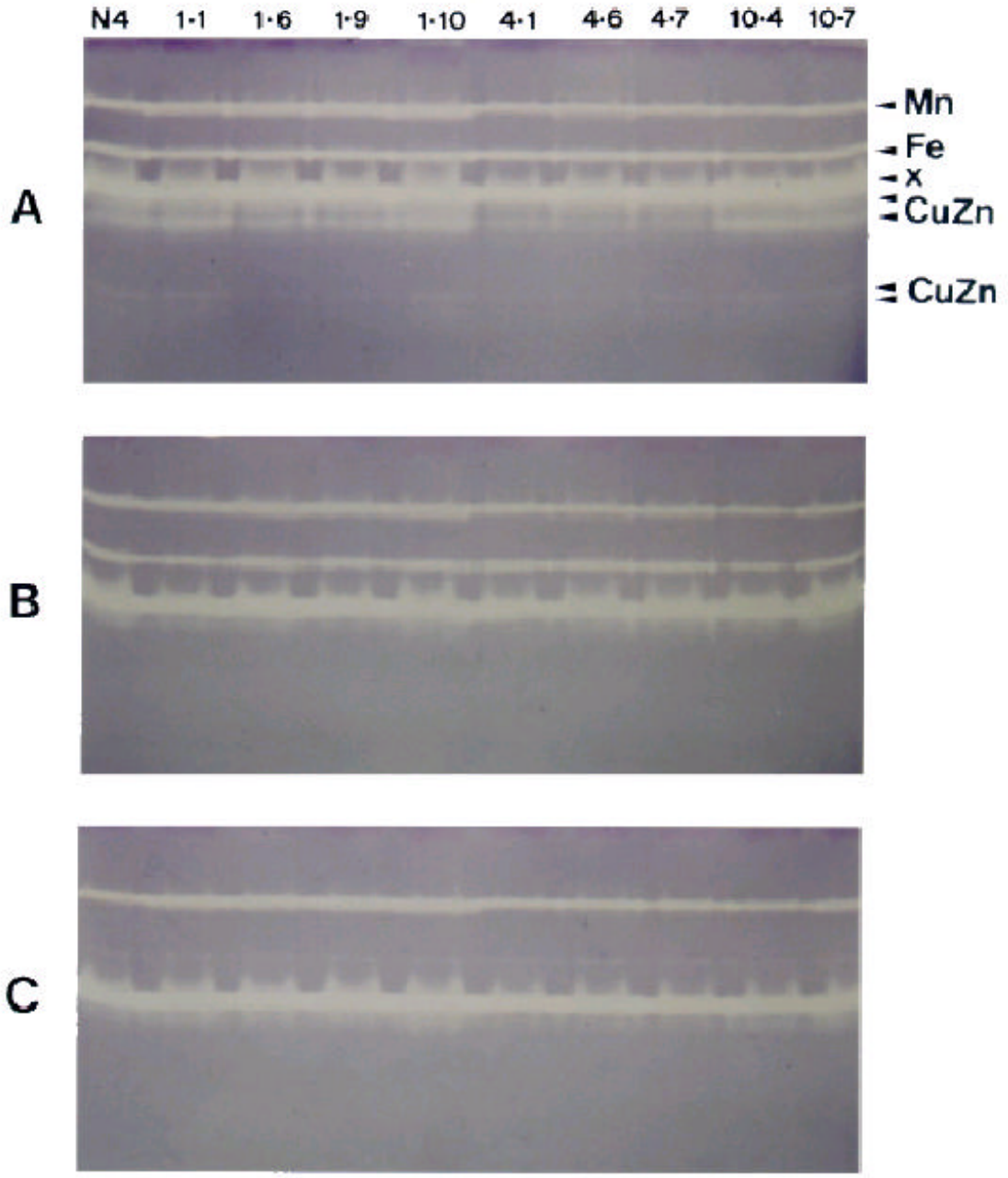

Figure 1. SOD isozyme composition of nodules from alfalfa line N4 and derived transgenic lines. These were designed to overproduce MnSOD in mitochondria (lines 1-1, 1-6, 1-9, and 1-10), MnSOD in the chloroplasts (lines 4-1, 4-6, and 4-7), or FeSOD in the chloroplasts (lines 10-4 and 10-7). Prior to activity staining, the gels were incubated with (A) potassium phosphate buffer, (B) buffer plus 3 mM KCN, or (C) buffer plus 5 mM H_2O_2. Lanes were loaded with 40 μg protein.

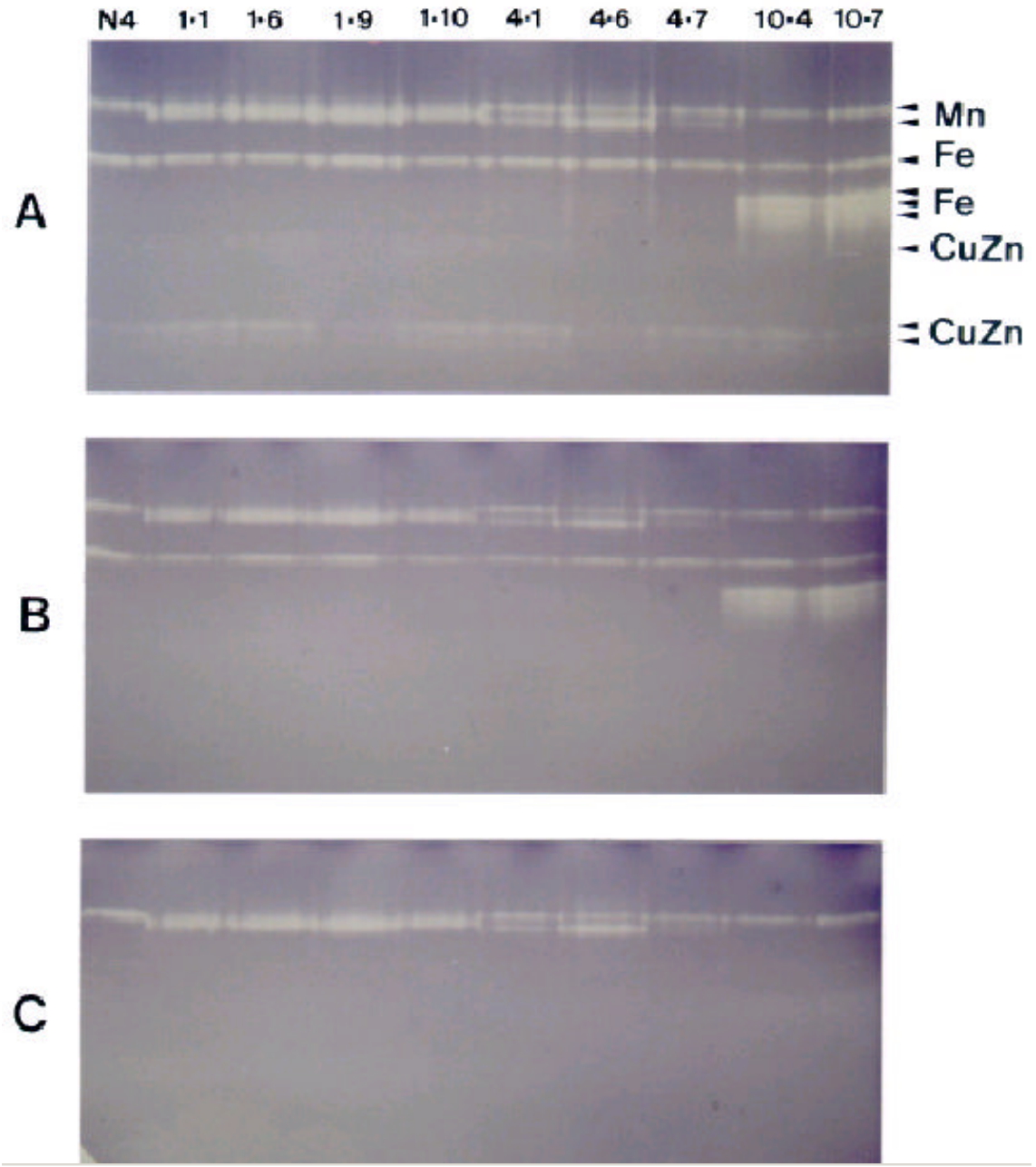

Figure 2. SOD isozyme composition of leaves from alfalfa line N4 and derived transgenic lines. Panel description is identical to that of Figure 1. Lanes were loaded with 40 µg protein.

These corresponded, in order of increasing mobility, to the MnSOD (KCN-insensitive, H_2O_2-insensitive), FeSOD (KCN-insensitive, H_2O_2-sensitive), and CuZnSOD (KCN-sensitive, H_2O_2-sensitive) isozymes. Nodule extracts of all lines produced a single band of mitochondrial MnSOD activity (Fig.1). However, in nodules and leaves of lines transformed to overproduce *Nicotiana plumbaginifolia* MnSOD in mitochondria (1-1, 1-6, 1-9, and 1-10), the MnSOD activity band was considerably more intense than in the nontransgenic line (Figs. 1 and 2), which probably reflects the inability of these gels to separate alfalfa and *Nicotiana* MnSODs. In contrast, in leaves of lines producing MnSOD in the chloroplasts (4-1, 4-6, and 4-7), two MnSODs were resolved (Fig. 2).

Nodules of all lines examined contained a distinct FeSOD isozyme, which was also present, albeit at lower levels, in the leaves (compare Figs. 1B and 2B). The presence of abundant FeSOD activity in alfalfa nodules and leaves is in clear contrast with previous reports showing no FeSOD in leaves (McKersie et al. 1999; 2000). Extracts of leaves, but not of nodules, of lines transformed to produce *Arabidopsis thaliana* FeSOD in the chloroplasts (10-4 and 10-7) showed three extra bands of FeSOD activity (Fig. 2). In contrast, in nodules there were four CuZnSOD isozymes, the first two of them (according to relative mobility) at higher levels than in the leaves (Fig. 1). Nodules also had a major band showing SOD activity, which was labeled as X. This band accounted for 70% of the total activity (calculated from data in Table 1) and was assigned to the MnSOD type (KCN-insensitive, H_2O_2-insensitive). However, its electrophoretic mobility (similar to that of plant FeSODs) and apparent molecular mass (45 kDa) are clearly different from those of typical plant MnSODs (Sevilla et al. 1982). It is possible that at least part of the activity of band X is due to bacteroid MnSOD. Thus, activity stain gels of extracts from highly purified bacteroids, broken by sonication, revealed a single MnSOD isozyme which had identical mobility to band X (Fig. 1).

Transcripts of SOD isozymes in alfalfa nodules and leaves

Out of the nine lines initially screened, we selected line N4 and three transgenic lines (1-10, 4-6, and 10-7) for further study. These lines were representative of each different construct. Expression of *MnSOD* and *FeSOD* genes in nodules and leaves of the four selected lines was analyzed by reverse transcription (RT)-PCR and northern blots. Gene-specific primers were used to distinguish the mRNAs encoded by the endogenous genes (alfalfa *MnSOD* and *FeSOD)* and the transgenes *(Nicotiana MnSOD* and *Arabidopsis FeSOD)*.

Consistent with activity data (Fig. 1), RT-PCR analysis revealed that the absence of *Arabidopsis* FeSOD activity in nodules of line 10-7 was due to lack of message, whereas the absence of *Nicotiana* MnSOD activity in nodules of line 4-6 was probably due to inactivation or degradation of the enzyme because the corresponding mRNA was detectable at levels similar to those found in line 1-10 (Fig. 3). The analysis also showed that the leaves of all four lines

express endogenous *MnSOD* and *FeSOD*, that the leaves of lines 1-10 and 4-6 express *Nicotiana MnSOD*, and that the leaves of line 10-7 express *Arabidopsis FeSOD* (Fig. 3).

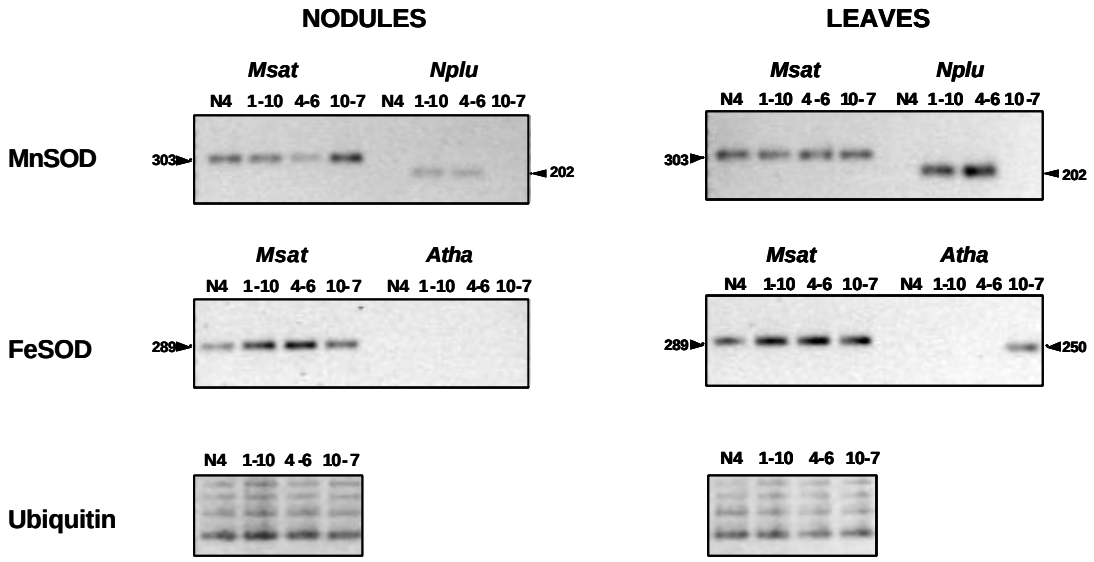

Figure 3. RT-PCR analysis of SOD isozymes of alfalfa nodules and leaves. Gene-specific primers were used to distinguish mRNAs of alfalfa (*Msat MnSOD* and *Msat FeSOD)* and mRNAs encoded by the transgenes (*Nplu MnSOD* and *Atha FeSOD)*. Abbreviations are as follows: *Msat, Medicago sativa; Nplu, Nicotiana plumbaginifolia; Atha, Arabidopsis thaliana.* Transgenic lines overproduced MnSOD in mitochondria (line 1-10), MnSOD in the chloroplasts (line 4-6), or FeSOD in the chloroplasts (line 10-7). Size of PCR products is indicated in bp. To ensure uniform amounts of template, PCR reactions were performed simultaneously using ubiquitin primers (Horvath et al. 1993).

Table 1. Antioxidant enzyme activities in nodules of line N4 and three derived transgenic lines of alfalfa[a]

Enzyme	N4	1-10	4-6	10-7
Total SOD	11.29 ± 1.59 a	13.43 ± 0.66 a	13.03 ± 1.03 a	12.09 ± 0.38 a
MnSOD	2.02 ± 0.15 a	2.93 ± 0.38 b	2.22 ± 0.33 ab	2.32 ± 0.16 ab
FeSOD	1.30 ± 0.14 ab	1.59 ± 0.24 ab	1.71 ± 0.03 b	1.08 ± 0.19 a
APX	92.7 ± 6.8 a	99.1 ± 11.2 a	104.3 ± 12.5 a	87.8 ± 11.8 a
GPX	683 ± 45 a	825 ± 111 a	820 ± 101 a	805 ± 69 a
Catalase	8.9 ± 0.8 a	9.1 ± 1.1 a	8.4 ± 1.5 a	7.1 ± 1.0 a
Protein	182 ± 6 a	198 ± 16 ab	212 ± 10 ab	220 ± 18 b

[a] Units are as follows: SOD (units mg^{-1} dry weight), APX and GPX (µmol min^{-1} g^{-1} dry weight), catalase (mmol min^{-1} g^{-1} dry weight), and protein (mg g^{-1} dry weight). Transgenic lines overproduced MnSOD in mitochondria (line 1-10), MnSOD in the chloroplasts (line 4-6), or FeSOD in the chloroplasts (line 10-7). Means ± SEM (n=4-7) were compared using one-way analysis of variance and the Duncan's multiple range test. For each parameter, means followed by the same letter do not differ significantly at $P<0.05$.

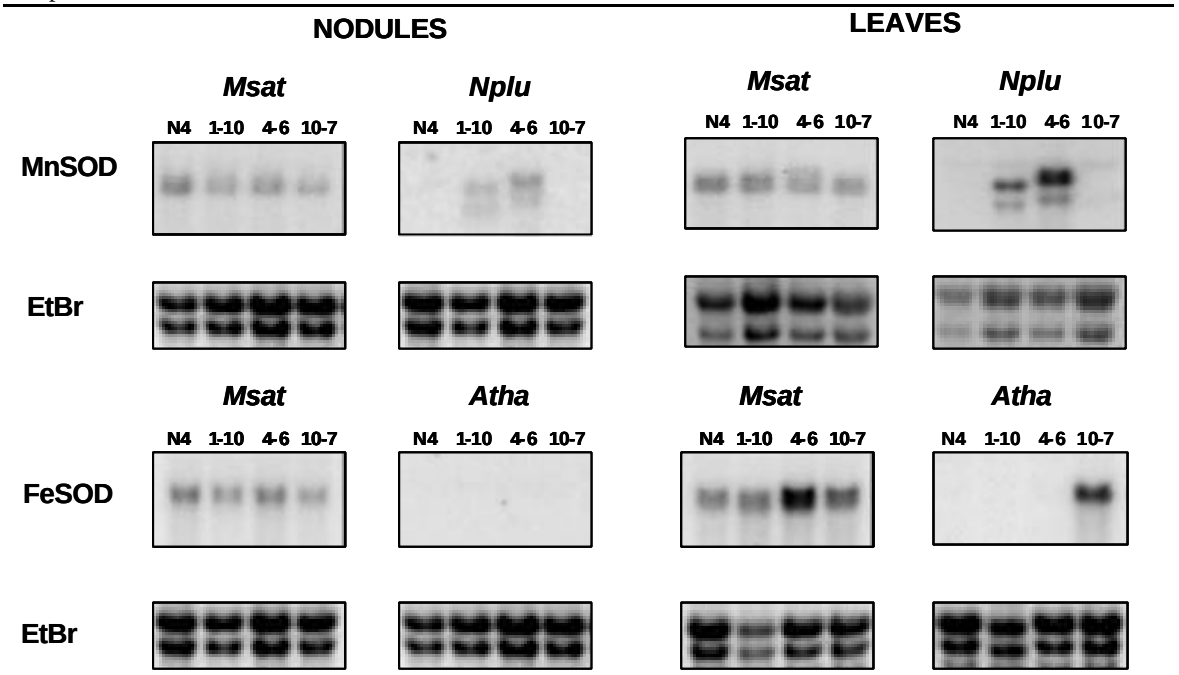

Figure 4. Northern blot analysis of SOD isozymes of alfalfa nodules and leaves. Transgenic lines overproduced MnSOD in mitochondria (line 1-10), MnSOD in the chloroplasts (line 4-6), or FeSOD in the chloroplasts (line 10-7). Probes were generated from the primers used for RT-PCR. All the bands had the expected size of 1100 to 1200 bp according to RNA molecular weight markers. Uniform loading and integrity of RNA was verified by spectrophotometry and visualization of RNA with ethidium bromide (EtBr).

Table 2. Antioxidant enzyme activities in leaves of line N4 and three derived transgenic lines of alfalfa[a]

Enzyme	N4	1-10	4-6	10-7
Total SOD	2.86 ± 0.14 a	2.77 ± 0.15 a	2.96 ± 0.15 a	3.98 ± 0.11b
MnSOD	1.52 ± 0.14 a	1.80 ± 0.13 b	1.88 ± 0.21 b	0.96 ± 0.10 c
FeSOD	1.18 ± 0.11 a	0.64 ± 0.08 b	0.75 ± 0.31 b	2.40 ± 0.14 c
APX	63.2 ± 5.4 a	49.1 ± 8.3 a	67.1 ± 7.2 a	63.9 ± 10.2 a
GPX	259 ± 11 a	188 ± 14 b	233 ± 11 a	238 ± 19 a
Catalase	0.67 ± 0.22 a	0.63 ± 0.20 a	0.58 ± 0.14 a	0.58 ± 0.09 a
Protein	163 ± 6 a	169 ± 8 a	204 ± 15 b	209 ± 11 b

[a] Enzyme units and statistical analysis of means ± SEM (n=4-5) are as in Table 1. Transgenic lines overproduced MnSOD in mitochondria (line 1-10), MnSOD in the chloroplasts (line 4-6), or FeSOD in the chloroplasts (line 10-7). For each parameter, means followed by the same letter do not differ significantly at $P<0.05$.

Northern blot analysis of the steady-state mRNA levels confirmed the presence of abundant message for endogenous MnSOD and FeSOD in nodules and leaves of all four lines, as well as the absence of *Arabidopsis* FeSOD mRNA in nodules of line 10-7 and its presence in the corresponding leaves (Fig. 4). Most interesting, northern blots showed abundant mRNA for *Nicotiana* MnSOD in the leaves of line 4-6 and less abundant mRNA in the leaves of line 1-10. The two mRNAs were also present in the corresponding nodules (Fig. 4). The differences in mRNA size between the *Nicotiana* MnSODs targeted to chloroplasts (line 4-6) and to mitochondria (line 1-10) reflect the differences in the original constructs, with the chloroplast transit peptide being significantly larger than the mitochondrial peptide (Bowler et al. 1991).

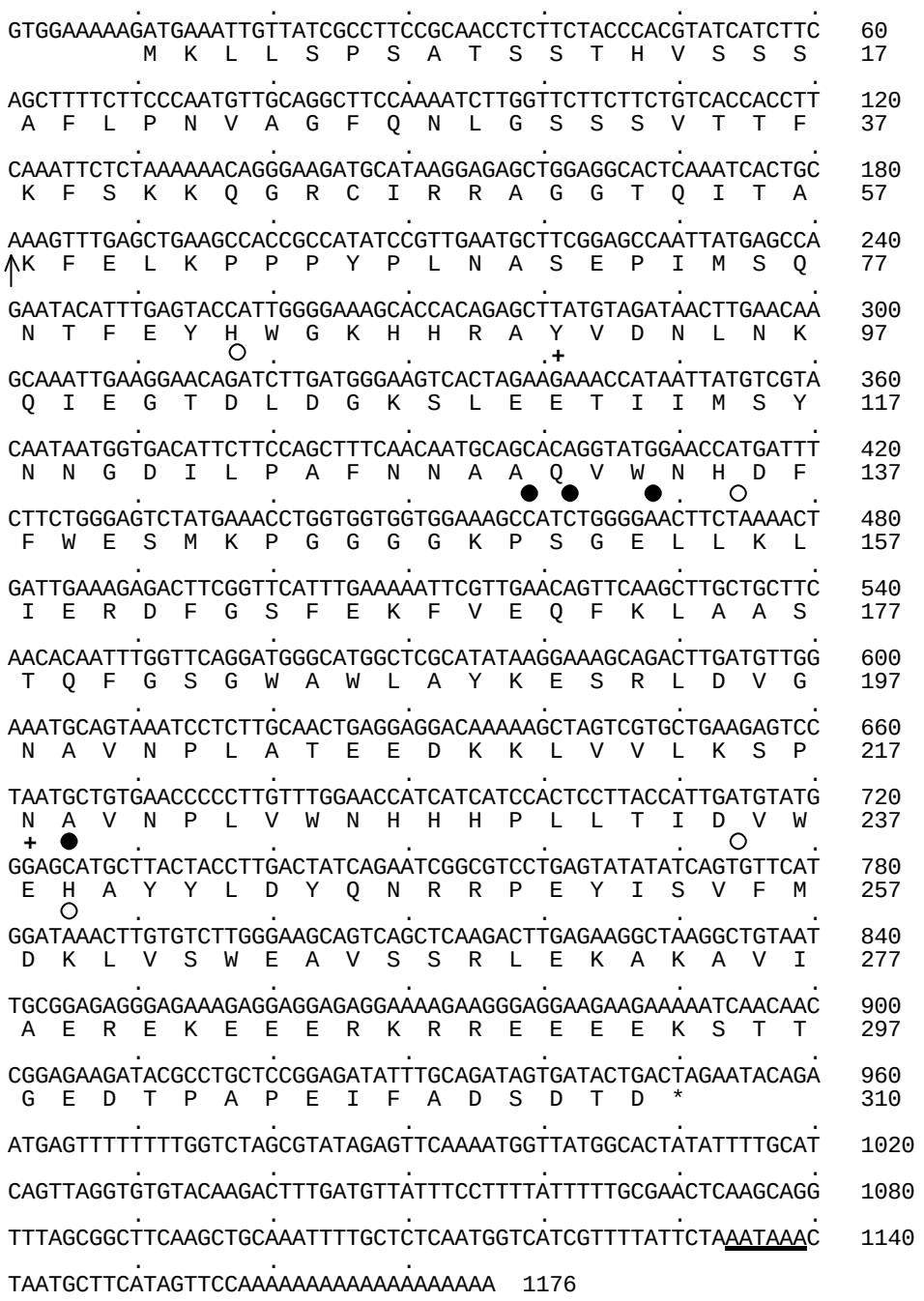

Figure 5. Nucleotide and deduced amino acid sequences of FeSOD from alfalfa nodules. The arrow indicates the putative cleavage site of the signal peptide. Residues that distinguish FeSOD from MnSOD are indicated with a black circle. Residues essential for catalytic activity and metal binding are indicated with a plus mark and a white circle, respectively. A putative polyadenylation sequence at the 3' end is underlined.

Antioxidant enzymes of alfalfa nodules and leaves

The activities of SOD and other antioxidant enzymes were measured in the four selected lines. The antioxidant activities of nodules from control plants were measured with optimized methods

including specific controls to ensure measurement of genuine activities in the presence of leghemoglobin, which may interfere with determination of guaiacol peroxidase (GPX) or SOD activity under certain conditions (Puppo et al. 1982). There were no significant differences in the total SOD activity or in the activities of the H_2O_2-scavenging enzymes in nodules for any of the four lines. However, when MnSOD and FeSOD activities were individualized by densitometry, MnSOD activity in line 1-10 was found to be 45% higher than in line N4 and FeSOD activity of line 4-6 was 32% higher (Table 1).

Total SOD, APX, GPX, and catalase activities were similar in leaves of all lines, except in line 10-7, which exhibited a total SOD activity 39% higher than in line N4 (Table 2). Separate quantification of SOD isozymes showed that the leaves of lines 1-10 and 4-6 had approximately 20% more MnSOD activity and 40% less FeSOD activity. Conversely, the leaves of line 10-7 had 37% less MnSOD activity and twice as much FeSOD activity. These results confirmed that the leaves of the transformed plants expressed the MnSOD or FeSOD encoded by the transgenes.

Characterization of the FeSOD cDNA and deduced protein of alfalfa nodules

The finding of abundant FeSOD activity in alfalfa nodules and the absence of sequences for any FeSOD from nonphotosynthetic tissue prompted us to isolate and characterize the corresponding cDNA. Degenerate primers were used to screen an alfalfa nodule cDNA library. As expected, the positive clones contained the 5' and 3' regions of an FeSOD cDNA, judging by the high homology (>70% identity) with other cDNAs encoding FeSODs from higher plants. The complete cDNA sequence of alfalfa nodules (Fig. 5) contains 10 nucleotides within the 5'-untranslated region (UTR), 942 nucleotides within the open reading frame (ORF), and 205 nucleotides within the 3'-UTR that precedes a poly(A+) tail. The 3' end also contains a consensus signal for polyadenylation.

The ORF encodes a protein of 313 amino acids (Fig. 5), with a calculated molecular mass of 35.4 kDa and isoelectric point of 5.79. The enzyme contains the residues thought to be essential for FeSOD activity (Tyr-91, Trp-133, and Asn-218) and for metal binding (His-83, His-135, Asp-235, and His-239), as well as the residues (Ala-130, Gln-131, Trp-133, and Ala-219) proposed as primary candidates to distinguish FeSODs from MnSODs (Van Camp et al. 1990; Bowler et al. 1994). The deduced protein of alfalfa nodules also contains a signal peptide that, based on the N-terminal sequences of other FeSODs (Van Camp et al. 1990), is 57 amino acid residues long (Fig. 5). The signal peptide is very rich in Ser (19%) and Thr (11%) compared to Arg (5%), and is devoid of Glu, Asp, and Tyr. All these characteristics are typical of plastid transit peptides (von Heijne et al. 1989). The mature protein has a calculated molecular mass of 29.4 kDa and isoelectric point of 5.09, which is significantly different from the values predicted (molecular mass of 23.0 to 25.3 kDa; isoelectric point of 5.42 to 6.07) for the FeSODs of other

plants. The deduced amino acid sequence of alfalfa nodule FeSOD has high homology (>70%) with the FeSODs from other higher plants and lower homology (<70%) with the enzymes from cyanobacteria and green algae. However, there are two exceptions: the nodule enzyme showed only 58% identity with *Arabidopsis* FeSOD isozyme-1 and 50% identity with rice FeSOD.

Because FeSODs are detected only ocassionally in plants, we screened extracts of other legume nodules for the presence of FeSOD. This provided information on the distribution and abundance of the enzyme and allowed a comparison with the alfalfa nodule FeSOD. Using SOD activity gels, we found FeSOD in nodules of cowpea, mungbean, pea, bean, and soybean, but not in those of lupine and broad bean (data not shown). The same primers used for alfalfa served to isolate clones and obtain the complete cDNA sequence of cowpea nodule FeSOD as well as partial sequences for pea and soybean nodule FeSODs. All derived protein sequences were used to construct a phylogenetic tree of known FeSODs (Fig. 6). It is interesting to note that two isozymes have been described for *Arabidopsis* and that the partial sequence we found for soybean nodules differs from that reported for leaves, suggesting that FeSOD may be present as a multigenic family at least in some plants. Phylogenetic analysis revealed three large clusters, which, as expected, are fully consistent with homology data: a cluster including the FeSODs of cyanobacteria, green algae, rice, and *Arabidopsis* isozyme-1; a cluster including the FeSOD of *Raphanus* and *Arabidopsis* isozyme-2; and a cluster including legume FeSODs. Interestingly, in this third cluster two groups can be recognized (Fig. 6) and this appears to be correlated with subcellular localization. Prediction programs reveal that the FeSODs of alfalfa and pea nodules contain a plastid signal peptide, whereas the FeSODs of cowpea nodules and soybean leaves and nodules lack any recognizable plastid peptide. The presence of two types of FeSODs in nodules is confirmed by the absence of cross-reactivity between an antibody raised to cowpea nodule FeSOD and alfalfa or pea nodule FeSODs (data not shown).

DISCUSSION

In this work we report that all three types of SODs are present in nodules and leaves of alfalfa (Figs. 1 and 2). The isozymic composition of SOD in our extracts (low CuZnSOD activity, high FeSOD activity) clearly contrasts with that reported by other authors (McKersie et al. 2000) for alfalfa leaves (high CuZnSOD activity, virtually no FeSOD activity). These differences may be due to variations in plant development or to nutritional factors, such as the nitrogen source (N_2 versus combined nitrogen) and the micronutrient supply (which is known to differentially affect expression of SOD isozymes; eg. Kurepa et al. 1997). Whatever the reason, our results indicate that important variations in the SOD isozyme pattern do not affect the health and growth of plants.

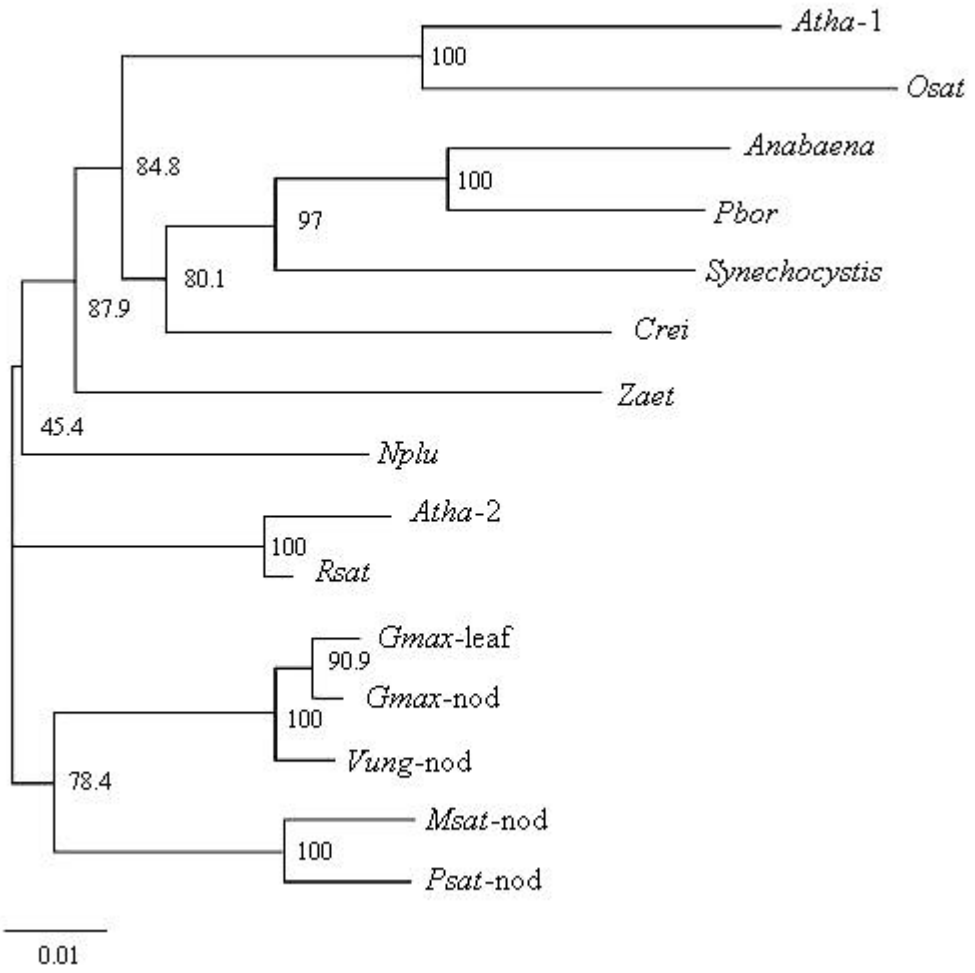

Figure 6. Unrooted phylogenetic tree of FeSOD proteins from cyanobacteria, green algae, and higher plants. The tree was calculated using the neighbor-joining method of the CLUSTAL W suite of programs. The numbers correspond to percentages of 1000 'bootstraps'. The bar represents 0.01 substitutions per site. Abbreviations and GenBank accession numbers are as follows: *Anabaena*, *Anabaena* strain PCC7120 (AF173990); *Atha*-1, *Arabidopsis thaliana* isozyme-1 (AF061852); *Atha*-2, *Arabidopsis thaliana* isozyme-2 (P21276); *Crei*, *Chlamydomonas reinhardtii* (JC4611); *Gmax*-leaf, *Glycine max*, leaf isozyme (M64267); *Gmax*-nod, *Glycine max* nodule isozyme (partial sequence); *Msat*-nod, *Medicago sativa* nodules (AF377344); *Nplu*, *Nicotiana plumbaginifolia* (A39267); *Osat*, *Oryza sativa* (AB014056); *Pbor*, *Plectonema boryanum* (P50061); *Psat*-nod, *Pisum sativum* (partial sequence); *Rsat*, *Raphanus sativus* (AF061583); *Synechocystis*, *Synechocystis* strain PCC6803 (P77968); *Vung*-nod, *Vigna unguiculata* nodules (AF077224); *Zaet*, *Zantedeschia aethiopica* (AF094831).

This may well reflect compensation in the activities of the SOD isozymes, which would require their expression to be tightly regulated. Thus, overproduction of MnSOD in the leaves of lines 1-10 and 4-6 was matched by lower FeSOD activities, resulting in similar total SOD activities in the leaves of the two lines and in the nontransgenic line (Table 2). Conversely, overproduction of FeSOD in line 10-7 was paralleled by a lowering in MnSOD activity, although in this case the 2-fold excess of the former was not compensated for completely and the total SOD activity of leaves in line 10-7 remained 40% greater than in line N4. Northern analysis indicated that the

compensation of activities is not due to changes in transcript abundance. Thus, the mRNA level of endogenous MnSOD in leaves of line 10-7 (which expresses *Arabidopsis* FeSOD) is even higher than in line N4, and the mRNA level of endogenous FeSOD in lines 1-10 and 4-6 (which express *Nicotiana* MnSOD) is similar to or higher than in line N4 (Fig. 4). Therefore, the compensation between MnSOD and FeSOD activities occurs, at least in part, at the post-translational level.

In nodules, only line 1-10 produced the transgene-encoded SOD (Fig. 1). However, the *Nicotiana* MnSOD mRNAs were found in both lines 1-10 and 4-6, albeit at low levels (Figs. 3 and 4). The absence of *Nicotiana* MnSOD activity in nodules of line 4-6, despite expression of the gene, can be attributed to degradation of the enzyme, perhaps as a result of the inability of nodule plastids to process the protein bearing a chloroplastic prepeptide. Another surprising observation is the absence of transcript for *Arabidopsis* FeSOD in nodules of line 10-7, despite its abundance in the corresponding leaves. This may be ascribed at least in part to a weak activity of the 35S promoter in alfalfa nodules. However, this would still leave unexplained why constructs with identical promotor and chloroplastic targeting sequences are expressed (MnSOD in line 4-6) or not expressed (FeSOD in line 10-7) in nodules. To verify the differences in expression among the three transgenic lines, nodules of exactly the same plant were used to extract RNA (for northern analysis) and protein (for activity gel analysis). This experiment confirmed that nodules of line 1-10 expressed transcript and activity, nodules of line 4-6 expressed transcript but not activity, and nodules of line 10-7 did not express the transcript.

The abundance of FeSOD in alfalfa nodules deserved special attention, as this type of SOD, in contrast to CuZnSOD and MnSOD, is not ubiquitous in plants. Furthermore, there are contradictory reports about the presence of FeSOD in leaves of some species, such as common bean and cowpea. Early studies reported that FeSODs were confined to a few families of higher plants (Bridges and Salin 1981). However, it seems now that the gene is more widely distributed than previously thought but remains silent due to lack of appropriate inducing conditions (Bowler et al. 1994). Our finding of FeSOD in nodules of healthy plants of alfalfa and other legumes is consistent with that hypothesis. The FeSODs of alfalfa and pea nodules are closely related and are synthesized as precursor plastidic proteins, which suggests that these enzymes play a role in plastid metabolism other than the scavenging of superoxide radicals associated with photosynthesis. The occurrence of abundant ferritin in the plastids may ensure an adequate Fe supply for the synthesis of FeSOD, especially under conditions in which this isozyme is up-regulated (reviewed by Becana et al. 1998). The presence of at least two types of FeSODs further underscores the multifaceted nature of antioxidant protection in nodules and suggests that the various SOD isozymes may have evolved to perform specific defensive or regulatory roles that are essential for optimal nodule functioning. Clearly, further work is necessary to characterize these roles.

MATERIALS AND METHODS

Plant material and propagation

Alfalfa (*Medicago sativa* L.) clones used in this study were generously provided by Dr. Bryan McKersie. Clone N4 is the nontransgenic parental line. Clones 1-1, 1-6, 1-9, and 1-10 were transformed with pSOD1, which was generated as a transcriptional fusion of the MnSOD preprotein from *Nicotiana plumbaginifolia* to the cauliflower mosaic virus 35S promoter (Bowler et al. 1991). Clones 4-1, 4-6, and 4-7 were transformed with pSOD4, which harbors the mature MnSOD-encoding sequence of *N. plumbaginifolia* fused to a chloroplast transit peptide sequence (small subunit of Rubisco from pea) under the control of the 35S promoter (Bowler et al. 1991). Clones 10-4 and 10-7 were transformed with pSOD10, which encodes FeSOD from *Arabidopsis thaliana* and is coupled to the same chloroplast targeting sequence under the control of the 35S promoter (Van Camp et al. 1996). All three binary vectors (pSOD1, pSOD4, and pSOD10) contained the *nptII* gene as a selectable marker, under the control of the nopaline synthase *(nos)* promoter (Herrera-Estrella et al. 1983).

Briefly, the transformation procedure was as follows. Alfalfa petiole explants from the parental line were co-cultivated with *Agrobacterium tumefaciens* C58C1 pMP90 for 3 d in the dark on SH induction medium (Van Camp et al. 1996; McKersie et al. 2000). The explants were plated on SH medium containing kanamycin (50 mg l^{-1}) and somatic embryos were matured on BOi2Y development medium containing sucrose but no growth regulators or antibiotics (McKersie et al. 2000). Putative transformants were screened for the presence of T-DNA by PCR. Positive plants were transplanted to growth medium in a greenhouse and were screened by determining SOD activity in leaf extracts and isozyme composition on nondenaturing gels (McKersie et al. 2000). Clones were vegetatively propagated by cuttings taken from plants before flowering, briefly dipped into commercial hormone rooting powder, and placed in beakers containing nutrient solution supplemented with 1 mM KNO_3. Cuttings were left to root in the growth cabinet under low light conditions for approximately 25 d. At that time, plants were transferred to pots containing a perlite/vermiculite mixture and watered once with nutrient solution supplemented with 2 mM KNO_3. After 3 d and 7 d, plants were inoculated twice with *Sinorhizobium meliloti* 102F78. Growth conditions were as previously described (Gogorcena et al. 1997). No obvious phenotypic differences between the transgenic and the parental line were observed.

Isolation of FeSOD cDNA of alfalfa nodules

An alfalfa nodule cDNA λZAP library (kind gift of Dr. Carroll P. Vance) was PCR-screened with degenerate oligonucleotide primers (forward: 5'-GC[A/T]TTCAACAA

[T/C]GC[T/A]GC[T/A]CAGG-3'; reverse: 5'-TC[A/C]AG[G/A]TAGTAAGCATGCTC CCA-3') based on conserved sequences of FeSODs (GenBank accession numbers in parentheses) from *Chlamydomonas reinhardtii* (U22416), *Nicotiana plumbaginifolia* (M55909), *Arabidopsis thaliana* isozyme-2 (M55910), and soybean leaves (M64267). The 5'- and 3'-end regions were amplified using the above primers in combination with T3 (5'-GCAATTAACCCTCACTAAAGGG-3') and T7 (5'-GCGTAATACGACTCACT ATAGGGC-3') primers. The PCR mix contained 0.2 µM of primers, 240 µM dNTPs, 1.5 mM $MgCl_2$, 0.05% W-1 detergent (Life Technologies, Paisley, U.K.), and 1.25 units of *Taq* polymerase (Life Technologies), in a final volume of 25 µl of the PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl). The PCR cycling protocol consisted of an initial denaturation step at 95°C for 3 min, 40 cycles (62°C for 45 s, 72°C for 60 s, 95ºC for 45 s), and a final elongation step at 72°C for 10 min.

The resulting PCR products were size-fractionated on 1% agarose gels, purified (Concert; Life Technologies), and subcloned into the linearized vector pGEM-T Easy (Promega, Madison, WI). The DNA inserts from several clones were sequenced on both strands with an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA). Homology searches were done with the BLAST algorithm (Altschul et al. 1997). Sequence alignments and analyses were performed using the Gap and PileUp programs of the Genetics Computer Group package (Madison). Predictions of subcellular localization and signal peptide analyses were performed using the programs MitoProtII (Claros 1995), PSORT (Nakai and Kanehisa 1992), ChloroP and TransitP (Center for Biological Sequence Analysis, Department of Biotechnology, Technical University of Denmark, Denmark), along with criteria stated by von Heijne et al. (1989).

RT-PCR and northern analyses

Total RNA (approximately 100 µg) was extracted from 200 to 400 mg of leaves or nodules by a phenol-LiCl procedure (Verwoerd et al. 1989), using RNaseOut (Life Technologies). RNA concentration and purity was determined by spectrophotometry and visualized by electrophoresis in agarose-formaldehyde gels.

For RT-PCR, the RNA (5 µg) was primed with oligo$(dT)_{20}$ and reverse-transcribed in a total volume of 25 µl using Moloney murine leukemia virus reverse transcriptase (Promega). PCR was carried out using gene-specific primers based on internal sequences of *Medicago truncatula MnSOD* (AW688895), alfalfa *FeSOD* (AF377344), tobacco *MnSOD* (X14482), and *Arabidopsis FeSOD-2* (M55910). For alfalfa *MnSOD*, the forward primer was 5'-TGTCATCAGCGGCGAAATC-3', and the reverse primer 5'-TTCTGCATTAACCTTTTGTATC-3'. For alfalfa *FeSOD*, forward: 5'-AAGGAAAGCA GACTTGATG-3', reverse: 5'-TCTCCTCCTCTTTCTCC-3'. For *Nicotiana MnSOD*, forward: 5'-GCGGCTTGCAGACCTTTT-3', reverse: 5'-GATAGCGCTATGCAATT TGG-

3'. For *Arabidopsis FeSOD*, forward: 5'-AATGAAAAACTCAAAGTAGTG-3', reverse: 5'-ACTCACTGTCACTGAAGTC-3'. PCR reactions (25 µl) contained 0.75 to 2 µl of first-strand cDNA (taken from a resuspension of the cDNA in 100 µl of water), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of primers, and 2.5 units of *Taq* polymerase (Life Technologies) in a total volume of 25 µl. Reactions were initiated by a denaturation step at 94°C for 4 min, followed by 25 cycles (94°C for 30 s, 54°C for 45 s, and 72°C for 45 s), and a final extension step at 72°C for 10 min. After amplification, the reaction products were resolved by electrophoresis on a 1% (w/v) agarose gel and stained with ethidium bromide. In all cases, preliminary runs were used to verify that the number of amplification cycles was below signal saturation. Images were captured using a Gel-Doc 2000 DNA Gel Analysis and Documentation System (Bio-Rad, Hercules, CA) using Quantity One 4.1.1 software.

For northern hybridization, RNA (10 µg) was separated on 1.2% agarose denaturing (formaldehyde) gels and capillary transferred overnight in 20 x SSC to Hybond-N^+ nylon filters (Amersham Pharmacia Biotech, Uppsala, Sweden). The RNA was fixed to the filter by exposure to UV light for 5 min and then at 80ºC for 2 h. Probes were obtained directly by gel purifying the products of the RT-PCR experiment. Random priming was used to ^{32}P-label the probes (Megaprime, Amersham Pharmacia Biotech). Filters were prehybridized at 42ºC for 2 h with 50% formamide, 125 mM sodium phosphate (pH 7.2), 250 mM NaCl, 1 mM EDTA, and 7% SDS. Hybridization was performed overnight at the same conditions as for prehybridization with addition of the ^{32}P-labeled probes. The filters were washed succesively with 2 x SSC for 10 min, 0.5 x SSC for 10 min, and 0.1x SSC for 3 min (all SSC media contained 0.1% SDS). Signals were detected using a storage phosphor screen (Imaging Screen-K; Eastman Kodak, Rochester, NY) and quantified with a Molecular Imager FX (Bio-Rad) using Quantity One 4.1.1 software.

RNA extractions and RT-PCR and northern experiments were repeated twice using nodules and leaves from two series of plants grown independently. Similar results were obtained and representative data are shown.

Assay of antioxidant enzymes

Samples of nodules or leaves to be used for biochemical analyses were flash-frozen in liquid N_2 and stored at -80°C. All enzymes were extracted at 0-4°C and activities were measured spectrophotometrically at 25°C within the linear region for both time and enzyme concentration. Total SOD activity was assayed by the inhibition of the reduction of ferric cytochrome *c* by the superoxide radicals generated with a xanthine/ xanthine oxidase system. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of ferric cytochrome *c* by 50% (McCord and Fridovich 1969). A low concentration of KCN (10 µM) was included in the assay medium of total SOD to inhibit mitochondrial cytochrome *c* oxidase without affecting

CuZnSOD activity. To assay KCN-insensitive SOD activity, the final KCN concentration was increased to 3 mM. Boiled extracts showed < 4% residual SOD activity.

The isozymic pattern of SODs in nodule and leaf extracts was analyzed by activity staining following electrophoresis on nondenaturing polyacrylamide gels (0.75-mm thick, 15% resolving gel, 4% stacking gel). The activity stain was based on the inhibition by SOD of the reduction of nitroblue tetrazolium by superoxide radicals generated photochemically (Beauchamp and Fridovich 1971). Identification of isozymes was based on the differential inhibition of SOD activity on gels preincubated with 3 mM KCN or 5 mM H_2O_2 for 1 h. Isozymes were quantitated by densitometry using National Institutes of Health software.

APX, GPX, and catalase were extracted with optimized media essentially as described by Gogorcena et al. (1995). APX and catalase activities were assayed by following the disappearance of ascorbate at 290 nm (Asada 1984) and of H_2O_2 at 240 nm (Aebi 1984), respectively. GPX activity was measured by following the oxidation of pyrogallol at 430 nm with preincubation for 5 min with 0.5 mM *p*-chloromercuriphenyl-sulfonic acid (Amako et al. 1994). Because APX may also catalyze pyrogallol oxidation to some extent, *p*-chloromercuriphenyl-sulfonic acid was included in the assay medium to inactivate APX and thus ensure the accurate measurement of GPX activity.

Statistical analyses

Six series of plants grown independently under identical environment conditions were required to obtain sufficient nodule and leaf material. Each series comprised plants of all four lines. All measurements were made on plants belonging to at least two series and were pooled for statistical analysis. All data were subjected to analysis of variance and, when this was significant, means were compared with the Duncan's multiple range test. The number of samples used for the calculation of the means is stated in each table. Experiments to analyze gene expression by RT-PCR and northern blot were performed in duplicate, each replicate corresponding to extracts of nodules and leaves from two series of plants.

ACKNOWLEDGEMENTS

This work was supported by grant 2FD97-1101 from the Comisión Interministerial de Ciencia y Tecnología and the European Commission and by grant PB98-0522 from the Dirección General de Investigación Científica (Spain). The Institute of Grassland and Environmental Research is grant aided by the Biotechnology and Biological Sciences Research Council. M.C.R. was the recipient of a predoctoral fellowship from the Ministry of Education and Culture (Spain). M.C.R. and J.R. were also supported by contracts on grant 2FD97-1101. We thank Gloria Rodríguez for help with harvesting of plant material, Iñaki Iturbe-Ormaetxe for help with Figure 4, and Jose F. Moran for help with Figure 6.

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Chapter 5

Concluding Remarks

Biological N_2 fixation constitutes the main natural input of nitrogen into the biosphere. This process is carried out by prokaryotes either in the free-living form or in mutualistic symbioses with green algae, legumes and actinorhizal plants. In particular, the rhizobia-legume symbiosis is the major source of fixed N_2 into agricultural systems and represents an economical and environmentally-friendly alternative to chemical fertilization. Taking into account that legumes provide 25-35% of the worldwide protein intake (Hernández 2002), the improvement of legume N_2 fixation by genetically manipulating the symbiotic partners will be of considerable benefit for agriculture.

Legume root nodules are formed as a result of a complex exchange of molecular signals between the rhizobia and the plant. During the last few years, the very early stages of nodule formation are being dissected at the molecular level. It is now clear that the signal pathway involves, for example, Ca^{2+} spiking in root hair cells (Ehrhardt et al. 1996; Walker et al. 2000), ethylene (Penmetsa and Cook 1997), and a number of kinases (Endre et al. 2002; Stracke et al. 2002; Ané et al. 2004; Lévy et al. 2004). Also, several genes encoding putative Nod factor receptors have been identified in the model legumes *M. truncatula* (Ben Amor et al. 2003; Limpens et al. 2003) and *L. japonicus* (Radutoiu et al. 2003).

There are now numerous lines of evidence that reactive oxygen species (ROS) and their associated enzymes also participate in signal transduction during symbiosis, as occurs with the plant-pathogen interaction. However, either the plant is able to discriminate between symbiotic and pathogenic bacteria, or the former elude the defense response. Compatible rhizobia suppress the accumulation of salicylic acid (a defense response) in alfalfa, whereas the *nodC*$^-$ mutant does not (Martínez-Abarca et al. 1998). Also, wild-type rhizobia increase several antioxidant activities in alfalfa roots during the preinfection period, whereas the *nodC*$^-$ mutant decreased them but enhanced H_2O_2 content. This strongly suggest that a defense response of the plant occurs in the incompatible interaction (Bueno et al. 2001). The two sets of experiments led the authors to conclude that Nod factors are essential to suppress the defense response. Along the same line, Shaw and Long (2003) have shown that Nod factors inhibit the efflux of H_2O_2 from roots, and hence that ROS formation is decreased as a result of infection.

On the other hand, Vasse et al. (1993) showed that rhizobia elicit a hypersensitive reaction in the host legume which is similar to that observed in the incompatible plant-pathogen interaction. Furthermore, they suggested that this defense response is part of the autoregulatory mechanism by which the plant controls nodule number. In *M. truncatula*, Nod factors induce expression of a peroxidase ("Rhizobium-induced peroxidase", Rip) three hours after inoculation (Cook et al. 1995) and elicit ROS production (Ramu et al. 2002). Interestingly, transcription of the *rip* gene and ROS production have the same tissue-specific pattern, and treatment with exogenous H_2O_2 is sufficient to activate *rip* transcription (Ramu et al. 2002). Also, production of superoxide radicals and H_2O_2 has been detected in the infection threads in indeterminate nodules (Santos et

al. 2001; Matamoros et al. 2003). In *Sesbania*, inhibitor studies have shown that formation of intercellular infection pockets require ethylene and H_2O_2, which act downsteam from the Nod factors (D'Haeze et al. 2003). However, the accumulation of ROS observed in some cases does not necessarily reflect a genuine oxidative burst (Santos et al. 2001), but rather the ability of rhizobia to modulate ROS production by the plant, probably due to the requirement of ROS for cell growth. This hypothesis is supported by inhibitor studies showing that ROS are generated by NADPH oxidase in the cell walls of infection threads (Rubio et al. 2004) and are directly involved in the elongation of roots and root hairs (Foreman et al. 2003). Taken together, the results described above indicate that the oxidative burst is suppressed during the symbiotic interaction (contrary to what occurs in the pathogenic response), but also point out that ROS play a critical role in infection thread growth and probably other stages of nodulation. Consequently, the production and scavenging of ROS need to be tightly regulated in time and space.

Another stage of nodule development in which ROS formation has been detected is in the senescent zone (IV) of indeterminate nodules (Rubio et al. 2004) and in senescing determinate nodules (Alesandrini et al. 2003). In the former case, H_2O_2 was found to accumulate surrounding degrading bacteroids and in the latter case large amounts of H_2O_2 were observed in the cytoplasmic and apoplastic compartments of the central zone. Based on the accumulation of H_2O_2 and on the detection of programmed cell death events, Alesandrini et al. (2003) proposed that senescent determinate nodules undergo programmed cell death starting in the periphery of the infected zone. Additional evidence that ROS production is enhanced during both natural and stress-induced senescence is the accumulation of oxidized lipids and proteins and the decrease of antioxidant defenses in the nodules (Gogorcena et al. 1997, Evans et al. 1999).

The overall results described above indicate that ROS are generated at various stages of symbiosis, probably at different concentrations. Evidence is also emerging on the essential role of ROS for succesful nodulation, probably by participating in the signal transduction pathway. At this point, there are many critical questions to be answered. It is not known what specific ROS (or related molecules) and at what specific step(s) of nodulation ROS are required. Also, it is uncertain if there is an oxidative burst during symbiosis (Shaw and Long 2003) and how specifically Nod factors and maybe other specific rhizobial molecules can suppress the plant's defensive response. Finally, it is important to know how ROS are kept under control to avoid deleterious effects while allowing some beneficial effects (growth of infection threads and cell walls, signal transduction, redox control) during nodule organogenesis, and how ROS production escapes control, ensuing oxidative stress during nodule senescence.

To resolve these crucial questions, the use of highly precise methodologies to dissect nodule formation and senescence at the molecular and cellular levels is imperative. In this Thesis we have devised three strategies that may prove to be useful for the detailed study of the genes and

enzymes associated with ROS production and scavenging.

Isolation of root hairs for biochemical and genetical analysis. Transformation of legumes for specific targeting of proteins to the root epidermis

Nodule organogenesis is triggered by the binding of rhizobial Nod factors to the corresponding receptors on root hairs. Consequently, biochemical and molecular studies on the implication of ROS in the early stages of the plant-rhizobial interaction would be greatly facilitated if root hairs can be isolated with high yield and purity. This type of studies are most conveniently performed with the model legumes *M. truncatula* and *L. japonicus*, which have small diploid genomes that will be completely sequenced in the near future. To purify root hairs of *M. truncatula*, we tried to adapt the protocol initially established for pea (Röhm and Werner 1987, Gloudemans et al. 1989). The root hair fraction obtained was, however, heavily contaminated with other plant material and did not lead to an enrichment of root hair protein. Therefore, we developed a new method to increase root hair yield while minimizing root fragmentation. Roots were grown on the surface of agar plates, so that the root hairs stick out and could be collected in liquid nitrogen. The yield was typically 25-40 µg of total root hair protein from a single plate containing 50-100 root tips after three weeks. Root tips were able to remain alive on the plates for several weeks, even months.

The efficiency of the root hair isolation procedure was tested taking advantage of the specific expression of the *PsRH2* gene in the pea root epidermis (Mylona et al. 1995). We first verified that this gene was also specifically expressed in the root epidermis of *M. truncatula* using constructs of the *PsRH2* promoter and the *GFP* reporter gene. As expected, transgenic plants expressed GFP specifically in the root epidermis. Further, roots of *M. truncatula* plants transformed transiently by *A. rhizogenes* (Boisson-Dernier et al. 2001) harbouring a *PsRH2::GUS* construct confirmed this expression pattern (R. Geurts, *unpublished results*). Then, we exploited this property to monitor the root hair isolation procedure and to estimate the high purity and yield of the root hair preparations.

In this work, we also showed that there is a protein homologous to PsRH2 in *M. truncatula*. The presence of the MtRH2 protein and the corresponding gene was proven with immunoblots and RT-PCR, respectively. These analyses are also consistent with a root epidermis-specific expression of MtRH2. Expression of transgenic proteins can, therefore, be specifically targeted to *M. truncatula* root hairs with the use of the endogenous *MtRH2* promoter or homologous promoters such as *PsRH2*.

In summary, a new method has been developed for the isolation of root hairs from *M. truncatula*. The method is very efficient, provides highly purified root hair preparations and is especially suitable when plant material is a serious limiting factor. For instance, transgenic plants can be generated and roots can be used well before seed becomes available, thus providing

relatively large amounts of homogeneous material in less than three weeks. Another advantages of the method are the possibility to select different root hair populations and its applicability to other legumes, such as *L. japonicus*, by using *A. rhizogenes*-mediated transformation. However, a drawback of the method is that detached roots, whether or not induced by *A. rhizogenes*, do not form nodules. This work also shows that *M. truncatula* contains a protein, MtRH2, that is homologous to PsRH2, which is constitutively and specifically expressed in the pea root epidermis. The MtRH2 protein proved to be a useful endogenous marker to monitor root hair isolation since it is also specifically expressed in the root epidermis. This method may be of valuable use for the study of ROS involvement in the early stages of the legume-rhizobia interaction at least for two reasons. First, it will allow expression analyses (transcripts, proteins, activities) of antioxidant enzymes and associated metabolites in purified root hairs (treated or not with Nod factors or rhizobia). Second, it will allow the specific targeting of superoxide- or H_2O_2-scavenging enzymes, such as SODs, peroxidases and catalases, to the root epidermis. Furthermore, a knock-down approach based on RNAi (see below) could be also followed. Together, it will facilitate analyses on the role of ROS in signal transduction pathways leading to nodule formation or in defense responses.

Transformation of legumes to inactivate genes: RNAi technology

In plants several reverse genetic techniques are being used to study gene function, such as co-suppression and antisense suppression (Tavernarakis et al. 2000, Elbashir et al. 2001). Thus, formation of double stranded RNA can lead to effective and sequence-specific post-transcriptionally degradation of homologous mRNA. RNA intereference (RNAi) is an important tool in the analysis of gene function in both plants and animals. RNAi can be triggered by generating (stable) transgenic plants that express RNAs capable of forming a double stranded hairpin (Waterhouse et al. 1998, Chuang and Meyerowitz 2000, Wesley et al. 2001).

Most studies on RNAi (Schweizer et al. 2000; Johansen and Carrington 2001) have focused on targeting genes in the aerial parts of the plant. Recently it was shown that RNAi can also be used to effectively silence (trans)genes in primary transformed roots in *M. truncatula* (Limpens et al. 2003) and *L. japonicus* (Kumagai and Kouchi 2003) by using *A. rhizogenes*-mediated transformation. The transformed roots are morphologically indistinguishable from untransformed roots and, in the case of legumes, can be nodulated by rhizobia and infected by mycorrhizal fungi. *A. rhizogenes*-mediated transformation offers a fast alternative to generate genetically transformed roots, especially in species where generating stable transgenic lines is very time consuming. Furthermore, this method has the advantage that root cultures can be clonally propagated without the requirement of additional plant hormones.

By targeting the *KOJAK* gene (which encodes a cellulose synthase-like protein involved in cell growth), we show that endogenous genes can efficiently be silenced in roots of *Arabidopsis*

via RNAi by using *A. rhizogenes*-mediated transformation. A high percentage (91%) of the homogeneously transformed roots showed phenotypes identical to the described mutant (Wang et al. 2001). Quantification of mRNA levels by qPCR confirmed the knock-down of the corresponding gene. However, residual mRNA could still be detected and also some variation in the level of expression was detected between independently transformed roots. This variation in mRNA levels could be an explanation for the observed variation in phenotypes. The occurrence of intermediate phenotypes as a result of RNAi has also been reported for stable transformed *Arabidopsis* plants (Chuang and Meyerowitz 2000) and can be an additional tool to gain insight into the function of a gene.

Generally it is thought that a specific mobile silencing signal exists that can travel between cells via plasmodesmata and long distances via phloem (Palauqui et al. 1997, Voinnet et al. 1998, Jorgensen 2002, Mlotshwa et al. 2002). For example, in *Arabidopsis*, biolistic delivery of dsRNA into leaf cells triggered silencing capable of spreading locally and systemically. It was reported that systemic spreading of the silencing signal could be detected two weeks after biolistic delivery starting in the veins of non-bombarded leaves and was clearly evident in non-vascular tissues one month after bombardment (Klahre et al. 2002). Strikingly, *A. rhizogenes*-mediated RNAi of trans *GFP* in *Arabidopsis* or *Medicago* roots showed that systemic transport of the silencing signal does not occur to non co-transformed roots. Targeting *GFP* in the Gal4 enhancer trap lines J0661 and J0781 also did not show any systemic spread to the non-transgenic shoot. However, targeting of *GFP* in a *35S::GFP* transgenic line did result in systemic transport of the silencing signal, but the extent of silencing was more limited and greatly variable. Similar results are reported in *L. japonicus* where *A. rhizogenes*-mediated silencing of a *35S::gusA* transgene did not spread to non co-transformed roots and was limited and variable in the shoots (Kumagai and Kouchi 2003). The lack of systemic spread of the silencing signal to non co-transformed roots is in agreement with grafting experiments performed in tobacco, which suggested that silencing is unidirectional from the base to the top of the plant (Palauqui et al. 1996). The observed variation in spatial patterns of silencing in the shoot of the *35S::GFP* line has also been observed in different plant species and for different transgenes under the control of the 35S promoter (Boerjan et al. 1995, Jorgensen et al. 1996, Kunz et al. 1996, Palauqui et al. 1996). The extent of systemic silencing in the shoot could depend on the regulation of the transgene, since no systemic silencing was observed in the shoot of transformed enhancer trap line J0661 and J0781, which express *GFP* under the control of an endogenous enhancer element.

The use of the reporter gene, *DsRED1* (which encodes a protein that exhibits red fluorescence), as selection marker enabled us to select chimaeric roots to examine the extent of systemic spread of the silencing signal within root tissue. The *Arabidopsis* line J0781 shows strong *GFP* expression in the cortex and stele of the root. Chimaeric J0781 roots partly

transformed with pRR-GFPi showed silencing of *GFP* in the entire cortex and vascular tissue, indicating that the silencing signal is able to spread systemically in the cortex and stele. Strikingly, lateral roots that formed on these chimaeric roots and were not co-transformed, regained *GFP* expression. This suggests that, within one root system, the silencing signal does not spread to non co-transformed lateral roots. This is most likely a result of the unidirectional movement of the silencing signal. In contrast to systemic spread in the cortex and vascular tissue in *Arabidopsis*, no cell-to-cell movement of the silencing signal was observed in the epidermis of *Medicago*, demonstrating that silencing in the root epidermis is cell autonomous. One explanation for the fact that spreading of the silencing signal is not observed in the epidermis of *A. rhizogenes* mediated roots could be that epidermal cells become symplastically isolated. By dye-coupling experiments in *Arabidopsis* roots it was shown that cells in the meristem and epidermal cells in the elongation zone are symplastically connected through plasmodesmata, but gradually become symplastically isolated as the epidermal cells differentiate. By the time root hair outgrowth is visible the epidermal cells are symplastically isolated (Duckett et al. 1994). Similarly, it was shown that symplastically isolated stomatal guard cells do not silence systemically (Voinnet et al. 1998). So, the symplastic isolation of cells could cause the immobility of the silencing signal.

RNAi via *A. rhizogenes*-mediated root transformation is a valuable tool to study genes involved in root development and root-microbe interactions. It is a very fast and efficient system to silence genes in roots. In *Arabidopsis*, silenced *A. rhizogenes*-transformed roots can already be obtained within 10 days. Especially for plant species with very time consuming regeneration times this methods offers a big advantage. The fact that silencing is triggered cell autonomously in the root epidermis provides the possibility to use inducible and tissue-specific promoters to more specifically regulate RNAi. At the same time it requires a thorough inspection of the chimaeric nature of *A. rhizogenes* transformed roots in order to correctly interpret the observed phenotypes. The pRedRoot vector provides this possibility.

In summary, we show that *A. rhizogenes*-mediated RNAi is a fast and effective tool to study the function of genes involved in root biology. The *Arabidopsis* gene *KOJAK*, involved in root hair development, was efficiently targeted by *A. rhizogenes* mediated RNAi and was efficiently knocked down. As selection marker for co-transformed roots, the *DsRED1* gene was used as nondestructive selectable marker. This marker offers the additional advantage to discriminate between chimaeric and homogeneously transformed roots. The identification of chimaeric roots allowed us to examine the extent of systemic spread of the silencing signal in the composite plants of both *Arabidopsis* and *M. truncatula*. We show that RNA silencing is not spreading to non co-transformed (lateral) roots and only with limited efficiency to the non-transgenic shoot of composite plants. Furthermore, we provide evidence that RNA silencing is cell-autonomously induced in the root epidermis. This technology will greatly facilitate also the study of ROS involvement during nodule development and senescence. Thus, RNAi constructs for antioxidant

genes driven by the 35S or nodule specific promoters can be used to transform model legumes and to knock-down the corresponding genes. In this way, the loss-of-function effect in signaling (early stages of the legume-rhizobia interaction), organogenesis (young and mature nodules), or oxidative stress (natural and stress-induced nodule senescence) can be determined.

Transformation of legumes to induce ectopic gene expression

The SOD family of enzymes represents a primary line of defense against the superoxide radicals and derived ROS in all organisms. Three types of SOD, differing in the metal at the active site, may coexist in plants. Typically, the CuZnSOD isozymes are localized in the cytosol and chloroplasts, the MnSOD isozymes are localized in the mitochondria and the FeSOD isozymes are primarily localized in the chloroplasts (del Río et al. 1992; Bowler et al. 1994; Ogawa et al. 1996). Complete cDNA clones encoding some enzymes of each type have been isolated and used to construct transgenic plants overexpressing SODs in the chloroplasts, mitochondria, and cytosol (Bowler et al. 1991; Scandalios 1993; Van Camp et al. 1996). These studies were performed using model plants for transformation, such as tobacco, but important crop legumes can now be transformed and regenerated with relative ease (Christou 1994). Because legumes are unique amongst crop plants in their ability to fix atmospheric N_2 in symbiosis with rhizobia, transformation of these plants with antioxidant genes could provide additional advantages related to its protective role in nodule activity (Puppo et al. 1982; Dalton et al. 1998; Matamoros et al. 1999).

The SOD isozymes were studied in the elite genotype (N4) and three derived transgenic lines of alfalfa *(M. sativa)*. The transgenic plants were designed, respectively, to overproduce MnSOD in the mitochondria (line 1-10), MnSOD in the chloroplasts (line 4-6), and FeSOD in the chloroplasts (line 10-7). Constructs were made with the MnSOD sequence of *Nicotiana plumbaginifolia* or the FeSOD sequence of *Arabidopsis*, under the control of the 35S promoter. We found that the nodules and leaves of alfalfa contain CuZnSOD, MnSOD, and FeSOD. However, only the nodules of line 1-10 produced the transgene-encoded SOD. The *Nicotiana* MnSOD mRNAs were also found in nodules of both lines 1-10 and 4-6, albeit at low levels. The absence of *Nicotiana* MnSOD activity in nodules of line 4-6, despite expression of the gene, can be attributed to degradation of the enzyme, perhaps as a result of the inability of nodule plastids to process the protein bearing a chloroplastic prepeptide. Another surprising observation is the absence of transcript for *Arabidopsis* FeSOD in nodules of line 10-7, despite its abundance in the corresponding leaves. This may be ascribed at least in part to a weak activity of the 35S promoter in alfalfa nodules. However, this would still leave unexplained why constructs with identical promotor and chloroplastic targeting sequences are expressed (MnSOD in line 4-6) or not expressed (FeSOD in line 10-7) in nodules. To verify the differences in expression among the three transgenic lines, nodules of exactly the same plant were used to

extract RNA (for northern analysis) and protein (for activity gel analysis). This experiment confirmed that nodules of line 1-10 expressed transcript and activity, nodules of line 4-6 expressed transcript but not activity, and nodules of line 10-7 did not express the transcript.

We observed a compensation in the activities of the SOD isozymes. Thus, overproduction of MnSOD in the leaves of lines 1-10 and 4-6 was matched by lower FeSOD activities, resulting in similar total SOD activities in the leaves of the two lines and in the N4 line. Conversely, overproduction of FeSOD in line 10-7 was paralleled by a lowering in MnSOD activity, although in this case the 2-fold excess of the former was not compensated for completely and the total SOD activity of leaves in line 10-7 remained 40% greater than in line N4. Northern analysis indicated that the compensation of activities is not due to changes in transcript abundance. Thus, the mRNA level of endogenous MnSOD in leaves of line 10-7 is even higher than in line N4, and the mRNA level of endogenous FeSOD in lines 1-10 and 4-6 is similar to or higher than in line N4. Therefore, the compensation between MnSOD and FeSOD activities occurs, at least in part, at the post-translational level.

The abundance of FeSOD in alfalfa nodules also deserved special attention, as this type of SOD, in contrast to CuZnSOD and MnSOD, is not ubiquitous in plants. Furthermore, there are contradictory reports about the presence of FeSOD in leaves of some species. Early studies reported that FeSODs were confined to a few families of higher plants (Bridges and Salin 1981). However, it seems now that the gene is more widely distributed than previously thought but remains silent due to lack of appropriate inducing conditions (Bowler et al. 1994). Our finding of FeSOD in nodules of healthy plants of alfalfa and other legumes is consistent with that hypothesis. The FeSODs of alfalfa and pea nodules are closely related and are synthesized as precursor plastid proteins, which suggests that these enzymes play a role in plastid metabolism other than the scavenging of superoxide radicals associated with photosynthesis. The occurrence of abundant ferritin in the plastids may ensure an adequate Fe supply for the synthesis of FeSOD, especially under conditions in which this isozyme is up-regulated (reviewed by Becana et al. 1998). The presence of at least two types of FeSODs further underscores the multifaceted nature of antioxidant protection in nodules and suggests that the various SOD isozymes may have evolved to perform specific defensive or regulatory roles that are essential for optimal nodule functioning. Clearly, further work is necessary to characterize these roles.

In summary, we have analyzed the SOD composition of nodulated plants in three transgenic alfalfa lines. All four lines displayed low CuZnSOD activities and abundant FeSOD and MnSOD activities in nodules and leaves. Transgenic alfalfa showed a novel compensatory effect in the activities of MnSOD (mitochondrial) and FeSOD (plastidic) in the leaves, which was not caused by changes in the mRNA levels. These findings imply that SOD activity in plant tissues and organelles is regulated at least partially at the post-translational level. All four lines had low CuZnSOD activities and an abundant FeSOD isozyme, especially in nodules, indicating that

FeSOD performs important antioxidant functions other than the scavenging of superoxide radicals generated in photosynthesis. This was confirmed by the detection of FeSOD mRNAs and proteins in nodules of other legumes. The full-length cDNA encoding alfalfa nodule FeSOD was characterized and the deduced protein found to contain a plastid transit peptide. Comparison of sequences and other properties reveals that there are two types of FeSODs in nodules, which can be distinguished biochemically and immunologically and that are probably located in different subcellular compartments. This work has addressed important questions on ROS metabolism, such as the critical protective role of SODs and their sophisticated post-transcriptional regulatory mechanisms in leaves and nodules. However, it also makes clear that SODs may perform additional roles in nodules and this has been further supported by the differencial tissue expression of each type of SOD. Finally, our work also recommends the use of specific nodule promoters (such as lbc3) rather than the constitutive 35S promoter (which shows strong activity in leaves) for overexpression and knock-down studies in *Medicago* species.

Conclusions

During the last few years, the very early stages of nodule formation are being dissected at the molecular level. The signal transduction pathway involves calcium, ethylene, and kinases. But there is now evidence that ROS and their associated enzymes also participate in signal transduction during symbiosis. However, in clear contrast to pathogen systems, the Nod factors of the rhizobia allow them to elude the plant's defense response. ROS are generated not only at the early stages of the symbiotic interaction but also during nodule development and senescence. At this stage, however, many critical questions on the roles of ROS in symbiosis need to be answered. What specific ROS are required for nodulation and at what specific steps of the signal transduction pathway do they participate? How do Nod factors suppress the plant's defense response? How are ROS kept under control during nodule organogenesis and why do they escape such control during nodule senescence?

These crucial questions require to be approached by methodologies that allow the molecular and cellular dissection of nodule formation and senescence. In this thesis we have devised three strategies that may prove to be useful for the detailed study of the genes and enzymes associated with ROS production and scavenging. First, a new method for the isolation, at high yield and purity, of root hairs from *M. truncatula*. This method can be used for expression analyses of antioxidant enzymes in purified root hairs and their variation after Nod factor binding, and also for specific targeting of the enzymes to the root epidermis in transgenic legumes. Second, we have used RNAi technology and *A. rhizogenes*-mediated transformation as a fast and effective tool by which gene function can be studied. This technology can be used to make constructs of RNAi for antioxidant genes, transform model legumes, knock-down the corresponding genes, and determine the loss-of-function effect. Third, we have used constructs for ectopic

overexpression of SODs in leaves and nodules. This work has addressed important questions on ROS metabolism and has made clear that SODs may perform additional, and rather specific, roles in nodules.

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Summary

Biological N_2 fixation is carried out exclusively by prokaryotes, either in the free-living form or in mutualistic symbioses with green algae, legumes and actinorhizal plants. The most agronomically relevant symbiosis is, by far, that formed between soil rhizobia and legume roots. In addition, the input of fixed N_2 by this symbiosis into agricultural systems represents an economical and environmentally-friendly alternative to chemical fertilization. Therefore, the improvement of the efficiency of symbiotic N_2 fixation, both under physiological and stressful conditions, is highly desirable.

The process of N_2 fixation occurs in specialized organs, called nodules, that are formed as a result of a complex exchange of molecular signals (flavonoids, Nod factors) between the rhizobia and the host plant. During nodule formation, bacteria differentiate into bacteroids, which express the nitrogenase enzymatic complex. This catalyzes the reduction of N_2 to ammonium, requires ATP, Mg^{2+} and an electron donor, and is irreversibly inactivated by O_2. During the last few years, the very early stages of nodule formation are being dissected at the molecular level. It is clear that the signal transduction pathway, which is triggered after binding of compatible Nod factors to receptors, involves oscillatory changes in cytoplasmic calcium, ethylene and protein kinases. Several lines of evidence indicate that reactive oxygen species (ROS) are also involved in signal transduction during nodule formation as well as in natural and stress-induced nodule senescence. However, critical questions remain to be answered: what specific ROS and at what specific step(s) of nodulation do they participate, how can Nod factors suppress the plant's defensive response, or how ROS production escapes control during nodule senescence.

In this thesis we have devised three strategies that may be of value for the cellular and molecular study of symbiosis and in particular ROS metabolism in nodules. First, we report a novel method that allows the isolation of root hairs, with high yield and purity, from the model legume *M. truncatula*. We also found in *M. truncatula* a protein, MtRH2, homologous to pea PsRH2, that is specifically localized in the root epidermis. This feature was exploited to monitor the root hair isolation procedure and can also be used for specific expression of antioxidant enzymes in root hairs. A second strategy that may be helpful to study the function of ROS is gene inactivation. We show that *A. rhizogenes*-mediated RNA interference (RNAi) is a fast and effective tool for this purpose. The introduction of RNAi in legumes and *Arabidopsis* using *Agrobacterium rhizogenes* based transformation was set up with genes with a known knock-out phenotype. The efficiency of the technique was demonstrated by using RNAi to knock-down the *KOJAK* gene of *Arabidopsis,* which is involved in root hair development. We also show that RNAi silencing is restricted to the epidermal cells where RNAi constructs are expressed. A third strategy to study ROS metabolism is the overexpression of important antioxidant enzymes, such as superoxide dismutases (SODs), in transgenic legumes. Three lines of transgenic alfalfa *(M.*

sativa) were designed to overexpress, respectively, MnSODs in chloroplasts, MnSOD in mitochondria and FeSODs in chloroplasts. Analysis of SOD composition in these plants led us to discover a novel compensatory effect in the activities of MnSOD and FeSOD in the leaves, which was not due to changes in mRNA levels. We also provide evidence that SOD activity in plants is regulated at least partially at the post-translational level and that FeSOD performs important antioxidant functions other than the scavenging of superoxide radicals generated in photosynthesis. Finally, in this thesis we discuss the significance, applications and prospects of the work done in the light of current research on nodule formation and senescence.

Samenvatting

Alleen sommige prokaryoten zijn in staat om stikstof te binden. Zij doen dit of in een vrij-levende vorm of in een symbiotische interactie met b.v. algen, vlinderbloemige planten of zgn. actinorhiza planten. De symbiose van Rhizobium bacteriën en vlinderbloemige planten is voor de landbouw veruit de meest belangrijke. Verder is deze biologische stikstofbinding vanuit milieukundig als ook economisch oogpunt een aantrekkelijk alternatief voor kunstmest. Daarom is de verbetering van biologische stikstofbinding onder normale en stres condities gewenst.

De Rhizobium bacteriën zetten hun gastheer plant aan tot het maken van wortelknolletjes en in deze organen worden ze gehuisvest en zijn ze in staat stikstof te binden. De vorming van deze knolletjes wordt geïnitieerd door een uitwisseling van moleculaire signalen. In deze wortelknol differentiëren de bacteriën tot de stikstofbindende vorm die bacteroid genoemd wordt. Deze bacteroiden vormen het enzym complex nitrogenase en dit is verantwoordelijk voor de omzetting van stikstofgas in ammonia. Deze enzymatische reactie vereist veel energie in de vorm van ATP en ook een elektronen donor en verder wordt dit enzym geïnactiveerd door zuurstof.

Gedurende de afgelopen jaren heeft het onderzoek zich in het bijzonder gericht op een ontrafeling van de moleculaire processen die een rol spelen bij de vroege stadia van knolvorming. Het signaal van de bacterie dat knolvorming initieert is de Nod factor. Na herkenning van de Nod factor door een receptor worden er verschillende elektrofysiologische veranderingen geïnduceerd. Echter verschillende studies maken duidelijk dat reactieve zuurstof moleculen (reactive oxygen species (ROS)) ook betrokken zijn bij deze processen. Het is echter nog onduidelijk welke ROS hierbij een rol spelen en een aantal sleutelvragen moeten eerst beantwoord worden; welke ROS spelen een rol? Bij welke stappen zijn ze betroken? Zijn ROS betrokken bij afweer? hoe kunnen Nod factoren een dergelijke afweer onderdrukken en hoe zijn ze betrokken bij verouderingsprocessen in de knol?

In dit proefschrift hebben we 3 strategien ontwikkeld die van betekenis kunnen zijn voor toekomstig moleculair celbiologisch onderzoek aan dze symbiose en in het bijzonder aan de rol van ROS in knollen en wortelharen. Allereerst beschrijven we een nieuwe methode om wortelharen van de model plant Medicago te isoleren. Deze methode is efficiënt en resulteert in een hoge opbrengst van zeer zuiver wortelhaar materiaal. In Medicago hebben we een gen, MtRH2 geïdentificeerd dat homoloog is met het eerder beschreven erwten PsRH2 gen. Dit gen komt specifiek tot expressie in de wortelepidermis. Van deze eigenschap werd gebruik gemaakt om de zuivering van de wortelharen te volgen. De promotor van dit gen lijkt verder zeer geschikt om genen in wortelharen.

Een tweede strategie die gebruikt kan worden om de functie van ROS te bestuderen is gen-inactivatie. Hier laten we zien dat geïnduceerde RNA interferentie (RNAi) in wortels, gevormd

middels transformatie met Agrobaterium rhizogenes, een snelle en efficiënte methode is om gen expressie specifiek uit te schakelen. Bij het opzetten van deze RNAi methode in Medicago en Arabidopsis werd gebruik gemaakt van genen waarvan het knock-out fenotype bekend is. Dit was b.v. het KOJAK gen van Arabidopsis dat essentieel is voor wortelhaarvorming. Door gebruik te maken van transgene planten die het GFP gen tot expressie brengen kon worden aangetoond dat specifiek in de epidermis geïnduceerde RNAi zich niet verspreidt naar andere weefsels.

Een derde strategie om de functie van ROS tijdens knolvorming te bestuderen is overexpressie van belangrijke antioxidant enzymen zoals superoxide dismutase (SODs). Drie alfalfa lijnen werden gemaakt waarin verschillende SODs tot overexpressie zijn gebracht, namelijk; MnSOD in chloroplasten, MnSOD in mitochondrien en FeSOD in chloroplasten. Analyse van SODs die voorkomen in deze transgene lijnen liet zien dat de niveaus van MnSOD en FeSOD activiteit elkaar beïnvloeden. Deze compenserende interactie wordt geregeld op het mRNA niveau. Verder werden er aanwijzingen verkregen dat SOD activiteit in planten ten dele geregeld wordt op een post translationeel niveau. Verder bleek FeSOD naast het onschadelijk maken van superoxide radicalen ook een andere antioxidant werking te hebben.

Uiteindelijk wordt in dit proefschrift de toepassingsmogelijkheden van de 3 ontwikkelde methode in onderzoek aan knolvorming en veroudering bediscussieerd.

List of Publications

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Curriculum vitae

Javier Ramos Escribano was born on 4 December 1967 in Madrid (Spain). When he was six years old he went with his family to live in Orense and at eight they moved to Zaragoza. In 1986 he started his studies in Biology at the University of Salamanca, being the son and grandson of biologists, and graduated in July 1991. Immediately he started a M.Sc. thesis on DNA synthesis in rat liver mitochondria at the Department of Molecular Biology in the University of Zaragoza under the supervision of Prof. Julio Montoya. From May 1992 he took part in a six month Erasmus exchange with the University of Heidelberg (Germany), working in the Institut Fachrichtung Physiologie (Prof. R. Zwilling), the European Molecular Biology Laboratory (Prof. Luis Serrano), and the Physiologisches Institute II (Prof. Caspar Rüegg).

From October 1992 to September 1993 he lost his time with an offset printing machine during compulsory military service. After getting the M.Sc. degree and being temporarily unemployed, in April 1994 he moved to Wageningen with the idea of accomplishing a Ph.D. The title of the original project was "Immunolocalisation of the early nodulin Enod5 by a tagging approach" with Prof. Ton Bisseling as supervisor. The project involved the expression, in transgenic nodules, of the early nodulins Enod5 and Enod12 fused to the HA antigen from the influenza virus, and its immunolocalization in partially-transformed *Vicia hirsuta* plants. Between 1994 and 1997 two research lines were approached under Dr. Henk Franssen's supervision: comparative studies of promoter activity (Enod12, 35S and NOS fused to the 35S enhancer) in transgenic nodules from Vi*cia hirsuta* using GUS as reporter; and expression of fragments from the early nodulin Enod40 fused to GFP in *Vigna unguiculata* protoplasts under the 35S promoter. Under Prof. Bisseling's supervision, five constructs for the overexpression of cytoskeleton proteins actin and profilin, and their fussions to GFP were introduced in transgenic *M. truncatula*, driven by the specific root hair promoter of the *RH2* gene. Transgenic *M. truncatula* plants were created carrying the calcium reporters "aequorin" and "cameleon" (1997-2000). From May 2000 he has been working in the study of antioxidants in legumes, especially in their involvement in symbiotic nitrogen fixation, in the laboratory of Prof. M. Becana at the Estación Experimental de Aula Dei, CSIC, Zaragoza (Spain).

Acknowledgements

I want to thank my supervisor, Ton Bisseling, for not losing the hope that a thesis could be finished one day, and to Maria Augustijn for keeping alive the link with Wageningen. Special thanks should go to Manuel Becana who put the energy to push the thesis into completion, and to Carmen Pérez-Rontomé, who gave shape to it. I want to thank Jan Verver and Jan Hontelez for all the help and useful tips at the bench during many years, and to my student Ralf Regeer for giving me in 1999 again a glimpse of enthusiasm for science. Also, I thank Erik Limpens, who finally decided to give use to one of my transgenic plants.

The place where I received the best teaching at Wageningen was indeed the SSR studentenvereiniging, which I joined in 1995. Its kroeg is always holding friendly philosophers. My best Dutch still comes out only after drinking "Oranjeboom" beer. The university fencing association, "De Schermutselaers", where I practiced violently for five years, was the best answer to frustration, and this is a collective thanks to all the members I met, wherever they are now. For helping me to keep the right (southern) viewpoint of reality, I have to thank Gustavo Gualtieri, with whom I shared many dinners at Unitas mensa. Also, thanks go to all the people that have been living at Haarweg 27, my home for years. Thanks MC and Vincent, my paranymphs. Thanks to Fred and Jessica too. And, of course, I cannot forget Fewry.