Studies on legume root hair development: correlations with the infection process by *Rhizobium* bacteria

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Statements

1. Polar growth of different types of plant cells involves common mechanisms.

   This thesis.

2. The studies of Cook et al. do not prove that induction of expression of the peroxidase gene *rip1* upon inoculation of *Medicago truncatula* with *Rhizobium meliloti*, is restricted to the epidermis.

   Cook et al., 1995, Plant Cell 7, 43-55.

3. The conclusion of Staehelin et al. that Nod factors activate the same signal transduction pathway in cultured tomato cells and legumes roots is not correct, since the observed alkalinization in the cultured medium is in contrast with the proton efflux described in root hairs.

   Staehelin et al., 1994, Proc. Natl. Acad. Sci. USA 91, 2196-2200.
   Allen et al., 1995, Adv. Mol. Genet. Plant-Microb. Inter. 3, 107-113.

4. The demonstration that cytoplasmic dynein is required for nuclear segregation in yeast, does not exclude a role of the same protein in retrograde transport.

   Eshel et al., 1993, Proc. Natl. Acad. Sci. USA 90, 11172-11176.
   Li et al., 1993, Proc. Natl. Acad. Sci. USA 90, 10096-10100.

5. It cannot be excluded that substoichiometric quantities of polypeptides present in the complex of seven proteins promoting posttranslational protein translocation across the ER with reconstituted proteoliposomes from yeast have a regulatory function in translocation.

Panzner et al., 1995, Cell 81, 561-570.

6. Bureaucracy exists in many if not all countries; the only thing that differs is the salary of the bureaucrats.

7. Het verlangen om zo goedkoop mogelijk te sporten en het milieu bewustzijn hebben geresulteered in het enorme aantal fietsen in Nederland, waardoor het transport in dit land een relatief lage graad van automatisering heeft.

8. The only thing I know is that I know nothing.

Sokrates.

Statements from the thesis entitled:
"Studies on legume root hair development: correlations with the infection process by *Rhizobium* bacteria"
Panagiota Mylona, Wageningen, 11 June 1996.

CONTENTS

SCOPE

Rhizobia-legume interaction leading to the formation of specific organs, namely root nodules, starts at the epidermis of the root. Bacteria interfere with the develomental programme of the epidermal cells by inducing a number of responses, as new root hair growth, root hair deformation and curling, and formation of special structures, the infection threads, via which the bacteria enter the plant. It has been postulated that infection threads grow in a similar manner as root hairs, but to the opposite direction. We have initiated a programme aiming originally at studies on the development of root epidermis/root hairs. That resulted in the isolation of a number of cDNA clones that represent genes involved in different aspects of epidermis/root hair development. These clones were used as molecular tools to unravel whether infection thread growth employs the same programmes as growing root hairs and germinating pollen and play a role in polar growth. To further understand the mechanisms of infection thread formation we performed studies on epitope-tagged early nodulins involved in the infection process.

In chapter 3, 4, and 5 we report the characterization of three root hair specific cDNA clones and the expression pattern of the corresponding genes. In chapter 2 we describe the exploratory studies on the potentialities of using epitope tagging of early nodulins to examine the role of early nodulins in the infection process and the formation of infection threads.

The various steps involved in the infection of legume roots by rhizobia and the formation of nitrogen fixing root nodules are described in the review presented in chapter 1, while in the concluding chapter 6 is discussed to what extent infection thread formation is compatible to growth of root hairs and pollen tubes.

CHAPTER 1

Symbiotic Nitrogen Fixation

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Symbiotic Nitrogen Fixation

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INTRODUCTION

Biosphere nitrogen is subjected to rapid turnover, and because it is eventually lost as nitrogen into the atmosphere, its maintenance requires continuous replenishment with reduced nitrogen from atmospheric nitrogen. Biological reduction of nitrogen to ammonia can be performed only by some prokaryotes and is a highly oxygen-sensitive process. The most efficient nitrogen fixers establish a symbiosis with higher plants in which the energy for nitrogen fixation and, in general, the oxygen protection system are provided by the plant partner. In two groups of symbiotic interactions, the prokaryotic partners are soil bacteria (rhizobia in legume symbioses and *Frankia* bacteria in actinorhizal symbioses), whereas in the case of symbiosis of *Gunnera*, nitrogen is fixed by the cyanobacterium *Nostoc*. In *Gunnera*, the symbionts reside in already existing stem glands, whereas in legumes and actinorhizal plants, new organs, the root nodules, are formed by the plant upon infection with the symbiont. In all three systems, the prokaryotes fix nitrogen inside the cells of their host, but they are separated from the plant cytoplasm by membranes derived from the plant plasmalemma (Figure 1).

Because research on legume symbiosis is the most advanced of these three symbiotic systems, in this article we concentrate mainly on this system. The interaction of rhizobia and legumes begins with signal exchange and recognition of the symbiotic partners, followed by attachment of the rhizobia to the plant root hairs. The root hair deforms, and the bacteria invade the plant by a newly formed infection thread growing through it. Simultaneously, cortical cells are mitotically activated, giving rise to the nodule primordium. Infection threads grow toward the primordium, and the bacteria are then released into the cytoplasm of the host cells, surrounded by a plant-derived peribacteroid membrane (PBM). The nodule primordium thereupon develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, which is known as the bacteroid (Figure 1A). Bacteroids, together with the surrounding PBMs, are called symbiosomes. At this stage, bacteria synthesize nitrogenase, which catalyzes the reduction of nitrogen. The product of nitrogen fixation, ammonia, is then exported to the plant.

All of the steps of nodule development involve the expression of nodule-specific plant genes, the so-called nodulin genes (van Kammen, 1984). The early nodulin genes encode products that are expressed before the onset of nitrogen fixation and are involved in infection and nodule development. The products of the late nodulin genes are involved in the interaction with the endosymbiont and in the metabolic specialization of the nodule (Nap and Bisseling, 1990).

In the first part of this review, we describe the early steps of the interaction between rhizobia and legumes that result in the formation of a nitrogen-fixing nodule. We focus on the role of specific lipooligosaccharides secreted by rhizobia in the induction of these early steps. In the second part, we describe nodule functioning and compare actinorhizal and legume nodules.

EARLY EVENTS OF NODULATION

Nod Factor Structure and Synthesis

The *Rhizobium* signal molecules that play a key role in the induction of the initial stages of nodulation are lipochitooligosaccharides known as Nod factors. The bacterial genes involved in Nod factor synthesis are the *nod* (nodulation) genes. These genes are not expressed in free-living bacteria, with the exception of *nodD*, which is expressed constitutively. NodD has the ability to bind to specific flavonoids secreted by the roots of the host plant (Goethals et al., 1992); upon flavonoid binding, it becomes a transcriptional activator of the other *nod* genes (Fisher and Long, 1992), which encode enzymes involved in the synthesis of Nod factors.

The structure of the major Nod factor of *Rhizobium meliloti* was determined in 1990 (Lerouge et al., 1990), and since then the structures of the Nod factors of most other rhizobia have been determined. (For detailed information on Nod factor structure and biosynthesis, see Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993; Carlson et al., 1995.) In general, Nod factors consist of a backbone of three to five β-1,4–linked *N*-acetylglucosamines bearing a fatty acid on the nonreducing sugar residue (Figure 2). In addition, the factors can have various substitutions on both the reducing and nonreducing terminal sugar residues.

Genetic and molecular analyses have shown that the synthesis of the Nod factor backbone is catalyzed by the products of the *nodA*, *nodB*, and *nodC* loci. NodC has homology with

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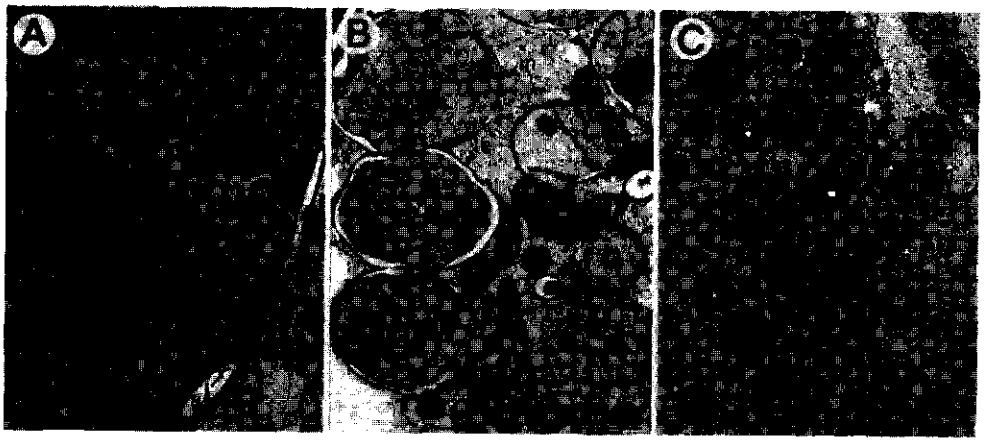


Figure 1. Nitrogen-Fixing Endosymbionts in Three Different Symbioses.

(A) Intracellular rhizobia in a nodule formed on clover by *Rhizobium trifolii*. This magnification of the region of the clover indeterminate nodule shows the transition of the prefixation zone to the interzone. In the right cell (prefixation zone), intracellular bacteria (ba) have not yet differentiated into their nitrogen-fixing form. The left cell (interzone) contains amyloplasts (a) and differentiated nitrogen-fixing bacteroids (b). In both cells, intracellular bacteria are surrounded by a plant-derived peribacteroid membrane (pbm).
(B) Intracellular *Frankia* in a nodule formed on *Alnus serrulata*. Vegetative hyphae (hy) and nitrogen-fixing septate vesicles (ve) can be seen. Vesicles are surrounded by a lipid envelope (le) that provides oxygen protection of the nitrogen fixation process. Both hyphae and vesicles are surrounded by the invaginated plasma membrane (m) of the host cell.
(C) Intracellular *Nostoc* in stem gland cells of *Gunnera*. Vegetative *Nostoc* cells (v) and nitrogen-fixing heterocysts (h) are surrounded by the invaginated plasma membrane (m) of the host.
Bars = 1 μm.

chitin synthases and therefore is the enzyme that most likely catalyzes the synthesis of the chitin oligomer (Geremia et al., 1994). The latter is further modified by the action of NodB, which de-*N*-acetylates the terminal nonreducing end of the molecule (John et al., 1993). At this position, NodA finally transfers a fatty acid from an acyl carrier protein (Röhrig et al., 1994). The backbone is further modified by the action of other Nod proteins that synthesize or add various substituents. These substitutions determine host specificity as well as the biological activity of the molecules. For example, in *R. meliloti*, the *nodH* and *nodPQ* genes are the major host range determinants (Roche et al., 1991). NodPQ proteins have been shown to represent enzymes that generate active forms of sulfate, and NodH is homologous to sulfotransferases. Therefore, these enzymes are probably directly involved in catalyzing the sulfation of *R. meliloti* Nod factors (Roche et al., 1991; Fisher and Long, 1992).

In general, rhizobia have the ability to interact with only a limited number of host plants. However, some rhizobia, for example, *Rhizobium* NGR234, have a more promiscuous nature. This *Rhizobium*, which can nodulate various tropical legumes, excretes 18 different Nod factors (Price et al., 1992). The production of this variety of factors is thought to be the basis for its broad host range (Price et al., 1992). In contrast, most rhizobia produce only a few different Nod factors.

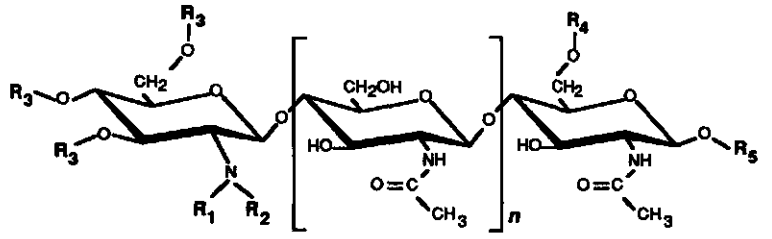
Interaction with the Root Epidermis

When rhizobia colonize legume roots, they induce deformation and curling of root hairs and the expression of several plant genes in the epidermis. In several systems, it has been shown that purified Nod factors induce the deformation of the root hairs at concentrations as low as 10^{-12} M (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993; Heidstra et al., 1994), but in general curling is not observed (Relic et al., 1993). Purified Nod factors can also induce the expression of certain plant genes (Horvath et al., 1993; Journet et al., 1994; Cook et al., 1995).

For *Vicia sativa* (vetch), a fast semiquantitative root hair deformation assay has been developed that allows the root hair deformation process to be characterized in detail. In this plant, root hair deformation is induced only in a small zone of the root, encompassing young root hairs that have almost reached their mature size (Heidstra et al., 1994). Deformation starts with

swelling of the root hair tips, which is already apparent within 1 hr after Nod factors are added. Subsequently, new tip growth is initiated at the swollen tips, resulting in clearly deformed hairs within 3 hr. Incubation with Nod factors for ~10 min is required to set the deformation process in motion (Heidstra et al., 1994); after this, even if the Nod factors are removed, the deformation process continues. These morphological changes are preceded by a depolarization of the plasma membrane (Ehrhardt et al., 1992), changes in the flux of calcium, proton efflux, rearrangements of the actin filaments (Allen et al., 1994), and increased cytoplasmic streaming (Heidstra et al., 1994). These changes occur within 5 to 30 min after Nod factors are added and may be part of a series of events that leads eventually to root hair deformation.

Several plant genes whose expression is activated in the epidermis during nodulation have been cloned and used to study the mode of action of Nod factors. The early nodulins *ENOD5* (Scheres et al., 1990b) and *ENOD12* (Scheres et al., 1990a), which encode proline-rich proteins, and *Mtrip1* (Cook et al., 1995), which encodes a peroxidase, represent such genes. The latter gene is expressed in the root pericycle of uninoculated roots, and all three genes are induced in the epidermis within a few hours after application of Nod factors (Horvath et al., 1993; Journet et al., 1994; Cook et al., 1995).



Species	R_1	R_2	R_3	R_4	R_5	*n*	References
Rhizobium meliloti	-H	-C16:2 (2,9)[a] or -C16:3 (2,4,9)	-$COCH_3$ (O-6)[b] or -H	-SO_3H	-H	1,2,3	Lerouge et al. (1990) Schultze et al. (1992)
Rhizobium leguminosarum bv *viciae*	-H	-C18:4 (2,4,6,11) or -C18:1 (11)	-$COCH_3$ (O-6)	-H or -$COCH_3$[c]	-H	2,3	Spaink et al. (1991) Firmin et al. (1993)
Bradyrhizobium japonicum	-H	-C18:1 (9), -C18:1 (9,Me), -C16:1 (9), or -C16:0	-$COCH_3$ (O-6) or -H	2-*O*-Methylfucosyl group	-H	3	Sanjuan et al. (1992) Carlson et al. (1993)
Bradyrhizobium elkanii	-H or Me	-C18:1	-$COCH_3$ (O-6), -H, or Cb[d, e]	2-*O*-Methylfucosyl or fucosyl group	-H or Gro[f]	2,3	Carlson et al. (1993)
Rhizobium sp strain NGR234	Me	-C18:1 or -C16:0	Cb (0-3 and/or 0-4)[b] or -H	Sulfated or *acetylated* 2-*O*-methylfucosyl group	-H	3	Price et al. (1992)
Azorhizobium caulinodans strain ORS571	Me	-C18:1 or -C18:0	Cb (0-6) or -H	D-Arabinosyl or -H	-H	2,3	Mergaert et al. (1993)
Rhizobium fredii	-H	-C18:1	-H	Fucosyl or 2-*O*-methylfucosyl group	-H	1,2,3	Bec-Ferté et al. (1994)
Rhizobium tropici	Me	-C18:1	-H	-SO_3H	-H	3	Poupot et al. (1993)

Figure 2. Structure of Nod Factors of Different Rhizobia.

The number of the *N*-acetylglucosamine residues can vary between three and five. The substitutions at positions R_1, R_2, R_3, R_4, and R_5 among the different rhizobia are indicated.

[a] The numbers in parentheses indicate the positions of the double bonds in the fatty acids.

[b] O-n indicates the position of the substitution on the *N*-acylglucosamine residue.

[c] This substitution is present only in Nod factors of *R. leguminosarum* bv *viciae* strain TOM.

[d] Cb indicates carbamyl group.

[e] The position of the carbamyl group could be 0-3, 0-4, or 0-6.

[f] Gro indicates glyceryl group.

Mtrip1 is not expressed during other steps of nodulation, whereas *ENOD12* and *ENOD5* are also expressed during infection and nodule development (see later discussion).

The induction of *ENOD12* and *Mtrip1* expression occurs in a relatively broad zone of the root, starting just above the root tip, where root hairs have not yet emerged, and extending to the region containing mature root hairs (Pichon et al., 1992; Cook et al., 1995). Cytological studies have shown that Nod factors elicit the expression of these genes in all epidermal cells (Journet et al., 1994; Cook et al., 1995) and that a direct contact between Nod factors and epidermal cells is required (Journet et al., 1994). Thus, it is likely that within the susceptible zone, root hair–containing cells as well as the other epidermal cells recognize Nod factors. However, the response is restricted to the epidermis, because *ENOD12* and *Mtrip1* are not even induced in the hypodermal cell layer.

The root hair deformation assay and tests of their ability to induce early nodulin genes have been used to analyze the structural requirements of Nod factors to elicit epidermal responses. When roots are treated with Nod factors, molecules containing three or fewer sugars are found in the growth medium and on the root. These molecules are probably generated by chitinases secreted by the plant, and they are at least 1000-fold less active in a deformation assay than the Nod factors with four to five glucosamine residues (Heidstra et al., 1994; Staehelin et al., 1994b). Therefore, the length of the sugar backbone plays an important role in recognition by the plant. Furthermore, the fatty acyl group present at the nonreducing end is required for recognition, because chitin molecules neither cause deformation (Heidstra et al., 1994) nor induce *ENOD12* expression (Journet et al., 1994). However, the structure of the fatty acyl group is not very important, because molecules with different fatty acid substitutions have similar activities.

In contrast, substitutions at the reducing terminal sugar can have a dramatic effect on recognition. For example, Nod factors of *R. meliloti* bear a sulfate group on this sugar residue. This sulfate moiety is important for induction of root hair deformation as well as for the elicitation of *ENOD12* expression in the *R. meliloti* host, alfalfa. Desulfation of the *R. meliloti* factors reduces their activity at least 1000-fold on alfalfa (Journet et al., 1994), whereas they attain the ability to deform root hairs of the nonhost vetch (Roche et al., 1991). Vetch can form nodules with *Rhizobium leguminosarum* bv *viciae*, which produces Nod factors that lack a substitution at the reducing terminal sugar residue. Hence, the sulfate substitution is a major host specificity determinant.

Thus, very low concentrations of Nod factors induce several responses in the root epidermis. If a receptor is involved in the elicitation of these responses, it must recognize the length of the Nod factors as well as the substitutions at the reducing end. Because the presence of a fatty acyl moiety is essential but its structure is not important, it is likely that this part of the molecule is not recognized by a receptor. Instead, the fatty acyl group might play a role in "docking" the Nod factors in the membranes and, in that way, facilitate binding to a putative receptor.

Infection

After attachment of rhizobia to the root hair tips, the tips curl tightly and bacteria become entrapped in the curls. A local hydrolysis of the plant cell wall takes place in the curled region (Callaham and Torrey, 1981; Van Spronsen et al., 1994), and the plasma membrane invaginates and new plant cell wall material is deposited (for reviews, see Bauer, 1981; Newcomb, 1981; Brewin, 1991; Kijne, 1992). This results in the formation of a tubular structure, the so-called infection thread, by which the bacteria enter the plant.

The ultrastructure of the wall of the infection thread is very similar to that of the normal plant cell wall, but the incorporation of certain nodulins may endow it with unique properties. The proline-rich early nodulins ENOD5 and ENOD12 are candidates for components of the infection thread wall, because cortical cells containing an infection thread express the corresponding genes (Scheres et al., 1990a, 1990b). The bacteria in the infection thread are surrounded by a matrix that seems to consist of compounds secreted by both the plant and the bacteria. For example, a 95-kD glycoprotein normally present in the intercellular spaces of the root cortex is localized in the infection thread matrix (Rae et al., 1992).

Concomitant with infection thread formation, cortical cells are mitotically reactivated, forming the nodule primordium (see later discussion). Infection threads grow toward this primordium and, once there, release bacteria into the cytoplasm. In those legumes that form indeterminate nodules, such as alfalfa and pea (see Nodule Functioning), nodule primordia arise from inner cortical cells. Hence, in the formation of this nodule type, the infection threads must traverse the outer cortex before they reach these cells. Before infection thread penetration, the outer cortical cells undergo morphological changes. The nuclei move to the center of the cells, and the microtubules and the cytoplasm rearrange to form a radially oriented conical structure, the cytoplasmic bridge, that resembles a preprophase band (Kijne, 1992). The infection threads traverse the cortical cells through the radially aligned cytoplasmic bridges, which are therefore called preinfection threads (Van Brussel et al., 1992).

Although the preinfection thread–forming outer cortical cells never divide, the induced morphological changes are reminiscent of those seen in cells entering the cell cycle. In situ hybridization experiments (Yang et al., 1994) showed that narrow rows of outer cortical cells express the S phase–specific histone *H4* gene (Figure 3A). However, a mitotic cyclin gene specifically expressed during the G2-to-M phase transition is not activated. Hence, the cells that form the preinfection thread reenter the cell cycle and most likely become arrested in the G2 phase, whereas the inner cortical cells progress all the way through the cell cycle and form the primordia (Figure 3A). This shows that part of the infection process is derived from a general process, namely, cell cycling. In some way, rhizobia have modified it and now exploit it for a completely different purpose, the infection process.

Purified Nod factors induce preinfection thread formation, but infection threads are not formed (Van Brussel et al., 1992).

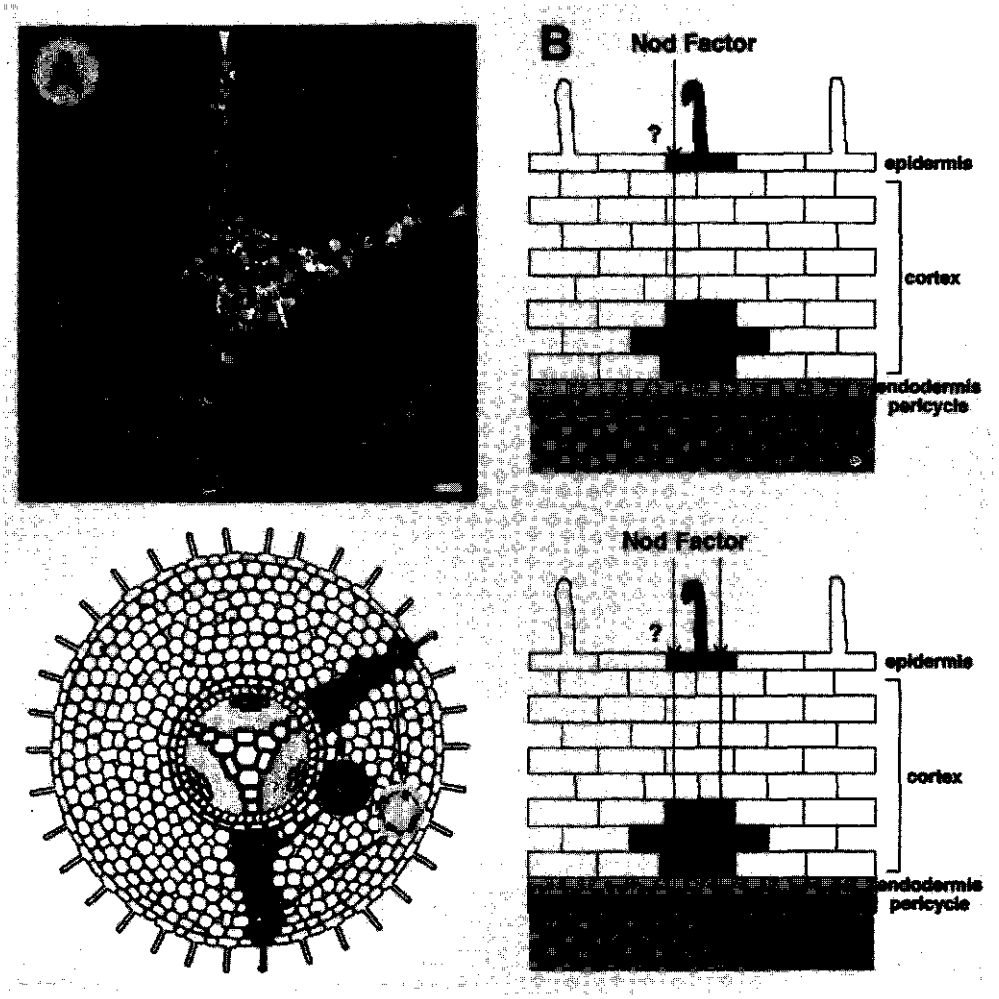


Figure 3. Events in the Cortex during Induction of an Indeterminate Nodule.

(A) Activation of the cortex after inoculation with rhizobia. The top panel is a dark-field micrograph of a cross-section of a pea root 1 day after inoculation with *R. leguminosarum* bv *viciae* that was hybridized with a histone *H4* gene probe. *H4* transcripts are localized in narrow rows of cortical cells in front of the infection sites, which are indicated by arrowheads. The silver grains represent the hybridization signal. Note that the infection sites are opposite to the protoxylem poles (arrow). Bar = 50 μm. The bottom panel is a schematic drawing showing the reactivation of cortical cells in pea roots after inoculation with rhizobia or application of Nod factors. The outer cortical cells, shown in lavender, reenter the cell cycle, proceeding from the G0/G1 phase to the S phase, and finally becoming arrested in G2, as indicated in the cell cycle in the yellow circle. In contrast, inner cortical cells, shown in purple, progress all the way through the cell cycle, as indicated by the cell cycle in the green circle, dividing and forming the nodule primordia. The activated cells are opposite the protoxylem poles of the root, which are shown in green. **(B)** Mode of action of Nod factors shown in a schematic depiction of a longitudinal section of a legume root. Application of Nod factors leads to root hair deformation, followed by an activation of the pericycle, due either to the action of Nod factors themselves or to that of second messengers (indicated by ?) generated in the epidermal cells. *ENOD40* expression in the pericycle cells may cause a change in the cytokinin/auxin ratio that results in cortical cell divisions (top panel). Alternatively (bottom panel), *ENOD40* expression in the pericycle may lead to susceptibility of the cortical cells, which then divide due to the action of either Nod factors or the second messengers generated in the epidermis. Cells shown in red are those that are sensitive to the action, direct or indirect, of Nod factors. In the bottom panel, the right arrow should point to the activated cortical cells instead of to the pericycle.

Thus, bacteria seem to be required for the formation of infection threads. It has been shown that pretreatment of clover root hairs with lipopolysaccharides of *R. trifolii* can improve the efficiency of infection thread induction by this strain, whereas pretreatment with lipopolysaccharides from a noninfectious *Rhizobium* strain leads to an increase in aborted infections (Dazzo et al., 1991). Furthermore, mutations in rhizobial exopolysaccharide biosynthesis can render the bacteria unable to induce infection threads (Dylan et al., 1986; Niehaus et al., 1993). Thus, interaction with bacterial surface compounds seems to play an important role in infection thread formation.

Cortical Cell Divisions

During mitotic reactivation of root cortical cells by rhizobia, genes that control the progression through the cell cycle, such as *cdc2* and mitotic cyclins, are induced (Yang et al., 1994). In addition, several nodulin genes are expressed, allowing a distinction to be made between nodule primordia and root or shoot meristems. Examples of such nodulin genes are *ENOD12* (Scheres et al., 1990a), *Gm93* (Kouchi and Hata, 1993), *ENOD40* (Kouchi and Hata, 1993; Yang et al., 1993; Asad et al., 1994; Matvienko et al., 1994), and *MtPRP4* (Wilson et al., 1994). These genes are activated in all cells of the primordia. Furthermore, *ENOD40* is also induced in the region of the pericycle opposite to the dividing cortical cells (Kouchi and Hata, 1993; Yang et al., 1993; Asad et al., 1994; Matvienko et al., 1994). Another early nodulin gene, *ENOD5* (Scheres et al., 1990b), is transcribed only in primordial cells that contain rhizobia.

Nod factors are sufficient for mitotic reactivation of the cortical cells (Spaink et al., 1991; Truchet et al., 1991; Relic et al., 1993). The early nodulins *ENOD12* and *ENOD40* are activated in such primordia (Vijn et al., 1993). In some host plants, purified Nod factors even induce nodule formation (Truchet et al., 1991; Mergaert et al., 1993; Stokkermans and Peters, 1994).

Interestingly, only certain cortical cells are susceptible to Nod factors. In tropical legumes, such as soybean, it is the outer cortical cells that are mitotically activated. In temperate legumes, such as pea, vetch, and alfalfa, it is the inner cortical cells, and especially those located opposite protoxylem poles, that divide (Kijne, 1992). The mechanism that controls the susceptibility of cortical cells is unknown. It has been postulated for decades that the susceptibility of cortical cells is conferred by an arrest in the G2 phase (Wipf and Cooper, 1938; Verma, 1992). However, use of cell phase–specific genes as probes in in situ hybridization experiments shows that this is not the case (Yang et al., 1994). Instead, susceptible cortical cells are, like other cortical cells, arrested in G0/G1.

The pattern of responding cortical cells provides some hints about a possible mechanism. Figure 3A shows that only narrow rows of cortical cells are activated to express the histone *H4* gene by rhizobia. At this time, the infection thread tips—the site where Nod factors are released—are still in the epidermis, indicating that Nod factors act at a distance. These rows of susceptible cells are located opposite protoxylem poles. More than 20 years ago, Libbenga et al. (1973) found that an alcohol extract of the stele could induce cell divisions in explants of the pea root cortex in the presence of auxin and cytokinin. A substance responsible for this activity, the so-called stele factor, has since been isolated and is thought to be released from the protoxylem poles. Such a compound might confer susceptibility to the cortical cells located opposite the protoxylem poles (Smit et al., 1993).

Which Nod factors can induce mitotic reactivation of cortical cells depends on the host plant. Rhizobia that induce cell divisions in the inner cortical layers, such as *R. leguminosarum* bv *viciae* and *R. meliloti*, produce Nod factors with highly unsaturated fatty acyl groups (Figure 2), whereas rhizobia that mitotically reactivate outer cortical cells, such as *Bradyrhizobium japonicum*, generally contain a C18:1 acyl group. The highly unsaturated fatty acyl group appears to be important for inducing cell divisions in the inner cortex. For example, only those *R. leguminosarum* bv *viciae* factors containing a C18:4 acyl group cause the formation of nodule primordia in vetch (Van Brussel et al., 1992). Whether the highly unsaturated fatty acyl moiety is recognized by a specific receptor and whether it is required for transport to the inner layers, for example, are unknown.

To unravel the mechanism by which Nod factors elicit cortical cell divisions, studies with compounds that can mimic their mitogenic activity have been performed. Two lines of evidence strongly suggest that Nod factors cause a change in the auxin/cytokinin balance. Both cytokinin (Cooper and Long, 1994) and compounds that block polar auxin transport (Hirsch et al., 1989) induce the formation of nodule-like structures in which early nodulin genes are expressed. Because some early nodulin genes are activated before cortical cells divide, an interesting question is whether such nodulins are involved in changing the phytohormone balance. The early nodulin gene *ENOD40*, which is induced by Nod factors in root pericycle as well as in dividing cortical cells, has a phytohormone effect when introduced into the nonlegume tobacco. This effect was examined in a protoplast assay in which the correlation between efficiency of cell division and auxin concentration was monitored. Tobacco protoplasts expressing a legume *ENOD40* gene under the control of the cauliflower mosaic virus 35S promoter divide efficiently at high auxin concentration, whereas in control protoplasts, this level of auxin suppresses their ability to divide (T. Bisseling and R. Walden, unpublished data).

Because *ENOD40* is sufficient to cause a phytohormone-like effect in tobacco and because induction of *ENOD40* expression in the pericycle precedes the first cortical cell divisions (T. Bisseling, unpublished data), we hypothesize that *ENOD40* expression in the pericycle of legume roots can cause the cytokinin/auxin ratio of the cortical cells to change, resulting in cell division (Figure 3B). In this model, mitotic reactivation would be induced in an indirect manner—that is, cortical cells would not themselves interact with the Nod factors. Alternatively, *ENOD40* expression in the pericycle might cause a

change in the cortical cells that renders them susceptible to Nod factors (or to second messengers generated in the epidermis), resulting in their mitotic reactivation (Figure 3B).

The role of *ENOD40* in altering phytohormone balance is not yet clear. *ENOD40* cDNA clones have been isolated from different legumes, and only in the soybean cDNAs could a long open reading frame be found (Kouchi and Hata, 1993; Yang et al., 1993; Crespi et al., 1994; Matvienko et al., 1994). Therefore, it has been postulated that this gene is active on the RNA level (Crespi et al., 1994; Matvienko et al., 1994).

Nod Factor Perception and Signal Transduction

As we have discussed, Nod factors induce responses in three different tissues of the root, namely, epidermis, cortex, and pericycle. Because Nod factors play a pivotal role in the early steps of nodulation, major efforts are being directed toward unraveling their mode of action. Nod factors are active at low concentrations, and their biological activity on a particular host is controlled by the presence of certain substitutions on the factor. These data suggest that Nod factors are recognized by a receptor in the host plant. However, it is unclear whether Nod factors interact directly with all three responding tissues or whether their interaction with epidermal cells results in the generation of second messengers that, after diffusion or transport, trigger responses in the inner tissues (Figure 3B).

The induction of certain host responses requires Nod factors with a very specific structure, whereas the demands for the induction of other responses are less stringent. For instance, for the induction of alfalfa root hair deformation, neither the structure of the fatty acid nor the presence of the *O*-acetyl group at the nonreducing end is important. On the other hand, the induction of infection thread formation in the same alfalfa root hairs requires a very specific structure. *R. meliloti* strains producing Nod factors that either are non–*O*-acetylated at the nonreducing end or do not contain the appropriate C16 unsaturated fatty acid initiate markedly fewer infection threads (Ardourel et al., 1994). A double mutant secreting Nod factors lacking the *O*-acetyl group and containing an inappropriate fatty acid has completely lost the ability to induce infection threads. This led Ardourel et al. (1994) to postulate that at least two different Nod factor receptors are present in the epidermis: a "signaling receptor" involved in the induction of root hair deformation, and an "uptake receptor" that is activated only by molecules with a very specific structure and that initiates the infection process.

The existence of distinct signaling and uptake receptors is supported by studies on the pea gene *sym2*, which controls nodulation. *sym2* originates from the wild pea variety Afghanistan. Afghanistan peas and cultivated peas carrying an introgressed *sym2* region nodulate only after inoculation with an *R. leguminosarum* bv *viciae* strain carrying an additional *nod* gene, namely, *nodX*. NodX catalyses the *O*-acetylation of *R. leguminosarum* bv *viciae* Nod factors (see Figure 2) at the reducing end (Firmin et al., 1993). *R. leguminosarum* bv *viciae* lacking *nodX* induces root hair deformation, but the ability to induce infection thread formation is strongly reduced. Therefore, Sym2 is a good candidate for an uptake receptor that interacts with NodX-modified Nod factors (T. Bisseling, unpublished data).

A biochemical approach to isolate a Nod factor receptor is feasible because large quantities of purified Nod factors, as well as chemically synthesized ones (Nicolaou et al., 1992), are available. A first report (Bono et al., 1995) on Nod factor binding proteins has revealed the occurrence of a binding protein that is present predominantly in the 3000*g* fraction of root extracts from alfalfa. However, the affinity of this binding protein for its ligand is lower than the concentration at which Nod factors are active. Furthermore, it binds to sulfated and nonsulfated factors in a similar way, whereas factors lacking the sulfate group are barely active on alfalfa. Therefore, it is unlikely that this protein is the Nod factor receptor. The availability of labeled Nod factors also creates the possibility of clarifying whether lectins play a role in binding of Nod factors, as has been postulated in the past (Long and Ehrhardt, 1989).

At present, genetic approaches to unravel Nod factor perception are restricted to legumes such as pea and soybean. Unfortunately, these species are recalcitrant to molecular genetic strategies leading to gene cloning. To study the mode of action of Nod factors, it might therefore be essential to develop new legume model systems (Barker et al., 1990; Handberg and Stougaard, 1992) or to explore the potential of nonlegume systems such as Arabidopsis. The latter may at first seem illogical, but a few observations show that Nod factors are recognized by nonlegumes. For example, expression of rhizobial *nod* genes in tobacco affects the development of these plants (Schmidt et al., 1993). Furthermore, a mutated carrot cell line that has lost the ability to form somatic embryos can be rescued by Nod factors (De Jong et al., 1993), and Nod factors trigger the alkalinization of the medium by tomato suspension-cultured cells (Staehelin et al., 1994a). Consequently, Nod factor receptors may be present in nonlegumes, a conclusion supported by the existence of a nonlegume, *Parasponia*, that can be nodulated by rhizobia (Marvel et al., 1987).

The availability of a root hair deformation assay and knowledge of some of the plant genes that are activated by the Nod factors, together with methods to inject root hairs (Allen et al., 1994), should make it possible to unravel the signal transduction cascades that are activated after Nod factor perception. These tools have been developed only recently, and therefore our understanding of Nod factor signal transduction is still in its infancy. Additional studies are required to determine the relevance of Nod factor–induced changes such as ion fluxes, membrane depolarization, and rearrangements of the actin filaments in the signal transduction pathways (Allen et al., 1994). Furthermore, it has now become possible to study whether, in addition to Nod factors, other rhizobial compounds are involved in facilitating Nod factor–induced responses. A

candidate is the NodO protein (Sutton et al., 1994). *nodO*, which is present in *R. leguminosarum* bv *viciae*, encodes a secreted protein that is not involved in Nod factor biosynthesis. When added to lipid bilayers, the purified protein can form channels that allow the movement of monovalent ions. Therefore, it has been suggested that NodO may amplify the Nod factor–induced responses by integration into the plant plasmamembrane (Sutton et al., 1994).

From Nodule Development to Nodule Functioning

As described previously, research on early stages of nodulation has emphasized the developmental steps of these processes. The achievements of rhizobial genetics allowed the dissection of the early stages and facilitated the characterization of the rhizobial signal molecules, the Nod factors, which play a key role in all early nodulation processes. The availability of purified Nod factors has now made the system accessible for biochemical approaches that should yield insight into the structure and distribution of Nod factor receptors and their signal transduction pathways.

Regarding the final steps of nodule formation, however, the proteins involved in nodule nitrogen, carbon, and oxygen metabolism have been studied on a biochemical level for decades, whereas research on the developmental aspects of the final steps of nodule formation is still in its infancy. Although several bacterial genes have been identified that, when mutated, cause a block in relatively late steps in nodule development, it has not been possible to identify the factors directly affecting differentation. Most mutants show pleiotropic effects or display host plant–dependent symbiotic phenotypes (see, for example, Gray et al., 1991; Hotter and Scott, 1991; Rossbach and Hennecke, 1991). The function of potential regulatory factors in nodule development, for example, in bacteroid differentiation, cannot be assessed because the technology for targeting these compounds to their sites of activity is not available. Thus, questions regarding signal exchange and developmental switches during the later steps of nodule formation have been difficult to address. For these reasons, in the following sections the major emphasis is on the biochemistry of nodule formation; developmental aspects are mentioned only briefly.

NODULE FUNCTIONING

Symbiotic nitrogen fixation takes place in specialized bacterial cells, in bacteroids in legume nodules, and in *Frankia* vesicles in actinorhiza. The bacterial enzyme nitrogenase catalyzes the following reaction:

$$N_2 + 8H^+ + 8e^- + 16\text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\text{Mg-ADP} + 16P_i \quad (1)$$

Nitrogenase consists of two components, the homodimeric Fe protein, encoded by *nifH*, and the tetrameric molybdenum–iron (MoFe) protein, encoded by *nifD* and *nifK*, which contains the MoFe cofactor. Hydrogen evolution is part of the nitrogenase mechanism; in the absence of other reducible substrates, the total electron flux through nitrogenase is funneled into hydrogen production (Hadfield and Bulen, 1969). Crystallographic structure analyses of the Fe protein (Georgiadis et al., 1992) and MoFe proteins of free-living nitrogen-fixing bacteria have shown structural similarities with other electron transfer systems, including hydrogenases and the photosynthetic reaction center (Kim and Rees, 1992; Kim et al., 1993; see von Wettstein et al., 1995, this issue).

In symbiosis, ammonium, the product of nitrogen fixation, is exported to and assimilated in the plant, which in turn supplies the bacteria with carbon sources to provide the energy for the nitrogenase reaction. The structure of a mature nodule has developed to meet the requirements set by this nutrient exchange between both symbiotic partners.

Nodule primordia differentiate into nitrogen-fixing nodules when bacteria have been released from the infection threads into the infected cells. Two types of legume nodules can be distinguished by their growth pattern—indeterminate and determinate nodules (Newcomb, 1981). Both types of nodule are characterized by peripheral vascular bundles and a central tissue containing infected and uninfected cells. In indeterminate nodules, a developmental gradient from the distal persistent meristem to the proximal senescence zone is present in which the central tissue is divided into specific zones (Figures 1A and 4; Vasse et al., 1990). The meristem is followed by the prefixation zone, where infection of the cells takes place. In the interzone, bacterial nitrogen fixation is induced, and it proceeds throughout the nitrogen fixation zone. In the senescence zone, bacteroids are degraded by the plant. In determinate nodules, the nodule meristem ceases to divide at an early stage of development. As a result, all of the cells of the central tissue are at a similar stage of development at any given time. Actinorhizal nodules display an indeterminate growth pattern, but in contrast to legume nodules, they represent coralloid structures composed of several modified lateral roots without root caps (lobes). Actinorhizal nodule lobes contain a central vascular bundle as well as infected and uninfected cells in the cortex (reviewed by Berry and Sunell, 1990).

Metabolite Exchange between Plant Cells and Intracellular Bacteria: The PBM as Interface

Root nodules provide the proper environment to allow efficient nitrogen fixation by the microsymbiont. Part of this specialization is the occurrence of plant-derived membranes that in all cases surround the "intracellular" microsymbiont (Figure 1). In legume nodules, these membranes are called peribacteroid membranes (PBMs; Figure 1A). They form the interface between the symbiotic partners across which signals and metabolites are exchanged and prevent a defense response by

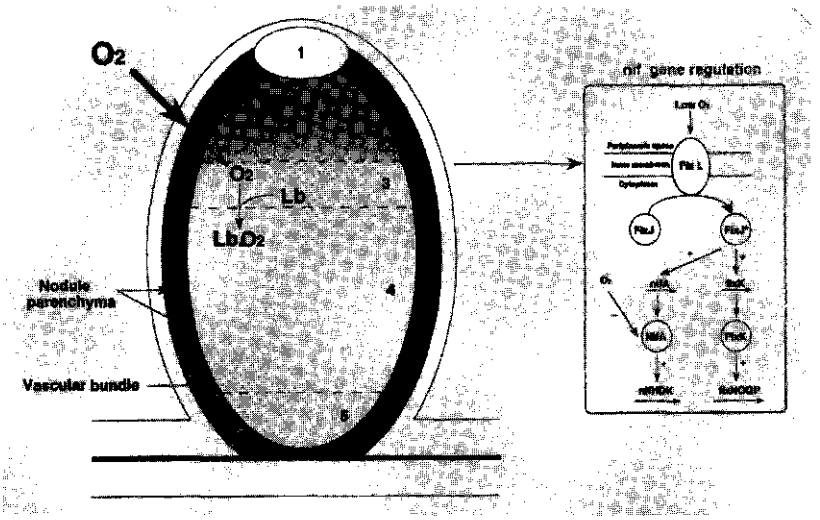


Figure 4. Oxygen Regulation in Indeterminate Legume Nodules.

Indeterminate legume nodules consist of five distinct regions: 1, nodule meristem; 2, prefixation zone; 3, interzone; 4, nitrogen fixation zone; and 5, senescence zone. An oxygen barrier is present in the nodule parenchyma surrounding the nodule vascular bundle (shown in red) that reduces oxygen access to the central tissue of the nodule. However, because this oxygen barrier is interrupted in the meristem, an oxygen gradient forms that extends from the distal to the proximal end of the nodule (shown by blue shading). In the first cell layer of the interzone (shown by the dashed green line), the low oxygen concentration leads to the events described in the green box. Low oxygen concentrations activate the *bacterial transmembrane oxygen sensor protein FixL, which in turn phosphorylates and thereby activates the transcriptional activator FixJ. The* activated FixJ protein (FixJ*) induces transcription of *nifA* and *fixK*, and the protein products of these genes induce the transcription of different genes encoding proteins involved in the process of nitrogen fixation. As an additional level of control, the NifA protein itself is oxygen sensitive. Leghemoglobin (*lb*) genes are expressed in the prefixation zone, the interzone, and the fixation zone. Leghemoglobin proteins transport oxygen to sites of respiration, thus enabling ATP production in a low-oxygen environment.

the plant against the "intracellular" bacteria (Nap and Bisseling, 1990; Verma, 1992; Werner, 1992).

Upon release from the infection thread, bacteria become internalized in legume nodules by a process resembling endocytosis (Basset et al., 1977). In actinorhizal nodules, however, *Frankia* hyphae penetrate the cell wall of cortical cells and start branching, while the plasma membrane invaginates and cell wall material is deposited around the growing hyphae. Thus, *Frankia* is not released into the plant cytoplasm and stays surrounded by encapsulating cell wall material throughout the symbiosis (Berry and Sunell, 1990). Subsequently, the endosymbionts multiply, enlarge, and eventually occupy most of the volume of the infected cell. During this process, growth of the microsymbiont and the surrounding membrane is synchronized by an unknown mechanism. This process of endosymbiont internalization and propagation requires massive membrane synthesis—in the case of legume nodules, 30 times the amount of plasma membrane synthesis (Verma, 1992).

The membrane surrounding the microsymbiont is derived from the host plasma membrane. The PBM of legume nodules has phospholipid (Perotto et al., 1995) and protein composition that are different from those of the plasma membrane (Verma, 1992) and that (presumably) endow it with specialized functions. The PBM contains several nodulins and may also contain a rhizobial protein (Fortin et al., 1985; Miao et al., 1992). Within the peribacteroid space between the bacteroids and the PBM, several proteins are present that are also found in vacuoles, for example, α-mannosidase II (Kinnback et al., 1987; Mellor and Werner, 1987), proteases (Mellor et al., 1984), and protease inhibitor (Garbers et al., 1988; Manen et al., 1991). Thus, the PBM may have adapted some properties of the tonoplast membrane (Mellor and Werner, 1987). Indeed, it has been proposed that the symbiosome (the PBM with enclosed bacteroids) has properties of a lytic compartment continuously being neutralized by ammonia exported by the bacteroids (Kannenberg and Brewin, 1989). According to this hypothesis, one would expect that the lack of bacterial nitrogen fixation

would lead to bacteroid degradation. In fact, there is evidence for premature bacteroid degradation of nonfixing *Rhizobium* mutants (for example, see Hirsch and Smith, 1987).

The extensive membrane biosynthesis in infected cells, together with the possibility to manipulate gene expression in root nodules without affecting other parts of the plant, has made the PBM an ideal system to study membrane biogenesis in plants. By using an antisense strategy in combination with nodule-specific promoters, it has been possible to show that homologs of the Ypt1 protein (Schmitt et al., 1986), which controls membrane biosynthesis in yeast, are involved in PBM biosynthesis in soybean nodules (Cheon et al., 1993). In nodules expressing antisense RNA of such a homolog, the number of bacteroids per cell was reduced and the infected cells did not expand.

Because the PBM constitutes the interface between bacteroids and host plants, it plays an important role in controlling the exchange of metabolites. These include ammonium, the product of nitrogen fixation, and heme, the prosthetic group of the oxygen transport protein leghemoglobin, which are exported by the bacteroids to the host cytoplasm (O'Gara and Shanmugan, 1976; Nadler and Avissar, 1977), as well as carbon sources and probably also assimilated ammonium, which are supplied by the host to the bacteroids (De Bruijn et al., 1989; Werner, 1992). Which proteins are involved in the transport of these compounds is largely unclear. Bacteroids express a dicarboxylic acid uptake system, isolated bacteroids take up dicarboxylic acids, and mutants in this uptake are symbiotically ineffective (Ronson et al., 1987; Werner, 1992), all of which indicates that dicarboxylic acids are likely to be the carbon source supplied by the plant to the intracellular bacteria. It has been suggested that nodulin-26 transports the dicarboxylic acids to the bacteroids (Ouyang et al., 1991). However, its low substrate specificity in vitro indicates that it is more likely to form a pore responsible for the uptake of ions or small metabolites in general (Weaver et al., 1994).

After division, the intracellular bacteria differentiate into bacteroids. Because both plant (Häser et al., 1992) and bacterial (Glazebrook et al., 1993) mutants have been identified that are specifically defective in bacteroid differentiation, this process may be independent of internalization of bacteria by the infected cells. Bacterial mutants specifically defective in the release of bacteria from the infection thread are known as well (De Maagd et al., 1989). Bacterial *nod* genes are expressed in the distal part of the prefixation zone (Figure 4; Schlaman et al., 1991), indicating that Nod factors may play a role in signal exchange within the nodule. However, because bacterial release and bacteroid development can be impaired in bacterial strains with functional *nod* genes, other bacterial and/or plant signals must also play a role in these steps of development.

Metabolite Exchange between Nodule and Plant: Nitrogen Transport

In the context of the whole plant, the root nodule functions as a nitrogen source and a carbon sink. In fact, it has been suggested that legume nodules evolved from carbon storage organs (Joshi et al., 1993). The carbon source transported from the leaves to the nodules is sucrose (Hawker, 1985), which is introduced into nodule metabolism through degradation by sucrose synthase. This enzyme is present at high levels in both legumes and actinorhizal nodules (Thummler and Verma, 1987; M. van Ghelue, A. Ribeiro, A. Akkermans, B. Solheim, A. van Kammen, T. Bisseling, and K. Pawlowski, unpublished observations). The form in which nitrogen is transported depends on the plant: temperate legumes, which generally form indeterminate nodules, export amides, whereas tropical legumes, which form determinate nodules, export ureides. Actinorhizal plants export mostly amides, with the exceptions of *Alnus* sp and *Casuarina equisetifolia*, which are citrulline exporters (Schubert, 1986; Sellstedt and Atkins, 1991). In all cases, ammonium is exported by the microsymbiont as the first product of nitrogen fixation and is assimilated in the cytoplasm of nodule cells via the glutamine synthetase (GS)/glutamate synthase pathway (Schubert, 1986; see Lam et al., 1995, this issue). Subsequently, glutamate is metabolized into nitrogen transport forms. The products of several late nodulin genes play a role in this metabolism.

In ureide-producing determinate legume nodules, the assimilation of ammonium by GS and the biosynthesis of ureides are spatially separated to some extent: whereas GS is expressed in both infected and uninfected cells of soybean nodules (Miao et al., 1991), uricase (nodulin-35), a key enzyme in purine oxidation that catalyzes the oxidation of uric acid to allantoin, has been found in peroxisomes of uninfected cells only (Hanks et al., 1981; Nguyen et al., 1985). Allantoinase, which catalyzes the next step in purine oxidation, has also been localized to uninfected cells (Hanks et al., 1981). The uninfected cells of determinate nodules also seem to be involved in the transport of fixed nitrogen. These cells constitute a more or less continuous network throughout the whole central tissue that facilitates the transport of assimilated ammonium to the nodule vascular bundle (Selker, 1988). An elaborate tubular endoplasmic reticulum system that is appressed to the peroxisomes, where ureides are produced, and continues through plasmodesmata connects all uninfected cells (Newcomb et al., 1985). In indeterminate nodules, by contrast, no specialized function has been assigned to the uninfected cells in the central tissue. Instead, efficient transport of fixed nitrogen is achieved by the presence of transfer cells in the pericycle of the nodule vascular bundles (Pate et al., 1969).

Oxygen Protection of Bacterial Nitrogen Fixation

Nitrogenase is highly oxygen sensitive because one of its components, the MoFe cofactor, is irreversibly denatured by oxygen (Shaw and Brill, 1977). On the other hand, the large amount of energy required for this reaction has to be generated by oxidative processes; thus, there is a high demand for oxygen in nodules. Different strategies are used in different symbiotic interactions to cope with this paradox. In legume nodules, a low oxygen tension in the central part of the nodule is achieved

by a combination of a high metabolic activity of the microsymbiont and an oxygen diffusion barrier in the periphery of the nodule, that is, in the nodule parenchyma (Figure 4; Witty et al., 1986). Because oxygen diffuses ~10^4 times faster through air than through water, it is generally assumed that oxygen diffusion in nodules occurs via the intercellular spaces. The nodule parenchyma contains very few and small intercellular spaces, and this morphology is thought to be responsible for the block in oxygen diffusion (Witty et al., 1986). In the nodule parenchyma, nodulin genes such as *ENOD2* are expressed whose protein products might contribute to the construction of the oxygen barrier (Van de Wiel et al., 1990). In the infected cells of the central part of the nodule, high levels of the oxygen carrier protein leghemoglobin facilitate oxygen diffusion. In this way, the microsymbiont is provided with sufficient oxygen to generate energy within a low overall oxygen concentration (Figure 4; Appleby, 1984).

In contrast to *Rhizobium*, *Frankia* bacteria can form specialized vesicles in which nitrogenase is protected from oxygen (Figure 1B; Benson and Silvester, 1993). However, vesicle formation during symbiosis does not take place in all *Frankia*–root interactions (Benson and Silvester, 1993) and does not always seem to provide full oxygen protection of nitrogenase (Tjepkema, 1983; Kleemann et al., 1994). In these cases, an oxygen diffusion barrier is established around groups of infected cells by lignification of the walls of adjacent uninfected cells (Berg and McDowell, 1988; Zeng et al., 1989). In addition, the oxygen transport protein hemoglobin, the equivalent of leghemoglobin, is found in the infected cells (Fleming et al., 1987; Tjepkema and Asa, 1987; Jacobsen-Lyon et al., 1995).

As in actinorhizal symbioses, in the *Nostoc–Gunnera* symbiosis, oxygen protection of nitrogen fixation is achieved by the formation of a specialized compartment containing nitrogenase: *Nostoc* forms heterocysts that are protected from oxygen by a glycolipid cell wall (Figure 1C; Bergman et al., 1992).

Gene Regulation in Nodules

To obtain nitrogen-fixing root nodules, several genes of both symbionts are specifically induced or repressed during nodule development. The use of reporter genes as well as in situ hybridization studies has provided detailed insights into the spatial and temporal regulation of such genes in indeterminate nodules. In such nodules, major, sudden developmental changes occur at the transition of the prefixation zone to the interzone: starch is deposited in the plastids of the infected cells, and the bacteroid morphology alters (Figures 1A and 4; Vasse et al., 1990). These events are accompanied by changes in bacterial gene expression: transcription of bacterial *nif* genes, which encode enzymes involved in the nitrogen fixation process, is induced, whereas expression of the bacterial outer membrane protein gene *ropA* is dramatically reduced (Yang et al., 1991; De Maagd et al., 1994).

All of these events, together with dramatic changes in plant gene expression (see later discussion), take place within a single cell layer. What plant factor causes this rapid change in bacterial differentiation? To answer this question, rhizobial *nif* gene regulation has been studied extensively and has generally been found to be induced by microaerobic conditions (reviewed in Merrick, 1992; Fischer, 1994). The regulation of *nif* gene expression in *R. meliloti* is described here (see Figure 4) because it can be correlated to morphological changes observed in an indeterminate nodule.

Transcription of *R. meliloti* nitrogen fixation (*nif/fix*) genes is controlled either by the transcriptional activator NifA together with the sigma factor RpoN (Gussin et al., 1986; Morrett and Buck, 1989) or, for some genes, by the transcriptional activator FixK. NifA activity is under oxygen control at two levels: the NifA protein itself is oxygen sensitive (Krey et al., 1992), and its transcription, together with that of *fixK*, is induced under microaerobic conditions by the transcriptional activator FixJ (David et al., 1988). FixJ is part of a two-component system that includes the oxygen-sensing hemoprotein FixL. FixJ is activated by FixL by phosphorylation upon microaerobiosis (see Figure 4; David et al., 1988; Gilles-Gonzalez et al., 1991; Da Re et al., 1994). It is the activated FixJ protein that in turn induces the transcription of *nifA* and *fixK* (Batut et al., 1989).

Although microaerobic conditions are essential for rhizobial *nif* gene transcription in symbiosis, it has long been debated whether the reduction of oxygen concentration is the sole regulatory factor for the induction of *nif* gene expression in the interzone. Recent results (Soupène et al., 1995) have shown that *R. meliloti nif* gene expression in plants can be modified by changing the external oxygen concentration: in nodules immersed in agar, *nif* gene expression is extended to a younger part of the nodule and now also occurs in the prefixation zone. This effect is controlled by the FixLJ system, because the same result is obtained by nodulation with a strain carrying a constitutively active mutant form of FixJ (FixJ*; see Figure 4). Thus, oxygen concentration seems to be a major factor in controlling symbiotic *nif* gene transcription during symbiosis. In contrast, *ropA* expression is not under oxygen control in free-living bacteria, and *ropA* repression can even be uncoupled from *nif* gene induction in the same cell layer. In mutant nodules induced by a *Rhizobium* strain whose host range had been manipulated, *ropA* mRNA distribution was equal to that in wild-type nodules, whereas bacteroid differentiation and *nif* gene induction did not take place (De Maagd et al., 1994). Therefore, further analyses are required to determine the other regulatory factors responsible for the changes in bacterial gene expression in the first cell layer of the interzone.

The expression of several plant genes is also controlled at the transition of the prefixation zone to the interzone as well as in other zones of the central tissue (Scheres et al., 1990a, 1990b; Yang et al., 1991; Kardailsky et al., 1993; Matvienko et al., 1994). However, the expression of these genes seems not to be controlled by the oxygen tension (Govers et al., 1986) but rather to be under developmental control. To analyze the regulators of plant nodulin gene expression, the expression of nodulin promoter–β-glucuronidase fusions has been studied in heterologous legumes (Forde et al., 1990; Szabados et al., 1990; Brears et al., 1991).

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The most extensive studies have been performed on the leghemoglobin genes. So far, promoter analysis of these genes has led to the identification of a so-called organ-specific *cis*-acting element (OSE; Ramlov et al., 1993), also called the nodule-infected cell-specific element (NICE; Szczyglowski et al., 1994), which has also been found in the promoter of the nodule-specific hemoglobin gene of the actinorhizal plant *Casuarina glauca* (Jacobsen-Lyon et al., 1995). A *C. glauca* hemoglobin promoter–β-glucuronidase fusion is expressed in the infected cells of *Rhizobium*-induced nodules from *Lotus corniculatus* (Jacobsen-Lyon et al., 1995), which implies that similar regulatory factors are involved in both legume and actinorhizal systems. However, the corresponding transcription factors that bind to these promoter elements have yet to be identified.

CONCLUDING REMARKS

Symbioses between higher plants and nitrogen-fixing microorganisms provide a niche in which the prokaryote can fix nitrogen in a very efficient manner. A comparison of the development and functioning of the three different nitrogen-fixing symbioses has provided and continues to provide insight into how both common and unique strategies have evolved to solve problems imposed by various requirements of nitrogen fixation. For instance, in all systems the plant copes with intracellular bacteria by enclosing them in a plasmalemma-derived membrane, whereas protection of the enzyme nitrogenase against oxygen is achieved in diverse manners.

An intriguing aspect of the nitrogen-fixing symbioses is their host specificity, whose strictness varies in the different systems. In the *Gunnera–Nostoc* system, only a single plant genus can establish the interaction, whereas rhizobia can interact with most members of the legume family. *Frankia* bacteria are the most promiscuous microsymbionts, because they can establish a symbiosis with plants belonging to different families; however, recent molecular phylogenetic studies have shown that these families are actually rather closely related (Chase et al., 1993; Maggia and Bousquet, 1994).

Host specificity provides a serious restraint in the application of symbiotic nitrogen fixation in agriculture, because most major crops are unable to establish such a symbiosis. Therefore, it is not surprising that since the development of plant genetic engineering techniques, an important goal has been to transfer the ability to form a nitrogen-fixing symbiosis to important crops, such as rice. However, molecular genetic research has shown that a relatively high number of specific host functions are involved in forming a nitrogen-fixing organ. Therefore, it has seemed impossible to achieve this aim with the methodology available.

The possibility of reaching this goal has become newly invigorated as a result of research indicating that mechanisms controlling nodule development might be derived from processes common to all plants. For example, Nod factors might be recognized by receptors that are also present in nonlegumes; preinfection thread formation appears to involve a mechanism derived from the cell cycle machinery; and several plant proteins that were thought to function exclusively in nodules appear to have nonsymbiotic counterparts, as has been described for soybean nodulin-26 (Miao and Verma, 1993) and *Casuarina* hemoglobin (Jacobsen-Lyon et al., 1995). Furthermore, actinorhizal nodules and nodules induced by rhizobia on the nonlegume *Parasponia* closely resemble lateral roots (Hirsch, 1992). Thus, the processes modified in the nodule developmental programs are common to all higher plants. Studies of how these common processes have been altered might therefore provide new means to design strategies by which nonlegume plants can be given the ability to establish a symbiosis with a nitrogen-fixing microbe.

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CHAPTER 2

Exploratory studies on epitope-tagged early nodulins

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INTRODUCTION

During the *Rhizobium*-legume interaction the expression of a number of nodule specific plant genes, called nodulin genes (Van Kammen, 1984) is induced. The genes involved in the early steps of the interaction, playing a role in the infection process and nodule development, are the so-called, early nodulin genes. Among the best studied early nodulin genes, are *ENOD5* and *ENOD12*, for which homologues have been isolated from different leguminous plants (Scheres et al., 1990a,b; Pichon et al., 1992; Allison et al., 1993; Vijn et al., 1995a).

PsENOD5 is expressed in both root hairs and root cortical cells containing the tip of an infection thread (Scheres et al., 1990a). *PsENOD12* is expressed in the epidermis and cortical cells containing infection threads, as well as in cortical cells before they are traversed by them (Scheres et al., 1990b). In the nodule primordia, *PsENOD5* transcripts are present only in the infected cells, whereas *PsENOD12* mRNA occurs in all cells of the primordia. In a mature nodule, *PsENOD5* mRNA occurs only in the infected cells of the prefixation zone, and it reaches its maximum level in the interzone. The amount of the transcript in the cells decreases suddenly at the transition of interzone into fixation zone where it remains at a constant low level. *ENOD12* is highly expressed in infected as well as uninfected cells of the prefixation zone, while *ENOD12* mRNA is absent in the interzone and fixation zone (Vijn et al., 1995a,b).

Both ENOD5 and ENOD12 have a proline rich nature and might be cell wall proteins involved in infection thread growth. They are probably useful tools to unravel the molecular mechanism of infection thread formation. As a first step in resolving the function of these proteins, we wanted to immunolocalize the proteins using antisera raised against, either purified proteins produced in *E.coli*, or synthesized peptides conjugated to a carrier protein. Despite the fact that the antisera recognized the *E. coli* expressed protein or the synthetic peptide, every trial to detect the ENOD5 and ENOD12 proteins has been unsuccesful so far (data not shown). For this reason we decided to initiate an alternative approach, namely epitope-tagging. In this method, one can make use of the commercial available antibodies against small peptides. A vector containing a gene encoding a protein, consisting of a peptide epitope for which antibodies are available, linked to the N-terminus, the C-terminus or inserted somewhere in the protein of interest, has to be constructed and introduced into plants. A fast transformation system for *Vicia*, giving rise to transgenic roots that can be inoculated by *Rhizobium*, is at the moment available (Quandt et al., 1993). We used this transformation system for an attempt to study epitope-tagged ENOD5 and ENOD12.

The nonapeptide YPYDVPDYA (HA) is often used as an epitope tag. This peptide was originally identified as a major antigenic determinant of the human influenza virus hemagglutinin, a glycoprotein required for infectivity of the human influenza virus (Green et al., 1982; Wilson et al., 1984). Monoclonal and polyclonal antibodies for this epitope, are

available commercially. Therefore, we studied whether HA can be used as a tag in plants. Data on these studies will be presented in this chapter.

RESULTS

Tissue-specific expression of the duplicated 35S promoter

For immunolocalization studies of epitope-tagged early nodulins in transgenic plants, a promoter is needed that drives the expression of the nodulin transgenes in the cells in which the early nodulin genes are normally transcribed, and gives a strong expression. The CaMV 35S promoter is one of the strong promoters that can be used for studies in roots and nodules (Quandt et al., 1993). The duplicated CaMV 35S promoter was shown to have an even stronger activity than the single CaMV 35S promoter (Kay et al., 1987; Fang et al., 1989). To find out whether this modified promoter can be useful for studies on early nodulins, its expression pattern in nodules was studied. The duplicated 35S promoter was fused to *β–glucuronidase*-intron (*gusA*-int) gene.

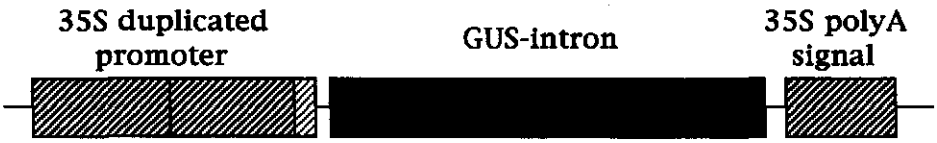

Figure 1. Schematic representation of the construct used to perform promoter analysis of the duplicated CaMV 35S promoter.

The densely shaded boxes depict the two copies of the CaMV 35S promoter enhancer (domain B, -343 to -90) followed by domain A containing the minimal promoter (-90 to 0) which is depicted with a light shaded box. *gusA*-int is depicted with a black box while the CaMV 35S polyadenylation signal is shown by a densely shaded box.

This construct (Figure 1) was introduced into *Vicia hirsuta* using an *Agrobacterium rhizogenes* transformation system (Quandt et al., 1993), resulting in a non-transformed shoot and transformed hairy roots. The hairy roots were inoculated with *Rhizobium leguminosarum* bv. *viciae* strain VH5e and pink nodules were formed after nine days.

As it can be seen from figure 2A, each shoot forms more than one hairy root and not all hairy roots of a single plant are showing GUS expression. The hairy roots lacking GUS activity might not contain the transgene. However, lack of staining might be also attributed to a low expression level or silencing of the transgene, due to positional effects. Furthermore, transformed roots form nodules with and without GUS activity (Figure 2B). This indicates

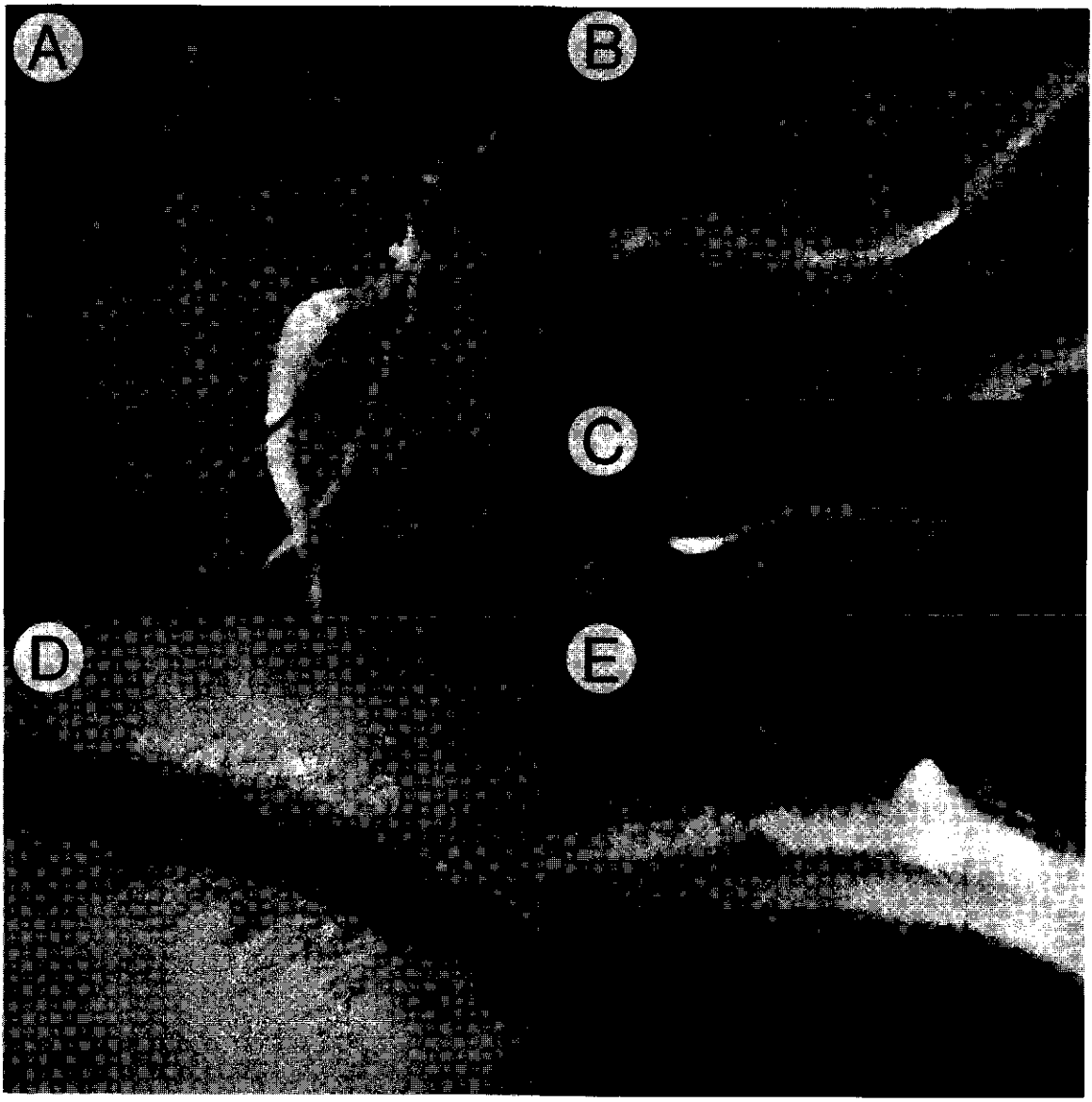

Figure 2. Histochemical localization of duplicated *35S-gusA*-int activity in transgenic *Vicia hirsuta*. The activity is shown as blue precipitate.

A. Overview of the hairy roots formed from a single plant after puncturing with *Agrobacterium rhizogenes* strain ARqua1. Note that there are roots that lack GUS activity.

B. GUS activity was strongly detected in the nodules. Note that not all nodules of a transformed root show the activity, indicating that the roots are chimaeric.

C, D, E. Close-ups of trangenic *Vicia hirsuta* roots. GUS activity is observed in the root vascular bundle (C, D, E), but not in the root cap and the root meristem of main (C) and lateral roots (E). The activity is also detected in nodule primordia (E) but not in lateral root primordia (D).

that the roots are actually chimaeric. In most experiments, 30%-70% of the roots contain GUS activity. 25%-40% of the nodules formed on the roots having GUS activity, expressed GUS at a detectable level (Table 1).

Period after inoculation with *R. leguminosarum*	GUS staining (%)	
	Roots	Nodules
7 days	62	42
14 days	32	24
21 days	44	39
29 days	68	24

Table 1. Transformation efficiency of plants transformed with duplicated 35S-*gusA*-int. Plants were stained for GUS activity, 7, 14, 21, and 29 days after inoculation of the hairy roots with *Rhizobium leguminosarum* bv. *viciae* strain VH5e. The percentages of the transformed roots showing GUS activity were scored. 80 plants were used in each time point. The percentages of the GUS expressing nodules, present on the roots having detectable levels of GUS activity, were also scored.

The duplicated 35S promoter is active at a variable level in the vascular bundle of both main and lateral roots (Figure 2C), but it is neither active in root meristem and root cap (Figure 2C, 2E), nor lateral root primordia (Figure 2D). In contrast, the duplicated 35S promoter is active at a high level in nodule primordia (Figure 2E, 3A). Furthermore, it is active in nodules of different ages (Figure 2B). A typical expression pattern within a nodule, can be seen in figure 3B. Longitudinal sections of plastic embedded nodules, showed that the promoter is active through all the zones of a mature nodule (15-21 days, Figure 3B). In most nodules though (Figure 3B), a higher expression level was observed in the prefixation zone and the nodule vascular bundle.
Since ENOD5 and ENOD12 are preferentially expressed in the prefixation zone, the duplicated 35S promoter is suitable for studies on the epitope-tagged early nodulins.

HA epitope-early nodulin constructs

Constructs encoding an HA-early nodulin fusion protein were made. PsENOD12B carries a signal peptide at the N-terminus, therefore the tag was introduced at the C-terminus (Figure 4, upper panel).

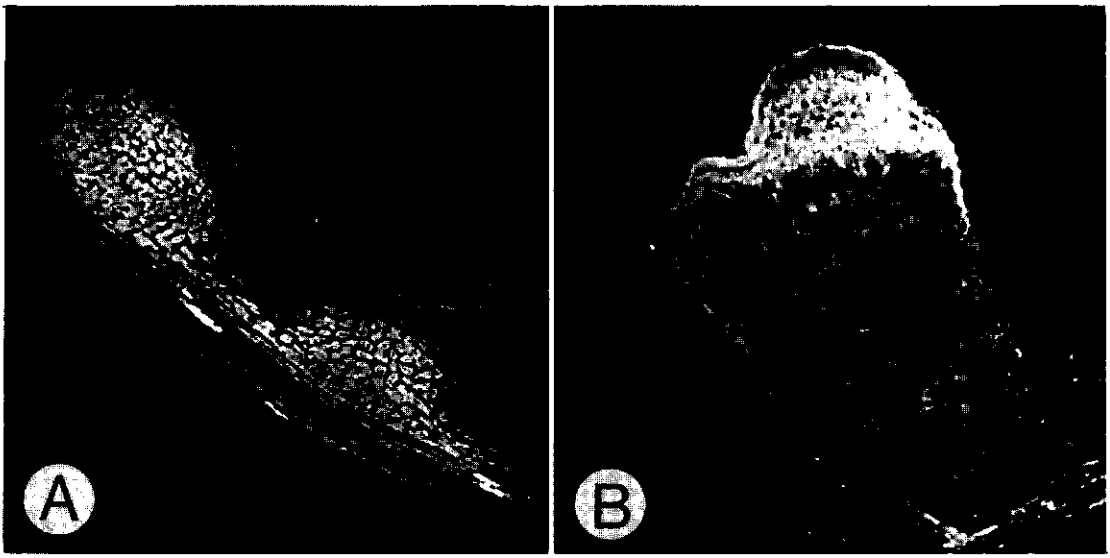

Figure 3. Histochemical localization of duplicated *35S-gusA*-int in nodules.

A, B. Dark field micrographs of cross-sections of plastic embedded nodules. The use of dark field results in visualization of GUS activity as purple precipitate (instead of blue when bright field is used). Blue precipitate though can be seen with dark field when the activity is too high.

GUS activity can be detected in nodule primordia (A) and the root vascular bundle (A). In B, the activity in a nodule of 15 days upon inoculation with *R. leguminosarum* bv. *viciae* strain VH5e, can be viewed. The purple precipitate can be observed in all zones. The highest GUS activity is in the nodule vascular bundle and some cells of the prefixation zone, where a blue precipitate is visualized. In the nodule meristem and the prefixation zone, -where a uniform deep purple colour can be seen, the activity is higher than in interzone and the fixation zone, -where the purple precipitate shows a more spotted appearance.

ENOD5 also contains an N-terminal signal peptide, while the C-terminus is hydrophobic and might serve as an anchor to the membrane, which could mask a C-terminal tag. Therefore, two constructs were made, one containing the HA tag at the C-terminus and another construct with the epitope inserted in the middle (Figure 4, upper and bottom panel). For the second construct we selected the area that has the highest hydrophilicity and antigenic index

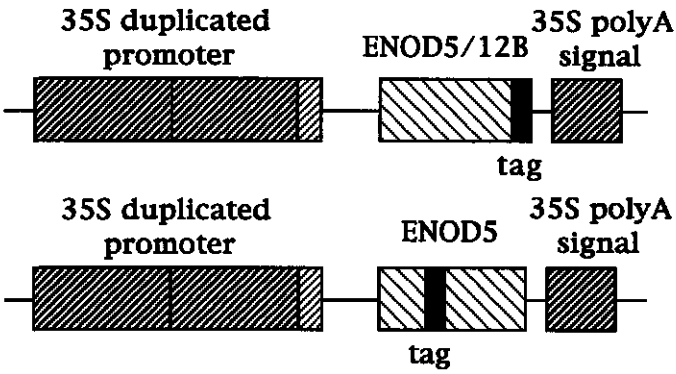

Figure 4. Schematic representation of the HA-early nodulin constructs.
All constructs were put under the control of the duplicated CaMV 35S promoter. The CaMV 35S polyadenylation signal was used. The epitope tag (HA, black box) is fused at the carboxyl-terminus of ENOD5 and ENOD12B (upper panel), or inserted in the middle of ENOD5 (between the 36th and 37th amino acids, bottom panel).

according to the Wisconsin Sequence Analysis Package^{TM} of the Genetics Computer Group, Inc (, 1986), and the tag was inserted between the 36th and the 37th amino acids.
All constructs were put under the control of the duplicated CaMV 35S promoter (Figure 4), and cloned into pBIN19, a vector appropriate for plant transformation with *Agrobacterium rhizogenes*.

Detection and characterization of epitope-tagged ENOD12 protein

Plants were transformed with a construct carrying the gene encoding the epitope-tagged PsENOD12B under the control of the duplicated 35S promoter (Figure 4, upper panel). As a control, plants were transformed with the vector. In order to confirm that, the transgene is expressed, and the fusion protein is stable in our plants, proteins were isolated and immunoblots were made. Since PsENOD12B might be a cell wall protein, a protein isolation protocol for cell wall proteins was used. Two protein fractions were isolated; the low salt fraction (LS), containing all the cytosolic proteins, and the high salt fraction (HS), containing the non-convalently bound cell wall and membrane proteins. The proteins were separated on a 20% SDS-PAGE gel and the western blot was incubated with monoclonal or polyclonal antibodies against the hemagglutinin nonapeptide (12CAT5). In the right panel of Figure 5, the result of an immunoblot with the polyclonal antibodies is presented. No differences were observed between the low salt protein fractions from nodules of plants transformed with either alone the vector or the construct (Figure 5). However, 2 extra polypeptides (arrow), of 14.4 kD and 15 kD, are present in the high salt fraction of the nodules expressing the HA-

ENOD12B gene. In addition, a lot of other bands common between the extracts of plants carrying the transgene or the vector are recognized by the polyclonal antiserum, indicating that either the antiserum is of bad quality or that proteins exist in plants that can crossreact with the particular antiserum. No crossreacting polypeptides could be detected with monoclonal antibodies, indicating that this antibody has a lower titer than the polyclonal HA antibody.

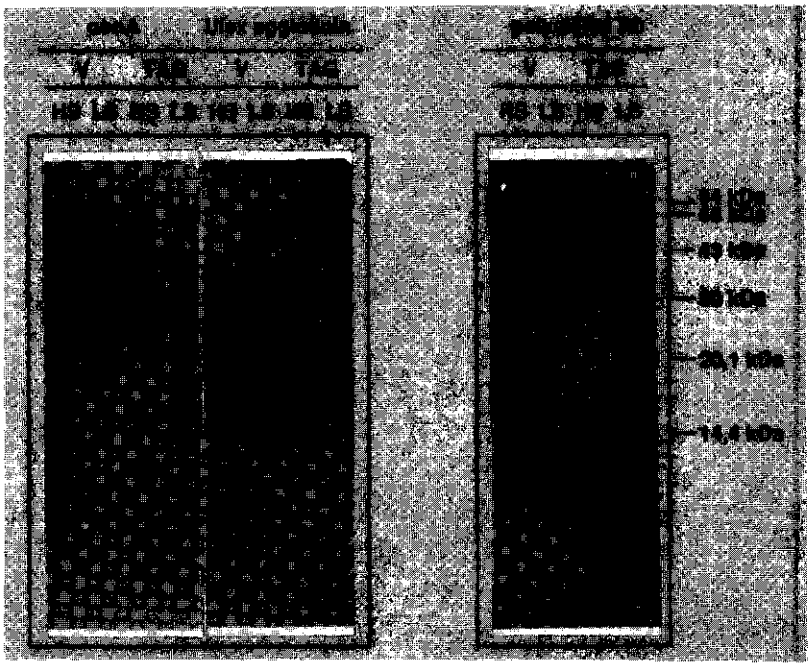

Figure 5. Western blots of protein isolates from transgenic plants transformed with ENOD12B-HA construct (TAG). As control, plants were transformed with the vector (V). Two protein extracts were obtained; LS, low salt fraction, and HS, high salt fraction. In the right panel, an immunoblot with the polyclonal antibody against HA is depicted. The arrow indicates the two extra polypeptides of 14.4 kD and 15 kD present in the HS fraction of plants transformed with the ENOD12B-HA. In the left panel, identical blots as in the one shown the right panel, were stained with Conconavalin A (conA), a mannose and glucose specific lectin and *Ulex europaeus* agglutinin A, a fucose specific lectin. The polypeptides recognized by the antibody are stained also with the lectins (arrows). A third polypeptide of 12 kD (arrowheads), present in the HS fraction of plants transformed by ENOD12B-HA, is also stained by the lectins but it is not recognized by the antibody.

The expected molecular weight of the mature PsENOD12B-HA fusion protein is 10,2 kD. In the immunoblots the detected extra bands have a larger molecular weight, indicating a shift that might be due to glycosylation. To determine whether the crossreacting proteins are glycosylated, similar blots as used for the immunodetection were stained with lectins (left panel of Figure 5). Conconavalin A, a mannose and glucose specific lectin, and *Ulex*

europaeus agglutinin I, specific for fucose, were used. Both polypeptides co-migrating with the 14.4 kD and the 15 kD polypeptides were stained with both lectins, indicating the presence of at least two sugar chains. Furthermore, a third polypeptide of approximately 12 kD was detected with the lectins (arrowhead) in the high salt protein fraction isolated from nodules of plants carrying the transgene. This polypeptide is not recognized by the antibodies. Nevertheless it seems likely that it is a protein derived from the tagged ENOD12B protein.
Hence, the recombinant protein is stable in plants and is found in the fraction of the cell wall proteins. The sequence characteristics of PsENOD12B has suggested that ENOD12B will be a cell wall protein. The occurrence of ENOD12 tagged protein in the high salt buffer fraction, appears to confirm this suggestion. In addition, preliminary data indicate that PsENOD12B is a glycoprotein.

Immunocytological studies

To check whether we are able to immunolocalize the epitope tagged proteins, we decided to transiently express the constructs in protoplasts. Cowpea protoplasts were isolated and transfected (Van Bokhoven et al., 1993) with the constructs carrying ENOD5-HA and ENOD12B-HA under the control of the duplicated 35S promoter, and the vector as a control. To localize the proteins that are transiently produced, the polyclonal or the monoclonal antibodies against the tag and FITC-conjugated goat-anti-rabbit or anti-mouse IgG respectively, were used. The use of the polyclonal antibodies resulted in a very high backround (data not shown). When the monoclonal antibodies were used as first antibodies no background was observed in the protoplasts transfected with the vector. But no recombinant protein was detected with the monoclonal antibodies when the protoplasts were transfected with the ENOD5-HA or the ENOD12B-HA constructs (data not shown). Bearing in mind that protoplasts lack cell walls, our failure to detect the recombinant proteins might due to the fact that they are secreted in the medium.
Localization studies performed on nodules of transgenic plants transformed with ENOD5-HA and ENOD12B-HA constructs, resulted in high backround when the polyclonal anti-HA antiserum was used (data not shown). No fusion protein was detected when the monoclonal anti-HA antibodies were used. These data confirmed the observations obtained with the experiments performed with the cowpea protoplasts.

CONCLUDING REMARKS

Epitope tagging has been succesfully used in *E. coli*, yeast and mammalian cell lines, and plant protoplasts. In particular, this approach can be employed to perform immunolocalization studies of tagged proteins, (Mieszczak et al., 1992), and to purify functional complexes by co-

immunoprecipitation of the tagged protein and the proteins associated with it (Field et al., 1988). Furthermore, structural or functional domains of proteins have been identified with this procedure, by insertion of the tag in different positions leading to disruption of the particular protein (Wadzinski et al., 1992; Thomas and Maule, 1995). Since this approach opens lots of possibilities, we decided to initiate similar studies in plants, focusing on early nodulins, and making use of the fast plant transformation system giving rise to transformed roots, that has been established for legumes (Quandt et al., 1993).

An important factor determining the success of such approach is the amount of fusion protein produced in the transgenic tissues, and therefore the procedure requires strong promoters to express the transgenes. The CaMV 35S promoter is one of the strongest promoters shown to be active in roots. Quandt et al. (1993) showed that this promoter is active in indeterminate type of nodules. Their work shows that the promoter confers high expression levels in the vascular bundle and the fixation zone of the mature nodule, whereas it is hardly active in the prefixation zone, the zone where infection thread growth occurs. This behaviour makes the promoter suitable for studies on late nodulins, but not suitable to overexpress early nodulins involved in the infection process.

The CaMV 35S promoter is comprised of domains that confer different developmental and tissue specific expression patterns (Benfey et al., 1989). When domain B (-343 to -90, enhancer) of the promoter was used in tobacco, strong expression was observed in the root vascular bundle. Domain A (-90 to -43) conferred expression in the root, with highest expression levels in root tip, root cap, epidermis and root hairs. Combination of the two domains though, showed expression throughout the whole root (Benfey et al., 1989). Our experiments with the combination of domains B and A confirmed the data obtained by Benfey et al. (1989). Comparison of the expression patterns using various combinations of *cis*-elements of the enhancer (domain B, -343 to -90) suggests synergistic interactions among the *cis*- elements that play a role in defining tissue-specific expression (Benfey et al., 1990a,b). Our studies revealed that the combination of two copies of the enhancer (position-343 to -90) CaMV 35S promoter changes indeed the expression pattern compared to the pattern obtained when only one copy of the enhancer is used. It actually behaves in a rather 'nodule specific' manner, showing low GUS activity in the vascular bundle of the roots and relatively very high GUS activity in nodules. The highest level of GUS activity was often seen in nodule primordia and in the prefixation zone of the mature nodules, both areas where infection takes place. These observations show that the duplicated 35S promoter is appropriate for studies on the epitope-tagged early nodulins ENOD5 and ENOD12.

The promoters of *PsENOD5* and *PsENOD12B* would be another choice, since the fusion proteins would then not be expressed ectopically. Since the *ENOD5* promoter is not available, we could not use it. Promoter analysis of the *ENOD12B* promoter revealed that it is active at a rather low level (Vijn et al., 1995b), therefore it was not attractive.

One of the major problems that our transformation system creates, is that only a minor part of the nodules express the transgene at a detectable level. Only 30%-70% of the hairy roots show GUS activity and furthermore only a subset of the nodules on such roots express the transgene at a detectable level. That emphasizes the need for a preselection of transformed nodules, before immunolocalization studies are performed. For that purpose the luciferase genes (Aflalo, 1989; Koncz et al., 1990) might be good candidates as reporter genes, because they can be employed in very sensitive non-destructive *in vivo* assays. In preliminary experiments we have shown that the firefly luciferase gene can indeed be used as an easy and fast screening marker enabling preselection of transformed nodules.

A number of experiments were performed with plants lacking the preselection marker, focusing mainly on studies on the ENOD12B-tag fusion protein, demonstrating that ENOD12B is a cell wall protein as it was predicted by the sequence characteristics. The immunolocalization studies, though performed with both polyclonal and monoclonal antibodies, gave no answer on the site of localization of either ENOD12B-tagged or ENOD5-tagged proteins. The major problem encountered with the use of the polyclonal antibodies is the high background on both transfected protoplasts and cryosections of nodules (data not shown). It seems that there are epitopes *in planta* that are recognized by the particular antiserum. The use of the monoclonal antibodies resulted in no background, neither the epitope-tagged proteins were detected so far. These data indicate that HA tag is not usable for our purposes.

The number of tags available is increasing, therefore a search for a combination of a tag and an antiserum that does not cause such backround problems *in planta* would be advisable. Alternatively, proteins like GFP (green fluorescence protein), isolated from jelly fish, that emits green light after excitation, can be tried as tag. In the latter case though, one should keep in mind that such tags might disturb proper sorting of the tagged proteins due to their relatively big size (around 250 amino acids).

MATERIAL AND METHODS

Plant growth conditions

V. hirsuta seeds were obtained from John Chambers Ltd., London. Seeds were sterilized and germinated according to Vijn et al. (1995b). Germinated seedlings were transfered to Petri dishes containing 2% B&D-agar medium (Broughton and Dilworth, 1971) and were grown at 22°C with a day/night cycle of 16/8 h.

Construction of nodulin-tag fusions and duplicated 35S promoter-*gusA*-int

Plasmid pHATO, kindly provided by Dr. W. Filipowicz, was used for the construction of the epitope fusion protein vectors (Figure 6).

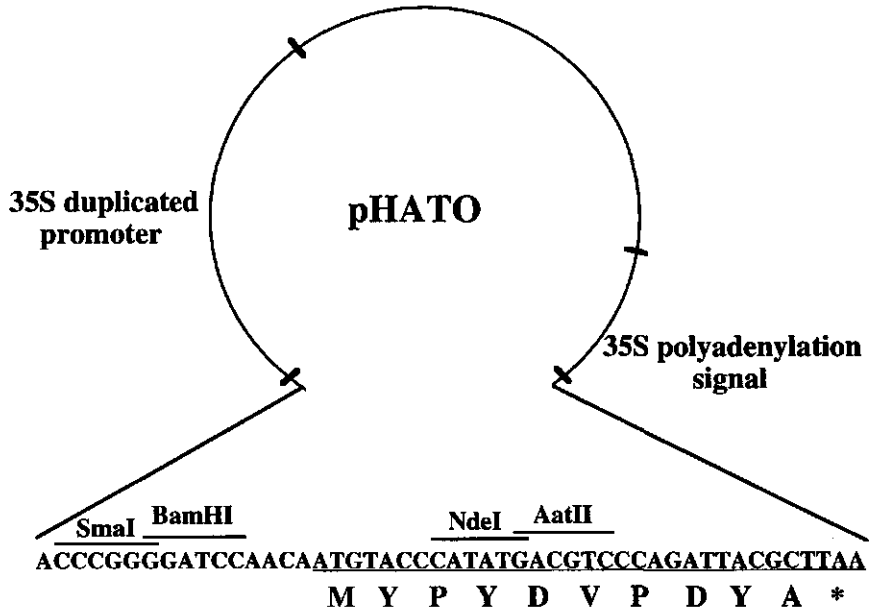

Figure 6. Schematic representation of pHATO.

This vector contains a duplicated CaMV 35S promoter and a CaMV polyadenylation signal, separated by the sequence encoding the influeza hemagglutinin nonapeptide, YPYDVPDYA. To obtain the C-terminus fusions the plasmid was cut with *BamHI* and *NdeI*. The cDNAs of PsENOD12B and PsENOD5 were amplified by PCR. The primers used in the PCR were:

PsENOD12B	forward:	5'-GGGGATCCACAATGGCTTCCCTTTTC-3'
	reverse:	5'-GGGACGTCATATGGGTACATGATATGGATGTTATG-3'
PsENOD5	forward:	5'-GCGGATCCTCAATATGGCTTCTTCTTCT-3
	reverse:	5'-CGTCATATGGGTATAGCCAAATTAAGAA-3'

The forward primers contain the *BamHI* site and the first 12 nucleotides of the coding region (from the starting methionine and downstream). The reverse primers cover the last 15 nucleotides of the coding region of both cDNAs and part of the beginning of the tag, including the *NdeI* site. The PCR products where digested with *BamHI* and *NdeI* and cloned into pHATO. The cassete (promoter+epitope tagged protein+polyadenylation signal) was cloned in pBIN19 (Bevan, 1984), a plant expression vector.

To obtain the PsENOD5 fusion protein carrying the tag in the middle (the tag was introduced between the 36th and the 37th amino acids of the protein), a based-PCR mutagenesis was followed. The primers used for the PCR reaction were:

PsENOD5	Forward:	5'-GAGAATTCATACCCATATGACGTCCCAGATTACGC TTGGAAGGTTAATTT-3'
	reverse:	5'-GCCTGCAGGCTATAGCCAAATTAAGAACAT-3'

The forward primer contains 9 nucleotides of the coding region of ENOD5 corresponding to the 34th, 35th and 36th amino acids of the encoded protein, followed by the sequence encoding the tag and ending with 15 nucleotides corresponding to 37th-41th amino acids of ENOD5. Between the 34th and 35th amino acids, there is an internal *EcoRI* site, which was used for cloning. The reverse primer contains the last 18 nucleotides of the coding region and an appropriate site for cloning (*PstI*). The PCR fragments were digested with *EcoRI* and *PstI*, and cloned in frame behind the cDNA of ENOD5 digested also with the same enzymes. Afterwards the cDNA of ENOD5 with the inserted tag was cloned in pBIN19, between the duplicated CaMV 35S promoter and the 35S polyadenylation signal.
The *β-glucuronidase*-intron (Vancanneyt et al., 1990) including the nopaline synthase terminator (NOS-ter) was cloned in pBIN19 behind the duplicated 35S promoter.
All DNA-constructs were introduced into *Agrobacterium rhizogenes* (ARqua1, Quandt et al., 1993) via electroporation (Mattanovich et al., 1989).

Plant transformation

Seedlings grown for 2 days were wounded with a 26 G needle containing *A. rhizogenes* (ARqua1) harbouring the chimaeric gene construct. The wounded plants were grown at 22°C, in the dark for the first 24h, followed by a day/night cycle of 16/8 h. After 10 days the main root was cut off, and the plants containing the transgenic hairy roots were transferred to another B&D-agar plate and inoculated with *R. leguminosarum* bv. *viciae* strainVH5e (Quandt et al., 1993).

Histochemical detection of GUS activity

GUS activity was detected according to Jefferson et al. (1987). The reaction was stopped with 70% ethanol. The stained nodules were further dehydrated in 100% ethanol and embedded in Technovit 8100, according to the manufactor's protocol (Kulzer GmbH, Germany). 4-6 µm thick sections of the embedded material were obtained with a microtome (Reichert-Jung

Biocut, 2035 Germany). Photographs were taken on a Nikon microscope equipped with a Nikon camera.

Protein isolation and western blotting

Nodules were harvested 10-20 days after inoculation of the trangenic plants with *R. leguminosarum* bv. VH5e and placed directly into liquid nitrogen. Nodules were ground in a mortar and pestle in a low salt buffer (3 mM EDTA, 10 mM DTT, 0.5 mM PMSF, and 10 µg/ml leupeptin, in 10 mM Tris pH 8.0). The extract was spun at 2500 X g for 10 min and the supernatant was retained as the low salt extract. The pellet was washed three times by resuspension in low salt buffer and centrifugation. Afterwards, the pellet was resuspended in high salt buffer (low salt buffer supplemented with 0.2 M $CaCl_2$), and allowed to extract for 2 hours. The extract was centrifuged for 10 min at 25.000 X g. The supernatant represents the high salt cell wall extract. All extraction steps were carried out at 4°C. Proteins were precipitated overnight at -20°C in 80% ethanol. Precipitated proteins were recovered by centrifugation and resuspended in sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 50 mM Tris pH 6.8, 0.05% bromophenol blue).
Protein concentrations were determined by a modified Bradford assay (BioRad microassay).
Proteins were further analysed by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Schleicher&Schuell).

Immunological techniques

Immunological detection of the proteins was carried out according to standard methods. Alkaline phosphatase-conjugated anti-rabbit goat IgG with Nitro Blue Tetrazolium (NBT; Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer Mannheim) were used for detection.
Concanavalin A, specific for glucose and mannose, and *Ulex europaeus* agglutinin I, a fucose-specific lectin were used for detection of glycostructures in proteins immobilized on nitrocellulose membrane, according to De Jong et al. (1995).

Immunocytochemistry

Nodules were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for 3 hours at RT. Afterwards they were washed twice in PBS (0.13 M NaCl, 7 mM Na_2HPO_4 (anhydrous), 3 mM NaH_2PO_4, 0.27 mM KCl) and transferred in a solution of 5% sucrose (in PBS) for 15

min. An incubation in 10% sucrose (in PBS) was followed and finally they were embedded in cryoblock frozen tissue medium (Boom/Wilten, Woltil, The Netherlands), and frozen in liquid nitrogen. 10 µm thick sections were obtained at -20°C with a cryotome (Bright, Instrument Company Ltd., Huntington England).

For the light microscopic localization studies, immunolabelling was performed as described by VandenBosch (1991). Briefly, sections were first incubated in blocking buffer (2% BSA, 2% normal goat serum, 0.2% Tween 20), followed by an incubation with the primary antibody (anti-HA, polyclonal or monoclonal), for 1 hour at RT or overnight at 4°C. The secondary antibody used for the cryosections was goat anti-rabbit (or mouse) alkaline phosphatase conjugate. Signals were visualized by incubation with Nitro Blue Tetrazolium (NBT; Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer Mannheim).

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CHAPTER 3

The root epidermis-specific pea gene RH2 is homologous to a pathogenesis-related gene

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The root epidermis-specific pea gene RH2 is homologous to a pathogenesis-related gene

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Abstract

Two-dimensional gel electrophoresis of pea root and root hair proteins revealed the existence of at least 10 proteins present at elevated levels in root hairs. One of these, named RH2, was isolated and a partial amino acid sequence was determined from two tryptic peptides. Using this sequence information oligonucleotides were designed to isolate by PCR an RH2 cDNA clone. *In situ* hybridization studies with this cDNA clone showed that *rh2* is not only expressed in root hairs, but also in root epidermal cells lacking these tubular outgrowths. During post-embryonic development the gene is switched on after the transition of protoderm into epidermis and since *rh2* is already expressed in a globular pea embryo in the protoderm at the side attached to the suspensor, we conclude that the expression of *rh2* is developmentally regulated. At the amino acid level RH2 is 95% homologous to the pea PR protein I49a. These gene encoding I49a is induced in pea pods upon inoculation with the pathogen *Fusarium solani* [12]. We postulate that *rh2* contributes to a constitutive defence barrier in the root epidermis. A similar role has been proposed for chalcone synthase (CHS) and chitinase, pathogenesis-related protein that are also constitutively present in certain epidermal tissues.

Introduction

The root epidermis is the outermost tissue of the root and in most plants it is a single cell layer [38]. It has a function in the absorbance of water and minerals from the soil and it is composed of two cell types: epidermal cells that have formed root hairs and epidermal cells which lack these outgrowths. The cells of the epidermis that form root hairs are called trichoblasts and the ones that do not, atrichoblasts [9].

During post-embryonic development the root epidermis is formed from the root meristem. In general root meristems of seed plants contain initial cells [5] at the apex of the meristem. From these initial cells the root cap and the three primary meristems – protoderm, ground meristem and procambium – are formed. The protoderm is the primary meristem that develops into epidermis. During the transition of protoderm into epidermis the protoderm cells stop dividing, they elongate and a central vacuole is formed and then the trichoblasts form root hairs.

Since the root epidermis is a relatively simple

tissue composed of only 2 cell types forming a single cell layer, it is an attractive system to study the molecular basis of a plant developmental process. Therefore several groups have initiated genetic studies on *Arabidopsis* root epidermis development [8, 30, 31]. Furthermore, this tissue plays a major role in the interaction between plants and microbes living in the soil. A well studied example is the *Rhizobium*-legume symbiosis [11]. At early stages of this interaction, *Rhizobium* interferes with root hair development [11, 14]. Lipo-oligosaccharides (Nod factors) secreted by *Rhizobium* stimulate root hair development, induce deformation of root hairs and elicit the expression of some early nodulin genes [10, 18, 20, 33, 36]. To study the molecular basis of root epidermal differentiation as well as the interaction of *Rhizobium* with the root epidermis at a molecular level, we initiated a programme on the isolation of pea cDNA clones of mRNAs involved in different steps of pea root epidermis development.

In this paper we describe the identification and characterization of a cDNA clone encoding the root epidermis-specific protein RH2.

Materials and methods

Plant growth

Pea seeds (*Pisum sativum* (L.) cv. Rondo; Cebeco, Netherlands) were grown in gravel [2]. Inoculation of plants with *Rhizobium leguminosarum* bv. *viciae* strain PRE was done as described [2]. Root segments containing root hairs were harvested from 5-day-old pea seedlings and immediately frozen in liquid nitrogen. Harvested plant material was stored at −70 °C until use.

Root hair isolation

Root hairs were harvested by the procedure of Röhm and Werner [26], with modifications as described by Gloudemans *et al.* [14].

RNA isolation, protein isolation and 2D gel electrophoresis

Frozen root hairs were ground in liquid nitrogen and resuspended in a hot (90 °C) mixture of RNA extraction buffer (0.1 M Tris-HCl pH 9.0, 0.1 M LiCl, 10 mM EDTA, 1% SDS) and phenol (1:1) [16].

After vortexing and centrifugation (30 min, 6000 × **g**) the water phase was collected and RNA was isolated as described by Govers *et al.* [16]. The interphase and phenol phase were collected and 2 volumes of 96% ethanol were added to precipitate root hair proteins. After extensive washing with 96% ethanol, proteins were dissolved and separated by 2D gel electrophoresis according to de Vries *et al.* [7]. Proteins were visualized by silver staining [24].

Total RNA was passed through an oligo-dT-cellulose column (Promega) to obtain poly(A)$^+$ RNA [28].

Purification of RH2 and determination of partial amino acid sequence

A 50 μg portion of protein isolated from root hairs was separated by 2D gel electrophoresis. Proteins were electroblotted onto polyvinylidene difluoride membranes (PVDF; Immobilon) as described [34], and proteins were stained by amido black. The spot representing RH2 was cut from the blot. Subsequently, this material was incubated in 0.2% polyvinylpyrrolidone in 100% methanol for 30 min and then washed by adding an equal volume of water. The solution was discanted and the membrane was washed three times in 0.1 M Tris-HCl pH 8.5.

The protein on the membrane was then digested by adding 1 μg (10 Units) of trypsin (Sigma) in 150 μl 0.1 M Tris-HCl pH 8.5 and incubated for 4 h at 37 °C. The liquid was collected and the membrane was washed with 100 μl aliquots of 80% HCOOH and water (4 times) which were added to the original incubation mixture. The final volume was separated by HPLC and the various peptides formed by trypsin digestion were collected [34].

The peptides were then applied to a 470A gas-phase sequencer equipped with a 120A on-line phenylthiohydantion (>phNCS) amino acid analyser (Applied Biosystems).

Oligonucleotides

Based on the amino acid sequence of two peptides obtained after hydrolysis of purified RH2, two oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer.

I: 5′-TTIAAITT(T/C)GA(G/A)GA(G/A)GA-(G/A)GCIAC-3′
corresponding to the peptide FNFEEEA

II: 5′-GTIACIGA(C/T)GCIGA(C/T)ATI(T/C)-T-3′
corresponding to the peptide VTDADI

III: 5′-CAGTAACCTTCAAGAG-3′
reverse primer corresponding to the region from base 428 to 443 in pRH2-1 (Fig. 4).

IV: 5′-AGTTCTTTCTCACAG-3′
corresponding to the region from base 25 to 39 derived from the nucleotide sequence of cDNA clone pI49a, which is missing in *drrg49c/rh2* [12] (Fig. 4).

Amplification of RH2 cDNA

Two microgram of root hair poly(A)$^+$ RNA was heated for 3 min at 80 °C in annealing buffer (250 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM EDTA) + 250 ng oligo-dT in a final volume of 9 μl. After 15 min incubation at 37 °C, 15 μl RT buffer (24 mM Tris-HCl pH 8.3, 16 mM $MgCl_2$, 8 mM DTT, 0.4 mM dNTPs) was added, 20 units of AMV reverse transcriptase (Life Science) and incubation was prolonged at 42 °C for 25 min. Subsequently 1 μl 10 mM dNTPs and 75 μl *Taq* polymerase buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 0.01% gelatine, 2 units *Taq* polymerase (Cetus)) and 250 ng of oligo I or oligo II was added.

The polymerase chain reaction (PCR) was performed on a PREMTM (LEP Scientific) using the following protocol: 5 min 92 °C, 2 min 40 °C, 2 min 72 °C followed by 20 cycles of 1 min 92 °C, 1 min 50 °C, 2 min 72 °C.

The PCR reaction mix was subsequently extracted with phenol/chloroform (1:1) and the DNA precipitated by addition of 1/10 volume of 3 M sodium acetate and 2 volumes of 96% ethanol. After washing and drying the DNA was dissolved into 50 μl Klenow buffer (50 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT) containing 0.5 mM dNTPs and 2 units of Klenow DNA polymerase and incubated for 15 min at 37 °C. After phenol-chloroform extraction and alcohol precipitation the DNA was ligated into the *Sma* I site of pBlueScript II KS/+ (pBs) and the ligation mixture was used to transform competent *Escherichia coli* JM109.

Oligos I and II were labelled with [γ-^{32}P]ATP and used to identify transformants containing RH2 sequences.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

An RT-PCR was started by mixing 2 μg total RNA and 250 ng oligo III in annealing buffer and reverse transcription was performed as described above. Subsequently a polymerase chain reaction was performed in a LEP-PREM PCR machine using 1 unit of *Taq* polymerase and oligo IV as sense primer. The protocol was 30 cycles of denaturation at 93 °C for 2 min, annealing at 42 °C for 2 min and elongation at 72 °C for 2 min. The synthesized DNA was separated on 1.5% agarose gels and transferred to GeneScreen *Plus* membranes. The membrane was hybridized with [^{32}P]-labelled insert of pRH2-1 [28].

Nucleotide sequencing

The nucleotide sequence of the insert of RH2-1 was determined by double-stranded sequencing using the dideoxy termination sequence method [29].

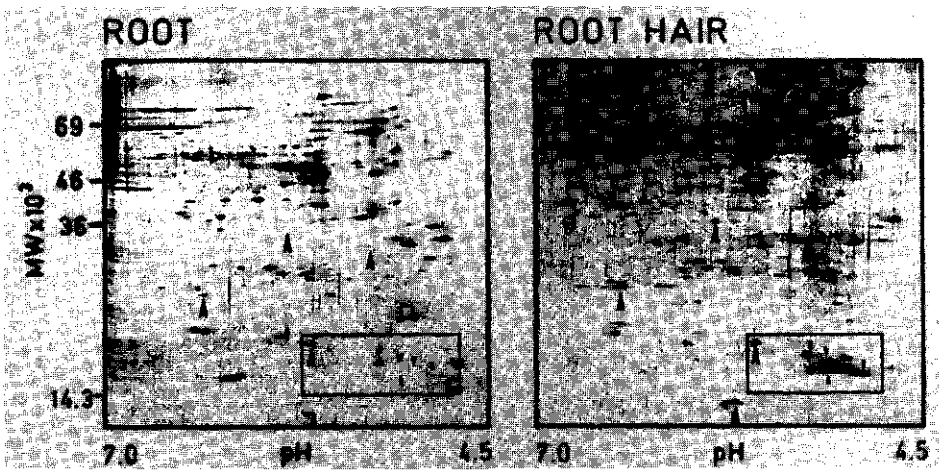


Fig. 1. 2D gel of proteins from roots and root hairs of 5-day-old pea seedlings. Proteins present at elevated levels in protein preparations of root hairs are indicated by big arrowheads. Within the rectangle in 'Root hair' the most abundant ones are indicated by small arrow-heads and RH2 is indicated by an arrow.

Northern blots

Total RNA was denatured in DMSO/glyoxal, separated on 1% agarose gels and blotted onto GeneScreen in 0.025 M NaH_2PO_4 pH 7.0 [28]. The blots were hybridized in 50% formamide, 1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 7, 10 × Denhard's solution at 42 °C. The pRH2-1 insert was labelled by random priming using [α-^{32}P]dATP (3000 Ci/mmol; Amersham) as radioactive label [28].

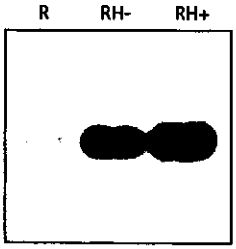


Fig. 2. Autoradiograph of a northern blot containing 20 μg of total RNA isolated from roots (R), root hairs of uninoculated plants (RH −), root hairs from *R. leguminosarum* bv. *viciae*-inoculated plants (RH +), respectively, and hybridized to pRH2-1.

In situ *hybridization*

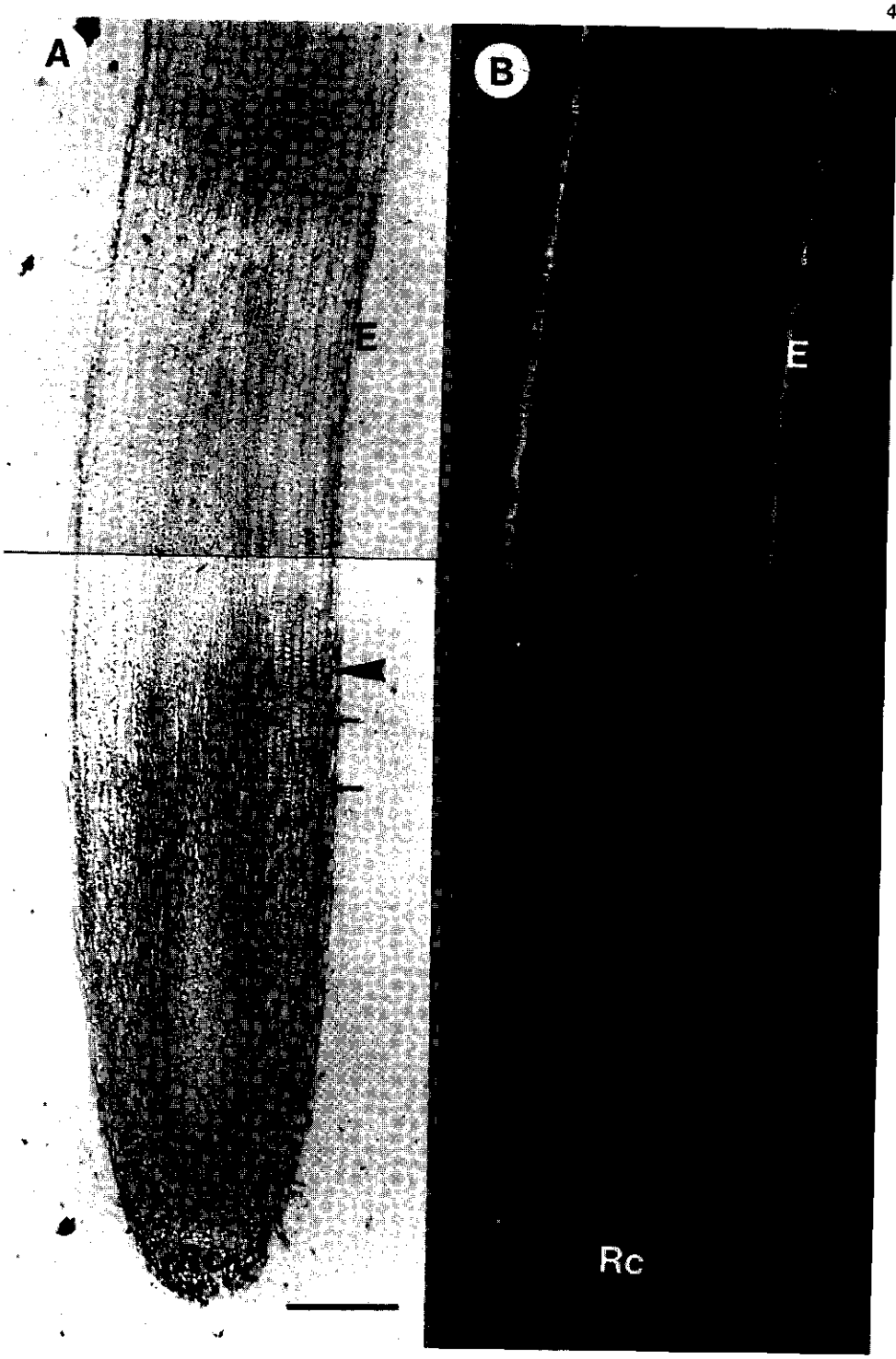
In situ hybridization was performed by a method derived from the procedure published by Cox and Goldberg [6] as described by van de Wiel *et al.* [35].

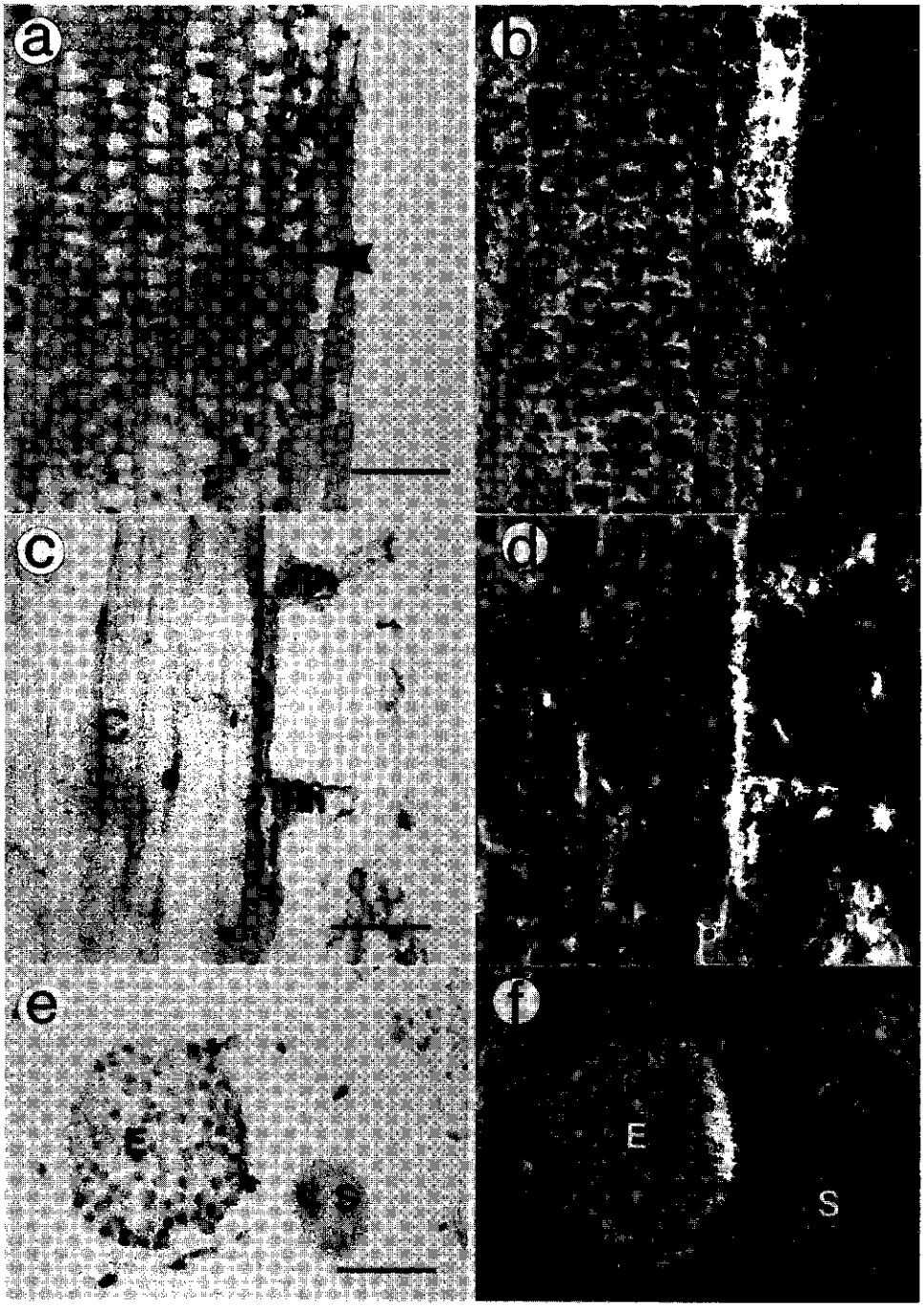
Results

Analyses of root hair proteins

As a first step towards the isolation of root epidermis-specific cDNA clones, we compared proteins of root hairs and roots of 5-day-old pea seedlings. The protein preparations were separated by 2D gel electrophoresis (Fig. 1), which showed that the majority of the proteins occurred in similar quantities in both preparations. Only 10 polypeptides were present in a significantly higher

Fig. 3. In situ localization of RH2 mRNA in the root tip. A. Bright field micrograph showing a longitudinal section of a pea root tip. (E, epidermis; Rc, root cap). B. Dark-field micrograph of A showing RH2 mRNA in the root epidermis (E) The arrow head shows the start of *rh2* expression in cells still covered by the root cap. Bar = 50 μm.


A
E
B
E
Rc


a
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amount in protein preparations from root hairs than in those from roots (marked by arrowheads in Fig. 1). This indicates that these proteins might be specifically present in root hairs. The most abundantly occurring 'root hair-specific' proteins form a group of five polypeptides with an apparent molecular mass of about 14 kDa and an isoelectic point of ca. 4.8 (enclosed within the rectangle in Fig. 1). Among these five polypeptides RH2 is the most abundant one (marked by an arrow).

RH2 cDNA clone

A 50 μg portion of root hair proteins was separated by 2D gel electrophoresis and subsequently blotted onto PVDF membranes. After staining the blot with amido black, the RH2 spot was cut from the membrane. The RH2 protein on the membrane was digested with trypsin and two peptides were isolated. From these peptides a partial amino acid sequence was determined, revealing the sequences VFNFEEEATSIVAP-ATLH and VTDADILTP, respectively. Based upon these amino acid sequences the oligonucleotides I and II (see Materials and methods) were designed.

To obtain an RH2 cDNA clone, cDNA was prepared from root hair poly(A)$^+$ RNA. Oligonucleotide I or II and oligo-dT (20 b) as a second primer were used to amplify the RH2 cDNA (see Materials and methods). Using oligonucleotide I and oligo-dT, a DNA molecule of 600 bp was amplified, whereas application of oligonucleotide II and oligo-dT resulted in the production of a 550 bp long DNA molecule. A Southern blot of the DNA obtained from these PCRs was hybridized with [^{32}P]-labelled oligonucleotide I or II. Oligonucleotide II hybridized to both the 600 bp and 550 bp DNA molecule, whereas oligonucleotide I only hybridized to the 600 bp long DNA molecule (results not shown). These observations indicated that the region of RH2 cDNA with the oligonucleotide II sequence is located downstream of the region with the oligonucleotide I sequence.

The 600 bp long RH2 cDNA was isolated from an agarose gel and cloned into the *Sma* I site of pBs. The cDNA clone that was isolated and studied in detail was designated as pRH2-1.

In situ *expression of* rh2

A northern blot containing RNA from root and root hairs was hybridized with [^{32}P]-labelled insert of pRH2-1. Figure 2 shows that the clone hybridized to an RNA with a length of 650 bases and that the RH2 mRNA is present at the highest level in root hairs (Fig. 2, lanes RH – and R). The amounts of RH2 mRNA in root hairs of plants inoculated with *Rhizobium leguminosarum* bv. *viciae* and uninoculated pea plants are identical (Fig. 2, lanes RH –, RH +).

To determine whether the expression of *rh2* is restricted to root hairs, or whether all root epidermal cells express this gene, we hybridized longitudinal sections of pea roots to [^{35}S]-labelled antisense RH2 RNA (see sequence of pRH2-1). Figure 3A shows a median longitudinal section of a segment of the root of a 5-day-old pea seedling including the root tip. The expression of *rh2* is restricted to the epidermis (Fig. 3B) and all cells of the epidermis contain RH2 mRNA. Figure 4C shows a magnification of a region of the root that contains root hairs (Rh) and *Rh2* is expressed in these hairs (Fig. 4D). Therefore, *rh2* is active in

Fig. 4. In situ localization of RH2 mRNA in roots and globular embryos of pea (panels A–F). A. Magnification of the region of a pea root tip containing the first cell showing *rh2* expression (just as in Fig. 3 indicated by an arrowhead; C, cortex). B. Dark-field micrograph of A showing the start of RH2 mRNA accumulation (arrowhead). C. Bright-field micrograph showing part of longitudinal section of a pea root including root hairs (Rh) hybridized with [^{35}S]-labelled RH2 antisense RNA. D. Dark-field micrograph of C showing hybridization in root hairs. E. Magnification of a pea ovule as shown in Fig. 5 showing the globular embryo (E) and suspensor (S). F. Dark-field micrograph of E showing hybridization at the suspensor attachment side. Bar = 10 μm.

both cell types that form the root epidermis. So *rh2* is not a root hair-specific but an epidermis-specific gene.

Since roots have an indeterminate growth pattern, the root tissues are of graded age implying that consecutive stages of development can be observed in a single longitudinal section. To study at which stage of development *rh2* is expressed, the expression of *rh2* in the vicinety of the root tip was carefully examined. Figure 3A and B show that *rh2* is first expressed when the epidermis is still covered with root cap cells. A magnification of this area of the section (Fig. 4A, B) shows that at this stage the *rh2*-expressing cells are still relatively small and root hairs have not yet formed.

These observations suggest that *rh2* expression is developmentally regulated. To find further support for this hypothesis, we hybridized zygotic pea embryos with antisense RH2 RNA. (Fig. 5A, B). Figure 5A shows a longitudinal section of a pea ovule harbouring an embryo (E) which is at the late globular stage. At the side where the future radicle will form, the cells of the suspensor (S) can be seen. RH2 mRNA is present in the cells of the embryonic protoderm, but only at the side of the suspensor as shown in a magnification (Fig. 4E, F).

Based on the expression pattern of *rh2* during embryonic as well as post-embryonic development of the root, we conclude that *rh2* expression is developmentally regulated.

Nucleotide sequence of pRH2-1

The nucleotide sequence of the insert of pRH2-1 was determined (Fig. 6). It contains at the 3′ end a poly(A) track preceded by an AATAA sequence

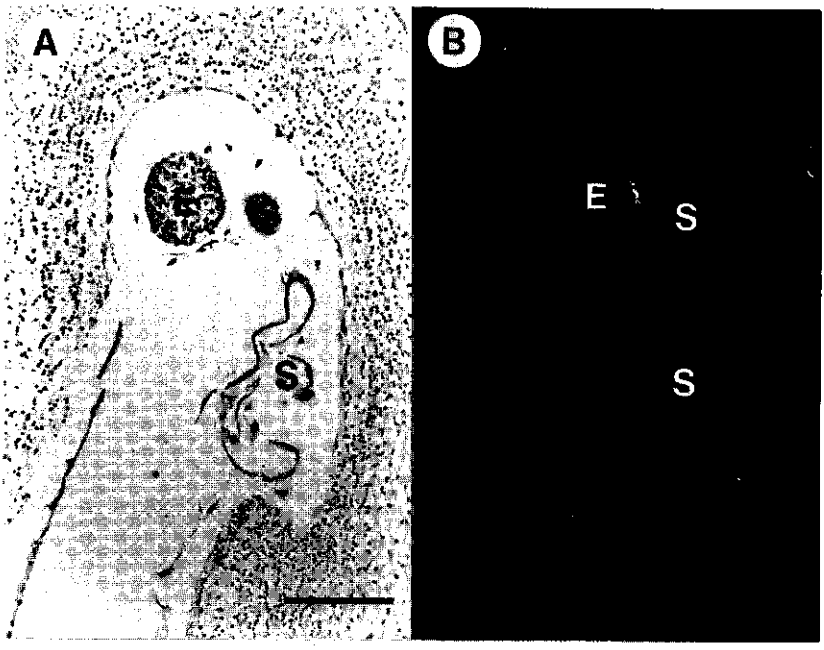

Fig. 5. In situ localization of RH2 mRNA in pea ovules. A. Bright-field micrograph of an ovule showing a globular pea embryo (E) and suspensor (S). Analysis of adjacent sections shows that the embryo is attached to the apical suspensor cell (data not shown). B. Dark-field micrograph of A showing the presence of RH2 mRNA in the protoderm at the side where the embryo is attached to the suspensor.

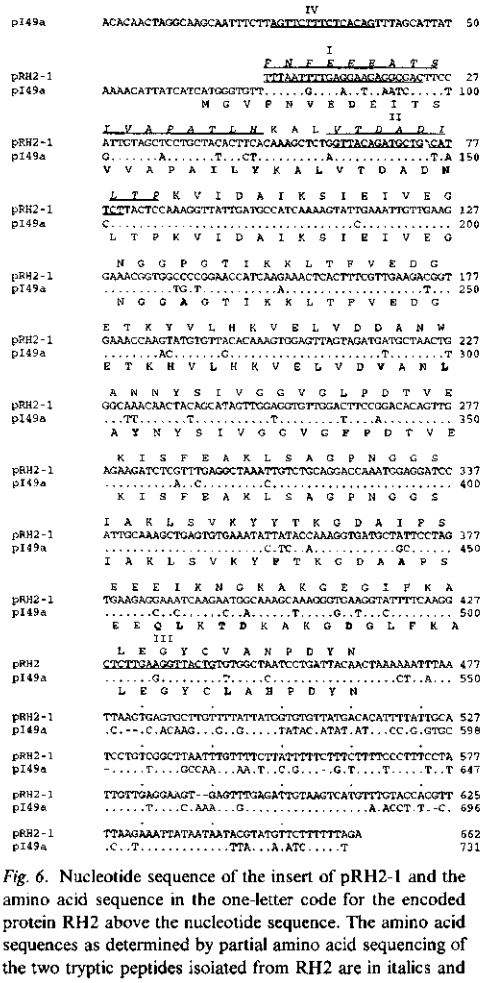

Fig. 6. Nucleotide sequence of the insert of pRH2-1 and the amino acid sequence in the one-letter code for the encoded protein RH2 above the nucleotide sequence. The amino acid sequences as determined by partial amino acid sequencing of the two tryptic peptides isolated from RH2 are in italics and underlined. For comparison the nucleotide sequence of cDNA clone pI49a and the amino acid sequence of the encoded protein are shown as well. Identical bases between pRH2-1 and pI49a are indicated by dots. Amino acids in the I49a-encoded protein that are different are displayed in bold type. In the nucleotide sequence of pRH2-1 the oligonucleotides that have been used in RT-PCR are underlined and numbered.

that probably is the polyadenylation signal. The largest ORF present in the nucleotide sequence starts at the beginning of the insert and ends of position 466. The polypeptide derived from this ORF contains the amino acid sequences obtained from the two peptides of the tryptic digest of the RH2 protein. This strongly suggests that the amino acid sequence derived from the ORF is the partial amino acid sequence of RH2. Since the amino acid sequence does not start with a methionine, pRH2-1 is not a full-size clone. However, since the molecular mass of RH2 and the amino acid sequence derived from pRH2-1 both are about 14 kDa we assume that only a small part of the coding region of the RH2 mRNA is lacking in pRH2-1. Comparison of the sequence of RH2 with proteins present in GenBank revealed that RH2 is 100% homologous to a pea protein encoded by *drrg49c* [4]. This gene was isolated from a genomic library using cDNA clone pI49a as a probe. This cDNA clone represents a gene that is induced in pea pods upon inoculation with the pathogen *Fusarium solani* or treatment with chitosan [12]. The proteins encoded by *rh2*/*drrg49c* and *I49a* exhibit 95% homology [4]. Since pI49a and *drrg49c* were isolated from libraries of the same pea cultivar these clones represent different members of a gene family. Together with the gene represented by cDNA clone pI76 [12] this gene family consists of at least three different members [4, 12]. If we assume that the N-terminal end of RH2 is fully homologous to that of DRRG49C only three triplets of the coding region, namely the start codon ATG and GGT and GTT, are missing in the sequence of pRH2-1. A hydropathy plot (data not shown) shows that RH2 has no typical N-terminal signal peptide and it is a hydrophilic protein, indicating that RH2 is a cytosolic protein.

Expression of the RH2 gene family

Rh2 is a member of a gene family of which the members are highly homologous. Therefore, the expression of other members might contribute to hybridization signals in northern blot analyses (Fig. 2) and *in situ* hybridization experiments (Figs. 3, 4, 5). One other member belonging to the gene family and of which the nucleotide sequence

has been elucidated is *I49a*. Hence, we studied whether this gene is expressed in the root epidermis. We used oligonucleotides specific for *I49a* (oligo IV and oligo III in Fig. 6 and Material and methods) in a RT-PCR experiment on RNA of roots and root hairs from uninoculated plants or from plants inoculated with *R. leguminosarum* bv. *viciae*. Figure 7A shows that similar amounts of *I49a* RNA are present in the three RNA preparations. To prove that this result is representative of mRNA levels, a RT-PCR experiment on the same RNAs was done using *rh2*-specific oligos I and III (Fig. 7B). The expression pattern for *rh2* obtained in the RT-PCR is identical as the result of the northern blot (Fig. 2). Both the northern blot analysis and the *in situ* hybridization experiments show that *rh2* homologous RNA is only present in the epidermis. This implies that *I49a* expression was not detected in non-epidermal root tissues by *in situ* hybridization, and can only be a minor part of the *rh2*-like transcripts present in roots.

Furthermore, the RT-PCR experiment shows that *R. leguminosarum* bv. *viciae* does not interfere with the expression of *I49a* in root hairs.

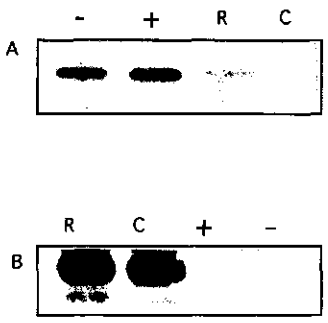

Fig. 7. A. RT-PCR experiment using *I49a*-specific oligonucleotides. DNA obtained in the RT-PCR was separated on agarose gels and blotted onto GeneScreen *Plus* filters. Filters were hybridized to pRH2-1. RNA from root hairs from uninoculated plants (−), *R. leguminosarum* bv. *viciae* inoculated plants (+) or roots (R) has been used. Lane C is RT-PCR on root RNA to which no reverse transcriptase has been added. B. Same as in A, using *rh2*-specific oligonucleotides.

Discussion

In this paper we describe the isolation and characterization of a pea cDNA clone pRH2-1. This cDNA clone was obtained by RT-PCR on root hair RNA using oligonucleotides based on partial amino acid sequence data of the most abundant protein present in root hairs as identified by 2D gel electrophoresis (Fig. 1). Using the latter approach several other proteins which are present at elevated levels in pea root hairs have been identified. Root hair-specific proteins have also been identified in clover, soybean and cowpea [13, 15, 19]. However, our results show that a root hair-specific protein identified in this way does not necessarily represent a gene whose expression is restricted to root hairs; *rh2* is expressed in the two cell types that form the root epidermis and also in the pea embryo.

During post-embryonic development *rh2* is induced in epidermal cells when elongation and vacuolization has started, but before root hairs have formed. Therefore, the induction of *rh2* more or less coincides with the transition of protoderm into epidermis. Thus *rh2* is induced at a specific stage of root epidermal development. However, since *rh2* is highly homologous to a PR gene, it can be questioned whether a 'stress' factor, like the physical contact with gravel, elicits *rh2* expression or whether *rh2* expression is developmentally controlled. *In situ* expression studies on pea embryos showed that *rh2* is expressed in the protoderm of a globular pea embryo, exclusively in the part that will form the radicle. This observation strongly supports the conclusion that *rh2* is regulated by a developmental cue.

During pea embryogenesis the protoderm is first formed at a late globular stage [22, 25]. We have not studied *rh2* expression in embryos at earlier stages of development and therefore, we do not know whether the start of *rh2* expression precedes or coincides with the formation of the protoderm. When the exact timing of *rh2* induction is known, *rh2* will be a good molecular marker for early embryogenesis and since only a few markers of early stages of embryo development are available [21], it is worthwile to study the

timing of induction in more detail. The different stages and genetics of zygotic embryogenesis are well studied in *Arabidopsis* and since *rh2* cross-hybridizes with *Arabidopsis* genomic DNA (data not shown), we plan to study the expression of *rh2* during *Arabidopsis* embryogenesis.

The exclusive expression of *rh2* in the protoderm of the part of the embryo that develops into the radicle, suggests that *rh2* expression is restricted to the roots. Preliminary RT-PCR experiments showed that *rh2* is not expressed in leaves or stems, but these studies have not been extensive enough to exclude that *rh2* is never expressed in aerial parts of pea plants.

Database searches revealed that pRH2-1 is 100% homologous to the pea gene *drrg49*c. Although this homology indicates that pRH2-1 represent the cDNA clone of *drrg49*c, it cannot be excluded that this homology might be the result of PCR-induced errors and thereby pRH2-1 might represent a cDNA clone of a *drrg49*c-related gene. pRH2-1 also is 95% homologous to the pea clone pI49a [4], representing a gene that is expressed in pea pods during pathogenic interactions. Furthermore, RH2 is 43% homologous to the major pollen allergen of white birch and 40% to proteins identified in potato [23] and bean [37], of which the genes are expressed during pathogenic interaction. Therefore, it is possible that in the root epidermis RH2 is also involved in a defence mechanism. The expression of *rh2* in all epidermal cells is consistent with such function. It is noteworthy that also other PR genes, *chitinase* [27] and *chs* [32, 39], are expressed in epidermal tissue. Therefore, it can be proposed that the formation of a constitutive defence mechanism is part of the root epidermal developmental program [17, 27, 32].

The observation that *rh2* expression pea plants is most likely restricted to the root epidermis makes the promoter of this gene a good candidate for genetic engeneering of this cell layer. Furthermore, the gene is induced at a stage preceding root hair development. Since the zone formed at this stage is susceptible to interaction with *Rhizobium* signals [1, 3], it will be especially suitable to study the role of certain plant genes in the *Rhizobium*-plant interaction, expressing these genes in sense or antisense orientation under the control of the *rh2* promoter.

Acknowledgement

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CHAPTER 4

rh4 expression is regulated by developmental and positional information

Panagiota Mylona, Arina Kippers, Ab van Kammen, and Ton Bisseling

INTRODUCTION

Growth and development of plants depends on the continuous formation and differentiation of cells in meristematic regions at the root and shoot apices. In the root apex the meristem contains initial cells that divide to give rise to the three primary meristems -protoderm, ground meristem and procambium-, and the root cap (Clowes, 1961). The root epidermis which is the outermost tissue of the root derives from the protoderm. During maturation of the epidermis each cell adopts one of the two altenative fates, it may develop into a cell capable of producing a root hair (trichoblast), or it may differentiate into a hairless cell (atrichoblast). Root hairs are tip-growing tubular outgrowths which help to anchor the roots in soil, are involved in the interaction with microorganisms, and assist in the uptake of water and nutrients (Clarkson, 1985).

Two apparently different mechanisms specify the fate of the epidermal cells. In one case, an asymmetric division of the epidermal cell gives rise to a small densely cytoplasmic cell, which subsequently forms a root hair, and a larger vacuolated cell which remains hairless (Sinnott and Bloch, 1936; Avers, 1963). As a result, a pattern of hairless epidermal cells interspersed with root hair containing cells throughout the epidermis, is obtained. This mechanism is utilized by a diverse group of plants, including pea.

A different pattern of epidermal cell differentiation has been identified in Brassicaceae, such as *Arabidopsis*, in which the fate of the epidermal cell is determined by its position relative to the neighbouring cortical cells (Dolan et al., 1993). This mechanism results in a distinctive pattern of hair-forming and hairless cells in the mature root, consisting of axial files of epidermal cells which are composed entirely of hairless cells or entirely of root-hair containing cells. A number of mutants impaired in the specification of the epidermal cell fate have been recently identified in *Arabidopsis*, producing roots that contain an increased or reduced number of root hairs (Galway et al., 1994).

The ectopic root hair formation observed in the mutant *ctr1* (*constitutive triple response*) of *Arabidopsis*, that shows a constitutive ethylene response, implicates a role of ethylene in root hair formation. Moreover, root hair formation is reduced in ethylene-insensitive mutants and by treatment with inhibitors of ethylene biosynthesis or action (Dolan et al., 1994). In addition, the identification of hairless mutants that can be complemented for hair growth by the application of exogenous ethylene confirms the essential role of ethylene in root hair ontogenesis (Dolan et al., 1994).

Prior to root hair emergence, trichoblasts undergo a number of changes. Their nuclei originally decrease in volume and take a place in the parietal cytoplasm. There, they regain a swollen appearance. Rearrangements in the cytoskeleton are observed followed by movement of the nuclei opposite to the site of the root hair emergence (Bakhuizen, 1988). A localized "bulge" is produced, due to a local loosening of the cell wall and reaction to internal pressure (Bakhuizen, 1988). The next step is the elongation of the root hair. In most root hairs the nucleus follows the expending root hair tip at a distance. After the root hair has ceased growing the nucleus

takes a random position in the parietal cytoplasm. Mutants in *Arabidopsis* impaired in different steps of root hair emergence have been identified. *rhd1* appears to regulate the degree of the epidermal cell wall loosening at the intial step of emergence, while other mutated genes (*rhd2,3,4*) were shown to play a role in later steps of elongation (Schiefelbein and Sommerville, 1990). In addition, the existence of an *Arabidopsis* root hair mutant impaired also in pollen tube tip-growth (*tip1*), indicates the presence of a common mechanism in all tip-growing cells (Schiefelbein et al., 1993). The way cell polarity, -required for tip-growth-, is established and maintained in root hairs or pollen tubes is not clear. Recently, it has been shown that the growth of these cell types, is associated with, and dependent on, a continuous influx of Ca^{2+} at the apex and an internal Ca^{2+} gradient (Clarkson et al., 1988; Schiefelbein et al., 1992). Furthermore, normal root hair and pollen tube growth require a functional actin cytoskeleton (Heath, 1990).
In this chapter data on the characterization of a root hair cDNA clone, namely pRH4 will be given.

RESULTS

Isolation of root hair/epidermis specific cDNA clones

To obtain cDNA clones corresponding to genes specifically expressed in root hairs, a root hair cDNA library from *Pisum sativum,* was differentially screened with root and root hair cDNA, respectively. One of the isolated clones, namely pRH4, was selected for further analysis.
The root hair specific/enhanced nature of *rh4* was confirmed by northern blot analysis. Roots were collected from 4-day old plants and the root segments containing root hairs were frozen in liquid N_2. Part of this root material was immediately used for RNA isolation, but the majority of the root segments were used to isolate root hairs by stirring the roots in the liquid N_2. RNA was isolated from the root hair samples. Northern blots were made with root (R), root hair (RH), leaf (L), and stem (S) RNA samples. Identical northern blots were probed with the insert of pRH4 and a cDNA corresponding to *ubiquitin* as control for the amount of RNA loaded in each slot (Figure 1).
rh4 is expressed highly in the root hairs (RH, Figure 1). A low amount of transcript was detected in the roots (R, Figure 1). A similar level of this transcript is present in the RNA samples isolated from stems (S) and leaves (L). Therefore, *rh4* is expressed at elevated levels in the root hairs.

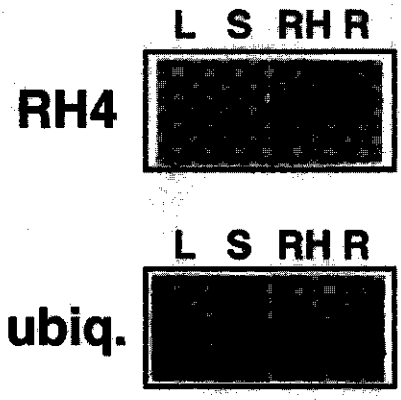

Figure 1. Autoradiograph of northern blots containing 20µg of total RNA isolated from roots (R), root hairs (RH), leaves (L), and stems (S), respectively, hybridized to pRH4 (upper panel). The amount of RNA on the filter was determined by a hybridization with a soybean ubiquitin probe (Kouchi and Hata, 1993), as it is shown in the bottom panel.

RH4 mRNA has a length of approximately 1.4 kb, whereas pRH4 bears an insert of 1197 bp. Thus pRH4 is not a full size clone.

Sequence characteristics

The nucleotide sequence of pRH4 was determined. The ORF lacks the starting methionine and encodes 322 amino acids of RH4. At the 969th nucleotide of the clone there is a stop codon followed by 230 nucleotides long 3'UTR, but the polyA tail is lacking. Since the *rh4* mRNA is about 200 nucleotides longer, we assume that only a short part of the ORF encoding RH4 is missing from pRH4. Figure 2 (upper panel) depicts the partial amino acid sequence of RH4. The protein contains several cysteines and leucines positioned in such a way, that a conformation of α-helixes followed by β-sheets might be formed (secondary structure predictions of Chou-Fasman and Garnier-Osguthorpe-Robson). Furthermore, RH4 contains five putative *N*-glycosylation sites, at positions 17, 67, 106, 205 and 296.

The amino acid sequence was used for homology searches in the sequence databases of the National Center of Biotechnology Information, and homologues were found among the randomly sequenced *Arabidopsis* cDNA clones (GenBank accession numbers T46201, Z46590, R65327, and T46192). No other homologies were found. One of these cDNA clones, (the only one available), was obtained from the Arabidopsis Biological Resourse Center.

The nucleotide sequence of the *Arabidopsis* cDNA clone, designated pAraRH4, was also determined. pAraRH4 encodes a polypeptide of 300 amino acids, lacks the starting methionine and has two putative *N*-glycosylation sites, at positions 37 and 221 (figure 2, bottom panel).

RH4

```
  1  IRHEHHWIFL RYHQPKNVTH NSQSGITFVR NSGFCQENVF GQYFGLGSET
 51  RGTNTYIPDP YGIEVGNPSE IPKGYVEKWM FNIHAIDTRG VEDKLGCIEC
101  KCDLYNVTKD EDGVALSPNY KGGLQCCPDN SKCKMLKGFL GKKRSIYLKY
151  TVMWMNWESF ILPAKIYIID ATDVLKISHK SKGKSLEHDC KIEYEVEPCS
201  KSNVNGSDCV DVKRSSFPMQ KGGYFIYGVG HMHVGSIGTT LYGKDGKVIC
251  SSIPIYGNRS EAGNEKGYVV GMSTCYPQLG SIKIHDGETL TLEAKYNNTI
301  RHSGVMGLFY FLVAEKLPHH HL
```

ARARH4

```
  1  VDHASDEFKW LLNIHAIDTR GVEDKKGCIE CLCDLYNVTI DEYGRAIRPG
 51  YKGGLYCCYD KTQCRVKSGF DNGEKTRTLY LKYTVRWVDW DSSVLPAKVY
101  IFDVTDSWER SKGDSQEHIC HVEYEVKPCK TNGDGCVDVK KKSLVMPFDG
151  YIVYGVAHQH AGGIGGALYR ENGEGICASM PKYGNGDEPG NEAGYIVGMS
201  SCYPADPVKV SYGETLTLES NYSNAVGHTG VMGLFYILVA QQLPEPDSSL
251  PNKQHFEAPA RSLSFLAIFA VTVVVAVVVL IAAVIYRRQK RGDGYQSLST
```

Figure 2. Amino acid sequence of the polypeptides encoded by the inserts of pRH4 (upper panel) and pAraRH4 (bottom panel). Putative *N*-glycosylation sites are shown by lines. The hydrophobic domain of ARARH4 that might span the membrane is enclosed in a rectangular. The arrowhead in the sequence of ARARH4 indicates the position where the homology between ARARH4 and RH4 stops.

Figure 3 depicts the comparison between RH4 and AraRH4. The two polypeptides exhibit 58% identity and 73% similarity throughout the whole sequence. Since pAraRH4 is not a full size clone, the homology between AraRH4 and RH4 starts at 65th residue of RH4. The positions of all cysteines that are present in both polypeptides are conserved. The first six cysteines (indicated by asterisks) are arranged in a motif similar to that of the EGF-like domain present in several proteins involved in protein-protein interactions ($CXXCXCX_3NX_{15}GGXXCCX_5C$). AraRH4 bears a carboxy-terminal domain of 53 amino acids, which is absent from RH4. This domain includes a 28 residues long hydrophobic region (enclosed in a rectangular, in Figure 2, bottom panel), that may span the membrane, followed by a carboxyl-terminal tail of 15 amino acids. Therefore, AraRH4 might be an integral protein, either inserted in the membrane or transmembrane with a small cytoplasmic tail at the carboxyl-terminal end, and an extracellular domain homologous to RH4. In contrast, RH4 does not have a hydrophobic domain at the carboxyl-terminus that is long enough to span the membrane, therefore it is not a transmembrane protein.

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                      .         .         .  *  * * .         .
RH4       65 VGNPSEIPKGYVEKWMFNIHAIDTRGVEDKLGCIECKCDLYNVTKDEDGV 114
             |:::|:       ||::||||||||||||| ||||| ||||||| || |
ARARH4     1 VDHASD.....EFKWLLNIHAIDTRGVEDKKGCIECLCDLYNVTIDEYGR 45
                      .**     *.         .         .         .
RH4      115 ALSPNYKGGLQCCPDNSKCKMLKGF..LGKKRSIYLKYTVMWMNWESFIL 162
             |:.|.|||| || |...|:: .||   :|.|.:||||||.|::|:| :|
ARARH4    46 AIRPGYKGGLYCCYDKTQCRVKSGFDNGEKTRTLYLKYTVRWVDWDSSVL 95
                      .         .         .         .         .
RH4      163 PAKIYIIDATDVLKISHKSKGKSLEHDCKIEYEVEPCSKSNVNGSDCVDV 212
             |||:||:|.|| :   .:|||.| || ||.:|||.||   ..||.:||||
ARARH4    96 PAKVYIFDVTDSW...ERSKGDSQEHICHVEYEVKPC...KTNGDGCVDV 139
                      .         .         .         .         .
RH4      213 KRSSFPMQKGGYFIYGVGHMHVGSIGTTLYGKDGKVICSSIPIYGNRSEA 262
             |:.|:.|. :||::|||:| |.|:||..|| .:|..||.|:| ||| .|:
ARARH4   140 KKKSLVMPFDGYIVYGVAHQHAGGIGGALYRENGEGICASMPKYGNGDEP 189
                      .         .         .         .         .
RH4      263 GNEKGYVVGMSTCYPQLGSIKIHDGETLTLEAKYNNTIRHSGVMGLFYFL 312
             ||| ||:||||.|||. :.:|:  |||||||..|.|.: |.|||||||:|
ARARH4   190 GNEAGYIVGMSSCYPA.DPVKVSYGETLTLESNYSNAVGHTGVMGLFYIL 238

RH4      313 VAEKLPHHH 321
             ||:.||...
ARARH4   239 VAQQLPEPD 247
```

Figure 3. Comparison of the amino acid sequence of pRH4 and pAraRH4 encoded polypeptides. Both cDNA clones are incomplete and they lack the starting methionine of the ORFs. Since pAraRH4 lacks a bigger part of the ORF than pRH4, homology between RH4 and ARARH4 starts at the 65th amino acid of RH4. Vertical lines denote identical amino acids, dots denote chemically similar amino acids. The position of the cysteines in both polypeptides is conserved as it is indicated by boxes. The cysteine residues of the EGF-like domain are marked by asterisks.

The position of *ararh4* on the *Arabidopsis* genomic map

ararh4 was positioned on the map of *Arabidopsis*, in order to determine whether the position of the gene coincides with a root hair mutation. These studies revealed that *ararh4* maps at the bottom half arm of chromosome 5 between the RLFP markers UM515 and m211 (C. Lister, personal communication; Lister and Dean, 1993). No root hair mutations mapping at this region have been reported so far.

rh4 expression pattern in pea roots

To detect *rh4* expression during root hair development, we used the *in situ* hybridization procedure. However, when sections of roots are prepared, the majority of the root hairs are 'decapitated' and hence are lost during the process. For this reason, we used the whole mount *in situ* hybridization approach. Whole mount *in situ* hybridization is ideal to study expression of genes in root hairs, since they are easily accessible to microscopy and hybridization is not hampered by uptake of the probes.

rh4 sense and antisense RNA was labelled with digoxigenin (DIG) and after hybridization the hybrids were detected with the use of anti-DIG antibodies conjugated to alkaline phosphatase, of which the activity is visualized as a purple precipitate. The labelled probes were hybridized to segments of lateral roots from pea, 1.5 cm long. These segments include the root tip, the zone preceding root hair emergence and the zones where root hairs emerge, elongate and have reached their mature stage. No hybridization was observed with the sense probe (data not shown).

Hybridizations with the antisense *rh4* RNA showed that expression occurs in epidermal cells in the zone preceding root hair emergence (Figure 4A, arrow) and in the zones containing emerging and elongating root hairs (Figure 4B, arrow). *rh4* RNA was not detected in mature root hairs.

Figure 4. Whole mount *in situ* localization of *rh4* in pea roots.

Segments of 1.5 cm pea lateral roots were hybridized with DIG-labelled antisense *rh4* RNA. Signal is visualized as a purple precipitate.

A. Micrograph shows *rh4* expression at the elongation zone of the root. The arrowhead indicates a trichoblast that expresses *rh4*. Note that the mRNA is localized in the transverse median level of the cell and coincides with the position of the nucleus. Arrows indicate the borders of the cell.

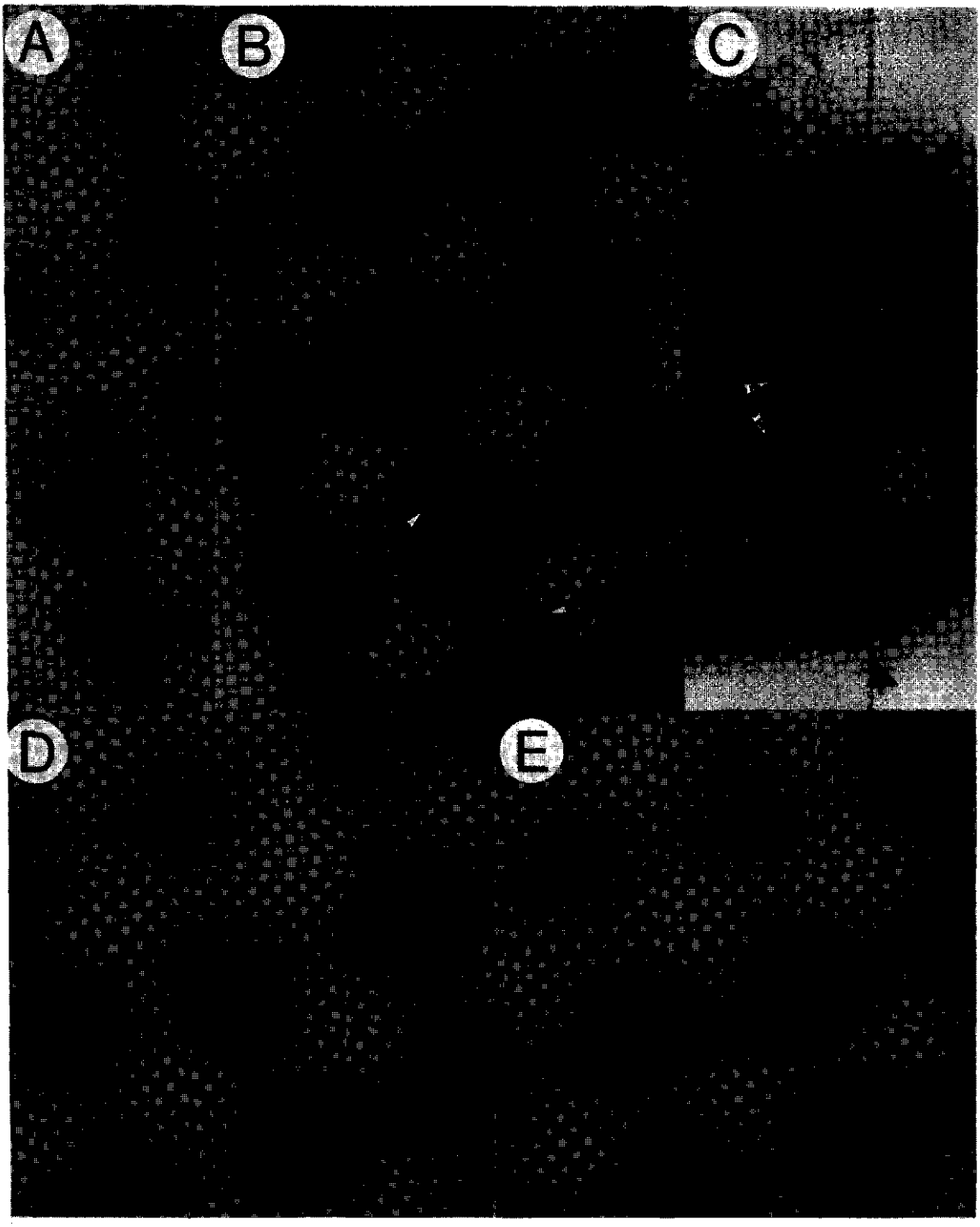
B. Micrograph showing *rh4* expression in the zone of root hair emergence and elongation. The arrow points to one of the *rh4*-expressing root hair-containing epidermal cells. The mRNA is localized in the outgrowth and not in the basal part of the cell. No signal is observed in the atrichoblasts, one of which is indicated by an arrowhead. Furthermore not all of the root hair-containing cells show expression, but they are arranged in files. At the left and the right side of the depicted root, files of cells not showing *rh4* expression can be seen.

C. Hand-made cross-section of a pea root hybridized with antisense *rh4* RNA. The cells that express *rh4* (black arrowheads) are located opposite to protoxylem poles (white arrowheads).

D&E. Close-up of a trichoblast expressing *rh4* prior to root hair emergence. The mRNA is localized at the site of the nucleus (big arrowhead). The small arrowhead points to the nucleus of a neighbouring cell, which contains an elongating root hair (arrow). Note that both nuclei have a swollen appearance. D and E depict the same area of the root, focused on different planes.

All micrographs were taken with the use of Nomarski optics.

Bar in A = 250μm; bar in D = 500μm. The same magnification was used in A/B/C and D/E respectively.


A
B
C
D
E

In the zones containing emerging and elongating root hairs, *rh4* transcripts are present in hair-containing cells, -but not in all of them-, and they are localized in the outgrowth itself and not in the basal part of the cell (Figure 4B, arrow). Furthermore, in these zones *rh4* RNA does not occur in the cells lacking a hair (atrichoblasts), which are interspersed with the root hair-containing cells (Figure 4B, arrowhead). Hence, these localization studies suggest that expression of *rh4* is induced only in cells bearing root hairs and this transcript is specifically located in the hairs.

In the zone preceding root hair emergence, *rh4* is transcribed as well (Figure 4A). Since at later stages of development *rh4* RNA is confined to root hair-containing cells, it is probable that the cells expressing the gene in an earlier developmental stage, will eventually form a root hair. This conclusion is supported by the position of the nucleus in the *rh4*-expressing cells (Figure 4A, 4D&E big arrowheads). Figures 4D&E are a close-up of a cell where *rh4* is expressed. No root hair is formed yet, but the nucleus has migrated to a position slightly out of the transverse median cell plane, close to the outer surface of the root (big arrowhead). It is known that in trichoblasts prior to root hair emergence, the nucleus leaves the parietal cytoplasm, regains a swollen appearence and migrates to the site of root hair emergence. For comparison, note the shape and the site of a nucleus of a neighbouring cell containing an elongating root hair (Figure 4D&E, small arrowhead). In both cases the nuclei are swollen and they are located to a position proximal to the site of root hair formation.

Furthermore, it is noteworthy that in the cells preceding root hair emergence, the *rh4* mRNA is not distributed uniformely in the cytoplasm. On the contrary, it is localized in the transverse median cell plane, coinciding with the position of the nuclei (Figures 4A, 4D&E). When the nucleus has migrated to the site of root hair emergence, a polarity has to be established in this cell, since the movement coincides with rearrangments in the cytoskeleton leading to the formation of nuclear envelope-radiating microtubule array. Therefore, it seems likely that the subcellular localization of the *rh4* mRNA reflects the polarity of the cell.

rh4 is expressed in specific rows of epidermal cells. To correlate the position of these rows with the vascular bundle, hand-made cross-sections from the hybridized roots were analysed. As it can be seen in figure 4C, the root hairs where *rh4* is transcribed (black arrowheads) are located opposite a protoxylem pole (white arrowheads). Therefore, it seems very probable that the developmental stage of the epidermal cells as well as positional information determined by the vascular bundle, are factors regulating *rh4* expression.

DISCUSSION

In this chapter we characterized a cDNA clone corresponding to a gene, *rh4*, specifically expressed in epidermal cells containing emerging and elongating root hairs, which are located opposite a protoxylem pole.

RH4 contains an EGF-like domain. A number of secreted or transmembrane proteins that play a role in signalling and cell-cell interactions, also contain EGF-like domains (Branden and Tooze, 1991). In most of these proteins the EGF-like domain is repeated several times. However, in the case of the lymph node homing receptor core protein, -a lymphocyte adhesion molecule-, the EGF-like domain is present once as in RH4 (Siegelman and Weissman, 1989). Whether a role in signalling and cell-cell interactions can be envisioned for RH4 is unclear at the moment. The *Arabidopsis* homologue of RH4, AraRH4, is a putative integral protein, whereas RH4 lacks such a domain that could act as an anchor in the membrane. Genomic blot analysis has shown that probably there is only one *rh4* gene in both *Arabidopsis* and pea, respectively. Therefore, it is possible that the genes have diverged during evolution and that the proteins have a slightly different mode of action. Thus, it is not clear whether *ararh4* plays a similar role in root hair development, as *rh4* does in pea. Since the expression pattern of *ararh4* has not been established yet, and *ararh4* does not map in a position of a known root hair mutation, further studies will be needed to elucidate its function.

rh4 is expressed in epidermal cells containing elongating root hairs. However expression was also observed at an earlier stage of development. To determine the identity of the *rh4*-expressing cells, certain morphological criteria were taken in account. In pea, it has been reported that an asymmetric division of an epidermal cell occurring in the elongation zone, gives rise to both a trichoblast and an atrichoblast. Trichoblasts prior to root hair emergence, undergo a number of changes (Bakhuizen, 1988). The nuclei, for instance, swell and leave their parietal position to take a predetermined position coinciding with the site of root hair emergence. In many plants, tip-growth initiation and root hair growth occurs in the proximal part of the trichoblasts, whereas pea root hairs were found to be positioned slightly out of the transverse median cell plane (Bakhuizen, 1988). In the *rh4*-expressing cells of the elongation zone, the nuclei are translocated to the transverse median plane of the cells. This observation supports the idea that *rh4* is expressed in trichoblasts prior to root hair emergence.

The whole mount *in situ* hybridizations show that the *rh4* mRNA is asymmetrically distributed within a cell. Prior to root hair emergence, it is located in the cytoplasm in the vicinity of the nucleus. When root hairs are formed, it is preferentially found in the hair, and not in the basal part of the epidermal cell. Examples of localized mRNAs have been found in several animal cells, as oocytes, fibroblasts, myoblasts, neurons, oligodendrocytes and epithelial cells (Wilhelm and Vale, 1993; Johnston, 1995). The common feature that these cell types share, is the fact that they are polarized. In plants, two cell types, root hair containing epidermal cells and germinating pollen, expand by polar growth named tip growth. Differential distribution of three mRNAs has been observed recently in germinating pollen tubes (Torres et al., 1995). While the mRNA of a hydroxyproline-rich glycoprotein is present in the tube, malic enzyme mRNA is only present in the body of the pollen cell, and α-tubulin mRNA is present in both parts of the cell (Torres et al., 1995). Thus in both types of plant cells showing polar growth, we find localized mRNAs.

In most cases studied so far, the site where the localized mRNAs are found, is determined by the polarity of the cells and often depends on the organization of the cytoskeleton (Johnston, 1995). *rh4* mRNA localization seems to be determined by the polarity of the trichoblasts. In trichoblasts, movement of the nucleus to the root hair emergence point, coincides with rearrangement of microtubules leading to the formation of a nuclear envelope radiating microtubule array (Bakhuizen, 1988). The *cis*-acting sequences required for localization of messengers reside in their 3'UTRs (Mowry and Melton, 1992; Dalby and Glover, 1993; Kim-Ha et al., 1993; Macdonald et al., 1993; Kislauskis et al., 1994). It seems that the localization often involves two separate steps, translocation to the site of localization and anchoring at that site, which might be controlled by different *cis*-acting elements. None of the so-far identified 3'UTR sequences responsible for localization of particular mRNAs in specific areas of a cell, are present in the 3'UTR of *rh4* messenger. The use of antisense oligonucleotides introduced via microinjection could reveal the sequences necessary for localization of *rh4* mRNA. It has been postulated that mRNAs are localized in the position where the encoded protein is needed (Johnston, 1995; Curtis et al., 1995). Therefore, the site of localization of *rh4* mRNA indicates that RH4 might be necessary in the position where the outgrowth is initiated originally, and later on during tip-growth, in the outgrowth itself.

The cells expressing *rh4* are arranged into files along the root, and are positioned opposite protoxylem poles. Similar behaviour has been observed with pea lectin, which has been localized in the elongating root hairs positioned also opposite to protoxylem poles (Diaz, 1989). Thus despite the fact that root hairs are morphologically identical, clear differences at a molecular level exist. Lateral roots arise from the pericycle cells located adjacent to a protoxylem pole. In *Arabidopsis*, cells in these files can be distinguished from cells in other files based on their length (Laskowski et al., 1995). Furthermore, it has been shown that an alcohol extract of the stele can induce cell divisions in explants of the pea root cortex in the presence of auxin and cytokinin (Libbenga et al., 1973). Therefore, it can be postulated that these morphogenes can influence gene expression in cells opposite the protoxylem poles.

In rhizobia-legume interaction, the nodules are preferentially formed opposite to protoxylem poles (Kijne, 1992). The interaction between the symbiont and the plant starts in the root hairs located in the same position, where the symbiont enters the plant via newly-formed structures, the infection threads (Kijne, 1992). It has been shown that lectin plays a role in the interaction between rhizobia and legumes (Diaz, 1989). Therefore, lectin might contribute to the susceptibility of the particular root hairs to rhizobia. Whether that is the case for the RH4 protein is not clear. However, one can postulate that *rh4* might play a role either directly, when rhizobia regulate its expression, or indirectly, by providing together with other genes, a certain 'identity' to the root hairs that enables them to interact with rhizobia.

METHODS

Plant material and growth conditions

Pea seeds (*Pisum sativum* cv. Finale; Cebeco, Netherlands) were grown in gravel (Bisseling et al., 1978). Root segments containing root hairs were harvested from 4-day old pea seedlings and immediately frozen in liquid nitrogen.
For whole mount *in situ* hybridization experiments, plants were grown on plates containing Fåhraeus medium (Fåhraeus, 1957) in 1% agar.

Root hair isolation and RNA isolation

Root hairs were harvested by the method described by Röhn and Werner (1987), modified as described by Gloudemans et al. (1989).
Frozen roots and root hairs were ground in liquid nitrogen and resuspended in a hot (90°C) mixture of RNA extraction buffer (0.1 M Tris-HCl pH 9.0, 0.1 M LiCl, 10 mM EDTA, 1% SDS) and phenol (1:1). After vortexing and centrifugation (30 min, 6000 x *g*) the water phase was collected and RNA was extracted as described by Pawlowski et al. (1994).

Differential screening

A cDNA library was constructed by Stratagene in λZAPII vector system, using poly(A)$^+$ RNA isolated from root hairs of uninoculated and inoculated with *R. leguminosarum* bv. *viciae* pea plants (*Pisum sativum* bv Finale).
This library was plated at a density of approximately 30000 plaques per 15-cm-diameter plate that were transferred onto nitrocellulose filters in duplicates. The filters were screened by differential hybridization with root-specific or root hair-specific radioactively labelled first-strand cDNA. Autoradiograms were screened for hybridization with the root hair specific cDNA probe only. The putative positive plaques were purified further with two rounds of differential screening. Inserts of the selected plaques were converted into plasmid vectors according to the established protocols (Stratagene).

Northern blot analysis

Total RNA was denatured in DMSO/glyoxal, separated on 1% agarose gels and blotted onto GeneScreen in 0.025 M NaH_2PO_4 pH 7.0. The blots were hybridized in 50% formamide, 1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 7.0, 10 x Denhard's solution at 42°C. The pRH4 insert was radiolabelled by random priming using [a-^{32}P]dATP (3000 Ci/mmol; Amersham) as radioactive label.

Nucleotide sequencing

The nucleotide sequencing of the insert of pRH4 was determined by double-stranded sequencing using the T7Sequencing kit from Pharmacia Biotech. The sequence of the insert of pAraRH4 was determined with an automatic sequencing apparatus of Applied Biosystems, Inc. by using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.).

Whole mount *in situ* hybridization

For the whole mount *in situ* hybridization, roots of *P. sativum* were fixed in PBS, 4% paraformaldehyde, 0.25% glutaraldehyde, 0.08 M EGTA, 10% DMSO, and 0.1% Tween 20, for 3 hrs at room temperature. *In situ* hybridization was performed essentially as described by Tautz and Pfeifle (1989), with modifications. The heptane washes were eliminated. Tissue was kept in ethanol, after fixation, at -20°C for 2 days. Before the proteinase K treatment, tissue was incubated for 30 min in 1:1 ethanol/xylene solution. This treatment was followed by a postfixation step in PBS, 0.1% Tween 20, and 5% formaldehyde. After the proteinase K treatment the same postfixation step was applied. Prehybridization and hybridization took place at 42°C. For the post-hybridization washes an RNase A treatment for 15 to 30 min, was included (40μg/ml RNase A in 500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). Before use, the anti-digoxygenin antibodies coupled to alkaline phosphatase (Boehringer Mannheim) were preabsorbed in an acetone extract of fixed roots (overnight at 4°C). The final concentration of the antibodies used, was 1:2000. Incubation with the antibody took place at 4°C overnight. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate, Boehringer Mannheim) and nitroblue tetrazolium (NBT, Boehringer Mannheim) was carried out for 30 min to several hours. A sense probe was used as control.

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CHAPTER 5

rh6 and *rh6-1* are two putative peroxidase genes involved in polar growth

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INTRODUCTION

Rhizobia form root nodules in a symbiotic interaction with legumes. The formation of nodules starts with the mitotic reactivation of root cortical cells leading to the formation of a nodule primordium that upon infection by rhizobia develops into a root nodule. The bacteria infect the plant by so-called infection threads. After bacteria attach to the root hair tip, the tips curl and bacteria become entrapped. There, a local hydrolysis of the plant cell wall takes place, and the plasmamembrane invaginates (Kijne, 1992; van Spronsen et al., 1994). New cell wall like material is deposited, resulting in the formation of an infection thread. The infection thread grows by incorporation of vesicles at the tip (van Brussel et al., 1992).

Plant cells can grow in two ways. Most cells have an intercallary growth in which case, vesicles are incorporated along the side walls and cells mostly elongate. A few plant cell types show tip growth; root hairs and pollen tubes. In these cells, vesicle incorporation only occurs at the tip of the cell. The involvement of polar growth mechanisms in infection thread formation is indicated by the cytoplasmic rearrangements that occur in the cells of the outer cortex. The nuclei swell and move to the center of the cell. The cytoplasm reorganizes to form a radially oriented conical structure, -the cytoplasmic bridge-, that resembles a preprophase band (Kijne, 1992). Furthermore, the cytoplasmic bridges are polarized, with the bulk of the cytoplasm and endomembranes located in the outer side, and all amyloplasts at the inner side of the bridge (van Brussel et al., 1992). The infection thread appears to grow via these cytoplasmic bridges, which therefore are called preinfection threads.

So, the infection threads grow at their tip by incorporation of vesicles and involve a polar organized cytoplasm. Hence, it is reminiscent of polar growth of e.g. root hairs, but the direction of growth is reversed. Based on the above mentioned observations, it has been postulated that infection thread formation uses mechanisms shared with polar growth of plant cells, in particular root hairs. We were interested to investigate this hypothesis, by testing whether genes specifically expressed in polar growing plant cells, are induced in root cortical cells forming infection threads.

Root hair growth and germination of pollen are processes that share certain common features. The growth of both cell types for instance, is dependent upon a continous influx of Ca^{2+} at the apex and an internal Ca^{2+} gradient (Schiefelbein et al., 1992; Pierson et al., 1994; Gilroy and Wymer, 1995). Furthermore, a functional actin cytoskeleton is required (Heath, 1990). However, the best support for common mechanisms in polar growth in plant cells comes from studies on the *Arabidopsis* mutant *tip1*. *tip1* plants (Schiefelbein et al., 1993) have branched root hairs that are also shorter than those of wild type plants. Furthermore, studies on pollen tube growth *in vivo* showed that the *tip1* pollen grow slower through the transmitting tissue of wild type flowers than the wild type pollen.

In this paper, we describe the isolation of two cDNA clones corresponding to genes expressed in both root hairs and germinating pollen. These cDNA clones have been used to test whether the formation of preinfection threads involves similar genes as other polar growing plant cells.

RESULTS

Isolation of cDNAs corresponding to root hair specific genes

To isolate cDNA clones corresponding to genes expressed in root hairs, a root hair cDNA library from *Pisum sativum,* was differentially screened with root and root hair cDNA, respectively. Two of the isolated clones, namely pRH6 and pRH6-1 were selected for further analysis.

To confirm that *rh6* and *rh6-1* are expressed at elevated levels in root hairs, we compared the amounts of the corresponding messengers in RNA preparations of roots and isolated root hairs. Roots were collected from 6-day old plants. The root tips were removed and root hairs were obtained by brushing roots frozen in liquid N_2. RNA was isolated from the root hair samples and from roots of 6-day old plants of which the root tips were removed. Identical northern blots were probed with the inserts of pRH6 and pRH6-1. The same northerns blots were subsequently hybridized with ubiquitin, to standardise the amount of RNA loaded in each slot (Figure 1).

rh6 mRNA was present in markedly higher levels in the root hair (RH) samples (Figure 1), than in RNA preparations of whole root (R). The low amount of *rh6* mRNA seen in the RNA sample of the root (R) could be due to the presence of root hairs or to a low level of expression in other cell types of the root. *rh6* mRNA was not detected in stems (S) and leaves (L), even after prolonged exposure times. pRH6 hybridized with a transcript of approximately 1,4 kb and carries an insert of 1359 bp.

rh6-1 mRNA occurs also at a higher level in the root hair RNA sample than in RNA preparations of total roots (Figure 1). *rh6-1* transcript was not detected in stems and leaves, even after prolonged exposure times. pRH6-1 hybridizes with a transcript of 1.1 kb and since it bears an isert of 974 bp, it is not a full size clone. *rh6-1* transcript is less abundant than *rh6,* since the northern blot of Figure 1, middle panel, had to be exposed 12 times longer to obtain the signal of the blot shown in Figure 1, upper panel.

So, both genes are expressed at elevated levels in root hairs when compared to the average expression level in root cells.

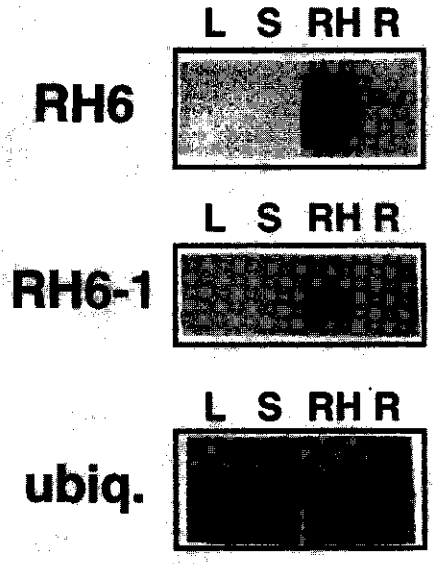

Figure 1. Autoradiograph of northern blots containing 20 µg of total RNA isolated from roots (R), root hairs (RH), leaves (L), and stems (S), respectively, hybridized to pRH6 (upper panel) and pRH6-1 (middle panel). The amount of RNA on the filter was determined by a hybridization with a soybean ubiquitin probe (Kouchi and Hata, 1993), as it is shown in the bottom panel.

Sequence characteristics

The RH6 cDNA contains an ORF encoding a polypeptide of 325 amino acids. The first 29 *N*-terminal amino acids constitute a putative membrane translocation signal sequence, with a hydrophobic core and a putative cleavage site at proline 29 (arrowhead, Figure 2; von Heijne, 1983). The putative mature polypeptide has an isoelectric point of 9.55 and a molecular weight of 32,8 kD. RH6 has a high homology with peroxidases available in databases. The most related to RH6 are, a cationic peroxidase from *Medicago truncatula* accumulating in the epidermis upon inoculation with *R. meliloti* (72% identity; Cook et al., 1995), a cationic peroxidase from peanut cells (59% identity; Buffard et al., 1990), and two cationic peroxidase isozymes formed in the stems of *Stylosanthes humilis* upon infection with *Colletotrichum gloeosporioides* (57% and 54% identity; Curtis et al., 1995; Harrison et al., 1995). Figure 2 shows an alignment of the protein sequence of RH6 with those peroxidases. The consensus sequences that are widely conserved among different peroxidases are present also in RH6. These include the context of the distal histidine at position 58 (GASLLRLHFHDCFV), that serves as an acid-base catalyst, and that of the proximal histidine at position 193 (DLVVLSGGHTIG) that is involved in the binding to heme (Tyson, 1991; 1992). The position of eight of the nine cysteines present in RH6, is also conserved in the different peroxidases. Furthermore, RH6 has three putative *N*-glycosylation sites (indicated by lines in Figure 2).
RH6-1 cDNA contains an ORF encoding a polypeptide of 281 amino acids and it lacks the *N*-terminal part including the starting methionine. The encoded polypeptide has an isoelectric point of 5.8. RH6-1 has also high homology with the plant peroxidases present in the

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            1                                                    50
      RH6   ...MDSHIQF YLVIFMVTVA TVLSPAVAKL TPNYYDRICP KALPIIKSVV
  Mtu16727  .MASSSPCQI FLVFVMVTLV TSLIPSNALL TPHFYDNVCP QALPTIKSVV
    Arcpnc1 MALPISKVDF LIFMCLIGLG S......AQL SSNFYATKCP NALSTIKSAV
     Ssncape ...MALIVPI SKVCFIIFMC LNIGLGSGQL SSNFYATKCP NALSTIKSAV
 Ssnperoxia ....MAILAI SKVCLIILVM SLIGLGSGQL SSNFYATTCP NALSTIRSGV

            51                                                   100
      RH6   KQAIYREPRI GASLLRLHFH DCFVNGCDAS VLLDDTPTFR GEKTAFPNIN
  Mtu16727  LHAILREKRI GASLLRLHFH DCFVNGCDGS VLLDDTPNFT GEKTALPNIN
    Arcpnc1 NSCVAKEARM GASLLRLHFH DCFVQGCDAS VLLDDTSNFT GEKTAGPNAN
     Ssncape NSAVSKEARL GASLLRLHFH DCFVQGCDAS VLLDDTSTFT GEKTAFPNVN
 Ssnperoxia NSAVSKEARM GASLLRLHFH DCFVQGCDAS VLLDDTSNFT GEKTARPNAN

            101                                                  150
      RH6   SIRGFEVVDQ IKAAVTKACR RDVVSCADIL AIAARDSVAI LGGNQYWYQV
  Mtu16727  SIRGFSVVDE IKAAVDKVCK GPVVSCADIL ATAARDSVAI LGGPQFFYNV
    Arcpnc1 SIRGFEVIDT IKSQVESLC. PGVVSCADIL AVAARDSVVA LGGA..SWNV
     Ssncape SARGFDVIDT IKSQVESLC. PGVVSCADIL ALAARDSVVA LGGP..SWNV
 Ssnperoxia SIRGFEVIDT IKSQVESLC. PGVVSCADIL AVAARDSVVA LGGP..SWTV

            151                                                  200
      RH6   LLGRRDARNA SWDAANANLP PPFFNFSQLI TNFNSHGLNL KDLVVLSGGH
  Mtu16727  LLGRRDARTA SKAAANANLP SPTFNFSQLI SNFKSQGLNV KDLVALSGGH
    Arcpnc1 LLGRRDSTTA SLSSANSDLP APFFNLSGLI SAFSNKGFTT KELVTLSGAH
     Ssncape QLGRRDSTTA SLNSANSDLP GPSFNLSGLI SAFSKKGFTA KELVTLSGAH
 Ssnperoxia QLGRRDSTTA SLSLANSDLA APTLDLSGLI SAFSKKGLST SEMVALSGGH

            201                                                  250
      RH6   TIGFAKCATF RDRIFNDTNI DTTFAANLQK TCPRI..GGD NNLAPFD.ST
  Mtu16727  TIGFARCTTF RNRIYNETNI DPIFAASLRK TCPRN..GGD NNLTPLDF.T
    Arcpnc1 TIGDAQCTAF RTRIYNESNI DPTYAKSLQA NCPSV..GGD TNLSPFDVTT
     Ssncape TIGDARCTTF RTRIYNESNI DPSYAKSLQG NCPSV..GGD SNLSPFDVTT
 Ssnperoxia TIGDARCTSF RTRIYTESNI DPNFAKSLQG NCPNTTGNGD NNLAPIDTTS

            251                                                  300
      RH6   PKKVDTAYYT SLLYKRGLLH SDQELFKGDG SQSDNLVLKY SKDSYAFAKD
  Mtu16727  PTRVENTYYR DLLYKRGVLH SDQQLFKGQG SESDKLVQLY SKNTFAFASD
    Arcpnc1 PNKFDNAYYI NLRNKKGLLH SDQQLFNGV. S.TDSQVTAY SNNAATFNTD
     Ssncape PNKFDNAYYI NLKNKKGLLH ADQQLFNGGG S.TDSQVTAY SNNAATFNTD
 Ssnperoxia PTRFDNGYYK NLLVKKGLFH SDQQLFNG.G S.TDSQVNGY ASNPSSFCSD

            301                                  331
      RH6   FGVSMIKMGN LKPLTGKKGE IRCNCRKVNS Y
  Mtu16727  FKTSLIKMGN IKPLTGRQGE IRLNCRRVR. .
    Arcpnc1 FGNAMIKMGN LSPLTGTSGQ IRTNCRKTN. .
     Ssncape FGNAMIKMGN LSPLTGTSGQ IRTNCRKTN. .
 Ssnperoxia FGNAMIKMGN ISPLTGSSGQ IRTNCRKTN. .
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Figure 2. Alignment of RH6 with *Medicago truncatula* (Mtu 16727), peanut (Arcpnc1) and *Stylonsanthes humilis* (Ssncape, Ssnperoxia) peroxidases.The conserved cysteines are shown with asterisk. The context of the distal and proximal histidines are enclosed within a rectangular, while the distal and proximal histidines are indicated by bold letters. The arrowhead indicates the putative cleavage site of the signal peptide of RH6 at proline (P) 29. The putative *N*-glycosylation sites of RH6 are marked with lines.

databases. The most related peroxidases, as it is shown in Figure 3, are the anionic peroxidase from *Populus kitakamiensis* (46,2% identity; Osakabe et al., 1995), two cationic isozymes of 38 kD and 40 kD found in the medium of cultured tobacco cells (44,3% and 43.6% identity respectively; Narita et al., 1995) and an acidic peroxidase associated with systemic acquired resistance in cucumber (44,6% identity, Rasmussen et al., unpublished).
The consensus sequences containing the context of the distal histidine (AASLLRLHFHDC, position 18) and the proximal histidine (DLVVLSGAHTIG, position 141) are also present in RH6-1. Furthermore, the position of the seven cysteines of RH6-1 is conserved among the different peroxidases. RH6-1 has one putative *N*-glycosylation site.
RH6 and RH6-1 show 43,2% homology and they belong to different classes of peroxidases since most likely *rh6* encodes a cationic peroxidase and *rh6-1* an anionic one. So, RH6 and RH6-1 are two putative peroxidases present in pea root hairs.

Expression of *rh6* and *rh6-1* in pea root hairs

Northern blot analysis indicated that *rh6* mRNA accumulates in root hairs. To check this, and to study in which epidermal cells *rh6* is expressed, whole mount *in situ* hybridizations were done.
Whole mount *in situ* hybridization experiments performed on pea roots confirmed that *rh6* mRNA is present in root hairs (Figure 4A). Expression of the gene was observed in young elongating root hairs as well as mature root hairs (Figure 4A) that have reached their maximum length. The *rh6* mRNA accumulates in the outgrowth but not in the basal part of the cell. Note that *rh6* transcripts are uniformely distributed along the hair (Figure 4A, arrowhead). No signal could be detected in the atrichoblasts, -epidermal cells that do not have a root hair-, which are interspersed between the root hair-containing cells. Furthermore, *rh6* mRNA was not detected in the root tip.

Figure 3. Alignment of RH6-1 with *Popolus kitakamiensis* (Poppa), tobacco (Tobcpi38ka and Tobcpi40kb) and cucumber (Cusprcpcra) peroxidases.
The conserved cysteines are indicated by bold letters. The context of the distal and proximal histidines are enclosed within a rectangular, while the distal and proximal histidines are shown with asterisk. The putative *N*-glycosylation site of RH6-1 is marked with a line.
A concensus sequence of the peroxidases (PERCON) is given in the bottom line of the aligned sequences.

```
              1                                                    50
     RH6-1    .......... .......... .......... .......... ......ARVE
     Poppa    .......... .......... .......... .......... FYDQTCPNVS
Tobcpi38ka    MRTAQLLLLS ..FLVFLSIV VCGVSGAGNN VPRKNFYKNT RCPNAEQFVR
Tobcpi40kb    MGTAQLLLLS NIFLVFLSIV VCGVSGAGNN VPRKNFYKST RCPNAEQFVR
Cusprepera    .......... .......... .......... .......... ......MTVE
   PERCON     .......... .......... .......... .......... ......qfV.

              51                            *                     100
     RH6-1    SKVKEWIKED YT....LAAS LLRLHFHDCG IRGCDGSILL KHEG...S..
     Poppa    TIIRDVITET LASDPRIGAS LIRLHFHDCF VNGCDGSLLL DNSDTIVS..
Tobcpi38ka    DITWSKAKND AT....LSAK LLRLHYHDCF VRGCDASILL DKVGTDQS..
Tobcpi40kb    DITWSKAKND ST....LGAK LLRLHYHDCF VRGCDASILL DKVGTDQF..
Cusprepera    FGVKRAIETD IR....AGAK LIRFHFHDCF VQGCDGSVLL EDPP...GFE
   PERCON     di..skik.d .t....lgAk LlRlHfHDCf vrGCDgSiLL dkvgt..s..

              101                                                 150
     RH6-1    .ERTAEA.SK TLRGYEVIDD IKAEVEKQCP KTVSCADILT TVARDA.TVE
     Poppa    .EKEAGGNNN SARGFEVVDR MKALLESACP ATVSCADILT IAAEES.EVL
Tobcpi38ka    .EKEARP.NL SLGGFDVIDD IKRQVEEKCP EIVSCADILA LAARDAVSFP
Tobcpi40kb    .EKEARP.NL SLGGFDVIDD IKRQVEEKCP GIVSCADILA LATRDAVSFR
Cusprepera    TELNGLG.NL GIQGIEIIDA IKAAVEIECP GVVSCADILA QASKDS.VDV
   PERCON     .Ekear..nl sl.GfeviDd iKaqvEekCP g.VSCADILa laarda.s..

              151                                 __              200
     RH6-1    LGGLYWTVPY GRKDGTI.SI DSE.TEIIPK GHENVTSLIE FFQSKGL.NV
     Poppa    AGGPNWTVPL GRRDSTTASR DAA.NAFLPA PNITLDQLRE SFTNVGLNNN
Tobcpi38ka    FKKSLWDVAT GRKDGNV.SF GSEVNGNLPS PFSDFATLQQ LFAKKGL.NV
Tobcpi40kb    FKKSLWDVAT GRKDGNV.SL ASEVNGNLPS PFSDFATLQQ LFAKKGL.NV
Cusprepera    QGGPSWRVLY GRRDSRT.AN KTG.ADNLPS PFENLDPLVK KFADVGL.NE
   PERCON     fgg.lW.V.. GRkDg...s. dse.ngnlPs pf....tLq. lFakkGL.Nv

              201       *                                         250
     RH6-1    LDLVVLSGAH TIGRTSCGSI QYRLYNYKDT GKPDPTIDPQ YLNFLKRKCR
     Poppa    SDLVALSGAH TFGRAKCSTF DFRLYDFNST GAPDQSLDPT LLAALQELCP
Tobcpi38ka    NDLVALSGAH TIGVAHCGAF SRRLFNFTGK GDMDPSLNPT YVESLKQLCP
Tobcpi40kb    NDLVALSGAH TIGVAHCGAF SRRLFNFTGK GDVDPSLSST YAESLKQLCP
Cusprepera    TDLVALSGAH TFGRSRCVFF SGRLSNFSGS GQPDPTLDPT YRQELLSACT
   PERCON     nDLVaLSGAH TiGrahCgaf srRL.nftg. GdpDpsldpt ylesLkqlCp

              251                                                 300
     RH6-1    ...WASE... YVDLDARTPK TFDEKYYINL EKKMGLLSTD QLLYS...DQ
     Poppa    QGGNGSV... LTDLDLTTPD AFDSNYYSNL QGNQGLLQTD QVLFSTPGAD
Tobcpi38ka    ...NPANPAT TVEMDPQSST SFDSNYFNIL TQNKGLFQSD AVLLT...DK
Tobcpi40kb    ...NPANPAT TVEMDPQSST SFDSNYFNIL TQNKGLFQSD AALLT...DK
Cusprepera    ...SQDT... RVNFDPTTPD KFDKNYFTNL RANKGLLQSD QVLHS...TQ
   PERCON     ...np.n... tv..Dp.tp. sFDsnYfnnL tqnkGLlqsD qvLls...d.

              301                                                 350
     RH6-1    ..RTSPLVSA MALESSVFKR QFAFSMSKFG AIDVLTGDDE GEIRTNCNFV
     Poppa    ..DVIALVNA FSANQTAFFE SFAESMIRMG NLRPLTG.TE GEIRLNCRVV
Tobcpi38ka    ..KSAKVVKQ LQKTNTFFS. EFAKSMQKMG AIEVLTG.NA GEIRKSCRVR
Tobcpi40kb    ..KSAKVVKQ LQKTNAFFS. EFAKSMQKMG AIEVLTG.NA GEIRKNCRVR
Cusprepera    GAKTVEIVRL MALKQETFFR QFRLSMIKMG NIKPLTG.SQ GEIRRNCRRV
   PERCON     ..k.ak.Vk. ...t.tfF.. .FakSM.kmG aievLTG.n. GEIRknCrvv

              351          365
     RH6-1    NAY....... .....
     Poppa    NANLAGPDSK LVSSI
Tobcpi38ka    N......... .....
Tobcpi40kb    N......... .....
Cusprepera    NDLGSETGHD VM...
   PERCON     N......... .....
```

Figure 4. *In situ* localizations of *rh6* and *rh6-1*.

A,B,C. Whole mount *in situ* hybridization performed on segments of 1.5 cm pea lateral roots (A) or pea germinating pollen (B&C) hybridized with DIG-labelled antisense *rh6* RNA. Signal is visualized as a purple precipitate.

A) *rh6* expression is observed in mature root hairs. *rh6* mRNA is distributed uniformly along the outgrowth as it is indicated by the arrowhead.

B&C) *rh6* is expressed in pollen tubes as soon as the tube emerges (arrow, Figure B). Furthermore, the gene is expressed throughout in germination process as it is shown in Figure C. Note that the messengers are distributed along the outgrowths (arrowheads), as in the case of root hairs.

D,E,F,G. Whole mount *in situ* hybridization performed on segments of 1.5 cm pea lateral roots (D&F) or pea germinating pollen (E&G) hybridized with DIG-labelled antisense *rh6-1* RNA. Signal is visualized as a purple precipitate.

D&F) *rh6-1* is expressed in emerging root hairs (D) and young elongating root hairs (F). *rh6-1* mRNA accumulates at the tip of the root hair (arrowhead).

E&G) *rh6-1* is expressed in emerging (arrow, Figure E) and elongating (Figure G) pollen tubes. The messengers are distributed preferentially at the tip of the tube (arrowhead).

All micrographs were taken with the use of Nomarski optics.

H) Bright field micrograph of a cross section of pea roots spot-inoculated with *Rhizobium leguminosarium* bv. *viciae* (after 7 days), hybridized with ^{35}S-UTP labelled *rh6* RNA.

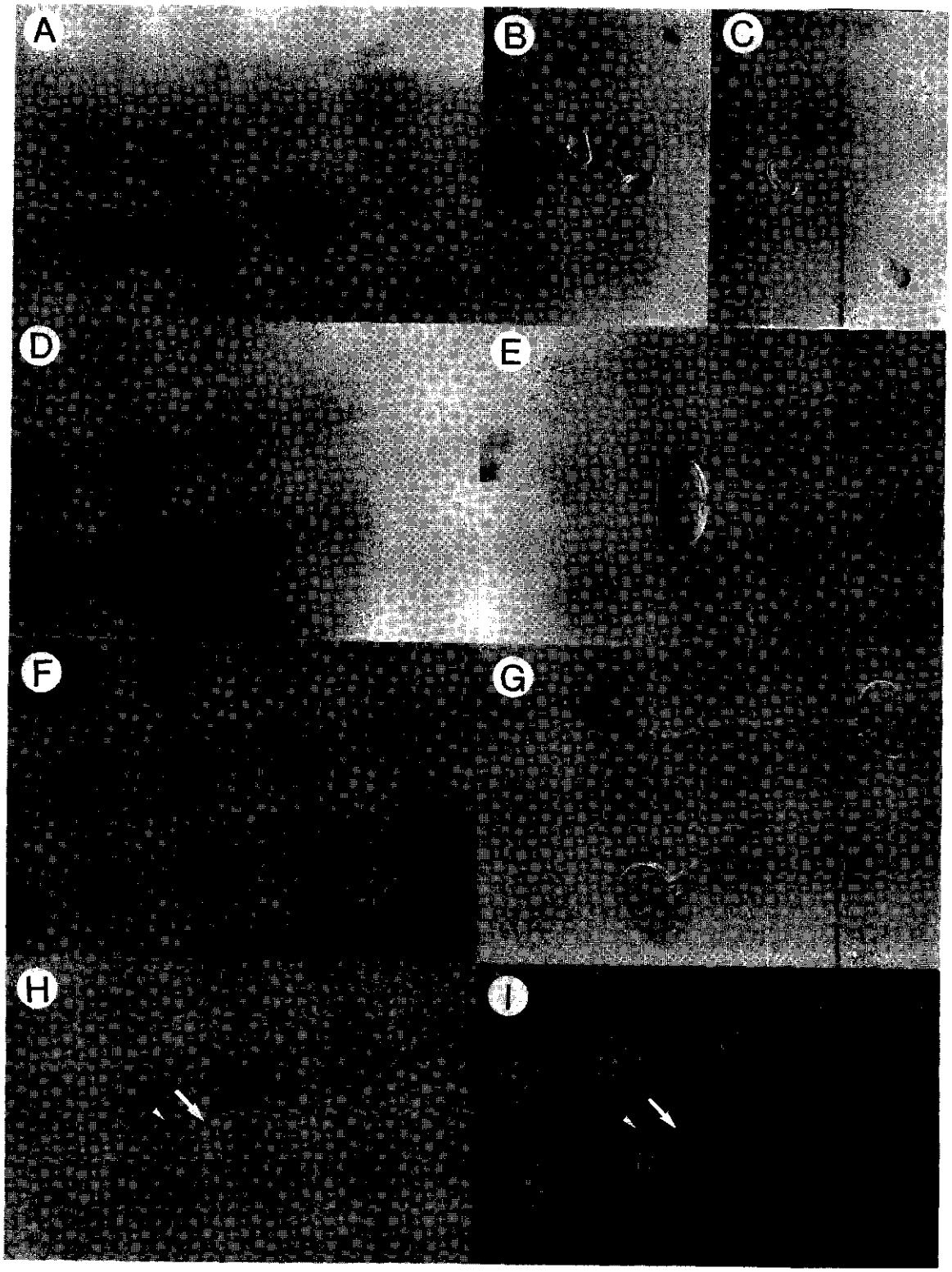
Morphological changes in the root cortical cells can be observed. The nucleus moves to the middle of the cell (arrowhead) and preinfection threads are formed (arrow).

I) Epipolarization of the same micrograph as in H. The signal is visualized as green dots. *rh6* mRNA is present in preinfection forming root cortical cells (arrowhead, arrow)

Bar in A = 250 μm; bar in E = 125 μm; bar in F = 500 μm. The same magnification was used in A/B/C/D/H/G, and E/G respectively.

rh6-1 mRNA is also present in the root hairs (Figure 4D&F). Expression of *rh6-1* was detected in all emerging (Figure 4D) and actively growing root hairs (Figure 4F). The mRNA as in the case of *rh6*, is present in the outgrowth and not in the basal part of the cell. Though, *rh6-1* mRNA is located at the tip of the root hair (Figure 4F, arrowhead), whereas *rh6* mRNA is equally distributed over the hair (Figure 4A, arrowhead). In addition, *rh6-1* is expressed in the epidermis, in the zone preceding root hair emergence (data not shown).

Thus, the two peroxidase genes show a different expression pattern. *rh6* expression is restricted to the root hairs, while *rh6-1* is already expressed at an earlier developmental stage preceding the emergence of the hair. Furthermore, the distribution of the messengers is different. *rh6* mRNA is equally distributed along the outgrowth while most of the *rh6-1* mRNA is at the tip of the hair.


A
B
C
D
E
F
G
H
I

rh6 and *rh6-1* expression correlates with polar growth

While most plant cells have an intercallary growth, root hairs and pollen tubes elongate by polar tip growth. To clarify whether RH6 and RH6-1 play a more general role in the mechanism of tip growth, pollen from pea flowers were isolated and germinated *in vitro*. Expression of *rh6* and *rh6-1* was studied using whole mount *in situ* hybridization. Figure 4B&C depict *rh6* expression in pollen. Expression is detected as soon as the tube emerges (Figure 4B, arrow) and it is sustained throughout pollen tube growth (Figure 4B&C, arrowheads). *rh6* mRNA is observed in the germinating pollen tube (Figure 4B&C, arrowheads) and not in the pollen grain. *rh6-1* has a similar expression pattern; *rh6-1* mRNA was detected in emerging (Figure 4E, arrow) and elongating pollen tubes (Figure 4G, arrowhead). No *rh6-1* messenger was localized in the pollen grain.

There is a striking difference in the location of the two peroxidase mRNAs in pollen tubes; *rh6* mRNA is located along the pollen tube (Figure 4B&C), while *rh6-1* mRNA appears to be located mostly at the tip of the growing tube (Figure 4E&G). This distribution of the mRNAs is strikingly similar to the distribution observed in root hairs (Figure 4A versus Figure 4B&C, Figure 4D versus Figure 4E, and Figure 4F versus Figure 4G).

Since both root hairs and germinating pollen have a corresponding pattern of *rh6* and *rh6-1* expression, it is likely that polar growth has common features. Furthermore, it is noteworthy that the *rh6* and *rh6-1* messengers have a similar cellular localization in both root hairs and pollen tubes.

rh6 is induced in preinfection thread containing cortical cells

Pea roots were spot-inoculated with *Rhizobium leguminosarum* bv. *viciae*. The bacteria enter the root hairs and the cortical cells via tubular structures called infection threads. Prior to infection thread penetration, a number of changes are observed in the cortical cells. The nucleus swells and moves to the middle of the cell (Figure 4H, arrowhead) and the cytoplasm obtains a polar organization to sustain the growth of the tip growing infection thread by forming a radially oriented conical structure designated as preinfection thread (Figure 4H, arrow; van Brussel et al., 1992). *In situ* hybridization with *rh6* ^{35}S-UTP labelled antisense RNA was performed on cross sections of root segments containing preinfection structures. Expression of the gene was observed in front of infection threads, in cells that contain preinfection threads (Figure 4H&I). Hybridizations with *rh6-1* have not been done yet.

Thus, the symbiont induces the expression of a gene in the cortex, that is normally expressed in growing root hairs and pollen tubes. It seems that expression of *rh6* is correlated with polar growth since the cells containing preinfection threads, root hairs and pollen tubes support tip growth.

DISCUSSION

We isolated two cDNA clones with differential screening of a pea root hair cDNA library that represent genes expressed specifically in root hairs, pRH6 and pRH6-1. Expression of both genes occurs also during germination of pollen tubes, indicating the proteins play a more common role during tip growth. In addition, it has been shown that *rh6* is involved in preinfection thread formation.

rh6 as well as *rh6-1* encode a putative peroxidase. Peroxidases are grouped in three classes depending on their isoelectric point; cationic (pI 8.1-11), moderate anionic (pI 4.5-6.5) and anionic (pI 3.5-4). RH6 belongs to the class of the cationic peroxidases and RH6-1 to the moderate anionic ones. Both cationic and moderate anionic peroxidases have been isolated from cell walls of different plants (Kerby and Somerville, 1992; Reimens et al., 1992). Cationic peroxidase isozymes though are often vacuolar (Madern, 1986). Whether RH6 is localized in the vacuole or the cell walls is not clear at present. Each plant species has a number of peroxidase isozymes which show differences in substrate specificity. Some isozymes are implicated in polysaccharide cross-linking, others in cross-linking of extensin monomers, while others in indole-3-acetic acid oxidation, or ethylene biosynthesis (Greppin et al., 1986). Cross-linking of cell wall proteins and polysaccharides has been attributed to cationic, anionic and moderate anionic isozymes present in the cell walls. The existence of different isozymes in the cell walls might be due to differences in substrate specificity. Vacuolar cationic isozymes have been implicated in hormone biosynthesis.

The expression of different peroxidase genes is tissue specific, developmentally regulated or modulated by environmental stress factors, and pathogen attack. *In situ* hybridization studies showed that both *rh6* and *rh6-1* are expressed in root hairs and germinating pollen, indicating that the genes are developmentally regulated. During root hair growth and germination of pollen, new cell wall biosynthesis takes place. Therefore, it is tempting to speculate that RH6 and RH6-1 play a role in this process, either by crosslinking extensins or by crosslinking polysaccharides. Our observation that *rh6* and *rh6-1* are expressed in both pollen tubes and root hairs, shows that common genes are used in tip growing plant cells. This is complementary to the analysis of the *tip1* mutant of *Arabidopsis*, where it has been demonstrated with a genetic approach that there is a common basis for tip growth of plant cells.

The availability of cloned sequences of genes expressed in tip growing cells, provided the means to check on a molecular level, whether infection thread formation involves mechanisms used by other tip growing cells. The observation that *rh6* is expressed in preinfection thread forming cells strongly supports this hypothesis that was originally based on cytological studies (van Brussel et al., 1992).

RH6 shows the highest homology to the *Rhizobium meliloti* induced peroxidase of alfalfa (*rip1*). *rip1* is induced in the epidermis by rhizobial Nod factors and like *rh6* is not induced in the nodule. In addition, no expression of *rip1* was reported in the cortical cells upon

in the nodule. In addition, no expression of *rip1* was reported in the cortical cells upon inoculation. However, induction of *rip1* in the cortex was studied by means of northern blot analysis. Therefore, it is still possible that the level of *rip1* induction is below the detection limits of this method, since in our experiments it was shown that *rh6* is expressed only in the cortical cells forming the preinfection threads and not in the cortical cells containing infection threads. Thus, even though the expression patterns of *rip1* and *rh6* are not identical, it is still possible that both peroxidase genes play a similar role in the interaction.

It is noteworthy, that in both root hairs and pollen tubes the messengers of *rh6* and *rh6-1* are localized in a similar manner. *rh6* mRNA is located uniformely along the outgrowths and *rh6-1* mRNA is located mainly at the tip. Localized messengers have been observed in other polar cell types (Johnston, 1995). It has been postulated that the messengers are localized in the position where the encoded protein is needed (Johnston, 1995). *rh6-1* is expressed in young root hairs and not in mature root hairs. Since the messenger is located at the tip of the outgrowth, -in root hairs and pollen-, one can speculate that RH6-1 plays a role in new cell wall biosynthesis. In contrast, *rh6* is expressed in both young and mature root hairs and the messenger is located along the outgrowth of both root hairs and pollen. Therefore, a role of the protein in secondary cell wall modifications of the outgrowths can be envisioned.

METHODS

Plant material and growth conditions

Pea seeds (*Pisum sativum* cv. Finale; Cebeco, Netherlands) were grown in gravel (Bisseling et al., 1978). Root segments containing root hairs were harvested from 6-day old pea seedling and immediately frozen in liquid nitrogen.

For whole mount *in situ* hybridization experiments, plants were grown on plates containing Fåhraeus medium (Fåhraeus, 1957) in 1% agar. Some of the plants were spot-inoculated with *Rhizobium leguminosarum* bv. *viciae*. Root segments of the spot-inoculated area were collected after 7 days.

Pollen tube germination

Pollen grains were collected from freshly opened flowers and spread into a liquid culture medium, containing 15%-20% sucrose, 0.03% $CaCl_2$, and 0.01% H_2BO_3. The isolation was followed by a centrifugation step at 800 rpm that was repeated twice. Pollen grains were incubated for 1h at 25°C in the dark.

Root hair isolation and RNA isolation

Root hairs were harvested by the method described by Röhn and Werner (1987), modified as described by Gloudemans et al. (1989).
Frozen roots and root hairs were ground in liquid nitrogen and resuspended in a hot (90°C) mixture of RNA extraction buffer (0.1 M Tris-HCl pH 9.0, 0.1 M LiCl, 10 mM EDTA, 1% SDS) and phenol (1:1). After vortexing and centrifugation (30 min, 6000 X *g*) the water phase was collected and RNA was extracted as described by Pawlowski et al. (1994).

Differential screening

A cDNA library was constructed by Stratagene in λZAPII vector system, using poly(A)$^+$ RNA isolated from root hairs of uninoculated and inoculated with *R. leguminosarum* bv. *viciae* pea plants (*Pisum sativum* bv Finale).
This library was plated at a density of approximately 30000 plaques per 15-cm-diameter plate that were transferred onto nitrocellulose filters in duplicates. The filters were screened by differential hybridization with root-specific or root hair-specific radioactively labelled first-strand cDNA. Autoradiograms were screened for hybridization with the root hair specific cDNA probe only. The putative positive plaques were purified further with two rounds of differential screening. Inserts of the selected plaques were converted into plasmid vectors according to the established protocols (Stratagene).

Northern blot analysis

Total RNA was denatured in DMSO/glyoxal, separated on 1% agarose gels and blotted onto GeneScreen in 0.025 M NaH_2PO_4 pH 7.0. The blots were hybridized in 50% formamide, 1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 7.0, 10 x Denhard's solution at 42°C. The pRH6 and pRH6-1 inserts were radiolabelled by random priming using [α-^{32}P]dATP (3000 Ci/mmol; Amersham) as radioactive label.

Nucleotide sequencing

The nucleotide sequencing of the inserts of pRH6 and pRH6-1 was determined by double-stranded sequencing using the ^{T7}Sequencing kit from Pharmacia Biotech.

In situ hybridization

In situ hybridization experiments with ^{35}S-UTP labelled antisense and sense RNA probes were performed according to van de Wiel et al. (1990).

For the whole mount *in situ* hybridization, roots of *P. sativum* were fixed in PBS, 4% paraformaldehyde, 0.25% glutaraldehyde, 0.08 M EGTA, 10% DMSO, and 0.1% Tween 20, for 3 hrs at room temperature. *In situ* hybridization was performed essentially as described by Tautz and Pfeifle (1989), with modifications. The heptane washes were eliminated. Tissue was kept in ethanol, after fixation, at -20°C for 2 days. Before the proteinase K treatment, tissue was incubated for 30 min in 1:1 ethanol/xylene solution. This treatment was followed by a postfixation step in PBS, 0.1% Tween 20, and 5% formaldehyde. After the proteinase K treatment the same postfixation step was applied. Prehybridization and hybridization took place at 42°C. For the post-hybridization washes an RNase A treatment for 15 to 30 min, was included (40μg/ml RNase A in 500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). Before use, the anti-digoxygenin antibodies coupled to alkaline phosphatase (Boehringer Mannheim) were preabsorbed in an acetone extract of fixed roots (overnight at 4°C). The final concentration of the antibodies used, was 1:2000. Incubation with the antibody took place at 4°C overnight. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate, Boehringer Mannheim) and nitroblue tetrazolium (NBT, Boehringer Mannheim) was carried out for 30 min to several hours. A sense probe was used as control.

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CHAPTER 6

Concluding remarks

The aim of this thesis was to obtain more insight in the *Rhizobium* infection process by testing the hypothesis that infection thread formation and root hair development share common mechanisms. We initiated studies on root hair development by the isolation and characterisation of cDNA clones of genes specifically expressed in the root epidermis. One of the root hair specific clones was used to check the working hypothesis. An evaluation of the obtained results is provided in this section.

Expression and function of the epidermis/root hair specific genes

Our studies resulted in the isolation of four cDNA clones which represent genes that play different roles in the development of the root epidermis. *rh2* is expressed in all epidermal cells in a region starting at the protoderm and extending into the zone with mature hairs. Furthermore, it is expressed in the protoderm of pea embryos in the part that will form the radicle. Thus, *rh2* is an excellent marker for the root epidermis and its promoter can be an attractive tool for manipulation of gene expression in the root epidermis.
rh4, *rh6*, and *rh6-1* are expressed in trichoblasts and appear to be active during three successive but overlapping stages of development. *rh4* is induced already in trichoblasts prior to the emergence of the hair and its expression is sustained during root hair growth. *rh6-1* expression takes place in emerging and growing root hairs, whereas *rh6* is expressed in growing and mature root hairs. Remarkably, expression of *rh4* is restricted to epidermal cells positioned opposite a protoxylem pole, whereas *rh6* and *rh6-1* are expressed in all hairs of their respective developmental zone. Thus, the developmental stage of the epidermal cell as well as positional information provided by the stele appear to control *rh4* expression, whereas expression of *rh6* and *rh6-1* is only regulated by the developmental stage of the root hair.
The sequence characteristics of the proteins encoded by the studied cDNA clones leaves some space for speculations on their putative function. *rh2* belongs to a gene family of which one member has been shown to be involved in a pathogen induced defense response. Since there are other pathogen induced genes that are expressed in the root epidermis like chalcone synthase (Schmidt et al., 1990; Yang et al., 1992) and chitinase (Samac and Shah, 1991), it is possible that a defence barrier is present constitutively in the root epidermis. *rh6* and *rh6-1* encode putative peroxidases. Thus we can speculate about a role of these peroxidases in cell wall biosynthesis in crosslinking of either cell wall proteins or polysaccharides. RH4 has no homology to any protein with a known function, except in that it contains an EGF-like repeat. EGF-like repeats are implicated in signalling and cell-cell communication. At present it is not clear whether RH4 is involved in such a process.

Localized mRNAs in root hairs and pollen tubes

In polar growing cells mRNAs can be located in a specific part of the cell. To determine the intracellular location of the *rh* mRNAs, we used whole mount *in situ* hybridisation with digoxigenin labelled probes. These studies showed that the *rh* mRNAs are located in the hair itself and not in the basal part of the cell. *rh4* and *rh6-1* mRNAs are found preferentially at the tip of the hair, whereas *rh6* mRNA is present in the complete outgrowth. The intracellular distribution of *rh6* and *rh6-1* mRNA was also studied in germinating pollen, revealing a strikingly similar distribution of both mRNAs in pollen tubes and root hairs. Whether *rh2* and *rh4* are also expressed in pollen is unknown. In trichoblasts that have not yet formed a hair, *rh4* mRNA is targeted to the future site of root hair emergence, whereas in similar epidermal cells *rh2* mRNA is found in all parts of the cytoplasm. Taken together these studies show that the *rh* mRNAs do not have an identical cellular localization in trichoblasts, but that some mRNAs must be targeted to a specific part of the cell.

The occurrence of a differential distribution of mRNAs in germinating pollen tubes has been reported recently (Torres et al., 1995). It was shown that the mRNA of a hydroxyproline-rich glycoprotein is present in the tube, and α-tubulin mRNA is present in both parts of the cell (Torres et al., 1995).

It has been speculated that the mRNAs are localized at the site where the proteins are needed (Johnston, 1995). Thus, we can presume that RH4, RH6 and RH6-1 function in the root hair, RH4 and RH6-1 specifically at the tip and RH6 along the outgrowth. mRNA localization can play a direct role in the establishment of polarity either within a single cell, as it has been reported for β-actin (Kislauskis et al., 1994), or in the polarity of embryos as it has been shown for the localization of certain messengers in *Drosophila* and *Xenopus* oocytes (Johnston, 1995). For instance, a small region of 53 bases called BLE1 element is sufficient for direct localization of the messenger to the interior site of the *Drosophila* oocytes (Macdonald et al., 1993).

The only RNA-binding protein that has been proven to play a role in mRNA localization is the product of the Drosophila maternal gene *staufen* (Johnston, 1995). Whether similar mechanisms are involved in the localization of the root hair specific mRNAs is not clear yet. None of the identified 3'UTRs involved in targeting mechanisms analysed to date, are present in our messengers. Therefore, it will be very interesting to map these regions in the *rh* mRNAs in order to define the localization sequences and afterwards to disrupt them in order to find out whether they play a role in the establishment of polarity, or whether their localization is a result of the existing polarity. Furthermore, it would be interesting to find out which cytoskeletal elements are involved in *rh* mRNA localization.

Correlation between root hair growth and infection thread formation

Genetic studies resulted in the isolation of a number of *Arabidopsis* root hair mutants. One of these mutants is affected in development of both root hairs and pollen tubes, by which it can be hypothesised that polar growth of different types of plant cells involve common mechanisms (Schiefelbein et al., 1993). Our observation that *rh6* and *rh6-1* are expressed in both root hairs and pollen tubes strengthens this hypothesis.

Cytological studies on the formation of infection and preinfection threads have indicated a relation between these processes and root hair development (Van Brussel et al., 1992). The observed expression of *rh6* , in cells forming preinfection threads supports the hypothesis that the infection process involves genes that are recruited from common plant development; in this case the mechanism controlling tip growth. Along the same line it has been reported that the root cortical cells involved in the infection process enter the cell cycle, but become arrested in G2 and therefore do not divide but form a track for the growing infection thread. Thus a common process is modified and used for a step in the symbiotic interaction (Yang et al., 1994). This notion holds also true for other steps of nodule development. For example, it has been shown that rhizobial Nod factors induce a response in the non-legume tobacco (Röhrig et al., 1995). Nod factors are able to induce cell division in tobacco protoplasts in the absence of phytohormones. This shows that a Nod factor perception mechanism is present in the non-legume tobacco and suggests that Nod factor like molecules are endogenous plant signal molecules. Taken together, these studies strongly indicate that the developmental program controlling root nodule formation uses elements that are recruited from common plant developmental processes.

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SUMMARY

The symbiotic interaction between *rhizobia* and legumes leads to the formation of a special organ, the root nodule. Bacteria enter the plant via the root hairs, inducing the formation of special structures growing inwards, called infection threads. Infection threads elongate by growth at the tip, a process that requires a polarized cytoplasmic organization. The aim of my work was to initiate a study on the development of legume root hairs and to use the molecular tools obtained in such study, to examine correlations between root hair formation and infection thread formation. For that purpose, we decided to isolate cDNA clones corresponding to genes expressed specifically in the root hairs/root epidermis. A dual approach was followed, namely PCR-based cloning and differential screenings of a pea root hair cDNA library.

The most abundant protein occurring in pea root hairs was purified and microsequensed. Specific oligos were designed as primers for a PCR-based cloning of the cDNA clone pRH2. *In situ* hybridization experiments showed that *rh2* is expressed in all cells of the epidermis, cells that contain root hairs and cells lacking these tubular outgrowths. Expression of the gene starts at a developmental stage preceding hair emergence, in the protoderm. *rh2* belongs to a gene family, members of which were shown to be induced in leaves, by the pathogen *Fusarium oxysporum*. Therefore, it is possible that RH2 is part of a constitutive defense barrier present in the root. The expression pattern makes the *rh2* promoter a good candidate for genetic engineering of the root epidermis (Chapter 3).

By differential screening of a pea root hair cDNA library, putative root hair specific cDNA clones were isolated. *rh4* a gene corresponding to one of these clones, encodes a protein that has one EGF-like repeat. *rh4* is expressed in trichoblasts prior to root hair emergence, and also during emergence and elongation of the root hairs. *rh4* mRNA shows a polar distribution being localized in the site of root hair emergence. Expression of the gene is restricted to the epidermal cells positioned opposite to protoxylem poles of the root vascular bundle. It was shown that lectins have a similar position. This indicates that epidermal cells around the root do not have a uniform character. It is noteworthy that nodules produced following infection by *Rhizobium* bacteria are also formed opposite protoxylem poles. Therefore, it is possible that RH4 plays a role in the interaction either directly, when the gene is regulated by the symbiont, or indirectly by providing to the root hairs, together with other proteins, the required identity for this interaction (Chapter 4).

rh6 and *rh6-1* are two putative peroxidase genes that are expressed in root hairs and germinating pollen, indicating that they may play a more general role in polar growth, since both root hairs and pollen exhibit tip growth. This expression pattern makes these clones good candidates to check whether infection thread formation shares common features with other polar growth processes. It was shown that *rh6* is indeed induced upon inoculation with *rhizobia*, in the cortical cells prior to infection thread penetration. The gene is expressed in polarized cells that have formed cytoplasmic bridges, called preinfection threads (pit). Infection threads grow

through the cytoplasmic bridges in a manner resembling tip growth. The expression of *rh6* in the pit containing cells indicates the existence of similarities on a molecular level between root hair formation, pollen tube germination and infection thread formation (Chapter 5)

In an attempt, to bypass the production of specific antibodies epitope tagging was initiated, in order to perform immunolocalization studies on the early nodulins ENOD5 and ENOD12. These genes encode putative cell wall proteins which have been speculated to be localized in the infection threads. Fusion proteins were made with an oligopeptide for which there are antibodies commercially available. The constructs encoding the fusion proteins were used to transform vetch. Western blot analysis showed that the trangenes are transcribed and translated in our trangenic plants. The epitope-tagged proteins were detected in the cell wall fraction of the protein isolates from transgenic nodules. Immunolocalization studies though, performed on sections of transgenic nodules and transfected with the constructs cowpea protoplasts, gave no clear answer on their precise location. The major problem encountered is the high background caused by the used antiserum, indicating that the selected tag is inappropriate for studies on nodules (Chapter 2).

SAMENVATTING

De symbiotische interaktie tussen rhizobia en vlinderbloemigen lijdt tot de formatie van een speciaal orgaan, de wortelknol. Bacteriën komen de plant binnen via de wortelharen en induceren de vorming van speciale strukturen die naar binnen groeien, de zogenaamde infectiedraden. Infectiedraden worden langer door middel van groei van de top, een proces waarvoor het cytoplasma gepolarizeerd moet zijn. Het doel van mijn werk was het beginnen van een studie naar de ontwikkeling van de wortels van vlinderbloemigen en het gebruik van moleculaire technieken, ontwikkeld tijdens deze studie, om de correlatie tussen wortelhaarvorming en de vorming van infectiedraden te onderzoeken. Voor dat doel hebben we besloten tot het isoleren van cDNA kloons, die corresponderen voor genen die specifiek tot expressie komen in de wortelhaar/wortel epidermis. Een tweezijdige benadering werd gevolgd, namelijk een op de polymerase kettingreaktie gebaseeerde klonering en er werd gezocht naar verschillen in erwtewortel cDNA banken.

Het meest voorkomende eiwit in erwtewortels is gezuiverd en er is een microsequentiebepaling op uitgevoerd. Specifieke oligo's werden ontworpen om te gebruiken als 'primers' voor een polymerase kettingreaktie gebaseerde klonering van de cDNA kloon pRH2. *In situ* hybridisatie experimenten tonen aan dat *rh2* tot expressie komt in alle cellen van de epidermis, cellen die wortelharen bevatten en cellen die deze uitgroeiingen missen. Expressie van het gen begint in het ontwikkelingsstadium dat vooraf gaat aan het uitgroeien van de wortelharen, in de protoderm. *rh2* behoort tot een genfamilie. Er is aangetoond dat de pathogeen *Fusarium oxysporum* leden van deze genfamilie kan induceren in bladeren. Het is dus mogelijk dat RH2 een onderdeel is van een constitutieve defensie barrière, die aanwezig is in de wortel. Het expressiepatroon maakt van de *rh2* promoter een goede kandidaat voor genetische verandering van de wortelepidermis (Hoofdstuk 3).

cDNA kloons die vermoedelijk specifiek zijn voor wortelharen zijn geïsoleerd met behulp van een erwte-cDNA bank. *rh4*, een gen dat correspondeert met een van deze kloons, codeert voor een eiwit met een 'EGF achtige' herhaalde sequentie. *rh4* komt tot expressie in trichoblasten, voordat de wortelhaar uitgroeit en ook tijdens het uitgroeien en langer worden van de wortelhaar. *rh4* boodschapper RNA vertoont een polaire verdeling, aangezien het gelocalizeerd is daar waar de wortelhaar uitgroeit. Expressie van het gen is beperkt tot epidermiscellen die zich bevinden tegenover de protoxyleempolen van de vaatbundel in de wortel. Er is aangetoond dat ook lectines zich daar bevinden. Dit wijst er op dat de epidermiscellen rond de wortel onderling verschillen. Het is opmerkelijk dat ook wortelknollen, die ontstaan na infectie met *Rhizobium* bacteriën, tegenover de protoxyleempolen gevormd worden. Dus het is mogelijk dat RH4 een rol speelt bij de interaktie, hetzij direkt indien het gen gereguleert wordt door de symbiont, hetzij indirekt door de wortelharen de juiste identiteit te verschaffen, samen met andere eiwitten, die nodig is voor deze interaktie (Hoofdstuk 4).

rh6 en *rh6-1* zijn twee vermeende peroxidase genen die tot expressie komen in wortelharen en kiemend pollen, hetgeen er op wijst dat ze een meer algemene rol zouden kunnen spelen bij polaire groei, aangezien zowel wortelharen als kiemend pollen 'top groei' vertonen. Dit expressiepatroon maakt van deze kloons goede kandidaten om te controleren of de vorming van infectiedraden dezelfde kenmerken vertoont als andere polaire groeiprocessen. Het is aangetoond dat *rh6* inderdaad geïnduceerd wordt na inoculatie met rhizobia en wel in cortexcellen, voordat de infectiedraad binnendringt. Het gen komt tot expressie in gepolarizeerde cellen die cytoplasmatische bruggen vormen, de zogenaamde preïnfectiedraden. Infectiedraden groeien door deze cytoplasmatische bruggen op een manier die lijkt op 'top groei'. De expressie van *rh6* in de cellen die preïnfectiedraden bevatten duidt op het bestaan van overeenkomsten op moleculair niveau tussen wortelhaar vorming, pollenbuis kieming en infectiedraad vorming (Hoofdstuk 5).

In een poging om de produktie van specifieke antilichamen te omzeilen werd er begonnen met epitoop 'tagging' om immunolocalizatie studies uit te voeren met de vroege nodulines ENOD5 en ENOD12. Deze genen coderen voor vermeende celwandeiwitten en er wordt gespeculeert dat ze gelocalizeerd zijn in infectiedraden. Er werden fusie-eiwitten gemaakt met een 'tag' waarvoor commerciële antilichamen verkrijgbaar zijn. De constructen die coderen voor de fusie-eiwitten werden getransformeerd naar wikke. Eiwitblot analyses hebben aangetoond dat de transgenen getranscribeerd en getransleerd worden in onze getransformeerde planten. De eiwitten met epitoop 'tags' werden aangetoond in de celwandfractie van eiwitten geïsoleerd uit transgene wortelknollen. Echter, immunolocalizatie studies, uitgevoerd op secties van transgene wortelknollen en protoplasten die getransformeerd waren met de constructen, geven geen duidelijkheid over de exacte localizatie. Het belangrijkste probleem is de hoge achtergrond, veroorzaakt door het gebruikte antiserum, hetgeen aantoont dat de gekozen 'tag' niet bruikbaar is voor studies met wortelknollen (Hoofdstuk 2).

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CURRICULUM VITAE

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