The *Lotus japonicus* *LjSym4* Gene Is Required for the Successful Symbiotic Infection of Root Epidermal Cells

Paola Bonfante,¹ Andrea Genre,¹ Antonella Faccio,¹ Isabella Martini,¹ Leif Schauser,² Jens Stougaard,² Judith Webb,³ and Martin Parniske⁴

¹Dipartimento di Biologia Vegetale dell'Università and CSMT-CNR 10125 Torino, Italy; ²Laboratory of Gene Expression, Department of Molecular and Structural Biology, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark; ³Institute of Grassland and Environmental Research, Aberystwyth, Ceredigion, SY23 3EB, U.K.; ⁴The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich, NR4 7UH, U.K.

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The role of the *Lotus japonicus* *LjSym4* gene during the symbiotic interaction with *Mesorhizobium loti* and arbuscular mycorrhizal (AM) fungi was analyzed with two mutant alleles conferring phenotypes of different strength. *Ljsym4-1* and *Ljsym4-2* mutants do not form nodules with *M. loti*. Normal root hair curling and infection threads are not observed, while a *nodC*-dependent deformation of root hair tips indicates that nodulation factors are still perceived by *Ljsym4* mutants. Fungal infection attempts on the mutants generally abort within the epidermis, but *Ljsym4-1* mutants allow rare, successful, infection events, leading to delayed arbuscule formation. On roots of mutants homozygous for the *Ljsym4-2* allele, arbuscule formation was never observed upon inoculation with either of the two AM fungi, *Glomus intraradices* or *Gigaspora margarita*. The strategy of epidermal penetration by *G. margarita* was identical for *Ljsym4-2* mutants and the parental line, with appressoria, hyphae growing between two epidermal cells, penetration of epidermal cells through their anticlinal wall. These observations define a novel, genetically controlled step in AM colonization. Although rhizobia penetrate the tip of root hairs and AM fungi access an entry site near the base of epidermal cells, the *LjSym4* gene is necessary for the appropriate response of this cell type to both microsymbionts. We propose that *LjSym4* is required for the initiation or coordinated expression of the host plant cell's accommodation program, allowing the passage of both microsymbionts through the epidermis layer.

Additional keywords: cell death, cell wall.

The symbiosis of plant roots with arbuscular mycorrhizal (AM) fungi is widespread both geographically and systematically, probably as a consequence of its presence in the earliest land plants (Remy et al. 1994) and the selective advantage that

plants gain from the symbiosis. The improved uptake of phosphate by AM is of crucial importance in many tropical soils and the impact of AM on ecosystem diversity in moderate climates is significant (Van der Heijden et al. 1998).

Plant mutants unable to form AM are a powerful tool to identify genetically defined steps in the development of the symbiotic interaction. The genetic dissection of AM development has been pioneered by the isolation of pea mutants impaired in AM symbiosis. These mutants were initially identified through their altered root nodule symbiosis with *Rhizobium* spp. Subsequently, it was found that a subset of the nodulation mutants were also affected in the AM symbiosis (Duc et al. 1989). This finding demonstrated an overlap in the genetic programs for the two diverse symbiotic interactions (Duc et al. 1989; Hirsch and Kapulnik 1998; Albrecht et al. 1999). Unfortunately, the isolation of the affected genes from pea is hampered by its large genome size. We therefore chose to isolate symbiotic mutants from the more amenable model legume *Lotus japonicus* (Handberg and Stougaard 1992) with the final goal to clone and functionally characterize the responsible genes. A large collection of symbiotic mutants of *L. japonicus* has previously been isolated. Phenotypically, these mutants do not form nodules after inoculation with *Mesorhizobium loti*, or they form nodules that do not fix nitrogen (Imaizumi-Anraku et al. 1997; Schauser et al. 1998; Szczygłowski et al. 1998; J. Webb, unpublished data). A subset of these mutants was tested by Wegel et al. (1998) for their interaction with the AM fungus *Glomus intraradices*, and a number of nodulation mutants that were also impaired in the AM symbiosis were identified. Mutants in either of the genes *LjSym2*, *LjSym3*, and *LjSym4* all displayed a similar phenotype. Fungal infection was blocked at a very early stage of the infection; however, rare successful infection events occurred, leading to the development of structurally intact arbuscules. Wegel et al. (1998) concluded that the *LjSym2*, *LjSym3*, and *LjSym4* genes played a role during the early stages of the symbiotic infection; however, they were not required for arbuscule formation.

We have now identified a *Ljsym4-2* allele in which arbuscule formation was never observed in the interaction with two diverged AM fungi, *Glomus* spp. and *Gigaspora* spp. The detailed microscopy of the *Ljsym4-2* phenotype, combined with

Corresponding author: P. Bonfante; Dipartimento di Biologia Vegetale dell'Università and CSMT-CNR, Viale Mattioli 25 10125 Torino, Italy; Telephone: 0039 011 6502927; Fax: 0039 011 6707459; E-mail: p.bonfante@csmt.to.cnr.it

immunolabeling of cell wall components, revealed that epidermal cell penetration by *G. margarita* was associated with localized host epidermal cell death. The rhizobial symbiosis is arrested before infection pockets are formed by root hair curling. This comparative study indicates the *LjSym4* gene is required for the epidermal interaction in both symbiotic systems and describes a hitherto unknown active involvement of epidermal cells in the mycorrhizal invasion process.

RESULTS

EMS1749 harbors a single recessive mutation affecting both the root nodule and AM symbiosis.

Among the non-nodulation mutants isolated after chemical mutagenesis of *L. japonicus* (J. Webb, unpublished data), one

mutant (*EMS1749*) was found to be strongly affected in its interaction with *Glomus intraradices*. The phenotype of this mutant was stable in the M_5 generation and the segregation of the mutant phenotype in progeny of the cross to wild-type (WT) *L. japonicus* ecotype Funakura was 3:1 (42:15; $\chi^2 = 0.053$; $P > 0.05$). The segregation data indicated that a single, recessive gene was affected in this mutant.

EMS1749 represents a novel allele of the *LjSym4* locus.

The *LjSym2*, *LjSym3*, and *LjSym4* loci were previously shown to be required for both root symbioses (Schauser et al. 1998; Wegel et al. 1998). We therefore performed complementation crosses between *EMS1749* and plants carrying mutations in these genes. Progeny from crosses of *EMS1749* to either *Ljsym2-2* or *Ljsym3-1* lines formed root nodules upon

Fig. 1. *Mesorhizobium loti* and *Gigaspora margarita* infection process on wild-type (WT) and *Ljsym4* mutant plants. **A**, Infection threads (arrowheads) in WT plant visualized by β -galactosidase staining of *hema-lacZ* bacteria. Insert: top view of a root hair curling around stained bacteria (*). **B**, Root hairs on *Ljsym4-2* mutants inoculated with the *M. loti* *nodC::Tn5* mutant strain unable to synthesize lipooligosaccharides. **C**, Balloon swelling (arrowheads) and curving (arrow) of root hair tips on *Ljsym4-1* mutants inoculated with *M. loti*. **D**, Balloon swelling (arrowhead) of root hair tips on *Ljsym4-2* mutants inoculated with *M. loti*. **E**, *G. margarita* colonization on WT roots: hyphae (arrowheads) form appressoria on root surface, penetrate epidermis (e), and reach the cortical cells, where they form the arbuscules (a). **F**, *G. margarita* penetration attempt on *Ljsym4-2* mutant roots: extraradical hyphae form a branched appressorium (arrowheads) from which various pegs (arrows) penetrate between two adjacent epidermal cells; eventually, one of them passes through the epidermal wall forming a swollen intracellular structure (*). Image obtained by overlapping two photographs taken at different focal planes, the first at the epidermis surface, the second inside the colonized epidermal cell lumen. Bars = **A**, 2 mm; **A**, insert, 0.5 mm; **B–D**, 1 mm; and **E** and **F**, 50 μ m.

inoculation with *M. loti*. In contrast, progeny of multiple crosses between EMS1749 and *LjSym4* consistently failed to form nodules under these conditions. We conclude that EMS1749 harbors a novel mutant allele of the *LjSym4* locus, hereafter referred to as *Ljsym4-2*. The original *Ljsym4* allele described by Schauser et al. (1998) is reassigned *Ljsym4-1* according to *Lotus* genetic nomenclature (Stougaard et al. 1999).

The *Ljsym4-1* and *Ljsym4-2* alleles confer different phenotypic strength.

To compare the early interactions between the two *Ljsym4* mutants and the symbiotic microorganisms, plants were inoculated with *M. loti* and *Glomus intraradices*. As both *Ljsym4-1* and *Ljsym4-2* are non-nodulating mutants, an *M. loti* strain carrying a *hemA-lacZ* marker was used to follow the bacterial infection of the root tissue. No infection threads were formed on the two mutant lines (data not shown), while infection threads passing through root hairs into the root cortex were clearly visible on WT control plants (Fig. 1A). On WT plants, lipochitin-oligosaccharide (LCO)-dependent formation of infection pockets by root hair curling precedes infection

thread formation (Fig. 1A, insert) (Schauser et al. 1999). Both *Ljsym4* mutants were therefore assayed for root hair curling with WT *M. loti* and a bacterial *nodC* mutant unable to produce LCO Nod factors. Root hair curling was not observed on either of the mutants. Both *Ljsym4-1* and *Ljsym4-2* mutants respond to WT *M. loti* with swelling of the root hair tips (Fig. 1C and D). Additional deformations like swelling and curving were observed on the *Ljsym4* mutants (Fig. 1C). No swelling was observed in response to the *nodC* bacterial mutant (Fig. 1B). Foci of dividing cortical cells developing into nodule primordia were only observed on the WT plants (data not shown). These results show that the interaction between *Ljsym4* mutants and the rhizobial symbionts is arrested before infection thread formation and cortical cell divisions are initiated. However, the initial perception of the LCO signal appears to be functional in the mutants. We conclude that the *LjSym4* gene is required for progression of the root hair response into curling and formation of infection pockets.

After 58 days from inoculation of chive nurse plants with *Glomus intraradices*, the entire root system of 20 plants of each mutant was analyzed. On roots of both mutants, abortive

Fig. 2. Development of *Gigaspora margarita* **A and B**, on *Lotus japonicus* wild-type (WT) line Gifu and **C and D**, on *Ljsym4-2* mutant, as seen in oblique root sections after staining with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA) and overlapping of about 20 confocal optical sections, sampled at 1- μ m steps. **A**, Fungal hyphae (h) developing at epidermis surface (e) and branched arbuscules (a) colonizing the cortex (c). A dashed line marks cutting edge separating outside (right part) from inside (left part) of root. **B**, Detail of an extraradical hypha (h) contacting the epidermis. On the root surface the fungus frequently grows above the grooves formed by two adjacent epidermal cells. **C**, Abundant fungal hyphae (h) are present on the root epidermis (e) of *Ljsym4-2* mutant, while the cortex (c) is not infected. Dashed line marks cutting edge separating outside (right part) from inside (left part) of the root. **D**, Detail of an appressorium, with hyphae (h) contacting the epidermal cells. Arrowheads: infection pegs penetrating deeper in the epidermis, between two adjacent cells. Bars = **A and C**, 40 and **B and D**, 20 μ m.

infection events were observed at a high density. On *Ljsym4-1* mutants, a small subset of infection attempts was, however, successful. The fungus was able to reach the cortical cell layer of the roots and arbuscules were formed. Lateral spread of the fungus led to the colonization of a long section of the root as the consequence of each single successful infection event. In each of the 20 *Ljsym4-1* plants, consistently more than 40% of the root system was colonized. Despite the very strong infection pressure conferred by the fungus nourished by the chive nurse plants, *Glomus intraradices* was unable to penetrate the roots of the *Ljsym4-2* mutants beyond the epidermis. This result was confirmed in several independent experiments involving plants of various ages grown in different seasons. Arbuscule formation by *Glomus intraradices* in roots of *Ljsym4-2* mutant was never observed in more than 50 plants analyzed. The same results were obtained when *G. margarita* was used as a fungal symbiont on WT and *Ljsym4* mutants (Fig. 1E and F).

Light microscopical analysis of fungal development on *L. japonicus* roots.

The mycorrhizal invasion process of *L. japonicus* is poorly described. We therefore subjected the WT line Gifu to a detailed, morphological analysis to characterize its symbiotic

development as a basis for comparison with the symbiotic mutant *Ljsym4-2*. We chose *Ljsym4-2* because of the strong and clear-cut phenotype, and, to analyze the early cellular interactions between AM fungi and plant roots, the millipore sandwich method was chosen. This method provides clean and sterile conditions, and restricts fungal growth to two dimensions, leading to a rapid infection. The inoculum can be reproducibly applied because the spore number can be determined. To ease the spore handling involved in the method the large spores of *G. margarita* were used in this study. During 3 weeks of growth in the millipore sandwich, *G. margarita* produced abundant, extraradical hyphae and auxiliary cells irrespective of the plant genotype (data not shown). Hyphae on the root surface were in loose contact with epidermal cells (Fig. 2A and B), developing appressoria that produced penetrating hyphae (Fig. 2B). On the WT plant, penetration hyphae grew through the epidermal cells and developed intracellular hyphae that, in turn, produced arbuscules (Fig. 2A) in the inner cortical layer, close to the endodermis.

A comparison between the number of penetration points in WT and mutant roots coming from three sets of experiments resulted in two similar patterns: 1.96 and 2.76 penetration points per cm in WT and mutant plants, respectively, with a very high variability

Fig. 3. Ultrastructural features of epidermal cells in *Lotus japonicus* wild-type (WT) line Gifu in the absence of the fungus. **A**, Transverse section of a root in the differentiating region. Epidermal cells (e) are tightly joined through their anticlinal walls (r). Central cell, rich in cytoplasm, is developing a root hair (n = nucleus, c = cortical cell). **B**, In a more basal part, epidermal cells (e) are highly vacuolated and tightly joined through their anticlinal walls (r). Immunolabeling with monoclonal antibody (MAb) JIM 5. **C**, Gold granules are mostly present in inner layer of the tangential wall (t) after immunolabeling with the MAb JIM 7 to detect methyl-esterified pectins. **D**, Material within triangular junction (j) between epidermal (e) and cortical cells is rich in nonesterified pectin (immunolabeling with the MAb JIM 5). Bars = **A and B**, 2 and **C and D**, 0.5 μ m.

(standard deviation = 1.73 and 2.04, respectively). The arbuscule rate (Trouvelot et al. 1986) in WT mycorrhizal roots was 57.5%.

At the root surface of the *LjSYM4-2* mutant, abundant hyphae proliferated (Fig. 2C) that, upon contact with root epi-

dermal cells, produced appressoria and penetration hyphae, from which hyphal projections (pegs) originated (Fig. 2D). These penetrated epidermal cells, but ceased growing inside this cell layer (Figs. 2D and 5–7). Consistent with the results

Fig. 4. Light and electronic microscopical features of epidermal cells in *Lotus japonicus* wild-type (WT) line Gifu in the presence of *Gigaspora margarita*. **A**, An appressorium (a) contacts outer tangential wall (t) of an epidermal cell (e). Appressorium produces two penetrating hyphae (p) that grow between anticlinal epidermal cell walls (r), causing their separation. **B**, A large channel separates two epidermal cells in the vicinity of fungal hypha (h). **C**, Light micrograph showing a hypha (h) that develops intercellularly between two epidermal cells (e1 and e2), penetrates one (e2), and then moves to the underlying cortical cell (c). **D**, Detail of a serial section showing the same hypha (h) passing from the epidermal (e) to the cortical (c) cell. **E**, Ultrastructural aspect of an intracellular hypha colonizing an epidermal cell. Immunolabeling with the monoclonal antibody (MAB) JIM 5. Gold granules are seen on the interface material (im) occurring between the hypha (h) and the invaginated membrane (arrowhead) of an epidermal cell (e). Bars = **A**, 2; **B**, 0.5; **C**, 10; **D**, 5; and **E**, 0.3 μ m.

Fig. 5. Ultrastructural features of contacts between epidermal cells in *Lotus japonicus* *Ljsym4-2* mutant and *Gigaspora margarita*. **A**, An appressorium (a) is positioned above two epidermal cells. An intercellular hypha (ih) penetrated between adjacent epidermal cells (e). A thickening (arrowhead) is present in the periclinal wall of the underlying cortical cell (c). **B**, Magnification of **A** showing cell wall structure of an epidermal cell (e) in close contact with a fungal hypha (ih) but not subject to fungal colonization attempts. It shows regular organelles. Central vacuole is electron transparent. n = Nucleus. **C**, Magnification of **A** showing contact between protrusion of intercellular hypha (ih) and anticlinal wall of an epidermal cell (e). Electron-dense material (dm), partly reactive to monoclonal antibody (MAb) JIM 5, surrounds the fungal peg. Wall of fungal peg shows electron-dense granules (arrowhead). **D**, Contact between plant wall (w) and fungus (ih) after treatment with MAb against β -1,3 glucans (arrowheads). **E**, Appressorium (a) produces a penetrating intercellular hypha (ih), which crosses anticlinal wall (r) of an epidermal cell (e). Penetrating hypha is abnormally swollen and exhibits a highly degenerated cytoplasm. Most of the fungal cell wall is not lined with cytoplasm. Host cytoplasm, organelles, and membranes are not detectable, suggesting plant cell death. Bars = **A**, 3; **B** and **E**, 2; **C**, 0.5; and **D**, 0.3 μ m.

obtained with *Glomus intraradices*, cortical cells were never found to be colonized upon inoculation with *G. margarita* (Fig. 2C).

Epidermal cell wall architecture of *L. japonicus* roots.

Since epidermal cells seem to play a role in the response of *L. japonicus* to symbiotic microorganisms, their ultrastructural morphology in WT and mutant plants was compared. The root surface of *L. japonicus* WT consisted of tightly joined, epidermal cells, often with root hairs (Fig. 3A). The morphology of epidermal cells varied according to their position. In the apical region, living cells had peripheral cytoplasm and a central vacuole, while highly vacuolated cells were present in the older, proximal regions (Fig. 3B). The thick outer wall of epidermal cells consisted of two layers, the outer one becoming looser mostly at the junction between two epidermal cells (Fig. 3A and B). The anticlinal walls were thinner by comparison (Fig. 3A and B). Immunofluorescence (data not shown) and immunogold experiments were performed to determine some cell wall components with JIM 5 and JIM 7 monoclonal antibodies (Mabs), which detect nonesterified and methylesterified pectins, respectively. They revealed a differential presence of these components in the epidermal walls (Fig. 3B–D). Nonesterified pectins were loosely present at the junction be-

tween epidermal cells and on the tangential wall (Fig. 3B) and in the intercellular material occurring between epidermal and cortical cells (Fig. 3D). Gold granules indicating the presence of esterified pectins were less abundant and were mostly located on the outer wall of the epidermal cells (Fig. 3C). β -1,3 glucans were not detected (data not shown). Control sections did not show any relevant labeling.

The appearance of the epidermal cells and their wall architecture was indistinguishable between *L. japonicus* WT and the *Ljsym4-2* mutants. Similarly, no epidermal cell wall alterations were observed when extraradical hyphae developed around WT or mutant plants (data not shown).

Development of *G. margarita* on *L. japonicus* roots.

On the surface of inoculated roots, appressoria were identified as swollen structures, 8 to 16 μ m in diameter, from which penetration hyphae were produced (Fig. 4A). The underlying epidermal cells, which were of comparable diameter to the appressorium, were often deformed. The appressorium cell wall was thick, electron dense, and rich in granules (Fig. 4A and B) already identified as melanin granules (Grippiolo and Bonfante 1984).

The penetrating hyphae separated two adjacent, epidermal cells, producing a deep furrow. The cell wall material joining

Fig. 6. Ultrastructural features of colonization events of epidermal cells in *Lotus japonicus* *Ljsym4-2* mutant by *Gigaspora margarita*. **A**, An appressorium (a) produces a swollen penetrating hypha that causes deformation of epidermal cells (e), anticlinal walls of which are broken (arrowhead). c = Cortical cells. **B**, Magnification of upper lined area of **A**. Appressorium wall (aw) is strongly pigmented, due to a number of granules (dg) closely packed in a thick, electron-dense layer. Host wall material (w) of epidermal cell (e) labeled by monoclonal antibody (MAb) JIM 5 surrounds the fungal peg. Wall of fungal peg shows electron-dense granules (arrowhead). **D**, Contact between plant wall (w) and fungus (ih) after JIM 5 to detect nonesterified pectins. **C**, Magnification of lower lined area of **A**. Immunolabeling with MAb JIM 5. Labeling is seen in the thickening occurring in the underlying cortical cell wall (c). Thickening projections (p) are also labeled. Bars = **A** and **B**, 1; and **C**, 0.5 μ m.

the epidermal cells was ruptured and the anticlinal cell walls connecting the adjacent epidermal cells were eventually fully separated (Fig. 4A and B). The MAb that detects β -1,3 glucans led to the deposition of a few gold granules at the epidermal surface (data not shown).

The separation of epidermal cells by the penetration hypha was followed by the production of a short branch that entered one of the two adjacent epidermal cells (Fig. 4C). The penetration site was preferentially located along the thin, anticlinal wall and at the base of the epidermal cell. From this position, the hypha passed through the epidermal cell into the underlying cortical cell (Fig. 4D), where it produced a long, nonbranched hypha that reached the inner cortical cells close to the endodermis. Here, it branched and produced terminal arbuscules.

The intracellular growth of the fungus was always associated with an invagination of the host membrane, as widely reported for AMs (Bonfante and Perotto 1995; Bonfante, *in*

press). Pectins were present in the material laid down by the host at the interface occurring between the perifungal membrane and the large hyphae colonizing the epidermal cells (Fig. 4E). β -1,3 glucans were limited to plasmodesmata (data not shown).

Development of *G. margarita* on *LjSYM4* mutant roots.

On roots of the *LjSYM4-2* mutant, *G. margarita* produced electron-dense appressoria, which originated hyphae, often successful in penetrating the *L. japonicus* walls (Figs. 5 and 6). Penetration hypha separated the adjoining anticlinal walls of two epidermal cells, and produced one or two infection pegs, located at the base of the epidermal cell (Fig. 5A). As a result, the membrane of the epidermal cell involved in the event disintegrated; electron-dense material, partly reactive to JIM 5, was accumulated around the fungal peg, and the vacuole appeared rich in granular contents (Fig. 5A). The wall of

Fig. 7. Ultrastructural features of colonization events of epidermal cells in *Lotus japonicus* *LjSYM4-2* mutant by *Gigaspora margarita*. **A**, A swollen hypha (h)—seen in a longitudinal section—fills up cell lumen. Electron-dense material (arrowhead) is present around hypha (e = epidermal cell, c = cortical cell). **B**, Detail of infected epidermal cell (e), which exhibits convolution of membrane system (arrowhead), and electron-dense granules. **C**, Masses of electron-dense material (arrowheads) are seen inside a dead epidermal cell (e). β -1,3 glucans are not detected on host cell wall after labeling with β -1,3 glucans monoclonal antibody (MAb). Fungal hypha (h) is empty. **D**, A large empty hypha (h) is present inside an epidermal cell (e) that contains degraded cytoplasm, suggesting plant cell death. Bars = **A** and **C**, 1 μ m; **B** and **D**, 0.5 μ m.

the fungal peg was layered with embedded, electron-dense granules (Fig. 5C). The morphology of the neighboring epidermal cell, which was not involved in the colonization attempt, did not reveal any changes (Fig. 5B). When the MAb was used to localize β -1,3 glucans, a few gold granules were seen at the contact between the penetration hypha and the wall of the epidermal cell (Fig. 5D). The position of these was similar to the situation in WT samples (data not shown). The wall between epidermal and cortical cells below the position of the fungus appeared thickened when compared with the other walls of the same cortical cell (Fig. 5A).

Another aspect of fungal penetration is shown in Figure 5E. The crossing of the anticlinal wall of the epidermal cell is first associated with a constriction of the penetration hyphae and then with its swelling (Fig. 5E).

In some samples, the swollen, intracellular hyphae deformed the epidermal cells, the anticlinal walls of which appeared broken (Fig. 6A) and reactive to JIM 5 (Fig. 6B). The appressorium wall was strongly pigmented, due to a number of granules closely packed in a thick, electron-dense layer (Figs. 6B). Only membrane and cytoplasm remnants could be seen around the hypha (Fig. 6B). The underlying cortical cell exhibited a localized wall thickening in the vicinity of the infection event (Fig. 6A and C). It showed a spongy texture where electron transparent areas were mixed with electron-dense ones. The wall thickening was irregularly labeled by JIM 5, while it was not labeled by the other two MAbs used to locate esterified pectins and β -1,3 glucans.

As a consequence of the swollen hyphae that filled up the cell lumen (Figs. 6A and 7A), the infected epidermal cell exhibited convolution of the membrane system—which became progressively osmiophilic—and electron-dense granules (Fig. 7A and B). Lastly, an accumulation of electron-dense material characterized the epidermal cell (Fig. 7C), while β -1,3 glucans were not detected within its wall (Fig. 7C and D). All of the epidermal colonized cells inspected by electron microscopy appeared morphologically dead.

The abortion of infection attempts was also associated with a localized death of fungal hyphae. While extraradical hyphae appeared structurally intact (Figs. 5E and 6A), they were in continuum with intracellular parts, which suffered from a degenerated cytoplasm (Figs. 5E and 7C). Eventually, empty hyphae were seen inside empty epidermal cells (Fig. 7D).

DISCUSSION

The root epidermis: An active checkpoint in symbiotic infection.

Root epidermal cells and their protrusions, the root hairs, form the first physical barrier encountered by symbiotic microorganisms on their way to the root cortex. On *L. japonicus* roots, *M. loti* reaches the root cortex via infection threads that pass through root hairs, while AM fungi gain access to a cortical cell by separating two adjacent epidermal cells. Hyphae, however, do not proceed directly into the cortex; instead, a detour is taken that involves the infection of an epidermal cell. This detour suggests that epidermal cell infection is a prerequisite for initiating the successful colonization of the cortex. A similar penetration mechanism has been described in leek (Garriock et al. 1989), maize (Bonfante, *in press*), and a *Colchicum* sp. (Gallaud 1905, quoted in Smith and Smith 1997).

Not only these morphological observations, but also our genetic data reveal the critical role of the root epidermis as a checkpoint for mycorrhizal invasion. The mycorrhizal phenotype of *Ljsym4-1* mutants suggests a cell type-specific requirement of *LjSym4*. The *Ljsym4-1* allele exerts a strong effect within epidermal cells, where the majority of infection attempts are aborted. However, some gene function appears to be retained, allowing a few invasions to proceed into the root cortex. In older *Ljsym4-1* roots, fungal colonization and arbuscule formation reach levels comparable to those in the WT, indicating that the limiting step in this interaction is the passage through the epidermis.

LjSym4 is required for the successful infection of epidermal cells by symbiotic microorganisms.

LjSym4 is essential for a step common to both root symbioses. Mutant analysis suggests that *LjSym4* is involved in a process occurring in epidermal cells and essential for infection by both symbiotic microorganisms. Root hairs of *L. japonicus* respond to *M. loti* with curling and formation of infection pockets. The root cortex is reached through the infection thread formed by the plant host (Szczylowski et al. 1998; Schauser et al. 1999). AM fungi also rely on the formation of an infection structure by the plant (Garriock et al. 1989). During all stages of symbiotic bacterial and fungal infection, intracellular microbes are surrounded by a perimicrobial membrane. In the space between this host-derived membrane and the invading microorganism, so-called interface material is deposited that is, at least in part, of plant origin. The plant-fungus interface is morphologically and immunologically related to the plant-bacterium interface formed during the *Rhizobium* symbiosis (Perotto et al. 1994; Bonfante and Perotto 1995; Harrison 1999a). Both the synthesis of the perimicrobial membrane and the deposition of interface material presumably require a specific set of metabolic activities of the host cell, which are part of the general accommodation program of the host required to support microbial development within the plant root (Parniske 2000). Our analyses of *Ljsym4* mutants show that infection threads are not formed upon *M. loti* contact and the perifungal membrane does not develop around *G. margarita*, while a fully developed interface compartment with the expected pectin components was identified in the parental line. These observations suggest that *LjSym4* is required for this accommodation process within the epidermis. According to this hypothesis, insufficient structural support is provided in *Ljsym4* mutant epidermal cells and consequently fungal infection attempts are aborted. Because of the lack of structural containment by the plant, the hyphae invading the mutant epidermal cells increase abnormally in diameter. In this scenario, the arrested rhizobial interaction as well as the host and fungal cell death at the site of infection would be consequences of the incompetence of the host to support normal symbiotic development. On the *Ljsym4* mutants, the rhizobial-triggered nodulation process was arrested prior to infection and only swelling and deformation of root hairs were observed. In *Vicia sativa*, root hair tip swelling was shown to be the first visible response to LCO addition and following this swelling the *V. sativa* root hairs initiate an asymmetric tip growth leading to root hair curling (Heidstra et al. 1994). Our data are consistent with the idea that a similar chain of events occurs in root hairs of *L. japonicus*. However, curling of root hairs to form the in-

fection pockets and initiation of infection thread formation was not observed on the mutants. Therefore, the *LjSym4* gene appears to be necessary for the developmental process setting up the invasion structures after the initial LCO perception. Epidermal cells of *Ljsym4* mutants are invaded by AM fungi but not by *M. loti*. This difference might be explained by the different forces that bacteria and fungi, thanks to their appressoria, are able to impose on plant cell walls. Due to a combination of physical forces and enzymatic activities (Bonfante and Perotto 1995), AM fungi are able to force entry into the mutant epidermal cell, whereas the smaller rhizobia cannot enter without the active contribution of the plant cell (Parniske 2000).

Is *LjSym4* epidermal cell death caused by a defense response?

Epidermal cell death upon infection with AM fungi could be the consequence of a hypersensitive response (HR), which is a commonly observed response of resistant plants to attack by pathogens (Heath 1997). A large number of so-called disease-lesion-mimic mutants of several plant species have been described (Dangl et al. 1996). These “paranoid plants” (Jones 1994) inappropriately respond with cell death to various biotic and abiotic stimuli. If *Ljsym4* mutants responded to signals of both microsymbionts inappropriately with cell death, the abolition of infection of both microsymbionts would be a plausible consequence. However, our morphological observations did not reveal any evidence of defense-related mechanisms specifically elicited by the symbionts in *Ljsym4* mutant cells. First, no cell death was observed when *Ljsym4* roots were inoculated with *M. loti*, indicating that the lack of infection thread formation was not a consequence of cell death. Second, β -1,3 glucans indicative of callose—observed at the contact between AM fungi and mutant peas (Gollotte et al. 1993)—were not revealed in the *Ljsym4-2* mutant upon epidermal cell entry by *G. margarita*. Detectable amounts were, however, present adjacent to superficial contacts of hyphae with roots of *Ljsym4-2* or the parental line (data not shown), probably another manifestation of the weak and transient induction of defense responses widely reported for AMs (Gianinazzi-Pearson 1996; Barker et al. 1998b). We therefore have no evidence in favor of the idea that mutations in *LjSym4* give rise to a disease-lesion-mimic phenotype.

The phenotype of *Ljsym4* mutants differs from previously described symbiotic plant mutants.

AM fungi form appressoria prior to root penetration (Giovannetti et al. 1996; Harrison 1999b). *G. margarita* produces appressoria on isolated epidermal cell walls (Nagahashi and Douds 1997), suggesting that the epidermal cell wall itself provides the signals that trigger appressoria formation. Our results show that epidermal cell wall architecture is similar in both WT and *Ljsym4-2* mutants. The localization of the analyzed cell wall components performed by the use of different MAbs is unchanged. Consistently, appressoria were produced on the surface of *Ljsym4* mutant roots, demonstrating that plant signals required for appressoria formation are provided. All plant mutants described so far that are affected in the interaction with AM fungi trigger appressorium formation (Bradbury et al. 1993; Duc et al. 1989; Sagan et al. 1995; Shirtliffe and Vessey 1996; Wegel et al. 1998; Barker et al. 1998a).

Four pea loci (*PsSym8*, *PsSym9*, *PsSym19*, and *PsSym30*) have been identified, mutations of which affect the early stages of both root symbioses (Albrecht et al. 1998; Balaji et al. 1994; Borisov et al. 1994; Schneider et al. 1999). AM fungi form appressoria on the corresponding mutant root surfaces, but subsequent penetration of plant root cells is blocked (Balaji et al. 1994; Duc et al. 1989; Gollotte et al. 1993; Gianinazzi-Pearson 1996). Whereas root hairs of *Pssym9* and *Pssym19* mutants respond with curling, root hairs of *Pssym8* do not deform upon inoculation with *Rhizobium* spp. (Markwei and LaRue 1992; Postma et al. 1988). In the interaction with rhizobia, the root hair response of our *Ljsym4* mutants is therefore intermediate between that of the *Pisum* mutants, and also their AM phenotypes differ. *Ljsym4* mutants appear to be impaired at a later step of the infection process, because epidermal cell penetration by AM fungi still occurs. An even later step in AM development is defective in the *RisNod24* mutant of *P. sativum* cv. Finale, which allows invasion of cortical cells, but only supports the formation of stumpy fungal branches instead of normal arbuscules (Gianinazzi-Pearson 1996).

Our observations define a novel, genetically controlled step in the fungal invasion process, not covered by the described phenotypes of pea. Our observations also demonstrate that plant responses to the fungus are cell layer dependent.

MATERIALS AND METHODS

Mutagenesis of *L. japonicus*.

Several symbiotic mutants of *L. japonicus* B-129-S9 Gifu (Handberg and Stougaard 1992) were generated in a chemical mutagenesis program that will be described in full elsewhere (J. Webb, *personal communication*). EMS 1749 (*LjSym4-2*) is a symbiotic mutant, failing to form nodules after inoculation with *M. loti* and to support colonization by AM fungi. To avoid confusion with symbiotic (*Sym*) genes identified in other plants, the two letter prefix *Lj* for *Lotus japonicus* is now consistently applied (Stougaard et al. 1999). *Ljsym4-1* mutant 282-227 was isolated from a tagging population, originated from transformation by an *Agrobacterium* sp. (Schauser et al. 1998).

Plant culture and *M. loti* interaction.

The visualization of infection threads by β -galactosidase staining for *hemAlacZ* carrying *M. loti* bacteria in root explants was as described by Boivin et al. (1990) and Schauser et al. (1999). To detect foci of cell division, roots were examined by fluorescence microscopy (Schauser et al. 1999). The root hair curling and deformation assay was as follows: scarified and sterilized seeds were imbibed for 2 days on wet filter paper. They were then transferred to plates containing 2% Agar Noble 1/4 B&D medium, and grown for 2 days before adding WT *M. loti* or an *nodC::Tn5* *M. loti* mutant strain (Handberg and Stougaard 1992). Roots were cut off 3 days later and placed on a microscopy slide with a drop of a 0.001% Tween 20 solution. The roots were examined by brightfield microscopy.

Plant cultures and fungal strains.

L. japonicus seeds (Gifu, WT, and mutants, *Ljsym4-21* and *Ljsym4-2*) were scarified and surface-sterilized for 5 min in concentrated sulfuric acid, washed three times with sterile wa-

ter, and germinated on water agar in petri dishes. Ten-day-old seedlings were inoculated with *Glomus intraradices* Schenk and Smith (a single-spore descendant line obtained from D. Redecker, Philipps-University, Marburg, Germany) with chive nurse plants, grown and analyzed as described by Wegel et al. (1998).

As an alternative method for cellular analysis, about 10 days after germination, seedlings were inoculated with *Gigaspora margarita* Becker and Hall (strain deposited in the Bank of European Glomales as BEG 34) by the millipore sandwich method (Giovannetti et al. 1993). Three seedlings were placed between two membranes (pore diameter 0.45 μm ; Sartorius, Goettingen, Germany), either with 10 to 15 fungal spores or without any spores. Seedlings with the membranes attached were planted in sterile acid-washed quartz sand in Magenta GA-7 vessels (Sigma Aldrich, Milan, Italy) and grown in a climatic chamber at 20°C, 60% humidity, with 14 h of light per day. After 3 weeks, samples from roots were cut after observation under a stereo microscope. Some segments were stained with 0.1% cotton blue in lactic acid and the infection was quantified as described by Trouvelot et al. (1986); other fragments were processed for immunofluorescence and electron microscopy. Root segments from six independent experiments and a total of 30 sandwich cultures were analyzed.

Staining of nuclei and fungal structures.

Root segments were fixed for 1 h at room temperature with 3% paraformaldehyde in PEM buffer (50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)]-Na₂, 5 mM EGTA, 5 mM MgSO₄, pH 7). Samples were subsequently washed with PEM, embedded in 10% agarose, and cut into 50- μm sections with a vibratome. Nuclei were stained by incubating the sections for 10 min with 50 μg of propidium iodide per ml in 0.02 M phosphate buffer (PB), pH 7. Fungal structures were stained by treating the sections for 10 min with NaHClO 1.5% in PB (pH 7, 0.02 M) and subsequent incubation for 1 h in PB (pH 7, 0.02 M) containing fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA) 10 $\mu\text{g}/\text{ml}$.

Samples were observed with a Nikon Optiphot-2 microscope equipped with a Bio-Rad View Scan DVC-250 confocal system, under an excitation light of 488 nm and 514 nm. Images collected with a Hamamatsu C5985 Chilled CCD Camera were processed on an Apple computer with Adobe Photoshop and NIH Image software.

Electron microscopy.

Root segments from six independent experiments were fixed in 2.5% (vol/vol) glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) for 2 h at 4°C. After being rinsed with the same buffer, samples were postfixed in 1% (wt/vol) OsO₄ in double-distilled H₂O for 1 h, washed three times with double-distilled water, and dehydrated in an ethanol series (30, 50, 70, 90, and 100%; 10 min each step) at room temperature. The root segments were infiltrated in 2:1 (vol/vol) ethanol/LR White resin (Polysciences, Warrington, PA, U.S.A.) for 1 h, 1:2 (vol/vol) ethanol/LR White for 2 h, and 100% LR White overnight at 4°C according to Moore et al. (1991). Semithin sections (1 μm) were cut from 15 to 20 root-embedded samples for each genotype (WT and mutant) in the presence and in the absence of the fungus. Sections were stained with 1% toluidine blue for morphological observations.

Immunocytochemistry.

Six independent immunogold experiments were performed for each treatment. For each of them, five LR White, embedded samples were selected. For the location of nonesterified and methylesterified pectins, rat MABs JIM5 and JIM7 (Knox et al. 1990) were used, respectively, while for detection of β -1,3 glucans, a mouse MAB was used (Biosupplies Australia, Parkville). Immunogold labeling was performed on thin sections as described by Balestrini et al. (1996). About 50 thin sections from each selected, embedded sample were handled as floating sections. The sections were incubated for 15 min in normal goat serum diluted 1:30 in 0.05 M 2-amino-2 (hydroxymethyl)-1-3 propanediol (Tris)-HCl buffer with 500 mM NaCl (TBS, pH 7.6) and 0.2% bovine serum albumin (BSA), and then treated overnight with undiluted MABs JIM5 or JIM7. After washing, sections were incubated with 15 nm of colloidal gold-Ig complex (goat anti-rat; Bio Cell, Cardiff, U.K.) containing 1% BSA (diluted 1:20 in TBS) for 1 h. MAB for β -1,3 glucans was diluted 1:100 and secondary antibody (goat anti-mouse; Bio Cell, Cardiff, U.K.) was diluted 1:20 in TBS. The specificity of immunolabeling reactions was determined by treating grids with MABs JIM5 or JIM7 preabsorbed with 1 mg $\cdot$ ml⁻¹ solutions of pectin or Na-polygalacturonate (Sigma Chimica, Milan, Italy) 12 h before the incubation at 4°C (Moore and Staehelin 1988). The MAB for β -1,3 glucans was preincubated with 100 $\mu\text{g} \cdot \text{ml}^{-1}$ laminarin (Sigma Chimica, Milan, Italy) for 72 h at 4°C. Other control sections were run, replacing the primary antibody with buffer. Thin sections were post-stained with uranyl acetate and lead citrate before observation with a Philips CM 10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Immunofluorescence was performed on semithin sections. They were washed with 50 mM PBS, pH 7.2, saturated 1 h with 1% (wt/vol) BSA in the same buffer, treated with the MABs (diluted 1:200) overnight at 4°C, and then washed with PBS. The secondary antibodies were FITC-conjugated (Sigma) and were used at the recommended dilution (1:60). Sections were then observed under a Nikon Optiphot-2 microscope equipped with a Bio-Rad View Scan DVC-250 confocal system, under an excitation light of 488 nm.

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