Resistance of pea roots to endomycorrhizal fungus or *Rhizobium* correlates with enhanced levels of endogenous salicylic acid

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Abstract

The analysis of SA accumulation in roots of plant symbiotic mutants revealed two independent phenomena associated with the inability of either the plant or the microsymbiont to form a compatible symbiosis. SA accumulation in roots of the wild type and symbiosis-resistant P2 (Nod−, Myc−) *Pisum sativum* genotypes was induced upon interaction with *Glomus mosseae*. The amplitude of this accumulation was higher in P2 plants and increased with time, an effect that was not observed in roots of the wild-type *P. sativum* genotype. Likewise, *Rhizobium leguminosarum* wild type or a mutant blocked in Nod factor biosynthesis induced SA accumulation in P2, whereas SA accumulation in roots of the wild-type plant was dependent on the inability of the bacterium to produce Nod factors. These results suggest that the sym30 gene, which is mutated in P2 plants, could be implicated in a common pathway that leads to the suppression of an SA-dependent defence mechanism in legume plants against *Rhizobium* and endomycorrhizal fungi, thus allowing establishment of symbiosis.

Key words: Arbuscular mycorrhiza, plant defence, resistance, rhizobia, salicylic acid.

Introduction

Among symbiotic associations between soil microorganisms and plants, the endosymbioses formed by plant roots and arbuscular mycorrhiza (AM) fungi are very important because they are widespread and involve most agriculturally important crops. Legumes are unique because they can establish beneficial symbioses with nitrogen-fixing bacteria, the rhizobia, as well as with AM fungi.

Although these two root endosymbioses involve very different and unrelated microorganisms, with strong differences in the morphology of symbiotic structures and host-specificities, there may also be important similarities at the molecular and genetic level (revised by Hirsch and Kapulnik, 1998). Some authors suggest a situation in which essential plant elements are conserved in both root endosymbiotic processes (Gianinazzi-Pearson and Dénarié, 1997). Thus, the identification of symbiotic genes in legumes will enable the isolation of homologous genes in other important crops.

Evidence for a genetic link between rhizobia-legume symbiosis and AM has been found. Plant mutants that are Nod− (absence of nodule formation) are also Myc− (absence of mycorrhization) (Bradbury et al., 1993; Duc et al., 1989). Diallelic crosses have shown that some Myc− and Nod− plants are derived from mutations in the same gene. In pea, there are at least five separate loci involved in both nodulation and AM formation (Gianinazzi-Pearson et al., 1991).

Since both of these above-mentioned symbioses require clear recognition by the plant host, an important question is how the plant recognizes the invading microorganism as a beneficial symbiont instead of a pathogen. Arbuscular fungi or *Rhizobium* do not seem to activate a host defence response or else they have developed some mechanism to suppress the host defences in the compatible and successful interaction. In this regard, an involvement of *Rhizobium* Nod factor in the inhibition of salicylic acid (SA)-mediated defence in alfalfa roots has been suggested (Martínez-Abarca et al., 1998). An accumulation of SA during an incompatible interaction was observed between

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alfalfa roots and a *Rhizobium* mutant that is blocked in 
Nod factor biosynthesis, as well as a SA-mediated reduc-
tion of Nod factor-induced nodulation.

In this work, the effect of a genetic mutation which 
causes plant resistance to endosymbiotic microorganisms 
(*Rhizobium* or AM fungi) on the plant defence response 
was examined, representatively measured as accumulation 
of free SA, which is an endogenous marker for plant 
disease resistance (Klessig and Malamy, 1994). Two pea 
genotypes, Frisson wild type (Nod+ Myc+) and P2 (Nod− 
Myc−) mutant affected in the sym30 gene (Duc et al., 
1989; Sagan, 1992) were inoculated with *Glomus mosseae* or 
*Rhizobium leguminosarum* bv. *viciae*, as well as with 
the pathogenic bacteria *Pseudomonas syringae*. The effect 
of inoculation with a nodulation-defective *R. leguminosa-
rum* Nod C− mutant was also tested.

**Materials and methods**

**Plant natural and fungal inoculation**

Experiments were carried out in 80 ml pots containing a sterile 
mixture of sand-vermiculite:peat (50:50:15, by vol.). The 
seedlings of *Pisum sativum* var. Frisson, wild type and P2 
mutants (Duc et al., 1989) were inoculated with 1 g of soil 
inoculum of *Glomus mosseae* (Nico. & Gerd.) Gerd. and Trappe 
per pot at the moment of transplanting. Control plants were 
mock inoculated with a distilled water filtrate corresponding to 
1 g of inoculum (McAllister et al., 1997). In order to prevent 
effects due to possible contamination of the soil inoculum with 
rhizobial cells, the following precautions were taken: (i) the 
inoculum used was obtained from the rhizosphere of mycorrhizal 
non-legume plants (tobacco) grown under controlled conditions, 
and (ii) before freezing the root, visual analysis was performed 
to confirm that no nodules had been formed.

Plants grown under greenhouse conditions were harvested at 
the time points desired and the root system of each replicate 
pot was divided into two portions to record the following: 
mycorrhizal colonization (measured as described by Ocampo et 
al., 1980), and SA content.

**Bacterial inoculation**

For bacterial inoculation, the Frisson wild type and P2 mutant 
pea plants were axenically grown in tubes as described (Oliveras 
et al., 1980). The seeds were previously surface-sterilized in 
10% sodium hypochlorite solution, and germinated at 28°C. 
After germination, one seedling per tube was grown. One week 
later, when plant roots were well developed, each tube was 
inoculated with 10⁶ cells ml⁻¹ of either compatible *R. leguminos-
arum* bv. *viciae* 248 or incompatible Nod C− mutant cells 
(Canter Cremers et al., 1988). Control plants were mock 
inoculated with sterile distilled water. Bacteria were grown in 
tryptone-yeast extract medium. Cells were washed twice to 
remove the culture medium and resuspended in sterile distilled 
water before inoculation. Plant roots were harvested and frozen 
in liquid nitrogen 0, 34, 48, and 72 h after inoculation.

The same procedure as for *Rhizobium* was used to inoculate 
plant roots with 10⁶ cells ml⁻¹ of *Pseudomonas syringae* ssp. 
*syringae*. The *P. syringae* used was obtained from the Spanish 
National Collection of Type Cultures (CECT). The CECT 
received this strain from the NCPPB (National Collection of 
Plant Pathogenic Bacteria Harpenden, UK) as *Pseudomonas 
psidi*. Plants were harvested at 0, 48 and 72 h after inoculation.

**Extraction and quantification of free SA**

Salicylic acid (SA) was extracted from 1 g of root tissue frozen 
in liquid nitrogen and the free SA content was analysed 
(according to Rasmussen et al., 1991), but the concentrated 
ethanol extracts were resuspended in 5% trichloroacetic acid 
and extracted into 2 vols of cyclopentane/ethyl acetate/ 
isopropanol (according to Malamy et al., 1992). The organic 
extract was dried under nitrogen, resuspended in 10 µl of 
ethanol and analysed by TLC. The samples were spotted onto 
 silica gel 60 A chromatography plates (Merck) and developed in 
toluene: dichloromethane: acetic acid (90:25:4, by vol.) 
(Rasmussen et al., 1991). The fluorescent band corresponding to 
salicylic acid was visualized and identified in the plate by viewing under 
UV light (302 nm). The band was eluted from the silica gel 
with 1 ml of 95% ethanol and used for fluorimetric quantification 
(excitation wavelength = 310, emission wavelength = 400 nm). 
As standard, different aliquots of ethanal solution salicylic acid 
(Sigma) were run in parallel, recovered from the silica gel and 
quantified. Each data point is the average of five replicate 
samples (roots of five different pots) from one representative 
experiment. The value of each replicate is the average of 10 
fluorescent readings taken over 10 s. The limit of detection for 
salicylic acid in a final volume of 1 ml was 1 nmol. Each 
experiment was performed at least three times with similar 
results. Data were analysed by one-way analysis of the variance. 
The standard error of means is given.

**Results and discussion**

P2 mutant plants have been identified as Myc−, in 
addition to Nod− (Duc et al., 1989) and a typical defence 
response associated with root resistance to infection by 
mycorrhizal fungi has been shown (Gollotte et al., 1993). 
The effect of the inoculation with the mycorrhizal fungus 
*G. mosseae* on SA accumulation has been analysed in 
Frisson wild-type and P2 mutant roots (Fig. 1). A 
progressive increase in free SA parallel to plant age 
was observed in the root of all plants. This increase was less 
pronounced in non-inoculated P2 plants. The inoculation 
with *G. mosseae* causes an increase in the level of free SA 
that was stronger in P2 plants. At 10, 15 and 20 d after 
inoculation, the amount of free SA in P2 inoculated (I) 
plants was 3, 3.3 and 4.5 times greater than in control, 
non-inoculated (NI) P2 plants, respectively. Only at 10 d 
after inoculation did wild-type Frisson roots show an 
I:NI ratio of SA accumulation similar to that found in 
P2. However, while the I:NI ratio of SA accumulation 
in P2 roots continuously increased with time, the 
wild-type ratio decreased after having reached its maximum at 
10 d after inoculation. The free SA content in inoculated 
P2 roots was about 1.3 and 1.7 times greater than in 
inoculated wild-type roots, at 15 and 20 d after inocula-
tion, respectively. The wild-type inoculated plants reached 
8%, 20% and 30% root length mycorrhizal colonization 
at 10, 15 and 20 d, respectively.

These results from Fig. 1 suggest that in wild-type and
mulation of some flavonoids, changes in phytoalexin production, increases in peroxidase and chitinase activities, and alterations in other defences (reviewed by Gianinazzi-Pearson et al., 1996). In the compatible G. mossae wild-type pea association the fungus colonized the root normally, and the accumulation of SA was transient. Nevertheless, the amplitude of SA accumulation in the roots of inoculated P2 mutant plants and the continuing increase in free SA with time suggest that SA could mediate the defence response of P2 resistance.

As the genetic mutation in the sym30 gene also causes plant resistance to Rhizobium, the effect on SA accumulation in pea roots inoculated with R. leguminosarum wild-type or R. leguminosarum Nod C− mutant was analysed. Chart A in Fig. 2 corresponds to wild-type Frisson pea roots, and chart B to the Nod−, Myc− P2 genotype. These results clearly show two independent effects. Firstly,

P2 mutant plants mycorrhzal infection stimulates the accumulation of free SA, but the amplitude of this accumulation is lower in wild-type than in P2 roots. In this sense, at 20 d after inoculation, when the mycorrhrizal infection is well established in wild-type plants (30% of root length), the differences between P2 and wild-type are more important. At 10 d after inoculation, which could be considered as an early stage of mycorrhrizal infection, there is no difference between P2 and wild-type content in SA root accumulation. This is the stage of plant–fungal contact and appressoria formation in both wild-type and P2 plants and these events could be implicated in the stimulation of SA accumulation. In P2 mutant plants the endomycorrhizal interaction is arrested at this stage of appressoria formation (Golotte et al., 1993).

The early accumulation of SA observed in the fungal–plant interaction could be considered as part of the weak, transient and unco-ordinated plant host defence response to the invading fungus that is characterized by the accu-
the inoculation with *R. leguminosarum* Nod C− mutant causes the same effect on SA accumulation in wild-type pea plants (Fig. 2A) as in P2 (Fig. 2B). Therefore, the block in Nod factor production led to an accumulation of SA in roots, independently of the plant genotype. The increase in SA accumulation in wild-type and P2 plants inoculated with *R. leguminosarum* Nod C− mutant was observed at 48 h and 72 h and reached a maximum (10–13 times) 48 h after inoculation. These results agree with those reported by Martinez-Abarca and co-workers (Martinez-Abarca et al., 1998) and support the idea of the involvement of Nod factor in the inhibition of SA-mediated defence in legumes. Secondly, the mutation in P2 pea plants leads to an SA response in the roots when they were inoculated with the wild-type *R. leguminosarum* (Fig. 2B) that was not observed in wild-type pea roots. This response is therefore dependent on the plant genotype and is independent of the presence of Nod factor in the medium since P2 root exudates stimulate Nod factor production in the same manner as do exudates from wild-type pea roots (Sagan, 1992).

It is important to note that the time scales for Figs 1 and 2 are different because of the different symbionts and experimental conditions used for *G. mosseae* or *R. leguminosarum* inoculation, but in both cases early stages in plant–microorganism interaction was analysed, and a similar result was observed.

The mutation in P2 affects the syn30 symbiotic gene and causes resistance to *Rhizobium* and AM fungus penetration in the root (Nod− Myc− genotype) (Duc et al., 1989). Present data demonstrate a relationship between induction of a marker of plant defence, SA, and resistance to mycorrhizal and nodulation symbiosis in legumes that is associated with the mutation of a common mycorrhizal and nodulation symbiosis-related gene. Considering the relationship between pathogenic-induced SA synthesis in plants and the expression of Systemic Acquired Resistance (SAR) (Klessig and Malamy, 1994), it is possible to argue that a defence mechanism associated with SA accumulation takes place in the establishment of the AM fungus and *Rhizobium* legume symbioses, as previously reported in the establishment of the *R. meliloti*-alfalfa symbiosis (Martinez-Abarca et al., 1998). The differential effect observed on SA accumulation in the incompatible interactions between P2 plants and the endosymbiotic microorganisms suggests that SA could provide a signal for defence gene induction, as proposed for the activation of SAR in other plants (Ryals et al., 1996). Nevertheless, the role of SA as a systemic signal in SAR is presently unresolved (Willits and Ryals, 1998) and there is increasing evidence for the existence of several SA-independent pathogen-induced signalling pathways in plants (Vidal et al., 1998) including compatible and incompatible plant–pathogen interactions (Valléian-Bindschedler et al., 1998).

To assess whether the effect of SA accumulation in the P2 mutant is associated only with mutualistic interactions, free SA in plant roots inoculated with the plant pathogen *P. syringae* was quantified in the same culture system used for *Rhizobium*.

Figure 3 shows the amount of free SA in roots of wild type and Nod−, Myc− mutant P2 plants after 0, 48 and 72 h of inoculation with *P. syringae*. No differences were observed in SA accumulation between roots of wild-type and P2 plants treated with the bacteria. A clear symptom of plant disease was the appearance of brown colour in both wild and P2 roots. Only at 72 h after inoculation was the amount of SA in inoculated plants 1.6 and 1.7 times greater than in non-inoculated wild-type and P2 plants, respectively. The increase in SA observed at 72 h in both genotypes could be due to an unspecific plant response to *P. syringae*. Possibly the SA-mediated response should be more evident in the interaction with an incompatible pathogen. Therefore, these results confirm that the mutation in P2 leads to a specific increase of SA in the roots upon interaction with mutualistic, but not pathogenic microorganisms. Thus, the mutation in syn30 has not led to a general increase in defence mechanisms. In this sense, the elicitation with UV light of defence reactions, quantified as phenylalanine ammonia-lyase (PAL) and pisatin production show only slight differences between Frisson wild type and the P2 mutant (Morandi and Paynot, 1994). On the other hand, the P2 mutant does not show a higher resistance to pea pathogens, like *Chaumia elegans* (Dassi et al., 1994) or *Aphanomyces euteiches* (Gianinazzi-Pearson et al., 1994) than the wild-type.

The mutation in the syn30 symbiotic gene therefore causes specific resistance to symbiotic microorganisms and is associated with the accumulation of free SA. It is probable that this gene could participate in some common
pathways that lead to suppression of host defences in the compatible interaction. In this sense, there is evidence that another symbiotic gene (Sym5) is essential for the induction of the early nodulin genes PsENOD5 and PsENOD12A in pea roots interacting with either *Rhizobium* or the endomycorrhizal fungus *Gigaspora margarita* (Albrecht et al., 1998). The existence of similar responses in pea symbiotic mutants challenged by *Rhizobium* or endomycorrhizal fungi, either in the activation of defence mechanisms (this paper) or lack of induction of early nodulin genes (Albrecht et al., 1998) support the hypothesis that both endosymbionts produce similar signal molecules. This implies the production of a symbiotic Myc factor by AM fungi with a similar mechanism of action to Nod factor. The comparative analysis of defence response in other symbiotic plant mutants affected in different genes (i.e. sym8 and sym9) at early stages of fungal and/or rhizobia colonization can be used to advance knowledge of the relationships between symbiotic partners and the potential function of plant symbiotic genes.

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