



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

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Received 6 April 1999; Accepted 21 July 1999

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 agronomically 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 reduction 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. leguminosarum* Nod C⁻ mutant was also tested.

Materials and methods

Plant material 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 followings 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 (Olivares 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 10⁸ cells ml⁻¹ of either compatible *R. leguminosarum* 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, 24, 48, and 72 h after inoculation.

The same procedure as for *Rhizobium* was used to inoculate plant roots with 10⁸ 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 pisi*. Plants were harvested at 0, 48 and 72 h after inoculation.

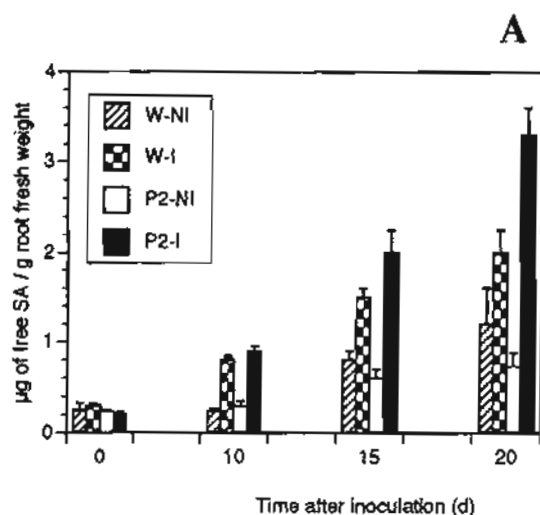
Extraction and quantification of free SA

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:dioxane: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 ethanol 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 inoculation, 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



B

Ratio of SA accumulation I/NI

Time (d)	W	P2
0	1.2	0.8
10	3.3	3
15	1.8	3.3
20	1.6	4.5

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. mosseae* 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,

Fig. 1. (A, B) Content of free SA in pea cv. Frisson wild-type (W) and P2 mutant roots non-inoculated (NI) and inoculated (I) with *G. mosseae* at different times after inoculation (A), and the ratio of SA accumulation in I:NI roots (B). Values are means of at least five replicates \pm SE.

P2 mutant plants mycorrhizal 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 mycorrhization 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 mycorrhizal 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 (Gollotte *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-

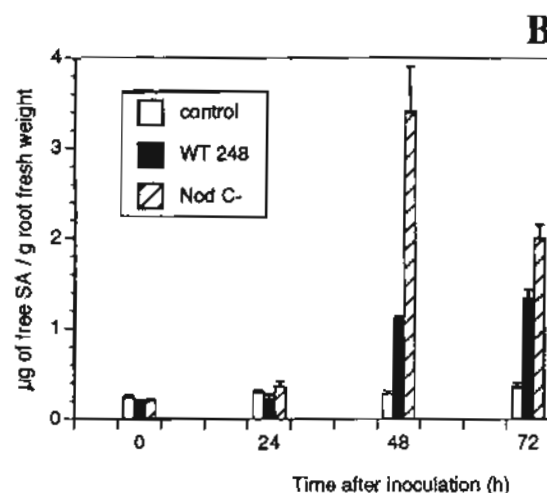
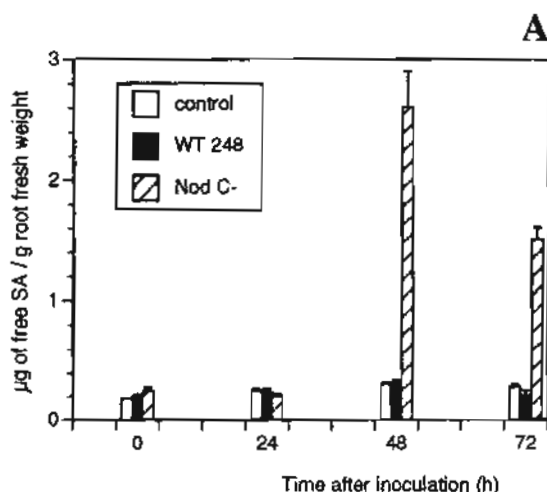


Fig. 2. (A, B) Content of free SA content in pea cv. Frisson wild-type (A) and P2 mutant (B) roots non-inoculated (control) and inoculated with *R. leguminosarum*, wild-type (WT248) or the isogenic *R. leguminosarum* Nod C⁻ mutant (Nod C⁻) at different times after inoculation. Values are means of at least five replicates \pm SE.

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 Martínez-Abarca and co-workers (Martínez-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 *sym30* 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 (Martínez-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 (Vallélian-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 *sym30* 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 *Chalara elegans* (Dassi *et al.*, 1994) or *Aphanomyces eutiches* (Gianinazzi-Pearson *et al.*, 1994) than the wild-type.

The mutation in the *sym30* 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

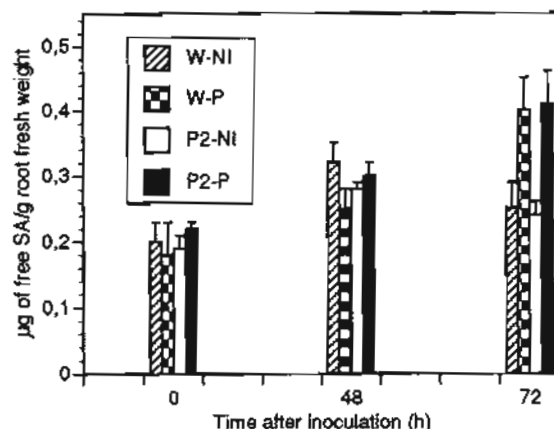


Fig. 3. Content of free SA in pea cv. Frisson wild-type (W) and P2 mutant roots non-inoculated (NI) and inoculated with *P. syringae* (P) at different times after inoculation. Values are means of at least five replicates \pm SE.

pathways that lead to suppression of host defences in the compatible interaction. In this sense, there is evidence that another symbiotic gene (*Sym8*) 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 fungus 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.

Acknowledgements

This work was supported, in part, by grants from the Spanish Instituto de Cooperación con el Mundo Árabe (ICMA) for I Blilou, and from MEC for JM García-Garrido. The authors wish to thank Drs Juan Sanjuan and José Olivares (EEZ-CSIC, Granada, Spain) for critical reading of the manuscript. We are also grateful to Dr Martínez-Abarca (EEZ-CSIC, Granada, Spain) for the gift of the *R. leguminosarum* 248 wild type and Nod C⁻ mutant, and Dr G Duc (Station de Génétique et d'amélioration des plantes, INRA, Dijon, France) for providing the seeds of wild-type Frisson and P2 mutant peas. The Comisión Interministerial De Ciencia y Tecnología, Spain (PB97-1202) provided financial support for this study.

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