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Root colonization by arbuscular mycorrhizal fungi is affected by the salicylic acid content of the plant

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Abstract

Wild type, transgenic NahG tobacco plants with reduced levels of salicylic acid (SA) and transgenic CSA (constitutive SA biosynthesis) tobacco plants with enhanced SA levels were inoculated with the arbuscular mycorrhizal fungi (AMF) *Glomus mosseae* or *Glomus intraradices*. In a time course study the effect of SA content on root colonization by the fungal symbiont was determined. Throughout the experiment in NahG plants an enhanced root colonization level could be detected, whereas in CSA plants mycorrhization was reduced. At the end of the experiment with *Glomus mosseae*, root colonization was similar in wild type and in transgenic plants (NahG and CSA plants), indicating that enhanced SA levels in plants can have an effect on delay AMF root colonization, but do not affect the symbiotic potential of plants in terms of changes in maximal threshold of root colonization. Compared to non-mycorrhizal plants, in mycorrhizal wild type and NahG the SA concentration was reduced. The role of SA in the regulation of mycorrhization is discussed.

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1. Introduction

Plants are endowed with various constitutive and inducible resistance responses to infectious agents. One general defence mechanism is known as systemic acquired resistance (SAR) [1]. SAR induction is strongly correlated with the coordinate expression of a set of PR genes and requires the signal molecule salicylic acid (SA), which accumulates in plants prior to the onset of SAR [2]. Thus the accumulation of SA has been proposed as an endogenous marker for plant resistance [3], linking enhanced SA levels in plants to a reduced susceptibility of these plants to pathogens.

Transgenic plants constitutively expressing a number of defence mechanisms have been shown as a valuable

tool to study plant defence responses against pathogenic [4] and symbiotic fungi such as arbuscular mycorrhizal fungi (AMF) [5,6]. In transgenic tobacco plants expressing the bacterial *nahG* gene, which encodes the enzyme salicylate hydroxylase that inactivates SA, levels of SA accumulation are reduced [7], whereas in transgenic CSA (constitutive SA biosynthesis) tobacco plants with two bacterial genes coding for enzymes that convert chorismate into SA, SA levels are highly enhanced [8], thus providing a tool to study the role of SA in pathogenic and symbiotic plant–microbe interactions. Testing these transgenic plants for their resistance to viral or fungal shoot pathogens showed that resistance levels and SA levels are linked [8,9].

Scarce information on the role of SA in the susceptibility of plant to symbiotic root micro-organisms is available. The application of SA to alfalfa plants inoculated with compatible *Rhizobium* strains caused a significant reduction in nodule formation [10] and in

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transgenic NahG *Lotus japonicus* plants with reduced SA levels, an increased number of nodules was formed [11]. To our knowledge no data are available on the effect of enhanced SA levels on root colonization by pathogenic or symbiotic soil-borne fungi.

Arbuscular mycorrhiza (AM) is a mutualistic symbiotic association between plants and root-colonizing fungi of the order Glomales, which improves plant nutrition and resistance towards a number of pathogens [12–14]. Findings in a number of studies indicate that in the host–AMF interaction plant defence responses are activated during early root colonization and are suppressed subsequently [15].

Despite the key role of SA in plant–pathogen interactions, its role during the formation of the AM association has received little attention. A transient accumulation of SA during the early stage of AM root colonization, reminiscent to the early activation of other plant defence responses [15], has been reported [16,17] and recently enhanced SA levels were linked to the inability of *Myc⁻ Pisum sativum* mutants to form the AM symbiosis [18].

In the work presented we used transgenic NahG and CSA tobacco plants with different SA levels as tools to study the role of SA during the AM association.

2. Material and methods

2.1. Biological material and growing conditions

Wild type and transgenic tobacco plants (NahG: *Nicotiana tabacum* cv. Xanthi-nc; CSA: *Nicotiana tabacum* cv. Samsun NN) were tested. Seeds were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated on a steam-sterilized (40 min, 120 °C) mixture of silicate sand, TurFace (baked clay substrate; Applied Industrial Materials, Corp.; Buffalo Grove, IL) and soil (v:v/v 1:1:1). Inoculated and non-inoculated plants were grown in a growth chamber (day/night cycle: 16 h; 23 °C/8 h; 19 °C; rel. humidity 50%).

Six weeks old plants were inoculated with one of the following two AMF.

The experiments with *Glomus mosseae* (BEG 12; La Banque Européenne des Glomales) was performed in the compartment system developed by Wyss et al. [19] in the substrate described above.

Inoculation with *Glomus intraradices* (DAOM 197198) was carried out in the same substrate in 80 ml pots. Each seedling was inoculated with a piece of Gel-Gro medium containing 50 spores and infected carrot roots. The monoxenic culture (*G. intraradices* and carrot roots) was made according to Chabot et al. [20].

Under our experimental conditions no visible differences between wild type and transgenic plants were

observed at the levels of percent of seeds germination, grown rate, and increase of biomass in mycorrhizal plants compared to non-colonized plants.

2.2. Estimation of root colonization

Several fresh roots from each plant were cleared by boiling in 10% KOH and stained according to the method of Vierheilig et al. [21] by boiling in a 5% ink (Shaeffer; black)/usual household vinegar (= 5% acetic acid) solution. Stained roots were observed with a light microscope and the percentage of root colonization was determined according to Ocampo et al. [22].

2.3. Extraction and quantification of SA

Free SA and its glucoside (SAG) were extracted and quantified as described by Malamy et al. [23]. One gram of frozen root tissue was ground in 3 ml of 90% methanol and centrifuged at 6000 × *g* for 15 min. The pellet was re-extracted with 3 ml of 100% methanol and centrifuged. Methanol extracts were combined, centrifuged for 10 min and dried at 40 °C under vacuum. For each sample, the dried methanol extract was resuspended in 5 ml of water at 80 °C, and the solution was divided into two equal portions. To one portion (SAG) an equal volume of 0.2 M acetate buffer (pH 4.5) containing 2 U/ml β-glucosidase (Sigma) was added, while to the other portion (free SA analysis) only buffer was added. Both portions were incubated at 37 °C overnight. After digestion, samples were acidified with HCl to pH 1 and SA was extracted and back extracted with 2 volumes of cyclopentane/ethyl acetate/isopropanol (50:50:1). The organic extract was dried under nitrogen, resuspended in 50 μl of 100% methanol and analysed by HPLC.

Ten microliter of methanolic extract was injected into a C18 column (Varian) and phenolics compounds were separated with 30% (v/v) methanol in 1% acetic acid with a flow rate of 1.0 ml/min at 40 °C. SA was identified with a fluorescence detector set (Varian) at 310 and 405 nm (excitation and emission, respectively) and quantified with a Data Module, using authentic standards. As has been described previously [24] the recovery of SA from tobacco using this methodology ranged between 30 and 50%.

2.4. Experimental design and statistical analysis

All experiments and measurements were repeated twice with at least three replicates per treatment. As results were similar in both experiments, data of only one experiment are given. Per experiment each replicate consist in a single tobacco plant takes from the total of plants line randomly distributed in the growth chamber. The percent values were arcsine non-linear transformed

and all the data were subjected to ANOVA. Means were compared using standard errors of means and Duncan's multiple range test ($P = 0.05$).

3. Results

3.1. Alteration on the AM colonization pattern by *G. intraradices* in transgenic NahG and CSA plants

The pattern of root colonization of wild type and transgenic plants was examined in a time course experiment. Wild type Xhanti-nc and transgenic NahG plants and wild type Samsun NN and transgenic CSA seedlings were inoculated with *G. intraradices*, and the percentage of root colonization was determined (Fig. 1).

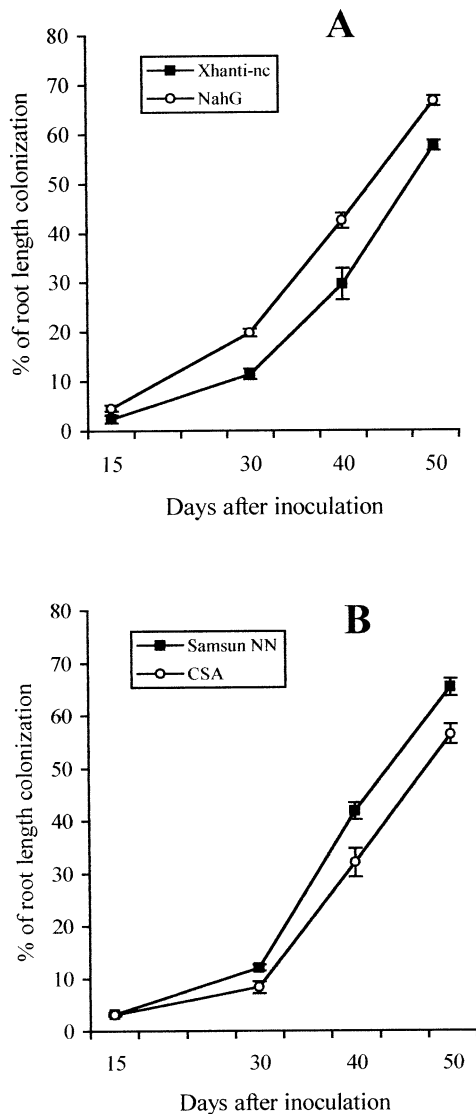


Fig. 1. Time course of root colonization by *G. intraradices* in wild type Xhanti-nc and NahG plants (A) and wild type Samsun NN and CSA plants (B). Values are means of at least three replicates \pm SE.

A typical pattern of root colonization was observed in all plants tested, showing after the initial phase of latency an exponential phase of mycorrhization. After the latency phase, at all time points NahG plants showed higher levels of mycorrhization than Xhanti-nc wild type (Fig. 1A). In contrast, throughout the experiment CSA plants (Fig. 1B) showed lower levels of mycorrhization than Samsun NN wild type plants. Samsun NN wild type plants and NahG Xhanti mutants revealed the same time course of root colonization, and Xhanti-nc wild type plants and CSA Samsun mutants exhibited a similar pattern of root colonization. The colonization of NahG and Samsun NN was higher than the colonization observed in Xhanti-nc and CSA.

The total SA content (free + glucosidate) in roots of NahG and CSA plants colonized by *G. intraradices* is shown in Fig. 2. At all time-points NahG plants had lower SA levels in the roots than wild type Xhanti-nc plants. In roots of CSA plants SA levels were higher compared to SA levels in roots of wild type Samsun NN. Interestingly, NahG mutant plants had similar SA content than Samsun wild type plants, and CSA and wild Xhanti-nc had similar SA levels.

3.2. AM colonization by *G. mosseae* in transgenic NahG and CSA plants

The pattern of root colonization of wild type and transgenic plants was examined in a time course experiment. Wild type Xhanti-nc and transgenic NahG plants and wild type Samsun NN and transgenic CSA seedlings were inoculated with *G. mosseae*, and the percentage of root colonization was determined (Fig. 3).

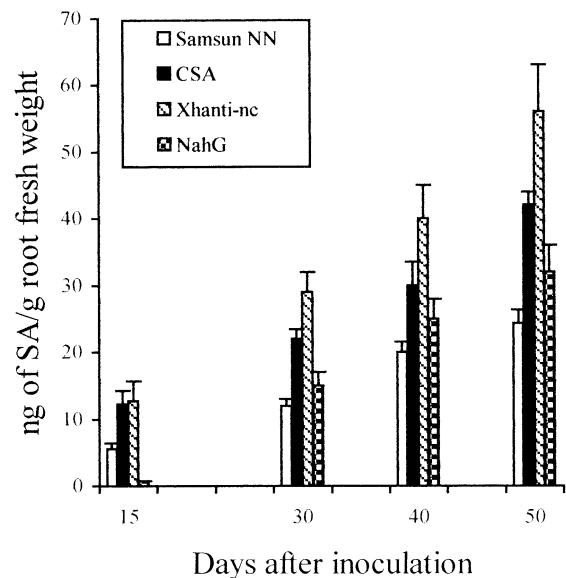


Fig. 2. Total SA levels (free SA + SA glucosidate) in a time course experiment of wild type and tobacco mutant plants inoculated with *G. intraradices*. Values are means of at least three replicates \pm SE.

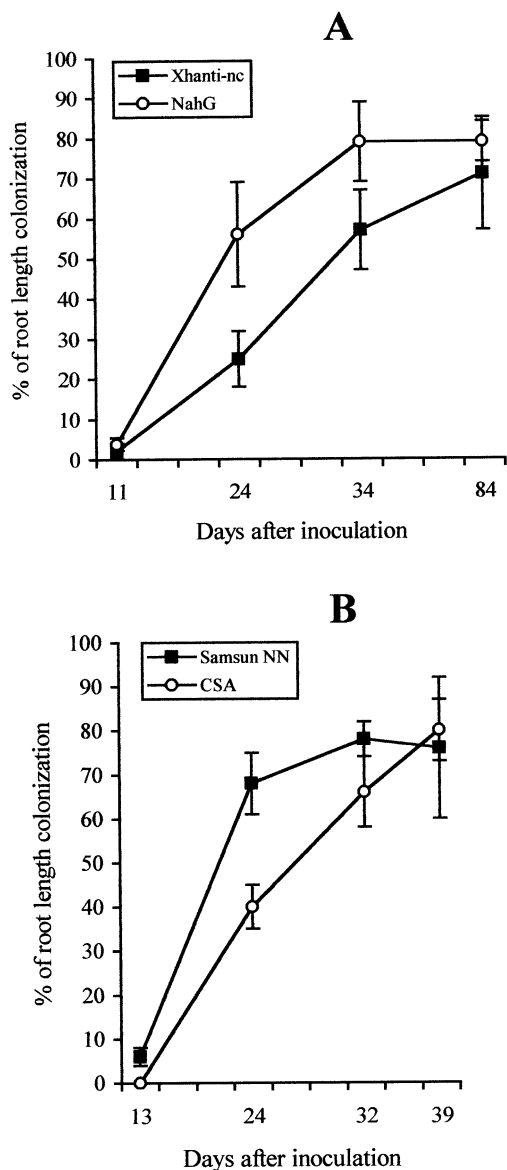


Fig. 3. Time course of root colonization by *G. mosseae* in wild type Xhanti-nc and NahG plants (A) and wild type Samsun NN and CSA plants (B). Values are means of at least three replicates \pm SE.

At the beginning of the experiment (11 days after inoculation) NahG plants showed similar levels of root colonization as wild type plants (Fig. 3A). Thereafter NahG plants were higher colonized (24 and 34 days after inoculation), whereas at the end of the experiment (84 days after inoculation) NahG plants showed similar colonization levels as wild type plants (Fig. 3A).

Throughout the experiment CSA plants showed lower level of root colonization than wild type plants, except at the end of the experiment (39 days after inoculation) when CSA plants showed similar colonization levels as wild type plants (Fig. 3B).

Interestingly at the end of both experiment (NahG and CSA) the levels of root colonization in the wild type

plants were adjusting to the colonization plateau in the NahG and the CSA plants.

Free SA and SA glucosidate were determined 34 days after inoculation in mycorrhizal and non-mycorrhizal roots Xhanti-nc and NahG plants. Free SA and SA glucosidate levels were lower in NahG plants compared to the wild type plants (Table 1). In mycorrhizal roots of NahG and wild type plants free SA and SA glucosidate levels were always lower compared to levels in non-mycorrhizal roots.

Moreover, in a second experiment with NahG plants the percentage of roots with arbuscules and of roots with infection units was determined (Table 2). At the beginning of the experiment (7 days after inoculation) NahG plants showed similar levels of root colonization as wild type plants. Thereafter NahG plants were higher colonized (14 and 21 days after inoculation), whereas at the end of the experiment (31 days after inoculation) NahG plants showed similar colonization levels as wild type plants (Table 2). Similar behaviour between both plants was obtained when the percentage of roots with arbuscules and of roots with infection units was analysed (Table 2). After the initial phase of latency, the exponential phase of mycorrhization (14 and 21 days after colonization) was characterized in NahG plants by a higher percentage of arbuscules and infection units than in wild type plants. Thirty-one days after inoculation similar levels of arbuscules and infections units were observed in wild type and NahG plants.

4. Discussion

During the establishment of the AM symbiosis the role of SA in the mediation of root colonization has been suggested before. Enhanced SA levels were linked to the inability of *Myc⁻ Pisum sativum* mutants to form the AM association [18] and SA exogenously applied to roots of AMF-inoculated rice reduced root colonization at the beginning of the interaction, but showed no effect on appressoria formation, thus excluding a direct inhibitory effect of the compound on AMF [16]. In contrast, application of SA to leaves of cucumber plants showed no effect excluding a systemic effect of the compound on mycorrhization [25].

In our study, with transgenic NahG and CSA tobacco plants, we could observe that SA levels are inversely correlated with the degree of root colonization. NahG plants with lower SA levels showed higher levels of root colonization, more infection units and more arbuscules, whereas CSA plants with higher SA levels than the control plants, showed lower root colonization levels. This root colonization pattern was nearly similar in both mutants inoculated with *G. intraradices* or *G. mosseae*, showing clearly that the observed effect of the SA level on mycorrhization is not AMF specific.

Table 1
Levels of free SA and SA glucosidate in wild type Xhanti-nc and NahG tobacco plants 34 days after inoculation with *G. mosseae*

Plant type	Free SA (ng/g root fresh weight)		SA glucosidate (ng/g root fresh weight)		% Root length colonization
	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal	Mycorrhizal
Xhanti-nc	23.3 b2	13.3 b1	172 b2	115 b1	57 a
NahG	9.7 a2	2.9 a1	73 a2	30 a1	79 b

Values are means of at least three replicate. Within free SA, SA glucosidate and % of root length colonization columns values followed by the same letter or row values followed by the same number are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 2
Time course of root length colonization, arbuscule and infection unit formation (% of roots) by *G. mosseae* in wild type Xhanti-nc and NahG plants

Days after inoculation	Plant type	% Root length colonization	% Roots length with arbuscules	% Roots length with infection units
7	Xhanti-nc	5.8 a	–	2.6 a
	NahG	9.7 a	0.8 a	5.9 a
14	Xhanti-nc	22.3 b	15.3 b	21.2 b
	NahG	44.8 c	31.1 c	34.7 c
21	Xhanti-nc	52.2 c	31.1 c	48.4 d
	NahG	64.6 d	52.8 d	60.7 e
31	Xhanti-nc	76.2 e	52.1 d	56.8 e
	NahG	75.3 e	54.0 d	61.3 e

Values are means of at least three replicate. Within columns values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

It is well known from a large variety of plants such as tobacco [17], and bean [19] that after a drastic increase the degree of root colonization by AMF reaches a final plateau. In the experiments with *G. mosseae* we found that at the end of the experiments when the colonization plateau in the plants with lower SA levels was reached, colonization levels were similar in wild type and mutants plants. This means that changed SA levels in plants have an effect on AMF during the establishment of the fungus, but they do not affect the level of final root colonization. No such effect could be observed in the experiments with *G. intraradices*. This might be due to the different root colonization pattern with this fungus in these experiments, thus, at the end of the experiment, the AMF colonization did not reach its plateau yet.

The importance of SA levels on root colonization could be observed in others root-microbe interactions. In transgenic NahG *Lotus japonicus* plants reduced SA levels resulted in an increased number of nodules [11], and correlation of SA with resistance to colonization also has been observed in the interaction of *Magnaporthe grisea* and rice [26]. These data suggest that SA may play a role as a constitutive defence compound in both mutualistic and pathogenic root interactions. The effect of accelerated mycorrhizal colonization observed in NahG plants has been showed in mycorrhizal *Medicago truncatula* plants over-expressing *enod40* early nodulin gene [27]. This is in line with the evidence that similar factors (signals, hormonal and gene products) control both rhizobial and mycorrhizal sym-

bioses. In this sense the function of the products of *sym* genes in the signal transduction pathway, including regulation of plant defences, should also be considered [15].

The suppression of a range of plant defence mechanisms during the AM association has been observed in several studies [15]. We could show, that SA levels as the signal molecule for the onset of SAR are also reduced by mycorrhization, however, we do not know whether reduced SA levels in mycorrhizal plants are responsible for the suppressed plant defence or whether a suppressional mechanism is affecting plant defence including SA accumulation.

To summarize, the data presented suggest a role of SA in the regulation of root colonization by AM fungi, however, whether enhanced SA levels are the symptom or the cause of a reduced colonization still has to be investigated.

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