Running title: Ethylene mediates *Sebacina vermifera*’s growth-promoting effects

**Corresponding author:**
Ian T. Baldwin
Department of Molecular Ecology
Max Planck Institute for Chemical Ecology
Hans-Knöll Strasse 8
07745 Jena
Germany

Phone: +49-3641-571100
Fax: +49-3641-571102
Email: baldwin@ice.mpg.de

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*Sebacina vermifera* Promotes the Growth and Fitness of *Nicotiana attenuata* by Inhibiting Ethylene Signaling

Oz Barazani‡†, Caroline C. von Dahl‡, and Ian T. Baldwin‡*

‡ Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll Strasse 8, 07745 Jena, Germany

†current address: Institute for Plant Sciences, Agricultural Research Organization, 50250 Bet Dagan, Israel

*corresponding author, Phone: +49 3641 571100, Fax: +49 3641 571102, Email: baldwin@ice.mpg.de

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Abstract

*Sebacina vermifera*, a growth-promoting endophytic fungus, significantly increases *Nicotiana attenuata*’s growth but impairs both its herbivore resistance and its accumulation of the costly, jasmonate (JA)-regulated, defense protein, trypsin proteinase inhibitor (TPI). To determine if the fungi’s growth-promoting effects can be attributed to lower TPI-related defense costs, we inoculated transformed *N. attenuata* plants silenced in their ability to synthesize JA, JA-isoleucine (Ile), and TPI by antisense (as-*lox3* and as-*td*) and inverted repeat (ir-*tpi*) expression, and found that inoculation promoted plant growth as in untransformed (WT) plants. Moreover, herbivore-elicited increases in JA and JA-Ile concentrations did not differ between inoculated and uninoculated WT plants. However, inoculation significantly reduced the morphological effect of 1-aminocyclopropane-1-carboxylic acid (ACC) on WT seedlings in a triple response assay, suggesting that ethylene signaling was impaired. Furthermore, *S. vermifera* failed to promote the growth of *N. attenuata* plants transformed to silence ethylene production (ir-*aco*). Inoculating WT plants with *S. vermifera* decreased the ethylene burst elicited by applying *Manduca sexta* oral secretions (OS) to mechanical wounds. Accordingly, OS-elicited transcript levels of the ethylene synthesis genes *NaACS3*, *NaACO1*, and *NaACO3* in inoculated plants were significantly lower compared to these levels in uninoculated WT plants. Inoculation accelerated germination in WT seeds; however, uninoculated WT seeds germinated as rapidly as inoculated seeds in the presence of the ethylene scrubber KMnO₄. In contrast, neither inoculation nor KMnO₄ exposure influenced the germination of ir-*aco* seeds. We conclude that *S. vermifera* increases plant growth by impairing ethylene production independently of JA signaling and TPI production.

**Keywords:** ethylene; endophyte; growth promotion; *Nicotiana attenuata; Sebacina vermifera*; trypsin proteinase inhibitor (TPI)
Introduction

Plants that associate with beneficial rhizosphere microorganisms, which include symbiotic and other endophytic and free-living rhizobacteria, often grow better than plants that don't (Glick, 1995; Varma et al., 1999; Strack et al., 2003; Barazani et al., 2005; Waller et al., 2005). The symbiotic associations of plants with arbuscular mycorrhizae (AM), ectomycorrhizal fungi, and nitrogen-fixing bacteria are referred to as mutualistic interactions. Symbiotic fungi or bacteria benefit from the plants' carbohydrates, while plants benefit when the supply of more stationary nutrients such as N, P, Ca, Mg, Zn, Cu, and Fe is increased. The sequence of events that leads to the development of symbiotic association involves regulating defense-related genes, which have been characterized during the early establishment of AM symbiosis (Kapulnik et al., 1996; Garcia-Garrido and Ocampo, 2002; Liu et al., 2003; Balestrini and Lanfranco, 2006). In addition, phytohormones, usually associated with plants' responses to biotic stresses, were shown to play a role in mycorrhizal development. In Allium sativum, for example, treatment with jasmonic acid (JA) was shown to stimulate mycorrhizal development (Regvar et al., 1996), and JA and its conjugated form JA-isoleucine (Ile) accumulated in the roots of Hordeum vulgare colonized with G. intraradices (Hause et al., 2002). Furthermore, silencing the AOC gene, which encodes allene-oxide-cyclase, an enzyme of the JA biosynthesis pathway, suppressed AM colonization, suggesting that jasmonates are associated with the establishment of a strong carbon sink in the roots (Hause et al., 2002; Strack et al., 2003; Isayenkov et al., 2005). In contrast, the mycorrhization of tobacco with G. mosseae reduced salicylic acid (SA) levels in the plant, and colonization by the fungus was suppressed by constitutive SA synthesis (Medina et al., 2003).

In addition to establishing symbiotic associations, plants are associated with a diverse range of free-living microorganisms which increase plant performance (Glick, 1995). This group of non-specific plant-growth-promoting rhizobacteria (PGPR), whose members can grow inside the root or on its surface, are known to increase plant fitness by secreting iron scavenging siderophores, reducing nitrates, fixing nitrogen, and producing plant growth regulators (Glick, 1995; Somers et al., 2004). In addition to supplying growth-limiting resources, plant-microbe interactions are often associated with increased resistance to plant pathogens (Pieterse et al., 2000; Borowicz, 2001; Pozo et al., 2004). Recently, Waller et al. (2005) related the increase
in grain yield and resistance among the pathogenic fungi of barley inoculated with *Piriformospora indica* to modifications in the anti-oxidative status of barley (*Hordeum vulgare*) (Waller et al., 2005). *P. indica*, a beneficial endophytic fungus (Sebacinales), was first isolated in India from the rhizosphere *Prosopis juliflora* and *Zizyphus nummularia* (Verma et al., 1998). *P. indica* was shown to increase the survival of regenerated tobacco plantlets (Sahay and Varma, 1999) and to increase the root and shoot biomass of *Zea mays*, *Nicotiana tabacum*, and *Petroselinum crispum* (Varma et al., 1999), as well as of *Spilanthes calva* and *Withania somnifera* (Rai et al., 2001).

Recently, we reported that *P. indica* and its genetically related species *Sebacina vermifera* increase the growth and fitness of *Nicotiana attenuata* (Barazani et al., 2005). However, the increased performance of inoculated *N. attenuata* came at the expense of the plant’s resistance to attack from the larvae of one of the plant’s most important lepidopteran insect herbivores, *Manduca sexta*. The decrease in herbivore resistance could be attributed to the down-regulation of trypsin protein inhibitor (TPI) activity (Barazani et al., 2005). Plants recognize that the specialist *M. sexta* is attacking when they are wounded and elicitors present in the larvae's oral secretions are introduced (OS) into the wounds during feeding (Halitschke et al., 2001). Applying OS to wounds is sufficient to induce a burst of two phytohormones, ethylene and JA, which activate a wide array of genes responsible for direct and indirect defenses, including the gene responsible for the accumulation of TPI. Consequently, the specialist larvae grow more slowly, presumably because the protein digestion in their gut is inhibited (Zavala et al., 2004b). However, the resistance benefits of TPI expression come at a substantial fitness cost for the plant. *N. attenuata* plants expressing TPIs produce 20% fewer seeds than do isogenic plants transformed to silenced TPI production; restoring TPI production by transforming an ecotype of *N. attenuata* naturally deficient in TPI production reduces lifetime seed production by 20% (Zavala et al., 2004a). Hence we hypothesized that the increase in growth and seed production that *N. attenuata* realizes from associating with *S. vermifera* results from the down-regulation of TPI production (Barazani et al., 2005).

Here we falsify this hypothesis with plants transformed in their ability to produce TPIs and in two steps in the JA signaling cascade required to elicit TPI production and demonstrate that *S. vermifera*’s growth-promoting effects result from
alterations in ethylene signaling. We show that: (i) increases in plant performance related to the fungus are independent of JA and TPI, but depend on the ability of the plant to produce ethylene; (ii) the beneficial effects of *S. vermifera* on seed germination and seedling growth are ethylene dependent; and (iii) the OS-induced ethylene emission and increased transcript accumulation of ethylene biosynthesis genes are reduced in *S. vermifera*-inoculated plants compared to uninoculated plants.

**Results**

*TPI activity and transcript accumulation is suppressed in OS-elicited inoculated plants*

TPI activity in OS-elicited rosette-stage leaves, 72 h after OS elicitation was nearly twice as high in uninoculated plants compared to *S. vermifera*-inoculated *N. attenuata* WT plants (Fig. 1). This significant (t-test, F1,6=6.67; P=0.04) difference in defense metabolite deployment was also detectable at the transcriptional level 6 h after elicitation (Fig. 1 Inset). In response to OS elicitation, TPI transcripts accumulated more rapidly in uninoculated plants than in inoculated WT plants (t-test, F1,6=7.63; P=0.04).

*Growth promotion by S. vermifera is unaffected in JA-, JA-Ile-, or TPI-silenced plants*

To determine whether the growth-promoting effect of *S. vermifera* resulted from the attenuation of the growth-related costs of TPI production or from the jasmonate signals that elicit TPI, we compared stalk lengths in inoculated and uninoculated WT plants and in plants transformed to silence JA and JA-Ile in antisense (as) orientation of lipoxygenase-3 (as-*lox3*), and threonine-deaminase (as-*td*), respectively; and TPI levels by inverted repeat construct (ir-*tpi*). *S. vermifera*-inoculation significantly increased stalk lengths of WT plants (ANOVA with repeated measures, F1,23=76.85; P<0.01). At the end of the growth phase, 56 d after germination, inoculated WT were 6.7% taller (t-test, F1,28=10.03; P<0.01) than uninoculated plants (Fig. 2A). In addition, inoculated WT plants started to flower 1 day earlier than uninoculated plants, a difference which was highly significant (Fig. 2A; t-test, F1,27=11.79; P<0.01).

Similarly, *S. vermifera* significantly increased the stalk lengths of as-*lox3* and as-*td* transformed plants (Fig. 2B,C; ANOVA repeated measures; as-*lox3*:
\[ F_{1,23}=107.60, P<0.01; \text{ as-td: } F_{1,19}=63.22, P<0.01. \]

\[ S. \text{ vermifera}-\text{inoculated as-}lox3 \text{ and as-td plants started to flower 1 to 2 days earlier (Fig. 2B,C; t-test as-}lox3: F_{1,28}=21.14; P<0.01; \text{ as-td: } F_{1,28}=12.39; P<0.01, \] and at the end of the growth phase, \( S. \text{ vermifera} \)-inoculated as-\( lox3 \) and as-\( td \) plants were 6.7 and 3.1\% taller than uninoculated plants, respectively (Fig. 2B,C; t-test, as-\( lox3 \): \( F_{1,28}=21.14; P<0.01; \) as-\( td \): \( F_{1,25}=4.79; P=0.03 \)). These results demonstrate that the growth-promoting effects are independent of the jasmonate signaling required to elicit herbivore defenses in \( N. \text{ attenuata} \).

Similar results were found in trials with ir-\( tpi \) plants. Inoculation significantly increased the growth of the inoculated transformed plants (Fig. 2D; ANOVA with repeated measures, \( F_{1,23}=84.04; P<0.01 \)) so that the final stalk lengths of \( S. \text{ vermifera} \)-inoculated ir-\( tpi \) plants were 6.5\% taller than those of uninoculated plants (t-test, \( F_{1,28}=13.40; P<0.01 \)). The day flowering began did not differ between the two inoculation treatments (Fig. 2D; t-test, \( F_{1,28}=0.18; P=0.67 \)). We conclude that the growth-promoting effects of \( S. \text{ vermifera} \) can not be attributed to an alleviation of the fitness costs of TPI production.

\( S. \text{ vermifera} \) inoculation does not affect the OS-elicited accumulation of JA and JA-Ile

Applying OS to wounded leaves elicits a dramatic JA burst which occurs in concert with a JA-Ile burst (Kang et al., 2006). These two factors have been shown to be responsible for most of the TPI transcript accumulation, as well as for the OS-induced increase in TPI activity (Halitschke and Baldwin, 2003; Kang et al., 2006). To verify the conclusions obtained from our observations of plant growth in as-\( lox3 \) and as-\( td \), we asked whether the JA and JA-Ile bursts were influenced by \( S. \text{ vermifera} \) inoculation. No quantitative or qualitative differences were observed between the amounts of OS-elicited JA (ANOVA with repeated measures, \( F_{1,7}=1.84; P=0.21 \)) and JA-Ile (t-test, \( F_{1,6}=0.29, P=0.61 \)) accumulated in the two inoculation treatments (Fig. 3).

\( S. \text{ vermifera} \) inoculation interferes with ethylene-signaling independently of ACC deaminase activity

The 'triple response assay' is a rapid means of estimating the sensitivity of plants to ethylene and has been successfully used to identify ethylene-insensitive mutants (Ecker, 1995). When dark-grown seedlings are exposed to ethylene, they...
display shortened root and hypocotyl growth, and a thickening of the hypocotyls, and the curvature of the apical hook becomes exaggerated. Since ACC synthase is frequently the rate-limiting step in ethylene biosynthesis, the germination media is often supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC) to accentuate the triple response phenotype. In the triple response assay of WT seedlings, root and hypocotyl growth were significantly inhibited by the presence of 5 μM ACC in the media (Fig. 4; ANOVA Student-Newman-Keuls multiple comparison test, P < 0.05). However, inoculating WT seeds with S. vermifera prior to the triple response assay significantly reduced the inhibitory effect of ACC on root and hypocotyl length (Fig. 4; ANOVA Student-Newman-Keuls multiple comparison test, P ≤ 0.05).

To determine whether the above effects are related to the ability of the fungus to degrade ACC by secreting ACC deaminase, the activity of the enzyme was assayed by measuring the amount of α-ketobutyrate produced during ACC cleavage (Penrose and Glick, 2003). By comparing the absorbance of α-ketobutyrate standard curve to the samples we found no evidence for ACC deaminase activity in cultures of S. vermifera, suggesting that the reduced inhibitory effect is not related to the fungus's use of ACC as a nitrogen source.

S. vermifera inoculation increases plant performance by inhibiting ethylene production

The altered growth performance of S. vermifera-inoculated seedlings observed in the triple response assay may be due to changes either in ethylene biosynthesis or in its perception. To examine how inoculation affects ethylene biosynthesis, we first compared the performance of inoculated and uninoculated N. attenuata plants transformed to silence ACC oxidase expression in inverted repeat constructs (ir-aco). S. vermifera did not increase the performance of inoculated ir-aco plants as it did with WT plants. Stalk lengths of ir-aco plants were also not influenced by inoculation with S. vermifera (Fig. 5A; ANOVA with repeated measures P>0.05). However, at the end of the growth phase, uninoculated ir-aco plants were 31.8% taller than uninoculated WT plants (compare Fig. 2A and Fig. 5A; t-test, F1,27=161.85; P<0.01 ). We therefore hypothesized that S. vermifera’s ability to reduce ethylene synthesis in inoculated
WT, as-\textit{lox3}, as-\textit{td}, and ir-\textit{tpi} plants was the reason for the increased growth performance of inoculated plants.

In the triple response assay, ACC significantly inhibited the root and hypocotyl growth of ir-\textit{aco} seedlings by 79.5 and 75.7%, respectively (Fig. 5B; root: \textit{t}-test, $F_{1,6}=21.04$; $P<0.01$; hypocotyl: \textit{t}-test, $F_{1,6}=3.43$; $P<0.01$). This demonstrates that ir-\textit{aco} plants still harbor sufficient ACO activity to induce a triple response. However, the inhibitory effect of ACC on both roots and hypocotyls was significantly reduced by pre-inoculation with \textit{S. vermifera} (Fig. 5B; multiple comparisons with Student-Newman-Keuls test, $P \leq 0.05$). These results are consistent with the hypothesis that the fungus inhibits ethylene production in plants. To test this hypothesis, we compared the amount of ethylene produced in response to OS-elicitation in inoculated and uninoculated plants. Measurements of ethylene emission from OS-elicited leaves revealed that uninoculated plants emitted 1.4 times more ethylene than did \textit{S. vermifera}-inoculated plants (Fig. 6 Inset; \textit{t}-test, $F_{1,14}=8.91$; $P<0.01$).

To learn how the fungus inhibits ethylene production, we measured the transcript accumulation of \textit{N. attenuata}'s ethylene biosynthetic genes by quantitative RT-PCR. OS elicitation in both uninoculated and inoculated plants resulted in the rapid accumulation of \textit{NaACS3a} transcripts, the first committed step of ethylene biosynthesis. Maximum transcript levels attained were not influenced by inoculation (\textit{t}-test, $F_{1,8}=0.01$; $P=0.90$). Six hours after OS elicitation, \textit{NaACS3a} levels began to decrease; final transcript levels of inoculated plants were significantly lower than those of uninoculated plants (Fig. 6; \textit{t}-test, $F_{1,6}=7.39$; $P=0.03$). In addition, we measured the transcripts levels of three ACO genes, which are involved in the second committed step of ethylene biosynthesis. Compared to transcript levels in uninoculated plants, those in inoculated plants of \textit{NaACO1} and \textit{NaACO3} were significantly reduced following OS elicitation: by 1.9-fold after 2.5 h and 3.0-fold after 6 h (Fig. 6; \textit{NaACO1 at 2.5 h l}: \textit{t}-test, $F_{1,7}=16.72$; $P<0.01$; \textit{NaACO3 at 6 h}: \textit{t}-test, $F_{1,8}=8.34$; $P=0.02$). No differences between the two inoculation treatments were measured in \textit{NaACO2} transcripts (Fig. 6). Unlike levels of ethylene biosynthetic genes, levels of the ethylene receptor gene \textit{NaETRI} were not affected by either OS elicitation or fungal inoculation (Supplementary Fig. 1).

\textit{Inoculation accelerates seed germination}
Whereas the germination of WT seeds on *S. vermifera*-inoculated media was significantly higher (85%) ($F_{1,5}=9.54; P=0.03$) than on uninoculated media (53%) (Fig. 7), the germination rate (70%) of ir-aco seeds was not influenced by the presence of the fungi (Fig. 7). The presence of the ethylene scrubber, KMnO$_4$, increased the germination rates of uninoculated WT seeds to the level found in *S. vermifera*-inoculated seeds (t-test, $F_{1,6}=5.85; P=0.05$). KMnO$_4$ had no effect on the germination of inoculated and uninoculated ir-aco seeds (Fig. 7). These results are consistent with the hypothesis that reducing ethylene in the headspace of germinating seeds accelerates germination and that inoculating plants with *S. vermifera* inhibits how much ethylene seeds produce during germination.

**Discussion**

*S. vermifera* (Sebacinales) increases the performance of *N. attenuata* plants by down-regulating ethylene production. Previously we showed that the association of *S. vermifera* increased the performance of inoculated *N. attenuata*. This growth benefit was accompanied by a decreased resistance to attack from *Manduca sexta* larvae, which could be attributed to the down-regulation of trypsin protein inhibitors (TPIs) (Barazani et al., 2005). Here we show that the association with *S. vermifera* also reduces the transcript levels of *NaTPI* (Fig. 1 Inset). Since the production of defense compounds provides a fitness benefit when plants are exposed to herbivores, but exacts fitness costs from the plant under normal growth conditions (Zavala et al., 2004a; Zavala et al., 2004b), growth promotion by *S. vermifera* could have resulted from reducing the costs of TPI production. However, inoculating transformed lines of *N. attenuata* impaired in their expression of *NaTPI* (ir-tpi) increased their growth performance just as it did in WT plants (Fig. 2A, D). This indicates that the beneficial effects of *S. vermifera* are not solely the result of down-regulating TPIs. Furthermore, ir-tpi plants flowered earlier than WT plants, and the flowering time of ir-tpi plants did not differ between the two inoculation treatments (Fig. 2D). Fitness benefits have been associated with the silencing of TPIs under constitutive conditions (Zavala et al., 2004a); these beneficial effects might be stronger than any effect of *S. vermifera* inoculation. Therefore, we can not exclude the possibility that earlier flowering time of *S. vermifera*-inoculated plants might be partially caused by down-regulating TPIs.
In addition to their effect on the nutritional status of a plant, its primary metabolism, and the plants' tolerance to stress, beneficial microorganisms can increase plant growth by modifying endogenous phytohormone levels in the plant (Smith and Read, 1997; Arkhipova et al., 2005; Ryu et al., 2005; Wang et al., 2005; Madhaiyan et al., 2006). Moreover, JA and its conjugated form, JA-Ile, were shown to be involved in the establishment of AM fungi (Hause et al., 2002; Isayenkov et al., 2005). To understand whether the increase in plant performance caused by S. vermifera is related to changes in phytohormone signaling, we measured the performance of S. vermifera-inoculated transgenic lines that had been independently silenced in two steps of the oxylipin pathways. Silencing the expression of lipoxygenase-3 (NaLOX3) (as-lox3) and threonine-deaminase (NaTD) (as-td) lowers TPI expression and increases plants' vulnerability to herbivores (Halitschke and Baldwin, 2003; Kang et al., 2006). The plant-growth-promoting effects of S. vermifera were as evident in these jasmonate-impaired transgenic lines as they are in WT lines (Fig. 2), demonstrating that the growth-promoting effects and down-regulation of TPIs in inoculated plants (Fig. 1) are not mediated by alterations in JA signaling by S. vermifera inoculation. Further support for this hypothesis was found in measurements of the JA and JA-Ile concentrations, which did not differ between the two inoculation treatments (Fig. 3). Similarly, P. indica (Sebacinales), which is closely related to S. vermifera, had no effect on the regulation of JA- and SA-related genes in barley (Waller et al., 2005).

O'Donnell et al. (1996) have shown that the expression of proteinase inhibitor genes in tomato is regulated both by JA and ethylene. In N. attenuata, ethylene emissions increased after feeding by M. sexta larvae or after treatments of wounded leaves with its oral secretions (OS) (Kahl et al., 2000). Although OS-induced nicotine levels are attenuated by ethylene (Kahl et al., 2000), a second well-described induced defense- the activity of TPI, is severely reduced in transgenic plants impaired in ethylene synthesis (ir-aco), constitutively and following OS elicitation (C.C. von Dahl and I.T. Baldwin unpublished data). Strikingly, growth promotion mediated by S. vermifera was lacking in fungus-inoculated ir-aco plants (Fig. 5A). These results are similar to those of Ryu et al., (2005), who demonstrated with several mutant lines of A. thaliana in an in-vivo experimental system that growth promotion by several beneficial bacteria required ethylene signaling. The effect of silencing of defense-
related genes on plant growth and fitness of *N. attenuata* has been previously discussed (Zavala and Baldwin, 2006). Here we show that inoculated as-"lox3" and as-td plants flowered earlier than uninoculated plants, which was not the case in ir-"aco" plants (Fig. 2, 5A). The fact that TPI is constitutively down-regulated in all the three transgenic lines is consistent with the hypothesis that ethylene signaling, rather than TPI production, mediates the growth promotion of *S. vermifera*-inoculated *N. attenuata* plants.

We further hypothesized that plants decrease their ethylene production when inoculated with *S. vermifera*. When ACC was added to germinating seedlings, the fungus inhibited the triple response of inoculated seedlings of both WT and ir-"aco" plants (Fig. 4, 5B). In addition, oxidizing ethylene with a KMnO₄ ethylene scrubber mimicked the effect of *S. vermifera* on the germination of WT seeds (Fig. 7), providing further evidence that the fungus promotes growth by manipulating ethylene production. Several beneficial microorganisms modify ethylene production by metabolizing ACC, and synthesizing and secreting ACC deaminase; the cleaved ACC can then be utilized as a nitrogen source by the fungus, and by reducing ethylene production in host plants, growth is promoted (Penrose and Glick, 2003; Madhaiyan et al., 2006). Because we did not find any evidence for ACC's deaminase activity in cultures of *S. vermifera*, we measured fungus-induced changes in the plant’s ethylene biosynthesis. Since OS elicitation dramatically stimulates ethylene production in *N. attenuata* (Kahl et al., 2000), we treated wounded leaves with OS and found in *S. vermifera*-inoculated plants a significant reduction in ethylene emission (Fig. 6 inset), as well as lowered transcript levels of the ethylene synthesis genes *NaACS3*, *NaACO1*, and *NaACO3* (Fig. 6), demonstrating a systemic down-regulation of ethylene biosynthesis in *S. vermifera*-inoculated plants. In a recent study by Waller et al. (2005), the ability of *P. indica* to increase plant tolerance to pathogenic attack and salt stress was associated with the increased concentration of ascorbate and the low concentration of dehydroascorbate (DHA) in inoculated barley roots. Since ACC oxidase converts ACC and ascorbate to ethylene and DHA, it is possible that down-regulating ACO genes in the inoculated roots increases the accumulation of ascorbic acid, consequently enhancing plants’ tolerance to biotic and abiotic stresses.

Several other reports have demonstrated the beneficial effects of *P. indica* on different plant species (Sahay and Varma, 1999; Peskan-Berghofer et al., 2004), but it...
was not clear how the fungus increases plant growth and fitness. We have shown that in the *N. attenuata*-*S. verminfera* interaction, inhibiting ethylene synthesis increases plants' susceptibility to herbivorous insects while promoting plant growth. Ethylene accumulates in plants in response to different biotic and abiotic stresses. In addition, ethylene production, an early response of pathogen attack, appears to help regulate defense responses (Knoester et al., 1998; Iniguez et al., 2005; Shan and Goodwin, 2006), including inhibiting mycelia growth (Chague et al., 2006). The JA/ethylene regulated proteinase inhibitors (O'Donnell et al., 1996) are a crucial defense response to both herbivores and pathogens (Mosolov et al., 1976; Ryan, 1989; Nakagami et al., 2005). Considering TPI's dual effects, the initial steps in the interaction of *N. attenuata* with *S. verminfera* may involve a suppression of the microbial-induced ethylene-regulated defense mechanisms. Additionally, a recent study has shown that in *P. indica*-inoculated barley roots, the programmed cell death hypersensitive reactions that are associated with the attack of biotrophic pathogens are repressed (Deshmukh et al., 2006), suggesting that upon colonization of plant roots with endophytic Sebacinales fungi, both ethylene and SA defense responses are suppressed. Whether this fungal-plant association is a true mutualistic interaction remains an open question which will be best addressed by experiments in *N. attenuata*’s natural habitat.

**Materials and Methods**

**Plant performance**

Seeds of an inbred line of *Nicotiana attenuata* Torr. ex. Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.) (WT) as well as of several genetically transformed antisense (as) and inverted repeat (ir) lines, as-\textit{lox3} A-300-1 (Halitschke and Baldwin, 2003); as-\textit{td} A-303-3 (Kang et al., 2006); ir-\textit{tpi} A04-186-1 (Steppuhn and Baldwin, in review; see supplemental material); and ir-\textit{aco} A03-321-10 (von Dahl et al., 2007) were germinated on Gamborg’s B5 medium (Krügel et al., 2002). Methyl jasmonate (MeJA)-induced levels of \textit{NaTPI} transcripts in ir-\textit{tpi} plants are below 1% of the transcript levels observed in elicited WT plants. Furthermore, no TPI activity is detectable in ir-\textit{tpi} plants regardless of the induction (Steppuhn and Baldwin, 2007; see supplemental material). Petri dishes were either pre-inoculated with *Sebacina verminfera* or left sterile. An axenic culture of *S. verminfera* (received from P. Franken,
Max Planck Institute for Terrestrial Microbiology, Marburg, Germany) was used to inoculate GB5 plates by pre-incubation in the dark at 26°C for 8 d (Barazani et al., 2005). During germination, plates were maintained at 26°C with an 11/13 h day/night cycle. Ten-day-old seedlings were transferred to Teku pots and 10 d later transferred to 1 L pots filled with B410 pot-soil mixture consisting of 95% turf and 5% clay, including 70 mg L⁻¹ N, 35 mg L⁻¹ P, and 75 mg L⁻¹ K with a pH between 5.5 and 6 (Stender, Lukau, Germany). Each of the genotype comparisons of uninoculated and *S. vermifera*-inoculated plants consisted of 10 to 15 pots with a single plant in each pot. About 1 month after germination, when had plants reached the elongation stage, stalk length was measured every second day and the start of flowering was recorded for each plant. About sixty days after germination, when plants stopped elongating, final stalk length was measured.

**OS-elicitation treatment**

Creating standardized puncture wounds and immediately applying *Manduca sexta* larvae oral secretions (OS) to the puncture wounds precisely mimics the transcriptional (Roda et al., 2004), proteomic (Giri et al., 2006) and metabolic (Halitschke et al., 2001) responses of *N. attenuata* to *M. sexta* attack. Moreover, with this method, the timing of the elicitation can be standardized precisely. To elicit TPI activity, transcript accumulation, and ethylene emission, puncture wounds on the leaf blade were created with a pattern wheel on each side of the midrib and diluted OS was immediately applied to the wounds. OS were collected from *M. sexta* larvae reared on *N. attenuata* leaf diet, diluted 1:5 (v/v) with water prior to each experiment.

**PI-activity assay**

To determine trypsin proteinase inhibitor (TPI) activity, leaf samples were harvested 3 d after OS elicitation, frozen in liquid nitrogen, and stored at -80°C until further processing. Samples were analyzed for TPI activity in an agar diffusion assay as described in (van Dam et al., 2001). Levels of TPI are expressed in nmol of inhibited trypsin proteinase molecules per milligram of total soluble protein, calculated by the clear zone of inhibitor-proteinase complex of the tested samples in reference to a standard soybean proteinase inhibitor curve (Jongsma et al., 1994). Protein concentration was determined according to Bradford (Bradford, 1976).
Phytohormone measurements

Leaf samples for hormone analysis were harvested at the indicated time points following OS elicitation. Approximately 300 mg of harvested leaf tissue were homogenized in 1 mL ethyl acetate spiked with 200 ng mL$^{-1}$ [$^{13}$C$_2$] jasmonic acid and para chlorogenic acid, as internal standards for JA and JA-Ile, respectively. After centrifugation at 13000 rpm for 20 min at 4°C, extraction was repeated with 1 mL ethyl acetate. The supernatants were combined and evaporated until dryness. The dried residue was re-dissolved in 500 µL 70% (v/v) methanol. Prior to analysis the samples were centrifuged for 10 min at 13000 rpm and 15 µL of the supernatant was analyzed using a Varian 1200L triple quadrupole MS (Varian, Darmstadt, Germany).

For the high-performance liquid chromatography, a Pursuit C8 column (150 mm x 2.0 mm, 3 µm particle size) was used and a gradient of water and methanol, both including 0.05% (v/v) formic acid, was the mobile phase with a flow rate of 0.2 mL min$^{-1}$. The mass spectrometer was operated in negative electro-spray ionization (ESI) mode with an argon pressure of 0.279972 Pa (= 2.1 mTorr) in the collision cell. A capillary voltage of -3200 V, a shield voltage of 600V and a detector voltage of 1800V was used. The pressure of the drying gas (N$_2$) was 131005 Pa (= 19 psi) at 300 °C and that of the nebulizing gas (air) was 379225 Pa (= 55 psi). The most abundant and characteristic fragment ion was chosen for quantification.

Ethylene emission was measured continuously and non-invasively in real time with a laser photoacoustic spectrometer. The light source consisted of a line-tunable infrared laser and the detection device was a resonant photoacoustic cell (INVIVO, Adelzhausen, Germany). For a detailed description, see von Dahl et al. (2007). “Stop-flow” measurements were performed with a 250 mL cuvette which was flushed with 130 to 150 mL min$^{-1}$ catalyzed air after the headspace of two fully mature, detached and OS-elicited leaves (+1 and +2 nodal positions) had accumulated in the cuvette for 3 h (n = 8).

Seedling performance assays

We used the triple response assay to measure the effect of 1-aminocyclopropane-1-carboxylic acid (ACC) supplementation and hence, ethylene, on the growth of uninoculated and S. vermifera-inoculated WT and ir-aco seedlings. Square (12 cm$^2$) Petri dishes were filled with 80 mL of GB5, with or without 5 µM
ACC (Fluka, Sigma, Taufkirchen, Germany), and the solidified agar was portioned out into two plates. Seeds (sterile or pre-inoculated with \textit{S. vermifera}) were placed on the agar to germinate. The plates were stored vertically in an incubator (26°C with an 11 h:13 h day/night cycle); after 3 days, when the radicles emerged, the light was turned off and seedlings were grown in the dark. Each inoculation and ACC treatment consisted of 4 plates each with 15 seedlings. After 10 d, the lengths of roots and hypocotyls were measured.

An ethylene scrubber (KMnO$_4$) was used to test the role of ethylene in \textit{S. vermifera}-mediated effects (Jayaraman and Raju, 1992). Seeds of WT and ir-aco plants were germinated on \textit{S. vermifera} pre-inoculated or sterile GB5 media in round Petri dishes ($r = 4.5$ cm) as described above. The open plates containing the seeds were placed in the center of a larger Petri dishes ($r = 7$ cm). The space of the larger Petri dish surrounding the smaller Petri dish was filled with 50 g KMnO$_4$ beads (‘Profresh’, Bioconservacion, Barcelona, Spain). Plates were maintained at 26°C with an 11 h:13 h day/night cycle. Germination was assessed every 24 h until all seeds were fully developed. Each treatment consisted of 4 replicate plates with 15 seeds per plate.

\textit{ACC deaminase analysis}

Measurement of ACC deaminase activity was performed following Penrose and Glick (2003). For fungus culture, mycelia of \textit{S. vermifera} were inoculated in 25 mL LB medium. Cultures were grown in the dark at 200 rpm, at 26°C. After 8 d, fungus mycelia were transferred to minimal medium with ACC as the only N source. Measurements of enzyme activity were conducted on two separate cultures as described by Penrose and Glick (2003).

\textit{RNA isolation and mRNA expression}

Fully mature leaves (at nodal position +1) of rosette-stage plants were elicited with OS as described above. Leaves were collected at different time points after the elicitation (for ethylene biosynthesis genes: 0 non-elicited, 60, 120, 180, 240, 300, and 360 min; for \textit{NaTPI}: 0, 6, 12, 24, 48, and 72 h), immediately frozen in liquid nitrogen, and kept at -80°C until further processing. For each time point, one leaf was harvested from 5 different elicited plants. Total RNA was extracted using TRI reagent (Sigma,
Taufkirchen, Germany). cDNA was synthesized from 20 ng of total RNA as described by (Schmidt et al., 2005) using the Taqman reverse transcription reagent kit (Applied Biosystems, Darmstadt Germany). Analysis of the relative expression of ethylene biosynthesis and perception genes was performed using primer pairs and fluorescent dye-labeled probes for NaACS3a (AY426752), NaACO1 (AY426756), NaACO2 (EF123109, NaACO3 (EF123111), and NaETR1 (EF203416), as described by (von Dahl et al., 2007). Analysis of NaTPI (AF542547) was performed using primers and fluorescent dye-labeled probes as described by (Zavala et al., 2004a). For each analysis, a linear standard curve, threshold cycle number (Ct) vs. Log (designated transcript level), was constructed using a series dilutions of a specific cDNA standard; the levels of the transcript in all unknown samples were determined according to the standard curve. A *N. attenuata* sulfite reductase (EC1), which is a house-keeping gene involved in plant sulfur metabolism and has been shown to have constant levels of transcript by both northern blotting and q-PCR, after W+W and W+OS treatments (Wu et al., 2007), was used as an internal standard for normalizing cDNA concentration variations. Real-time PCR was performed on a SDS7700 (Applied Biosystems, Darmstadt, Germany) using the qPCR™ reagent kit (Eurogentec, Seraing, Belgium); for a detailed description see (Schmidt et al., 2005).

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**Figure legends**

**Figure 1.** Association with *S. vermifera* reduces *Manduca sexta* oral secretions (OS)-induced trypsin proteinase inhibitors (TPIs). Mean ± SE TPI activity in leaves of wild-type (WT) *N. attenuata* plants of uninduced plants (0 h) and at different time points after elicitation by wounding and applying *M. sexta* OS. Open and filled symbols indicate uninoculated and *S. vermifera*-inoculated plants, respectively. **Inset:** Mean ± SE of the relative transcript levels of NaTPI of the same plants. Asterisk indicate significant differences (t-test, P<0.05) between inoculated and uninoculated plants at the respective times.

**Figure 2.** *S. vermifera* promotes growth of *N. attenuata*. Mean ± SE cm stalk length of uninoculated (open symbols) and *S. vermifera* inoculated (filled symbols) *N. attenuata* plants at the indicated days after sowing. Open and filled arrows indicate the first day of flowering of uninoculated and fungus-inoculated plants, respectively. We measured wild-type (A) *N. attenuata* plants as well as transgenic plants expressing NaLOX3 (B) or NaTD (C) in an antisense orientation and expressing NaTPI (D) as inverted repeat, all of which are impaired in either their JA, JA-Ile, or TPI accumulation, respectively. Repeated measures ANOVA revealed significant differences (P < 0.01) for all comparisons between inoculated and uninoculated plants within one genotype.

**Figure 3.** OS-induced accumulation of oxylipin-derivatives in *S. vermifera*-inoculated and uninoculated *N. attenuata* plants. Mean ± SE JA concentrations in leaves of uninoculated (open symbols) and *Sebacina vermifera* inoculated (filled symbols) WT plants. Fully mature leaves (at nodal position +1) of rosette-stage plants were OS-elicited and harvested from 15 to 90 min after inoculation. Untreated leaves were harvested at time 0 min. **Inset:** Mean ± SE JA isoleucine + JA leucine (JA-Ile/Leu) concentrations in leaves of uninoculated (open bars) and *Sebacina vermifera* inoculated (filled bars) WT *Nicotiana attenuata*. OS-induced samples were harvested 35 min after wounding and OS application to the leaves; control samples were taken from leaves at the same nodal position of non-elicited plants.
**Figure 4.** Triple response of uninoculated and *S. vermifera*-inoculated *N. attenuata* seedlings. Mean + SE hypocotyl and root length in mm of 10 d old uninoculated (open bars) and inoculated (filled bars) WT seedlings in a triple response assay. Inoculated and uninoculated WT seeds were germinated on media with and without the addition of 5 µM 1-aminocyclopropane-1-carboxylic acid (ACC). Different capital letters and lower case letters indicate significant differences among roots and hypocotyls, respectively (ANOVA Student-Newman-Keuls multiple comparison test, P < 0.05).

**Figure 5.** Growth promotion by *S. vermifera* of *N. attenuata* plants is ethylene dependent. (A) Mean ± SE stalk length of uninoculated (open symbols) and *S. vermifera*-inoculated (filled symbols) transformed *N. attenuata* plants expressing an ACO consensus region as an inverted repeat (ir-aco) on the indicated days after sowing. Open and filled arrows indicate the first day of flowering of uninoculated and fungus-inoculated plants, respectively. For comparison see stalk elongation of WT plants presented in Figure 2A. (B) Mean + SE hypocotyl and root length of 10 d old uninoculated (open bars) and *S. vermifera*-inoculated (filled bars) ir-aco seedlings in a triple response assay. Inoculated and uninoculated seeds were germinated on media with and without the addition of 5 µM 1-aminocyclopropane-1-carboxylic acid (ACC). Different capital letters and lower case letters indicate significant differences among roots and hypocotyls, respectively (ANOVA Student-Newman-Keuls multiple comparison test, P < 0.05).

**Figure 6.** OS-induced ethylene emission and ethylene biosynthetic transcripts in leaves of *S. vermifera*-inoculated and uninoculated plants. Mean ± SE of the relative transcript levels of *N. attenuata*'s ethylene synthesis genes, *NaACS3a, NaACO1, NaACO2,* and *NaACO3* in uninoculated (open symbols) and *S. vermifera*-inoculated (filled symbols) WT plants, at the indicated time points following OS elicitation. Asterisks indicate significant differences between fungus-inoculated and uninoculated plants (ANOVA, P < 0.05). Inset: Mean ± SE ethylene emitted by excised rosette-leaves from uninoculated (open bars) and *S. vermifera*-inoculated (filled bars) WT *N. attenuata* plants. Ethylene was accumulated in the headspace for 3 h after leaves were OS elicited. Asterisk indicates significant difference (t-test, P < 0.01).
**Figure 7.** *S. vermifera* induces germination of *N. attenuata* by inhibiting ethylene production. Mean ± SE germination rate of WT and ir-acco *N. attenuata* seeds on *S. vermifera*-inoculated (filled bars) and uninoculated (open bars) plates with and without the ethylene scrubber, KMnO₄. The number of germinated seeds was determined seven days after sowing. Asterisks indicate significant difference in germination between inoculated and uninoculated seeds within one genotype of each treatment (t-test, P < 0.05).

**Supplementary Figure**

**Supplementary Figure 1.** Transcript accumulation of ethylene receptor gene *NaETR1* in the leaves of OS-elicited uninoculated and *S. vermifera*-inoculated *N. attenuata*. Mean ± SE of the relative transcript levels in uninoculated (open symbols) and inoculated (filled symbols) WT plants, at the indicated time points following induction of the leaves by wounding and OS treatment. No significant differences were found between *S. vermifera*-inoculated and uninoculated plants at the indicated time points (t-test, P>0.05)
