The role of three small signal molecules in adaptation and herbivore resistance in *Nicotiana attenuata*

Dissertation

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Manuscript overview

Manuscript I

NaRALF, a peptide signal essential for the regulation of root hair tip apoplastic pH, is required for root hair development, and plant growth in its native soil

In this manuscript, we investigated the role of a peptide signal NaRALF in *N. attenuata*. Rapid alkalinization factor (RALF) is a 49-amino-acid peptide that rapidly alkalinizes cultivated tobacco cell cultures. In the native tobacco, *N. attenuata*, *NaRALF* occurs as a single copy gene and is highly expressed in roots and petioles. Silencing *NaRALF*'s transcript by transforming *N. attenuata* with an inverted-repeat construct generated plants (ir*RALF*) with normal wild-type (WT) above-ground parts but with roots that grew longer and produced trichoblasts that developed into abnormal root-hairs. Most trichoblasts produced a localized "bulge" without commencing root-hair tip growth; fewer grew, but were only 10% as long as those of WT plants. The root-hair phenotype was associated with slowed apoplastic pH oscillations, increased pH at the tips of trichoblasts, and decreased accumulation of reactive oxygen species in the root-hair initiation zone. The root-hair growth phenotype was partially restored when ir*RALF* lines were grown in a low-pH buffered medium and reproduced in WT plants grown in a high-pH buffered medium. When ir*RALF* plants were grown in pH 5.6, 6.7, and 8.1 soils and competed against WT plants in glasshouse experiments, they were out-competed by WT plants in basic but not acidic soils. When WT and ir*RALF* lines were planted into the basic soils of *N. attenuata*'s native habitat in the Great Basin Desert, ir*RALF* plants had smaller leaves, shorter stalks, and produced fewer flowers and seed capsules than WT plants. We conclude that NaRALF is required for the regulation of root-hair extracellular pH, the transition from root-hair initiation to tip growth, and plant growth in basic soils.

Dr. I. T. Baldwin and I designed all the experiments; Dr. A. Patankar cloned the *NaRALF* cDNA, E. L. Kurten and I screened the stable transformation lines; S. Gilroy and G. Monshausen measured the root hair apoplastic pH of ir*RALF* line 2. G. M. Hummel measured the root growth velocity of WT and ir*RALF* line 1 plants. I performed the remaining work.

Manuscript II

Methyl jasmonate (MeJA)-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA?

In this manuscript, we investigate how *N. attenuata* plants perceive outside MeJA signals. Treatment with methyl jasmonate (MeJA) elicits herbivore resistance in many plant species and over-expression of JA carboxyl methyltransferase (JMT) constitutively increases JA-induced responses in *Arabidopsis*. When wild-type (WT) *Nicotiana attenuata* plants are treated with MeJA, a rapid transient endogenous JA burst is elicited, which in turn increases levels of nicotine and trypsin proteinase inhibitors (TPIs) and resistance to larvae of the specialist herbivore, *Manduca sexta*. All of these responses are impaired in plants silenced in lipoxygenase 3 expression (as*LOX3*) but are restored to WT levels by MeJA treatment. Whether these MeJA-induced responses are directly elicited by MeJA or by its cleavage product, JA, is unknown. Using virus-induced gene silencing (VIGS), we silenced MeJA-esterase (*NaMJE*) expression and found this gene responsible for most of the MeJA-cleaving activity in *N. attenuata* protein extracts. Silencing *NaMJE* in as*LOX3*, but not in WT plants significantly reduced MeJA-induced nicotine and JA-Ile levels, and resistance to *M. sexta*, but not TPI levels. MeJA-induced transcript levels of threonine deaminase (*NaTD*) and phenylalanine ammonia lyase (*NaPAL1*), were also decreased in VIGS *MJE* (as*LOX3*) plants. Finally the performance of *M. sexta* larvae that fed on plants treated with JA or MeJA demonstrated that silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance in as*LOX3* plants. From these results, we conclude that the resistance elicited by MeJA treatment is not directly elicited by MeJA but by its de-methylated product, JA, either alone or after conjugation with Ile.

I isolated the *NaMJE* cDNA. Dr. I. T. Baldwin and I designed all the experiments. Together with Dr. L. Wang, we generated VIGS plants and analyzed their defense responses to *M. sexta*.

Manuscript III

***Narboh D*, a respiratory burst oxidase homolog in *Nicotiana attenuata*, is essential for resisting attack from both herbivores and pathogens**

In this manuscript, we investigate the roles of reactive oxygen species from Narboh D in the defense responses of *N. attenuata*.

Levels of ROS (reactive oxygen species) increase at wound sites when the specialist herbivore *Manduca sexta* attacks *Nicotiana attenuata*, as are transcripts of the respiratory burst oxidase homolog-*Narboh D*, which are also rapidly and transiently elicited by wounding and amplified when *M. sexta* oral secretions (OS) are added to the wounds. Silencing *Narboh D* significantly reduced ROS levels after OS elicitation, demonstrating that *Narboh D* is largely responsible for herbivore-elicited ROS production. In *Narboh D*-silenced plants, neither OS-elicited JA and JA-Ile bursts nor early transcripts (*NaJAR4* and *NaPAL1*) were influenced; however, the OS-elicited levels of trypsin proteinase inhibitors (TPIs), as well as of defense genes such as *polyphenol oxidase*, *TPI* and *Thionin*, and of herbivore resistance, especially to generalist *Spodoptera littoralis*, were dramatically reduced, demonstrating the importance of ROS derived from *Narboh D* in herbivore-elicited late responses. *Narboh D* transcripts are also strongly elicited by the challenges from *Trichoderma sp*. and *Pseudomonas syringae* (DC3000). Plants silenced in *Narboh D*, but not *COI1* (coronatine insensitive1) and *NPR1* (non-expressor of PR1), are highly susceptible to *Trichoderma sp.*, a very mild pathogen widely used as biological control agent. We also observed that *P. syringae* performed better in *Narboh D*-silenced plants; this increased susceptibility, which is SA-independent, is a result of the lower accumulation of defense proteins such as NaThionin. Based on these results, we conclude that ROS derived from *Narboh D* are essential in both herbivore- and pathogen-elicited defense.

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I isolated the *Narboh D* cDNA. Dr. I. T. Baldwin and I designed all the experiments. Together with Dr. L. Wang, we generated VIGS plants and analyzed their defense responses.

Introduction

Nicotiana attenuata and the challenges it faces in nature

Figure 1. *N. attenuata* and its natural habitat. (Photo courtesy by D. Kessler)
(A): Fire in Utah 2005.
(B): *N. attenuata* in Utah.
(C): *Manduca sexta*

The wild tobacco *N. attenuata* Torr. Ex Watson (synonymous with *Nicotiana torreyana* Nelson & Macbr.) is an annual diploid (2n = 24), largely selfing, which commonly grows after fires in the blackbrush, sagebrush and pinyon-juniper forests of

the Great Basin Desert of North American. Its short generation time and selfing properties make it a good system for both molecular and genetic studies.

N. attenuata plants germinate from long-lived seed banks after being exposed to cues from smoke (Figure 1A and B) (Baldwin and Morse, 1994; Baldwin *et al.,* 1994). Burning significantly increases not only the concentration of N, P, Mn, Ca, and Mg but also the pH of the soils (up to 7.89 ± 0.07 after burning) (Baldwin and Morse, 1994). The basic soil (around pH 8) of the Great Basin Desert requires plants to invest more energy in the active transport of nutrients. *N. attenuata* has evolved a complex mechanism to adjust its root hair cell wall pH to cope with such a situation (Wu et al. 2007).

In nature, *N. attenuata* plants are attacked by herbivores from more than 20 taxa, including mammalian browsers that can consume entire plants, intracellular sucking insects, and leaf-chewing insects, among which *Manduca sexta* and *M. quinquemaculata* are the major contributors to defoliation (Figure 1C). These native plants have evolved sophisticated means of coping with these herbivores, including the production of volatile signals that function as indirect defenses or a fine-tuned metabolic re-configuration and the expression of defense-related genes (Kessler *et al*., 2002).

RALF (rapid alkalinization factor)

RALF, a 49-amino-acid polypeptide isolated by Clarence Ryan and colleagues while searching for peptides that regulate the wound response of cultivated tobacco plants, elicits a stronger and more rapid alkalinization of the medium of tobacco suspension-cultured cells than do the tobacco systemins (Pearce *et al.,* 2001). Like the systemins, RALF also elicits MAP kinase activity in cultured cells (Pearce *et al.,* 2001). The apoplastic localization of RALF (Escobar *et al*., 2003), together with the discovery of 120 kDa and 25 kDa cell membrane proteins, which can specifically bind to RALF (Scheer *et al*., 2005), indicate that RALF may exert its biological activity through a (or some) specific interaction(s) with a cell membrane receptor.

When tomato and Arabidopsis seedlings were germinated and transferred to a medium containing micromolar levels of tomato RALF peptides, root growth was immediately arrested (Pearce *et al.,* 2001), suggesting RALF may play a role in root development.

Root hair

Root hairs, projections from the surface of root epidermal cells (trichoblasts), have been proposed to play critical roles in water and nutrient uptake, and in anchoring the plant in the soil (Ridge, 1995). To produce a root hair, a trichoblast undergoes two stages of development: root hair initiation and tip growth. During initiation, a highly localized bulge is produced in the outer surface of the trichoblast. Once initiated, the root hair commences tip growth. New cell wall material is deposited only on the expanding tip of the developing hair, leading to the elongated hair-like morphology (Schnepf, 1986).

Localized acidification of the cell wall and alkalinization of the cytoplasm are some of the first detectable indications of imminent root hair initiation (Bibikova *et al.,* 1998). The local apoplastic acidification of the cell wall can be prevented by treatment with high-pH buffers, which not only stop the trichoblast from bulging but also arrest the elongation of root hairs that have started tip growth (Bibikova *et al.,* 1998). These results suggest that not only root hair initiation but also tip growth requires tight regulation of apoplastic pH. This pH change may, for example, activate expansin proteins that catalyze the loosening of the cell wall and so promote turgor-driven expansion (Bibikova *et al.,* 1998; Baluska *et al.,* 2000). The mechanism responsible for this cell wall acidification is still unclear. One hypothesis evokes an unknown molecule, activated by developmental stimuli, that through receptors in the plasma membrane signals the acidification of the cell wall either by increasing a cell's ion exchange capacity or by activating a proton ATPase or other transporter.

Herbivory-induced defense responses in *N. attenuata*

Several studies have revealed the importance of fatty acid-amino acid conjugates (FACs) from herbivore oral secretions (OS) in eliciting herbivory-specific responses in plants (Alborn *et al*., 1997; Alborn *et al*., 2003; Halitschke *et al*., 2003). During herbivory, OS are introduced into wounds and elicit 1) jasmonic acid (JA) and ethylene bursts which are greater than those elicited by mechanical wounding (Kahl *et al.,* 2000); 2) high levels of trypsin proteinase inhibitors (TPIs), an important direct defense compound (Zavala *et al.,* 2004a); and 3) the release of volatile organic compounds (VOCs), which function as indirect defenses by attracting predators to feed on herbivores (Kessler and Baldwin, 2001). The functions of FACs are also demonstrated when FACs are removed from OS by ion-exchange chromatography,

abolishing *N. attenuata*'s herbivory-specific responses, i.e. cis-α-bergamotene emission, JA bursts, and extensive OS-specific transcript accumulation; moreover, adding synthetic FACs back to FAC-free OS restored all of the OS-elicited responses, and treating wounds with aqueous FAC solutions mimicked the effects of OS (Alborn *et al.,* 1997; Halitschke *et al.,* 2001; Halitschke *et al.,* 2003). Through a largely unknown signal transduction network, plants perceive FACs and rapidly initiate a suite of defense-related responses.

Methyl jasmonate (MeJA), jasmonic acid (JA) and its amino acid conjugates, collectively referred to as jasmonates, are important cellular regulators mediating diverse developmental processes including root growth, pollen production, and plant resistance to insects and pathogens (Creelman and Mullet 1997; Kessler and Baldwin 2002). Jasmonates are synthesized in plants via the octadecanoid pathway (Creelman and Mullet 1997). Briefly, linolenic acid is oxygenated by lipoxigenase (LOX) and then converted to 12-oxo-phytodienoic acid (12-oxo-PDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). JA is synthesized from 12-oxo-PDA through reduction and three steps of β-oxidation, and then catabolized further to form its volatile counterpart, MeJA (Seo et al. 2001), and numerous conjugates including JA-isoleucine (JA-Ile) (Staswick and Tiryaki 2004; Kang et al. 2006; Wang et al. 2007a).

JA has long been recognized as the main signaling molecule mediating a plant's defense system against herbivores (Creelman and Mullet, 1997; Reymond and Farmer, 1998; Halitschke *et al.,* 2003). In *N. attenuata*, applying OS to mechanically generated wounds induces a rapid JA burst with higher levels than does wounding alone (Kahl et al., 2000). The function of JA in *N. attenuata*'s defense against herbivory has been demonstrated by silencing *lipoxygenase3* (*NaLOX3*), which leads to greatly reduced levels of herbivory-induced JA and, therefore, lower levels of nicotine and TPI activity than those in wild-type (WT) plants (Halitschke and Baldwin, 2003).

Recently, JA-Ile rather than JA reportedly induced some resistant traits in *N. attenuata* (Kang *et al.* 2006; Wang *et al.* 2007a; Wang *et al.* 2007b), including TPIs and nicotine levels. After mechanical wounding or applying OS to wounds, JA-Ile is rapidly produced in wounded leaves, closely following the kinetics of the JA burst. *NaJAR4* and *NaJAR6* encode enzymes mainly for conjugating JA and isoleucine needed to produce JA-Ile. Transcripts of both *NaJAR4* and *NaJAR6* are rapidly and transiently increased by wounding and amplified when *M. sexta* OS are added to the

wounds. Plants silenced in the expression of both *NaJAR4* and *NaJAR6* produced only 15% as much JA-Ile as that of WT plants without influencing the JA burst. These plants were clearly more susceptible to *M. sexta* than any of the plants individually silenced in their *JAR4* and *JAR6* expression (Wang *et al.,* 2007b).

MeJA-induced plant responses

MeJA is one of the JA metabolites proposed to play an important role in inter- and intra-plant signaling (Farmer and Ryan 1990; Seo *et al.* 2001; Karban *et al*. 2000; Kessler *et al*. 2006; Baldwin *et al.* 2006). When plants are exposed to a certain amount of MeJA, a series of JA-mediated defense responses is elicited, and MeJA treatment is the most commonly used means of eliciting herbivore resistance in many plant species (McConn *et al.* 1997; Baldwin 1998; Li *et al.* 2002). However, it is still not known how plants elicit herbivore resistance traits in response to MeJA exposure.

When *NaLOX3*-silenced plants are treated with MeJA, their ability to produce nicotine and to resist herbivores is fully restored (Halitschke and Baldwin 2003), suggesting that the exogenous MeJA treatment is sufficient to elicit most JA responses. However, these results raise an important question: does the exogenously supplied MeJA function directly as a signal or must it first be hydrolyzed to JA?

Staswick's pioneering work on MeJA-insensitive *jar1* mutant in *Arabidopsis* suggests that exogenously applied MeJA is first demethylated and then conjugated to Ile before it becomes active in the inhibition of root growth (Staswick et al. 1992; Staswick and Tiryaki 2004). Indeed, MeJA hydrolyzing enzyme activity occurs in all the plant species so far tested (Stuhlfelder et al. 2002). It is also reported that MeJA was rapidly hydrolyzed to JA and further metabolized like JA when tobacco BY-2 cells were treated with MeJA (Swiatek et al. 2004). We tested the hypothesis that MeJA-elicited herbivore resistance is elicited after de-esterification to JA and subsequent conjugation to JA-Ile by investigating the MeJA-induced responses in *NaMJE*-silenced WT and as*LOX3* plants.

Reactive oxygen species (ROS) and *Narboh D*

The early production of reactive oxygen intermediates (ROS, largely O_2^- and/or its dismutation product H_2O_2) is a hallmark of defense responses to pathogens, especially in incompatible interactions between resistant plants and avirulent pathogens (Lamb and Dixon 1997). ROS can be produced by chloroplasts,

mitochondria, and peroxisomes, through the metabolic processes of photosynthesis and respiration (Apel and Hirt 2004), or by apoplastic cell-wall peroxidases, germin-like oxalate oxidases, and amino oxidases (Apel and Hirt 2004; Mittler *et al.* 2004). However, pharmacological and molecular data indicate that the enzymes responsible for most of the ROS generated during biotic interactions and in response to abiotic stresses are similar to the superoxide (O_2^-)-generating NADPH oxidases, originally characterized in mammalian phagocytes (Simon-Plas *et al.* 2002; Torres *et al*. 2002; Torres *et al.* 2005; Yoshioka *et al*. 2003; Sagi *et al*. 2004). NADPH oxidase-dependent H_2O_2 is also thought to function as a second messenger mediating the systemic expression of various defense-related genes in tomato plants (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas *et al.* 2001). Increased ROS levels have been reported at the attack site when herbivores consume soybean and lima bean (*Phaseolus lunatus*) leaves (BI and Felton 1995; Maffei *et al.* 2006). These results suggest that ROS produced by NADPH oxidase may play a role in plant-herbivore interactions. Here we explore this possibility in the *Nicotiana attenuata-Manduca sexta* interaction.

Narboh D is a homologue to *Ntrboh D* and *Atrboh D*, and can be rapidly and transiently wound- and herbivore-induced. Thus Narboh D is a good candidate NADPH oxidase for producing ROS in response to herbivory.

The objective of this thesis is to study 1) how *NaRALF* is involved in plant-environment interactions; 2) how plants perceive the exogenous MeJA signal; 3) how Narboh D-dependent ROS regulate *N. attenuata*'s resistance to *M. sexta*.

Manuscript I describes how NaRALF is involved in root hair development and plant growth in basic soil. We demonstrate that NaRALF is a peptide signal essential for root hair tip growth and plant adaptation to basic soil.

Manuscript II describes how MeJA-induced herbivore resistance is elicited by exogenous MeJA. We demonstrate that the resistance induced by MeJA-treatment is directly elicited not by MeJA but by its de-methylated product, JA, either alone or after conjugation with Ile.

Manuscript III describes how Narboh D-dependent ROS are involved in plant-herbivore interactions. We demonstrate that the OS-elicited ROS produced by *Narboh D* are essential for resisting attack from both herbivores and pathogens.

Manuscript I

The Plant Journal (2007)

Running title: Root hair apoplastic pH regulation by NaRALF

NaRALF, a peptide signal essential for the regulation of root hair tip apoplastic pH in *Nicotiana attenuata,* is required for root hair development and plant growth in native soils

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Abstract

Rapid alkalinization factor (RALF) is a 49-amino-acid peptide that rapidly alkalinizes cultivated tobacco cell cultures. In the native tobacco *Nicotiana attenuata*, *NaRALF* occurs as a single copy gene and is highly expressed in roots and petioles. Silencing *NaRALF*'s transcript by transforming *N. attenuata* with an inverted-repeat construct generated plants (ir*RALF*) with normal wild-type (WT) above-ground parts but with roots that grew longer and produced trichoblasts that developed into abnormal root hairs. Most trichoblasts produced a localized "bulge" without commencing root-hair tip growth; fewer trichoblasts grew, but were only 10% as long as those of WT plants. The root-hair phenotype was associated with slowed apoplastic pH oscillations, increased pH at the tips of trichoblasts, and decreased accumulation of reactive oxygen species in the root-hair initiation zone. The root-hair growth phenotype was partially restored when ir*RALF* lines were grown in a low-pH-buffered medium and reproduced in WT plants grown in a high-pH-buffered medium. When ir*RALF* plants were grown in pH 5.6, 6.7, and 8.1 soils together with WT plants in glasshouse experiments, they were out-competed by WT plants in basic but not acidic soils. When WT and ir*RALF* lines were planted into the basic soils of *N. attenuata*'s native habitat in the Great Basin Desert, ir*RALF* plants had smaller leaves, shorter stalks, and produced fewer flowers and seed capsules than did WT plants. We conclude that NaRALF is required for regulating root-hair extracellular pH, the transition from root-hair initiation to tip growth, and plant growth in basic soils.

Key Words: *NaRALF*, root hair, tip growth, extracellular pH oscillation, plant fitness, *Nicotiana attenuata*

Introduction

RALF, a 49-amino-acid polypeptide isolated by Clarence Ryan and colleagues while searching for peptides that regulate the wound response of cultivated tobacco plants, elicits a stronger and more rapid alkalinization of the medium of tobacco suspension-cultured cells than do the tobacco systemins (Pearce *et al.*, 2001). Like the systemins, RALF also elicits MAP kinase activity in cultured cells (Pearce *et al.*,

2001). The apoplastic localization of RALF (Escobar *et al*., 2003), together with the discovery of 120 kDa and 25 kDa cell membrane proteins, which can specifically bind to RALF (Scheer *et al*., 2005), suggest RALF may exert its biological activity through a specific interaction with a cell membrane receptor. Although alkalinization of the medium is often related to defense responses (Bolwell, 1995), little evidence supports the hypothesis that RALF has a defensive role: (1) unlike systemin, RALF does not elicit either the synthesis of tobacco trypsin inhibitors (Ryan *et al.,* 2002) or increases in *phenylalanine ammonia lyase* (*PAL*) transcripts (Haruta and Constabel, 2003); and (2) transcript levels of seven *RALF*s are not altered in either *mpk4* (constitutive systemic acquired resistance) or *ctr1* (constitutive ethylene response) mutant backgrounds in Arabidopsis (Olsen *et al.,* 2002). These results suggest that RALF may play roles that are not defense-related.

In Arabidopsis, RALF belongs to a large family of 34 genes (Olsen *et al.,* 2002). At least five RALF-like genes have also been identified in *Solanum chacoense* (Germain *et al.,* 2004). The large size of the gene family adds to the difficulty of identifying RALF's functions through genetic approaches. However, when tomato and Arabidopsis seedlings were germinated and transferred to a medium containing micromolar levels of tomato RALF peptides, root growth was immediately arrested (Pearce *et al.,* 2001), suggesting RALF may play a role in root development. We present evidence in this study consistent with an important role for RALF in root growth in general, and root hair development in particular.

Root hairs are projections from the surface of root epidermal cells (trichoblasts) which have been proposed to play critical roles in water and nutrient uptake, and in anchoring the plant in the soil (Ridge, 1995). Initial evidence for the function of root hairs in nutrient uptake comes from the observation that the number and density of root hairs increase under nutrient stress. The emerging molecular evidence is consistent with this idea: (1) high levels of transcripts of H^+-ATPase genes were observed in root hairs; the H^+-ATPase could provide the driving force for nutrient uptake (Moriau *et al.,* 1999); (2) a high-affinity P transport gene *LePT1* was found to be highly expressed in root hairs (Daram *et al.,* 1998); (3) the fitness of Arabidopsis mutant *act2-1*, which produces root hairs that are only 10 to 70% as long as those of WT plants, is reduced (Gilliland *et al.,* 2002).

To produce a root hair, a trichoblast undergoes two stages of development: root hair initiation and tip growth. During initiation, a highly localized bulge is

produced in the outer surface of the trichoblast. Once initiated, the root hair commences tip growth. New cell wall material is deposited only on the expanding tip of the developing hair, leading to the elongated hair-like morphology (Schnepf, 1986).

The transition to tip growth is a process genetically distinct from the process of root hair initiation. Analysis of mutants has revealed that many genes are essential for this phase of root hair development, such as *RHD2*, *SHV1*, *SHV2*, *SHV3*, *TRH1*, *KOJAK*, and *COW1* (Schiefelbein and Somerville, 1990; Parker *et al.,* 2000; Favery *et al.,* 2001; Rigas *et al.,* 2001; Foreman *et al.,* 2003; Bohme *et al.,* 2004). The *rhd2* mutant is unable to form the tip-focused calcium gradients required for hair growth. RHD2 has proven to be an NADPH oxidase, a protein that transfers electrons from NADPH to an electron acceptor, which leads in turn to the formation of reactive oxygen species (ROS) (Foreman *et al.,* 2003). The lesion in tip growth exhibited by *rhd2* strongly implicates ROS in the regulation of root hair development. The *TRH1* gene encodes a K^+ transporter (Rigas *et al.,* 2001), indicating that K^+ transport is required to cooperate with other localized transporters in driving the transition to tip growth. Indeed, ion fluxes have been intimately linked to the progression of root hair growth. It has been demonstrated that a tip-focused calcium gradient is essential for establishing tip growth (Bibikova *et al.,* 1997; Wymer *et al.,* 1997). Similarly, localized acidification of the cell wall and alkalinization of the cytoplasm are some of the first detectable indications of imminent root hair initiation (Bibikova *et al.,* 1998). The local apoplastic acidification of the cell wall can be prevented by treatment with high-pH buffers, which not only prevent the trichoblast from bulging but also arrest the elongation of root hairs that have started tip growth (Bibikova *et al.,* 1998). These results suggest that not only root hair initiation but also tip growth requires tight regulation of apoplastic pH. This pH change may, for example, activate expansin proteins that promote loosening of the cell wall and so facilitate turgor driven expansion (Bibikova *et al.,* 1998; Baluska *et al.,* 2000). The mechanism responsible for this cell wall acidification is still unclear. One potential hypothesis evokes an unknown molecule, activated by developmental stimuli, that through receptors in the plasma membrane signals the acidification of the cell wall either by increasing a cell's ion exchange capacity or by activating a proton ATPase or other transporters.

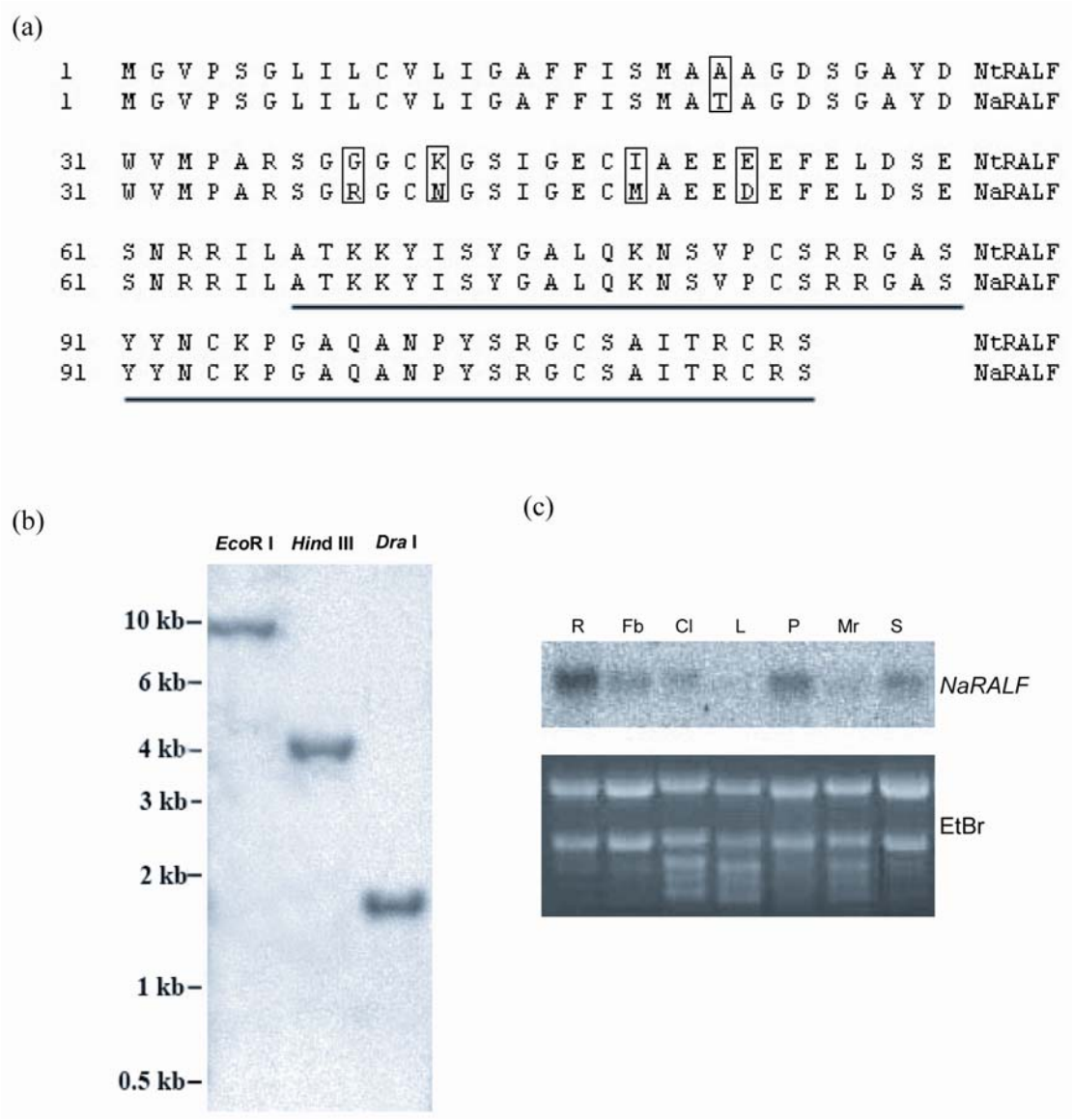

Figure 1. Isolation and characterization of *NaRALF* in wild-type *N. attenuata*.

(a) The amino acid sequence of NaRALF (AY456269) compared with tobacco RALF (NtRALF, AF407278). The mature peptide sequence of *NaRALF* is underlined, and the amino acids that differ are outlined in boxes.

(b) Southern blot analysis shows that only one copy of *NaRALF* is present in the *N. attenuata* genome. Five µg of genomic DNA were digested with *Eco*R I, *Hin*d III, and *Dra* I, and the ORF sequence of *NaRALF* was labeled with ^{32}P-dCTP for the probe.

(c) Transcript accumulation of *NaRALF* in different tissues of *N. attenuata*. Total RNAs (10µg) were extracted from roots (R), flower buds (Fb), cauline leaves (Cl), rosette-stage leaves (L), petioles (P), leaf mid-ribs (Mr) and stems (S) of 37-day-old WT plants, and analyzed by northern blotting, probed with a ^{32}P labeled *NaRALF* ORF sequence. An ethidium bromide (EtBr) stained gel serves as a loading control.

Here we report that NaRALF, which has the same mature peptide sequence as cultivated tobacco (Pearce *et al.*, 2001), affects root development in general and plays a role in the transition from root hair initiation to tip growth. When *NaRALF* is silenced, we observe that roots grow faster and show disrupted root hair development associated with disrupted apoplastic pH regulation. In addition, the fitness of plants

silenced in their *NaRALF* transcript is severely reduced when plants are grown in basic soils, either in the glasshouse or in *Nicotiana*'s natural habitat in the Great Basin Desert. These results are consistent with a role for RALF in coordinating root development through the regulatory role of proton fluxes associated with cell growth.

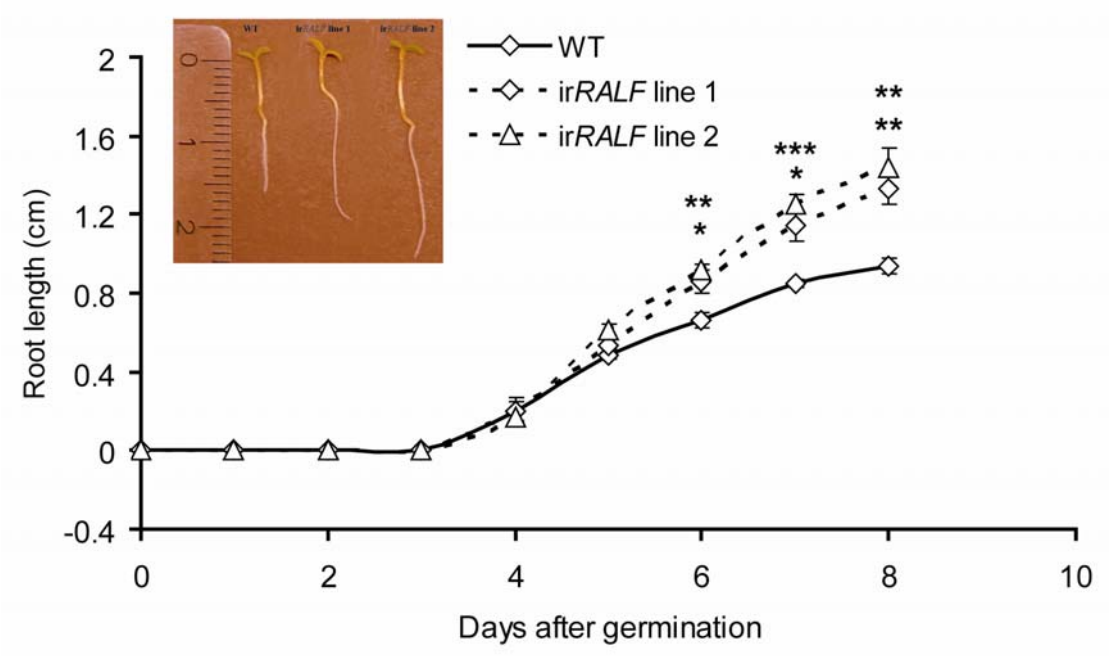

Figure 2. Root length of ir*RALF* and WT seedlings.

The root lengths (mean ± SEM) of 20 replicated WT and ir*RALF* seedlings were measured for 8 days after germination. All seedlings were grown vertically on the surface of Gamborg B5 medium (pH 6.8). **Inset:** Photograph of 8-day-old WT and ir*RALF* lines. The asterisks indicate the level of significant differences between WT and ir*RALF* lines 1 or 2 (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$).

Results

Cloning and characterizing the expression of *NaRALF*

To study the function of RALF in *N. attenuata*, we cloned the cDNA of *NaRALF*. The deduced peptide sequence shares 95.7% identity with that of RALF from cultivated tobacco (Pearce *et al.*, 2001); the predicted mature 49-amino-acid peptide is identical (Figure 1a).

We performed a Southern blot analysis, labeling the ORF sequence as a probe. Instead of several gene copies as in poplar (Haruta and Constabel, 2003), only one gene copy was found in *N. attenuata*'s genome (Figure 1b). Accordingly, we are unlikely to silence other homologous genes when plants are transformed with an RNAi construct composed of the same sequence.

Transcripts of *NaRALF* rapidly accumulate in leaves in response to mechanical wounding and applying water or *Manduca sexta* oral secretions (OS) to

the wounds (Figure S1a), but *M. sexta* did not gain more weight on ir*RALF* lines than on WT plants (Figure S3a), suggesting that *NaRALF* does not play a critical role in resistance to this adapted herbivore. *NaRALF* transcripts also rapidly respond to UV-B irradiation (Figure S1b), but ir*RALF* and WT plants do not differ in their morphologies after UV-B treatments (Figure S3b), suggesting that *NaRALF* does not play an essential role in resistance to UV-B damage. The analysis of different plant tissues revealed high levels of *NaRALF* transcripts in roots and petioles, but low levels in leaves, flower buds, and mid-ribs (Figure 1c). This result is consistent with the root hair phenotype in ir*RALF* lines.

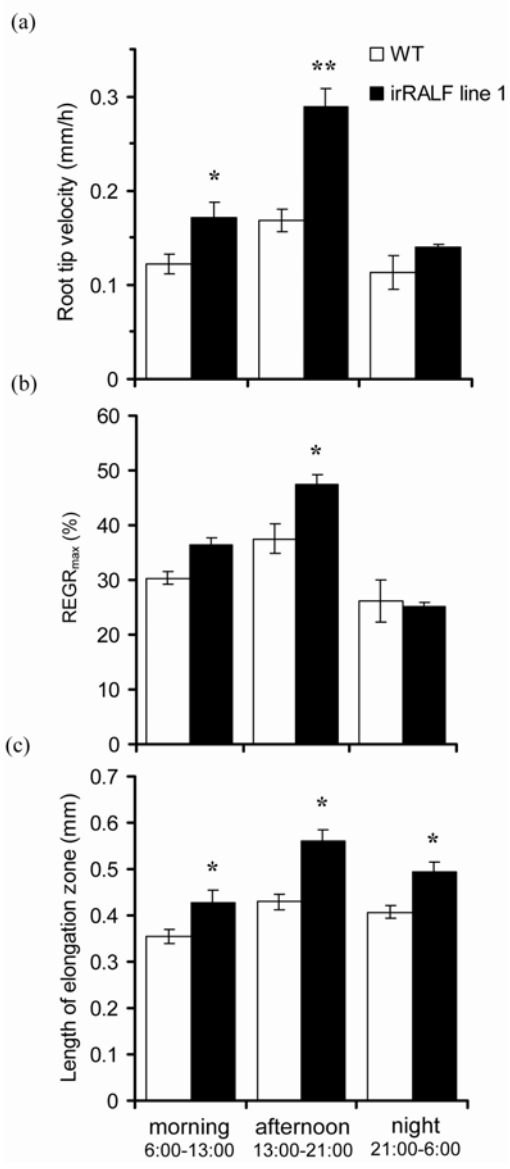

Figure 3. Longer root lengths of ir*RALF* lines result from faster growth.
The mean (± SEM) maximal root tip velocity (**a**), maximal relative elemental growth rate ($REGR_{max}$) (**b**), and length of root elongation zone (**c**) were measured in 5 replicated WT and ir*RALF* line 1 seedlings. The lights were switched on at 6:00 in the morning and switched off at 20:00 in the evening. The asterisks indicate the level of significant differences between WT and ir*RALF* line 1 (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$). All seedlings were grown vertically on the surface of Gamborg B5 medium (pH 6.8).

Silencing *NaRALF* leads to rapid root growth and abnormal root hairs

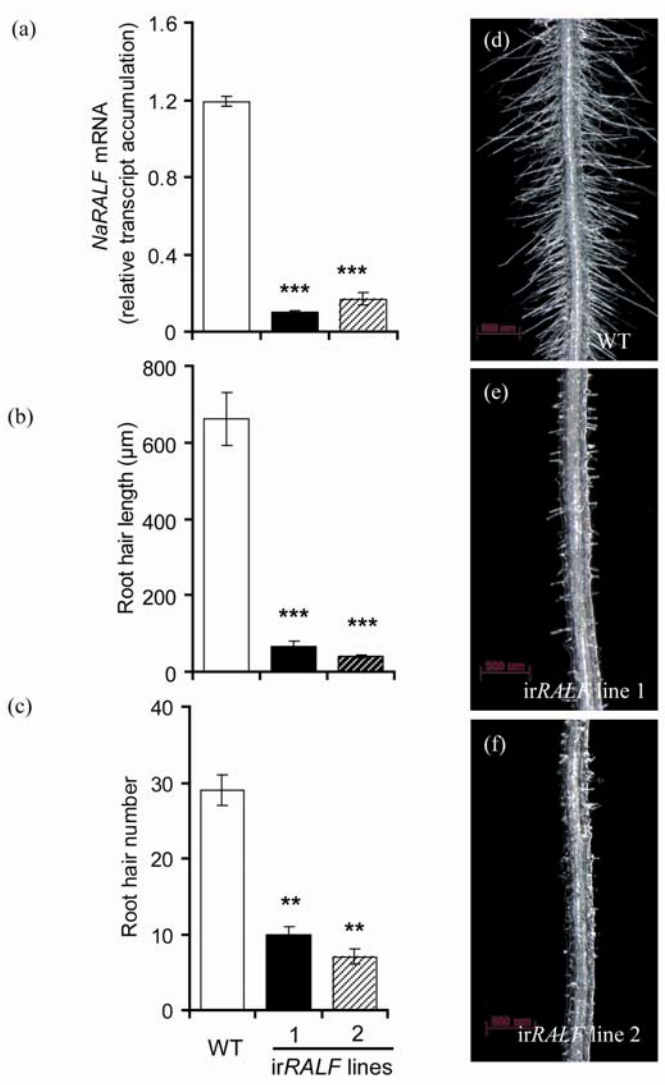

Figure 4. Relative *NaRALF* transcript accumulation, and root hair length and number in roots of WT and ir*RALF* seedlings.

Mean (± SEM) *NaRALF* transcript accumulation in roots as analyzed with qRT-PCR normalized to levels of an constitutively unregulated gene (sulfite reductase) (**a**), root hair length (**b**) and number (**c**) in 11 replicate 7-day-old WT (**d**) and ir*RALF* lines 1 (**e**) and 2 (**f**) seedlings. The root hair number was counted per 500µm root segment. The asterisks indicate the level of significant differences between WT and ir*RALF* lines 1 and 2 (unpaired *t*-test: **, $P<0.001$; ***, $P<0.0001$). All seedlings were grown vertically on the surface of Gamborg B5 medium (pH 6.8).

We silenced *NaRALF* in *N. attenuata* by *Agrobacterium*-mediated transformation (Krügel *et al.,* 2002) using a pRESC5 transformation vector (Bubner *et al*., 2006) (Figure S2a) containing a 263 bp *NaRALF* fragment in an invert-repeat orientation (Figure S2b). Two independently transformed F2 lines, each harboring a single insertion as confirmed by a Southern blot analysis (Figure S2c), were selected for all further experiments (ir*RALF* lines 1 and 2). The silencing of endogenous *NaRALF* gene expression was also confirmed by qRT-PCR (Figure 4a), which revealed that *NaRALF* expression was reduced by 90% in the roots of these lines compared to WT plants.

Because treatment with exogenous RALF has been reported to arrest root elongation (Pearce *et al.,* 2001), we examined the rate of root growth in ir*RALF* lines. Four days after germination, the root length in WT and ir*RALF* lines did not differ. However, by day 6, roots of both ir*RALF* lines were significantly longer than in WT

plants (Figure 2). To determine whether the longer roots resulted from higher root growth rates, we measured root tip growth velocity, maximal relative elemental growth rate ($REGR_{max}$), and the length of the elongation zone in WT and ir*RALF* line 1 plants. Because root growth correlates positively with temperature (Walter *et al*., 2002; Pahlavanian and Silk 1988), root tip velocity, $REGR_{max}$, and the elongation zone were measured during three time intervals, which bracketed the greatest changes in temperature: morning (6:00 to 13:00), afternoon (13:00 to 21:00), and night (21:00 to 6:00). The average root tip velocity in WT lines was 0.12 ± 0.01 mm/h in the morning and 0.17 ± 0.01 mm/h in the afternoon; however, the average root tip velocity in ir*RALF* line 1 was significantly faster both in the morning (142% of WT plants) and in the afternoon (171% of WT plants) but not in the evening (Figure 3a). The $REGR_{max}$, which characterizes the maximal expansion rate of cells, was also significantly higher in ir*RALF* plants during the afternoon measurements (Figure 3b) than in WT plants. The elongation zone of *irRALF* plants was significantly longer at all three times than in WT plants (Figure 3c). These results indicate that rapid root growth and a longer elongation zone can account for the longer roots of ir*RALF* plants.

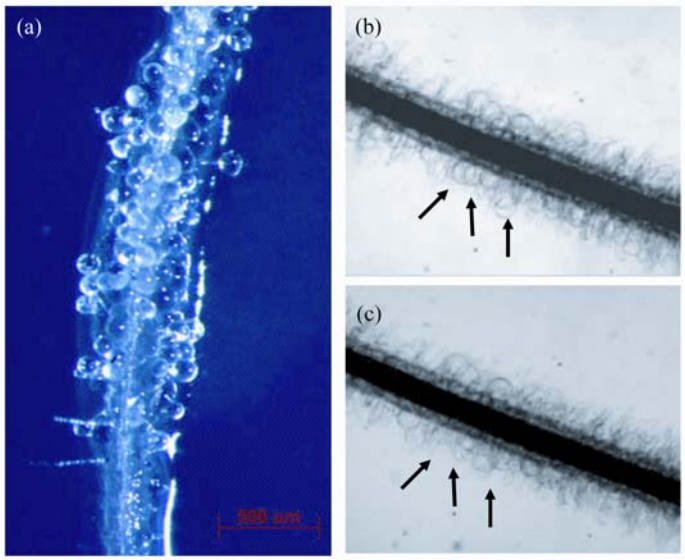

Figure 5. Reduced root hair number of ir*RALF* lines result from broken trichoblasts.
Root segments close to the root hair initiation zone of 5-day-old ir*RALF* line 1 seedlings, grown on the surface of Gamborg B5 medium (pH 6.8), are depicted. Most of the trichoblasts produced only a localized "bulge" without commencing tip growth (**a**); some of these grew into large "bulges" (**b**), which burst in (**c**), as indicated by the arrows. (**b**) and (**c**) are snapshots from a time-lapse movie (Supplementary Video Clip S1).

Root hairs grow in the differentiation zone of the root, perpendicular to the root axis, and result from polarized outgrowths of the basal ends of trichoblasts. Normally fully elongated root hairs of *N. attenuata* are 662 ± 70 µm long in WT plants (Figures 4b, d), grow at a rate of 0.58 ± 0.24 µm/min, and are produced at 29 ± 2 hairs per 500 µm of root length under our standardized growth conditions (Figures

4c, d). However, in ir*RALF* plants around two-thirds of trichoblasts produced only a localized "bulge" and never commenced tip growth (Figures 4c, e, f). The remaining third of these "bulges" successfully completed the transition to tip growth (Figures 4c, e, f); however, their growth rate was only 64% that of WT root hairs (unpaired *t*-test, $P < 0.001$), and their final root hair length was only 10% that of WT root hairs (Figures 4b, e, f). Most of the "bulges" we observed swelled until they ruptured (Figure 5 and Supplementary Video Clip S1).

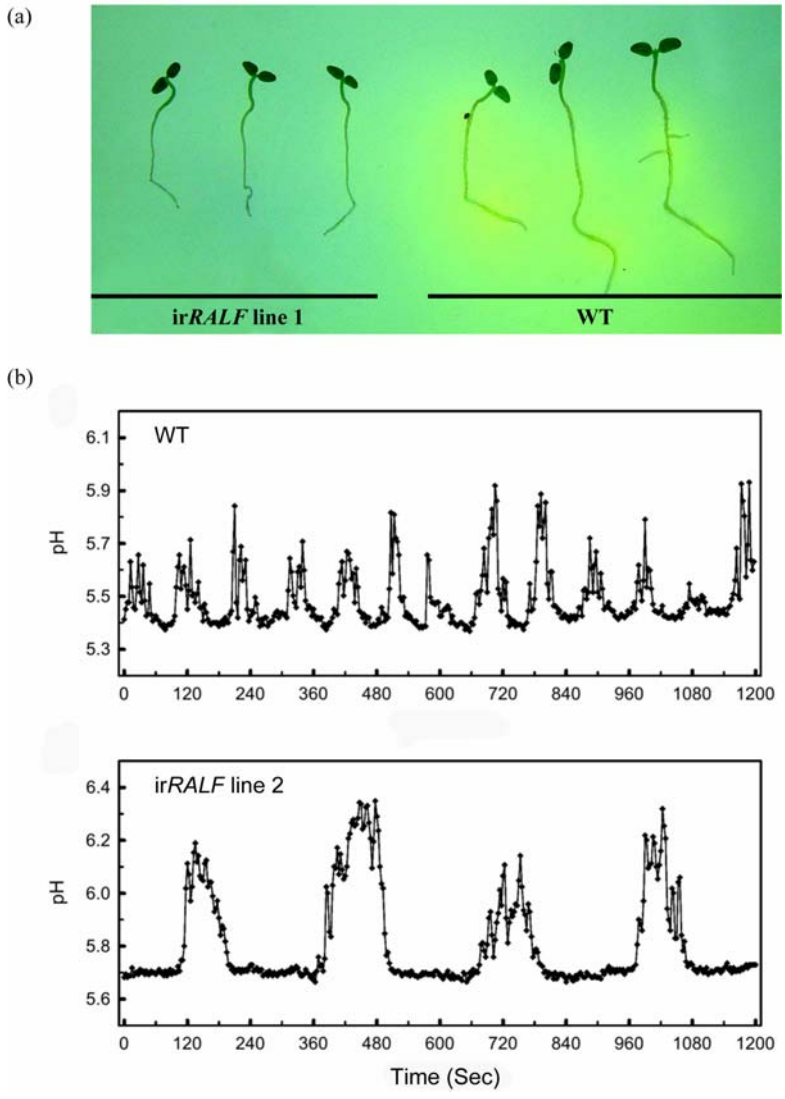

Figure 6. Extracellular pH of root hairs in *NaRALF* silenced plants oscillates more slowly at higher pH value than do those of WT plants

(a) *NaRALF*-silenced plants are unable to acidify the media. Seven-day-old seedlings of WT and ir*RALF* line 1 were pressed into pH indicator gel (pH 6.3), and images were taken after 48 h. The yellow color of the media surrounding the roots of WT seedlings illustrates their ability to acidify the media. Seedlings of neither ir*RALF* line 1 nor 2 (data not shown) were able to turn the media yellow.

(b) Typical extracellular pH oscillations at the tip of growing root hairs in WT and ir*RALF* plants.

Top: extracellular pH oscillations at the tip of one root hair in WT plant.

Bottom: extracellular pH oscillations at the tip of one root hair in ir*RALF* line 2 plants.

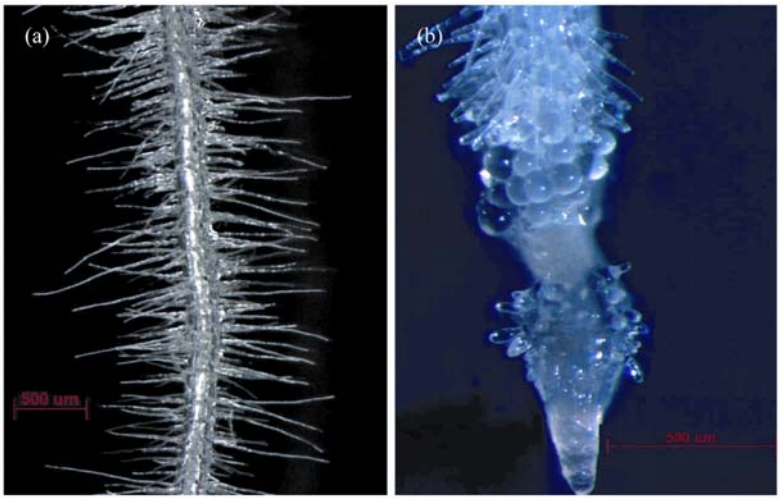

Figure 7. Root hairs of WT plants display the root hair phenotypes of ir*RALF* lines when grown in Tris (or HEPES) buffered medium (20mM pH 6.8).

WT seedlings grown in Gamborg B5 medium at pH 6.8 produce root hairs with an average length of 660 µm (**a**); however, when grown on the same medium but supplied with Tris or HEPES buffer (20mM pH 6.8), root hairs were short and some trichoblasts produced only a localized "bulge" (**b**), reminiscent of ir*RALF* lines grown on Gamborg B5 unbuffered media (pH 6.8).

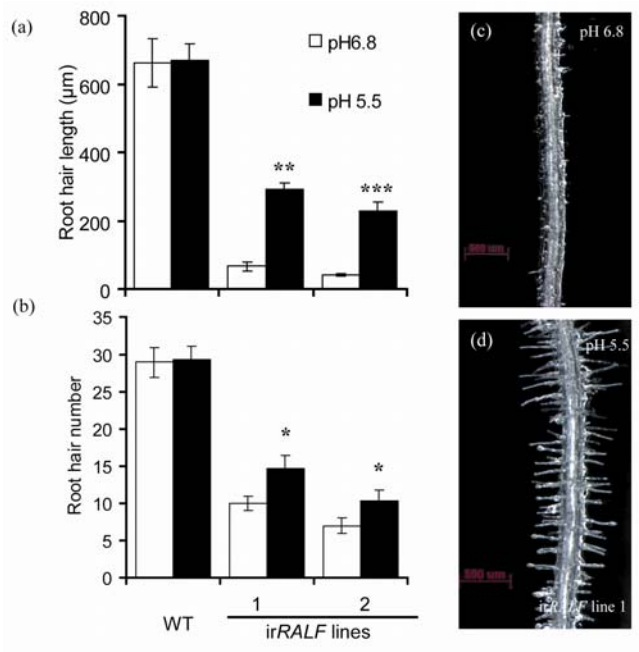

Figure 8. WT root hair phenotype is partially restored in ir*RALF* lines when grown on low-pH buffered medium.

Mean (± SEM) root hair length (**a**) and number (**b**) of 11 replicate 7-day-old WT and ir*RALF* plants measured in Gamborg B5 medium (pH 6.8) with or without MES buffer (or phosphate buffer) (20mM pH 5.5). The number of root hairs was counted per 500µm root segment. Examples of a root from a 7-day-old ir*RALF* line 1 plant grown in Gamborg B5 medium at pH 6.8 (**c**) and in medium supplied with 20mM MES/phosphate buffer at pH 5.5 (**d**). The asterisks indicate the level of significant differences between WT and ir*RALF* lines 1 and 2 (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$).

Apoplastic pH and root hair development in ir*RALF* plants

Clearly ir*RALF* plants are deficient in some aspect of root hair tip growth, which results in either short root hairs or a rupture of the root hair. To understand why ir*RALF* plants have such root hair phenotypes, we grew WT and ir*RALF* plants in a pH indicator gel and examined their ability to acidify the rhizosphere. After 2 days, the medium in which the WT plants grew turned yellow, demonstrating that they had gradually acidified the medium; this did not occur when ir*RALF* seedlings were grown on an identically prepared pH indicator gel (Figure 6a). Root hair tip growth in Arabidopsis has been characterized as involving oscillatory changes in extracelluar pH at the very apex of the elongating hairs that are likely regulating cell wall extensibility and so cell expansion (Monshausen *et al*., in review). We therefore investigated the pH of the tip of the root hair cell wall in WT and ir*RALF* plants with a pH-sensitive dye fluorescein-dextran. As the dye is dextran-conjugated, it is excluded from the cell and reports extracelluar pH. Confocal imaging of the pH-dependent fluorescence from the dye allowed us to measure the local changes in pH at the apex of the hairs in real time. The surface pH of WT plants oscillated with a period of 112 ± 28 sec (Figure 6b). While pH oscillations were also observed in plants of ir*RALF* line 2, the length of the period was twice as long as that of oscillations in WT plants (Figure 6b; ir*RALF* line 2 with 233 ± 35 sec; unpaired *t*-test, $P < 0.001$). The slower pH oscillation correlated with the slower root hair growth rate. Importantly, the extent of the pH oscillation in ir*RALF* plants was higher than that of WT plants (pH in WT plants increased to 5.79 ± 0.24 and in ir*RALF* line 2 to 5.94 ± 0.37; unpaired *t*-test, $P = 0.02$, n=6 separate roots). Furthermore, a large increase in alkalinization levels at the tip of the cell wall always preceded the rupturing of trichoblasts (Supplementary Video Clip S2).

Based on these results, we hypothesized that *NaRALF* plays a role in modulating extracelluar pH oscillations, which are required for tip growth during root hair development. We propose that in ir*RALF* plants, trichoblasts lose control of both timing and magnitude of their pH oscillations and attain higher apoplastic pH values than normally experienced. These changes in pH profile cause the normally highly controlled expansion of the very apex of the hair to become disrupted.

It is also possible that suppression of *NaRALF* expression leads to a range of growth effects on root hairs and so the altered pH profiles in the ir*RALF* lines might reflect a consequence rather than cause of the disrupted cell expansion in these plants.

In order to test whether the altered pH of the cell wall was primarily responsible for the altered growth seen in root hairs, we grew WT and ir*RALF* plants in media containing either a low-pH buffer (MES or phosphate buffer 20mM pH 5.5) or a high-pH buffer (Tris or HEPES buffer 20 mM pH 6.8) to determine if: 1) growing ir*RALF* plants on low-pH-buffered media could restore a WT root hair phenotype, and 2) growing WT plants on high-pH-buffered media could produce a root hair phenotype reminiscent of ir*RALF* plants. When grown on a low-pH-buffered medium, root hair length in ir*RALF* line1 increased from 67 to 292 μm, and in ir*RALF* line 2, from 41 to 228μm (Figures 8a, d). Root hair density was also partially recovered; for example, the number of root hairs in ir*RALF* line 1 increased from 10 to 14.8 (Figures 8b, d) per 500 μm root segment in the low-pH-buffered medium. Moreover, WT plants produced the same root hair phenotype as did ir*RALF* lines when they were grown in a high-pH-buffered medium (Figure 7): many trichoblasts produced only "bubble" structures; although some were capable of normal tip growth, they produced only short root hairs.

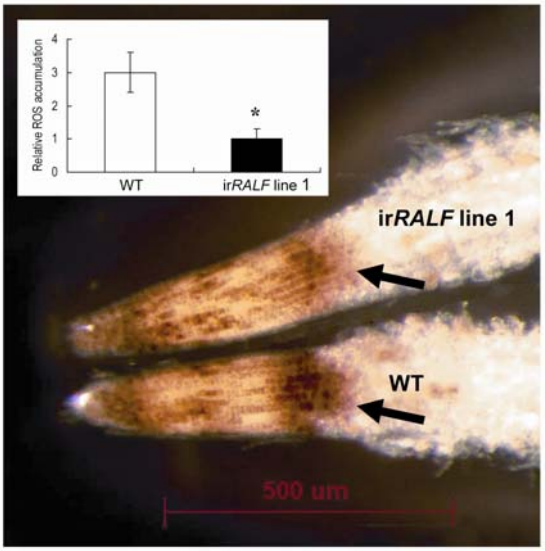

Figure 9. ROS accumulation decreased in roots of ir*RALF* lines.
Five-day-old seedlings were stained with 1mg/mL DAB for 2h. The root hair initiation zone of WT plants showed more intense DAB staining (as pointed by the arrows) than that of ir*RALF* line 1. Representatives of n>5 roots. **Inset:** Mean ROS measurement using the Amplex red hydrogen peroxide/peroxidase assay kit in 11 replicate 1-cm-long root tips of WT and ir*RALF* line 1; the intensity of ROS accumulation was defined as relative amounts in comparison to the ir*RALF* line 1 value, which was set as 1.0. The asterisk indicates the level of significant difference (unpaired *t*-test: *, $P<0.05$).

ROS accumulation decreased in ir*RALF* line 1.

Because ROS signals have been shown to be important for root hair development as indicated in the *rhd2* mutant (Foreman *et al.,* 2003), we examined ROS levels in the roots of ir*RALF* plants. 3,3'-diaminobenzidine (DAB) staining of the root tip revealed less ROS accumulation in the root hair initiation zone of ir*RALF* line 1 compared to in the zone of WT plants (Figure 9). Measurement of ROS

accumulation in root segments made as described previously in (Shaw and Long, 2003) revealed that the roots of ir*RALF* line 1 contained significantly less ROS relative to the roots of WT plants (Figure 9).

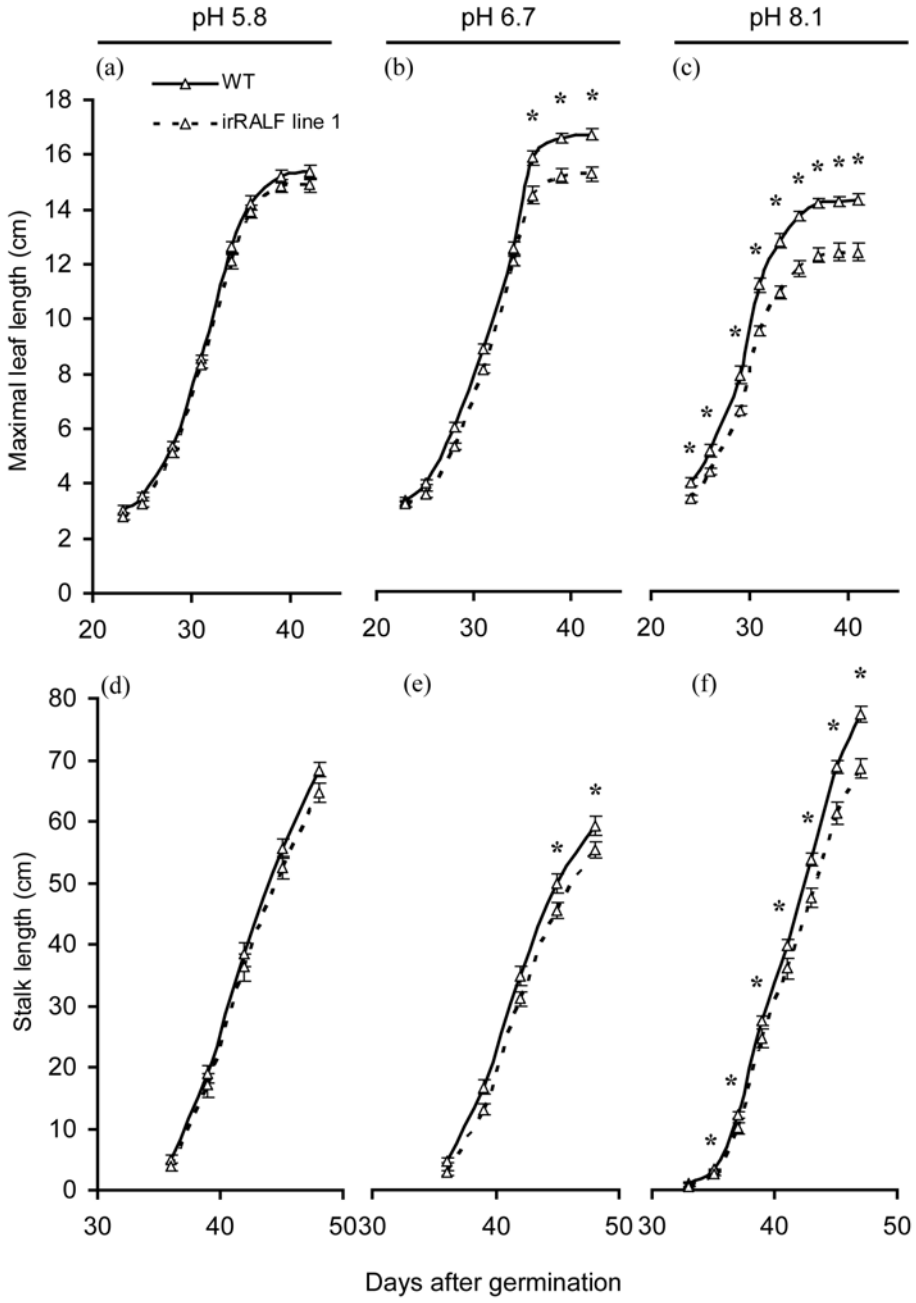

Figure 10. Leaf size and stalk length of ir*RALF* lines were reduced when they competed against WT plants in the glasshouse.

Mean (± SEM) maximal leaf length and stalk length were measured in 12 replicate pairs of size-matched WT and ir*RALF* line 1 plants grown in soil buffered to pH 5.8 (**a** and **d**), pH 6.7 (**b** and **e**), pH 8.1 (**c** and **f**). No differences were observed in symmetrical competition pairs (WT-WT or ir*RALF* line 1-ir*RALF* line 1 pairs; see Supplemental Figure S5, 6) and similar results were obtained in WT-ir*RALF* line 2 competitions (see supplemental Figure S4). The asterisks indicate the level of significant differences between WT and ir*RALF* line 1 (paired *t*-test: *, $P<0.05$).

ir*RALF* plants are out-competed by WT plants in basic soils

Root hairs are thought to be important for nutrient uptake. Because the driving force for most nutrient uptake is the proton gradient generated by the H^+-ATPase (Gilroy and Jones, 2000), the pH of the cell wall is thought to be crucial for nutrient uptake. The altered apoplastic pH and fewer root hairs of ir*RALF* lines led us to hypothesize that ir*RALF* lines would have lower fitness when competing with WT plants, especially when grown in basic soils, which are typical of *Nicotiana*'s native habitat in the Great Basin Desert. To test this hypothesis, we conducted competition experiments with size-matched pairs of competing WT and ir*RALF* plants grown in three different pH soils in the glasshouse, and in addition transplanted these WT-ir*RALF* pairs into a native population in Utah.

Figure 11

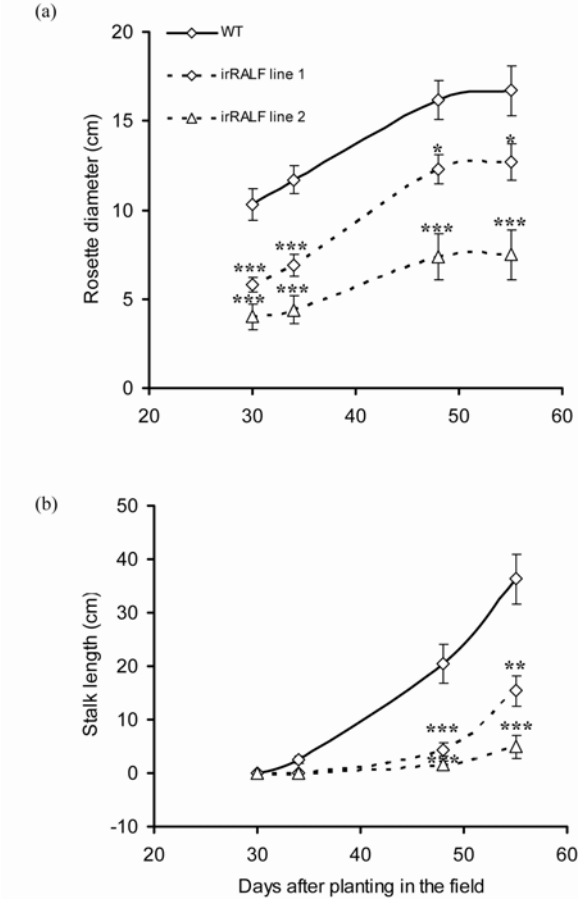

Figure 11. When grown in their native habitats in the Great Basin Desert, ir*RALF* plants attain smaller rosette sizes and shorter stalk lengths than WT plants.

Mean (± SEM) rosette diameters (**a**) and stalk lengths (**b**) of ten replicate WT and ir*RALF* lines 1 and 2 plants measured 30, 34, 48, and 55 days after planting in a native population in Utah. The asterisks indicate the level of significant differences between WT and ir*RALF* lines (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$).

Size-matched plants were planted into 2 L pots containing potting soil B410 at its normal acidic pH (approximately 5.8). No significant differences in leaf or stalk size (Figures 10a, d) or capsule production (WT plants with 15.6 ± 0.9 and ir*RALF* line 1 with 13.0 ± 1.1; unpaired *t*-test, $P = 0.1$) were found between WT and ir*RALF* plants. When plant pairs were grown in soil amended with 5% limestone, which

increased the soil pH to 6.7, the growth of ir*RALF* lines was significantly reduced in comparison to WT plants (Figures 10b, e) in the later stages of growth. 36 days after germination, the maximal leaf length was significantly reduced from 15.7 ± 0.2 cm (WT) to 14.3 ± 0.3 cm (ir*RALF* line 1) (unpaired *t*-test, $P = 0.0025$). When size-matched pairs were transplanted into potting soil amended with 20% limestone to produce a soil (pH 8.1) with a pH similar to the calcareous soils in which *N. attenuata* grows in Utah, the growth of ir*RALF* plants was severely reduced (Figures 10c, f). Large reductions in leaf size were even observed as early as 23 days after germination (reduced from 4.0 ± 0.2 to 3.3 ± 0.1 cm) (unpaired *t*-test, $P = 0.005$; Figure 10c) and dramatic delays in stalk growth were observed after 33 days of germination (reduced from 1.1 ± 0.1 to 0.5 ± 0.1 cm) (unpaired *t*-test, $P = 0.005$; Figure 10f). Reductions in leaf size and stalk length lowered the lifetime production of seed capsules: in soil of pH 8.1, ir*RALF* line 1 produced 9.4 ± 0.7 capsules and ir*RALF* line 2 produced 7.7 ± 0.8 capsules, about one-half the number of capsules produced by WT plants grown in these soils (16 ± 1 capsules). These data demonstrate that silencing *NaRALF* severely impairs the growth of *N. attenuata* in basic soils.

Genotype	buds	flowers	capsules
WT	7.7 ± 1.5	6.4 ± 2	4 ± 1.5
ir*RALF* line 1	4.2 ± 1 *	1 ± 0.5 **	0
ir*RALF* line 2	0.8 ± 0.5 **	0.1 ± 0.1 ***	0

Table 1. ir*RALF* lines produced fewer buds, flowers, and capsules than did WT plants in their native habitat.
Mean (± SEM) number of buds, flowers, and capsules of WT, ir*RALF* plants of ten matched triplicates after 55 days of growth. The asterisks indicate the level of significant differences between WT and ir*RALF* lines (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$).

In *Nicotiana*'s native habitat in the Great Basin Desert, rosette leaf diameter, stalk length, and production of flower buds, flowers, and seed capsules were recorded in WT plants and ir*RALF* lines 30, 33, 48, and 55 days after size-matched plants were planted in a recently burned juniper forest site near Santa Clara, Utah. The rosette leaf diameter of WT plants was 11.1 ± 0.9, 11.7 ± 0.8, 16.2 ± 1.1, and 16.7 ± 1.4 cm; this was significantly larger than that of both ir*RALF* line 1 plants (5.8 ± 0.4, 6.9 ± 0.6, 12 ± 0.8 and 12.7 ± 1 cm), and ir*RALF* line 2 (4 ± 0.7, 4.4 ± 0.8, 7.5 ± 1.3, and 7.5 ± 1.4

cm) (Figure 11a). The stalks of WT plants were taller than those of ir*RALF* lines (Figure 11b): 36.3 ± 4.7 cm high 55 days after plants were transplanted to the field compared to 15.4 ± 2.8 cm (unpaired *t*-test, $P = 0.002$) in ir*RALF* line 1 and 4.9 ± 2.1 cm (unpaired *t*-test, $P < 0.0001$) in ir*RALF* line 2. Most WT plants started producing flower buds 48 days after transplanting and after 55 days WT plants had produced significantly more flower buds, flowers, and seed capsules than had ir*RALF* lines (Table 1). These results are consistent with the hypothesis that *NaRALF* expression is essential for plant growth and reproductive performance in the basic soils of the plants' native habitat.

Discussion

Although the accumulation of *NaRALF* transcripts is rapidly increased by wounding and OS elicitation, since *M. sexta* larvae do not perform differently on ir*RALF* plants than on WT plants, we conclude that *NaRALF* does not play a central role in eliciting anti-herbivore defense responses. This result supports previous data showing that RALF peptides are more likely involved in plant development than in defense (Ryan *et al.,* 2002; Haruta and Constabel, 2003; Olsen *et al.,* 2002; Germain *et al.,* 2004). We also observed that *NaRALF* transcripts increased rapidly after exposure to UV-B radiation, but WT and *NaRALF*-silenced plants did not perform differently after UV-B exposure, suggesting that NaRALF does not play an important role in UV-B responses.

The ubiquity of RALF suggests it plays a fundamental physiological role. Previous work has shown that exogenous application of RALF can arrest root growth (Pearce *et al.,* 2001), suggesting that RALF may act as an endogenous negative regulator of root growth. Our characterization of the root phenotypes of ir*RALF* plants – longer root lengths, rapid growth rates, and longer root elongation zones, are consistent with this idea.

It's still unclear how root growth is regulated by NaRALF. Recently, it has been suggested that H_2O_2 is involved in root growth restriction and root hair formation (Dunand *et al*., 2007). Arabidopsis seedlings had longer roots when grown in the presence of KI, an efficient scavenger of H_2O_2; however, the elongation of roots was inhibited by exogenously supplied H_2O_2. These results provide a possible mechanism for NaRALF's effects on root growth that less ROS accumulation is the reason of longer roots in ir*RALF* plants. Another explanation for the effect of

silencing *NaRALF* could be that roots compensate for the loss of absorbing surface resulting from the absence of hairs by increasing their length.

Although the pH of the soil has long been proposed to strongly influence root hair formation (Ewens and Leigh, 1985), the reason has not been clear. More recently, the importance of the localized acidification of the cell wall during root hair initiation and tip growth has been demonstrated (Bibikova *et al.,* 1998), but how the process was regulated has until now remained unclear. Our results provide evidence that NaRALF is involved in regulating the apoplastic pH required for root hair formation. Silencing *NaRALF* led to a slower rate of oscillation in extracelluar pH, and in Arabidopsis such pH oscillations have been linked to the regulation of wall properties directly related to the control of tip growth (Monshausen *et al*., in review), suggesting possible reasons why ir*RALF* lines have disrupted root hair formation. Root hairs in the ir*RALF* lines often rupture, and the fact that a large increase in alkalinization levels always preceded a root hair rupture led us to hypothesize that the disrupted regulation of apoplastic pH at the root hair tips was one cause of the root hair phenotype of ir*RALF* lines. This hypothesis was confirmed by the partial recovery of the root hair phenotype (root hair length and number) when ir*RALF* lines were grown in low-pH buffered medium; moreover, WT plants produced a root hair phenotype similar to that of ir*RALF* lines when WT plants were grown in medium with the same pH (pH 6.8), but strongly buffered with Tris (or HEPES) buffer. Thus, irrespective of the precise mechanism whereby NaRALF is regulating the root hair growth, these results suggest an important element of NaRALF's action is its effects on proton fluxes associated with growth. Additional evidence came from the competition experiments: WT and ir*RALF* plants grew similarly in low pH soils; however, in high pH soil, where plants need more energy to generate a proton gradient across their cell walls for nutrient uptake, ir*RALF* plants were strongly out-competed by WT plants.

Silencing *NaRALF* in *N. attenuata* results in altered dynamics of pH oscillations and increased maximal pH at the tips of root hairs. This result may seem to contradict the fact that the RALF peptide elicits a rapid alkalinization of the medium of tobacco suspension-cultured cells. Cell cultures are expected to differ from intact plants, particularly in their cell-cell communication and given that *NaRALF* is highly expressed in roots, studies of intact roots are likely to provide a more accurate analysis of NaRALF's function.

The apoplastic localization of RALF (Escobar *et al*., 2003), together with the discovery of 120 kDa and 25 kDa cell membrane proteins, which can specifically bind to RALF (Scheer *et al*., 2005), suggest RALF may exert its biological activity through a specific interaction with a cell membrane receptor. We do not yet have direct evidence that NaRALF regulates H^+-ATPase activity; however, high levels of H^+-ATPase genes are expressed in developing root hairs in *Nicotiana plumbaginifolia* (Moriau *et al.,* 1999), which suggests the possibility that they are regulated by NaRALF.

ROS may be one of the down-stream signals regulated by NaRALF. Previous work with the *rhd 2* mutant demonstrated that the ROS produced by NADPH oxidase in the trichoblast is important for forming tip-focused calcium gradients required for tip growth. We also observed a decreased accumulation of ROS in the root hair initiation zone of the ir*RALF* lines, suggesting that NaRALF may also affect root hair development through this ROS-dependent series of events.

Root hairs are thought to be important for nutrient uptake. For example, a high-affinity P transport gene *LePT1* was found to be highly expressed in root hairs (Daram *et al.,* 1998) and the presence of root hairs significantly increased whole plant P uptake under P-limiting conditions (Bates and Lynch, 2000). Thus, alterations in root hair development should translate into changes in plant fitness. Indeed, the fitness of the Arabidopsis mutant *act2-1*, which produces root hairs that are only 10 to 70% as long as those of WT plants, is reduced (Gilliland *et al.,* 2002). When grown in pH 5.8 soils in the glasshouse, ir*RALF* lines showed leaf sizes and stalk lengths similar to those of WT plants. Low soil pH facilitates the active transport of nutrients, and the longer roots of ir*RALF* plants might compensate for the loss of nutrients that are normally absorbed by root hairs. When plants were grown in the basic soils in which *N. attenuata* plants are commonly found in nature, the fitness of ir*RALF* plants was severely reduced. Two factors may be responsible: either (a) the ir*RALF* lines have fewer and shorter root hairs, or (b) the trichoblasts have lost the ability to regulate cell wall pH dynamics. Additional work will be required to determine which if any of these mechanisms are responsible for the reduction in plant growth.

In summary, our results demonstrate that NaRALF is a peptide signal needed for regulating root growth and the apoplastic pH of the tip of trichoblasts. NaRALF is activated by unknown developmental stimuli, affects the levels of ROS accumulation and possibly the activity of H^+-ATPase in plasma, and subsequently influences the

periodicity of pH oscillations, and maintains an appropriate cell wall pH environment. When *NaRALF* is silenced, trichoblasts become "deaf" to developmental and environmental stimuli; the cell wall pH oscillates more slowly and attains higher pH values; these changes in pH profile cause the normally highly controlled expansion of the very apex of the hair to become disrupted, leading to "bulging", until the cells finally rupture.

Materials and Methods

Plant growth: We used seeds of the 21st generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts (synonymous with *Nicotiana torreyana*: Solanaceae) for transformation and as the WT genotype in all experiments. Seed germination and plant growth were conducted as described by Krügel *et al.* (2002). In brief, seeds were sterilized and germinated on agar with Gamborg B5 (Duchefa, http://www.duchefa.com) after soaking in a 1:50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NY, USA) and 1 mM of GA_3. After 10 days, seedlings were planted into soil in Teku pots (Pöppelmann, http://www.poeppelmann.com).

For the competition experiments in the glasshouse, after 10 days in Teku pots, two seedlings of similar size and appearance were transplanted 7 cm apart in 2-L pots under the conditions as described by Zavala *et al.* (2004). 180 pairs were assigned to the following five groups: (1) WT-ir*RALF* line 1; (2) WT-ir*RALF* line 2; (3) WT-WT (as a control); (4) ir*RALF* line 1-ir*RALF* line 1 (as a control); (5) ir*RALF* line 2-ir*RALF* line 2 (as a control). The five groups were grown in three kinds of soil: potting soil B410 (Stender, http://www.stender.de); B410 soil augmented with 5% limestone (95% $CaCO_3$, Trollius GmbH, http://www.trollius.femikal.de), which increased the soil pH to 6.7; B410 soil augmented with 20% limestone, which increased the soil pH to 8.1 to mimic the pH of soils usually observed in *Nicotiana*'s natural habitat. Additional fertilizers were supplied after the plants were transferred to soil: 2 g of Borax (Nic.Sosef International B.V., http://www.sosef.nl) were added to each pot on the day after transplanting; 1.25g Borax and 35 g of Peters General Purpose (Scotts International, http://www.scottsinternational.com) were supplied after one week; and finally 0.5 g of Borax and 50 g of Peters General Purpose were added after 2 weeks. The following fitness measurements were recorded for each plant: the longest leaf length and stalk length after the start of elongation for a period of 28 days; the total number of seed capsules (in soil of pH 5.8 and pH 8.1).

In the field, seeds were pre-treated and sown as described above. The petri dishes were kept at 25°C/16h light and 20°C/8h dark. After 10 days, seedlings were transferred to Jiffy 703 pots (1 ¾ inch x 1 ¾ inch, AlwaysGrows, http://www.alwaysgrows.com) which had been soaked in borax solution (0.4mg/ 45mL water). The seedlings were fertilized with an iron solution (stock solution: 2.78 g of $FeSO_4 \cdot 7H_2O$ and 3.93 g of Titriplex in 1L distilled H_2O, diluted 100 fold) after 7 days. After 3-4 weeks, WT and ir*RALF* plants were transferred to a native population of *N. attenuata* growing in a one-year-old burn near Santa Clara, UT, USA. Releases of the transformed plants were conducted under APHIS notification 06-003-08n. WT plants and ir*RALF* lines 1 and 2 were planted 0.5 m equidistant from each other in a triangular design with one plant randomly assigned to each corner of the triangle around a juniper tree that had burned the previous growing season. Rosette leaf diameter, stalk height, the numbers of flower buds, flowers, and capsules were recorded 30, 33, 48, and 55 days after plants were transplanted into the field**.**

Analysis of root growth and root hair phenotype: Seeds were germinated on agar with Gamborg B5 (Krügel *et al.,* 2002). After 3-4 days, roots started to grow vertically on the agar's surface and root lengths were measured daily from day 3 to day 8.

To measure the velocity of root tip growth, five-day-old seedlings were placed into a micro-rhizotron as described by Nagel *et al*, (2006) under a photoperiod of 14 h, 27 °C in light and 22 °C in dark phase. Every 30 seconds an image was taken of the root tip with a CCD camera (Sony XC-ST50; Sony, http://www.sony.com). Infrared illumination (λ = 940nm) allowed images to be acquired during the dark phase. The acquired images had a resolution of 700 x 480 pixels, which corresponds to an area of 3.6 x 2.5 mm^2. The camera was equipped with a lowpass infrared filter (RG, Schott, http://www.schott.com) to block visible irradiation. The CCD camera was trained on the growing root tips by means of a tracking algorithm that controlled the movement of the stage and centered the root tips in the focal field. The algorithms for root tracking and image sequence acquisition were written with a digital image sequence processing software package (Heurisko, Aeon, Hanau, Germany; Schmundt *et al.,* 1998). The image sequences were used to calculate root tip velocity and maximal relative elemental growth rate ($REGR_{max}$), a process which is described in detail in Walter *et al.* (2002, 2003). Because root growth correlated positively with temperature (Walter *et al.,* 2002; Pahlavanian and Silk, 1988), $REGR_{max}$ and root tip

velocity were averaged over 3 different time periods to compare WT plants and ir*RALF* line 1 (morning: 6:00 to 13:00; afternoon: 13:00 to 21:00; and night: 21:00 to 6:00).

The pH-buffered media were produced by adding MES (or phosphate) buffer (20mM pH 5.5) or Tris (or HEPES) buffer (20 mM pH 6.8) to the germination medium Gamborg B5, on which 20 seeds of each genotype were sown. Roots were grown vertically as described above. The number and length of root hairs were measured from photographs taken with a stereomicroscope.

To test the ability of WT and ir*RALF* plants to acidify their rhizosphere, three 7-day-old seedlings per genotype were pressed onto pH indicator gel (1 mM $CaSO_4$, 0.006% bromocresol purple, pH 6.3). After 2 days, the medium in which WT were growing turned yellow, indicating that WT plants had gradually acidified the medium; this did not occur when ir*RALF* seedlings were grown on identically prepared pH indicator gel. This experiment was repeated once.

Analysis of trichoblasts' extracellular pH by pH-sensitive dye fluorescein-dextran: Surface pH measurements were conducted as described (Monshausen *et al.,* in review). Five-day-old seedlings of WT and ir*RALF* line 2 were transferred to cuvettes and covered with 0.7% (w/v) low-temperature gelling agarose containing 10% (w/v) Gamborg B5 salts, 1% (w/v) sucrose, and 150 µg/ml pH-sensitive dye fluorescein conjugated to10 kDa dextran (Sigma, http://www.sigmaalsrich.com). As the dye is dextran-conjugated, it is excluded from the cell and reports apoplastic pH. Fluorescein shows a strong pH-dependent emission when excited at 488 nm and a much less pH-sensitive emission when excited at 458 nm, making it amenable to ratio analysis (Chen, 2002). After several hours of growth, newly formed root hairs were imaged with the Zeiss LSM 510 confocal microscope using the 458 and 488 nm lines of the argon laser and collecting emissions with a 488 nm dichroic mirror and 505 nm long pass filter. Images were collected every 3 s, with each individual image scan lasting 2.2 s. pH-dependent fluorescence was calibrated in 100 mM MES buffer at pH 5.0, 5.25, 5.5, 6.0, 6.5 and 7.0 using identical imaging parameters to the root imaging described above. Data were analyzed using the Zeiss LSM software.

Isolating *NaRALF* : We obtained the ORF of *NaRALF* using an RT-PCR with primers designed from the *N. tabacum RALF* cDNA sequence (forward primer: ATGGGAGTTCCTTCAGGTTT, and reverse primer:

TTAACTCCTGCAACGAGTGA). The ORF fragment was cloned into a pGEM-T EASY vector (Promega, http://www.promega.com) and sequenced.

Generation of the transgenic plants: A 263 bp fragment of the cDNA sequence (Figure S2b) was inserted into the pRESC5 transformation vector (Bubner *et al.*, 2006) (Figure S2a) in an inverted-repeat orientation. This vector was transformed into *N. attenuata* WT plants using the *Agrobacterium*-mediated transformation procedure described in Krügel *et al.* (2002). The number of insertions was determined by Southern hybridization of genomic DNA using a PCR fragment of the *hptII* gene as a probe. Two single-insertion lines (ir*RALF* line 1 and 2; Figure S2c) were identified, bred to homozygousity, and used in all experiments.

Caterpillar performance: Eggs of *Manduca sexta* were acquired from North Carolina State University (http://www.ncsu.edu) and kept in a growth chamber (Snijders Scientific, http://snijders-tilburg.nl) at 26℃ 16h light, 24℃ 8h darkness, until the larvae hatched. Freshly hatched neonates were placed directly on fully developed leaves of rosette-stage plants. Larvae were weighed after 3, 6, and 10 days of feeding.

Nucleic acid analysis: Extraction of total RNA and northern blot analysis were performed as previously described (Winz and Baldwin, 2001). Genomic DNA was extracted from leaves as described previously (Bubner *et al*., 2004). Five µg of DNA was digested with *Eco*RI, *Hind* III, and *Dra* I and then blotted onto a nylon membrane. A probe was prepared by labeling *NaRALF* ORF with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, http://www.amersham.com). A fragment of *hptII* (forward primer: 5'-CGTCTGTCGAGAAGTTTCTG-3', reverse primer: 3'- CCGGATCGGACGATTGCG-5') was amplified by PCR and used as a probe for Southern hybridization to confirm the single insertion of the transgenic lines.

To analyze the accumulation of *NaRALF* transcripts in WT plants in response to UV-B exposure and elicitation with *M. sexta* oral secretions (OS), we exposed rosette-stage plants to UV-B produced by one TL40W (Philips, http://www.philips.com) for 0, 2, 6 h (each with three replicates). For OS elicitation, leaves on 3 replicate plants were wounded with a fabric pattern wheel, water or *M. sexta* OS were immediately applied to the puncture wounds, and plants were harvested 0. 0.5, 1, 3, and 6 h after elicitation as described in Zavala *et al.* (2004). Leaf samples were harvested and immediately frozen in liquid nitrogen.

Real-time PCR assay (qPCR): Total RNA was extracted with TRI Reagent (Sigma, http://www.sigmaaldrich.com) according to the manufacturer's instructions, and cDNA was prepared from 500 ng total RNA with multiScribeTM reverse transcriptase (Applied Biosystems, http://www.appliedbiosystems.com). The primers and probes specific for *NaRALF* mRNA expression detection by qPCR were as follows:

NaRALF forward primer; 5'- TTCAATGGCGACCGCTG-3',
NaRALF reverse primer; 5'- TACTCCCGTTGCATCCCCT-3',
ECI forward primer; 5'-AGAAACTGCAGGGTACTGTTGG-3',
ECI reverse primer; 5'-CAAGGAGGTATAACTGGTGCCC-3',
FAM labeled NaRALF probe; 5'- GATTGGGTGATGCCGGCGAGA-3',
VIC labeled ECI probe; 5'-CGTCAAAATTCTCCACTTGTTTCAACTGT-3'.

The assays using a double dye-labeled probe were performed on an ABI PRISM® 7700 Sequence Detection System (qPCRTM Core Kit, Eurogentec, http://www.eurogentec.be) with *N. attenuata sulfite reductase* (ECI) for normalization and according to the manufacturer's instructions with the following cycle conditions: 10 min 95°C; 40 cycles: 30 sec 95°C, 30 sec 60°C.

ROS measurement and DAB staining: ROS was measured in roots as described by Shaw and Long (2003) using an Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes, http://probes.invitrogen.com). For in-situ staining, 6 days after germination, seedlings were stained with 1mg/ml DAB for 2 h, and photographed with a stereomicroscope.

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Supplementary Data

Video Clip S1. Time-lapse movie of root growth in ir*RALF* line 1.

Video Clip S2. Time-lapse movie of apoplatic pH increasing before a trichoblast bursts (Upper: fluorescence image; bottom: bright field image). The root hair was immersed in fluorescein-dextran. Increasing fluorescence intensity indicates increasing pH. Images were taken every 3 s.

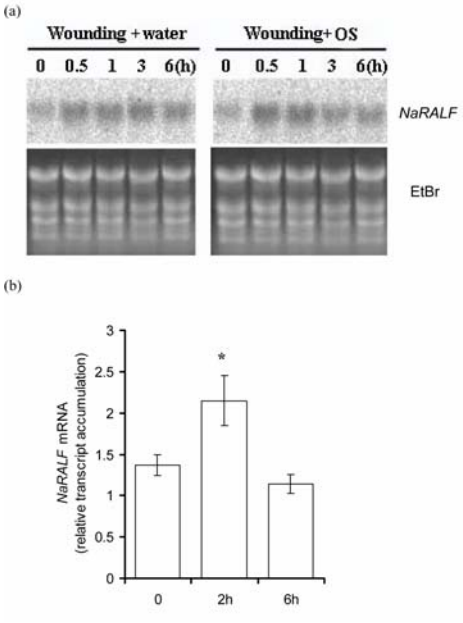

Figure S1. Transcript accumulation of *NaRALF* in response to different stresses.

(**a**) Northern blot analysis of *NaRALF* in response to wounding and water or OS (oral secretions of *M. sexta*). Samples were collected from rosette-stage leaves elicitated by puncture wounds and treated with water (wounding+water) or *M. sexta* oral secretions (wounding+OS) after 0, 0.5, 1, 3, and 6 h. Total RNAs (10µg) were loaded, probed with a ^{32}P labeled *NaRALF* ORF sequence. An ethidium bromide (EtBr) stained gel serves as a loading control.

(**b**) Mean (± SEM) *NaRALF* transcript accumulation in response to UV-B irradiation as analyzed by qRT-PCR normalized to levels of sulfite reductase transcripts. Three replicate rosette-stage plants were exposed to UV-B for 0, 2 and 6 h for each treatment time. The asterisk indicates the level of significant differences between control and treated plants (unpaired *t*-test: *, $P<0.05$).

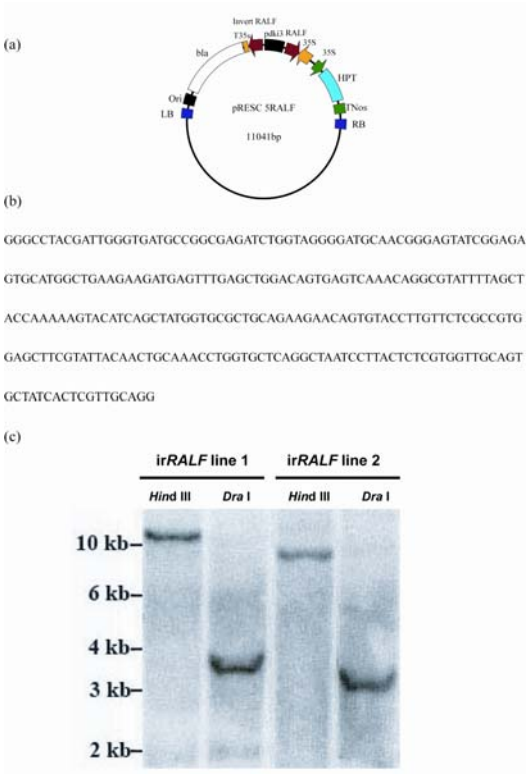

Figure S2. Generation of invert-repeat transgenic plants (ir*RALF* lines).

Transformation vector pRESC5 (**a**), described in Bubner *et al*. (2006), used to transform *N. attenuata* plants to silence *NaRALF* expression by transferring a 263bp-fragment (**b**) of *NaRALF* inserted twice in opposite directions and resulting in an inverted repeat construct.

(**c**) Southern blot analysis reveals that both ir*RALF* lines 1 and 2 harbor only one T-DNA as determined with an *hptII*-specific probe. 5 µg of genomic DNA were digested with *Hin*d III and *Dra* I.

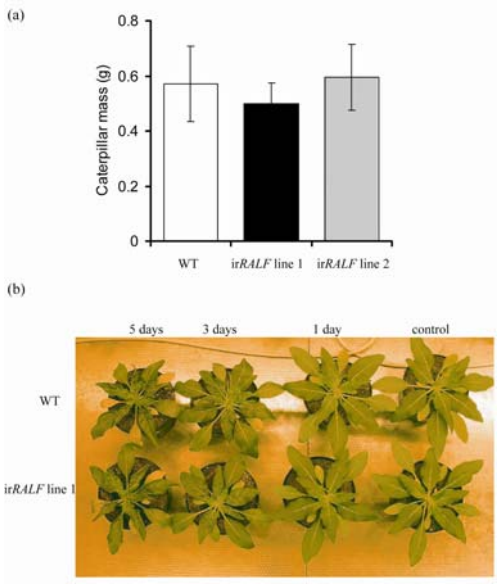

Figure S3. Caterpillar performance and morphologies of ir*RALF* and WT plants after UV-B treatments.
(**a**): Mean (± SEM) mass of *M. sexta* larvae after 10 days of growth on WT and ir*RALF* rosette-stage plants. A single freshly hatched larva was placed on a fully developed leaf of 12 replicates of WT and ir*RALF* plants.
(**b**): Morphologies of ir*RALF* line 1 and WT plants after UV-B treatment. ir*RALF* line 1 and WT plants did not differ in their morphologies after 0, 1, 3, 5 days of UV-B exposure. On each day the UV-B lamps were switched on at 9:30 and switched off at 15:30.

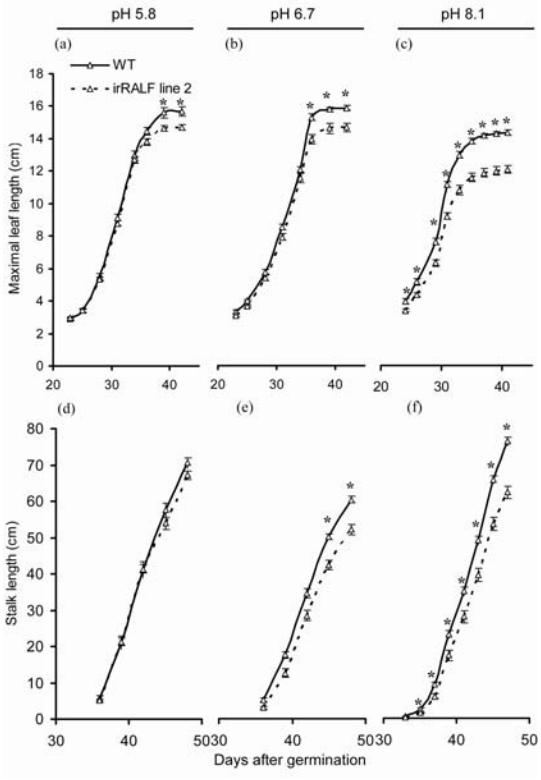

Figure S4. Leaf size and stalk length of ir*RALF* line 2 were reduced when they competed against WT plants in the glasshouse.
Mean (± SEM) maximal leaf lengths and stalk lengths were measured in 12 replicate pairs of size-matched WT and ir*RALF* line 2 plants grown in soil buffered to pH 5.8 (**a** and **d**), pH 6.7 (**b** and **e**), pH 8.1 (**c** and **f**). The asterisks indicate the level of significant differences between WT and ir*RALF* line 2 (paired *t*-test: *, $P<0.05$).

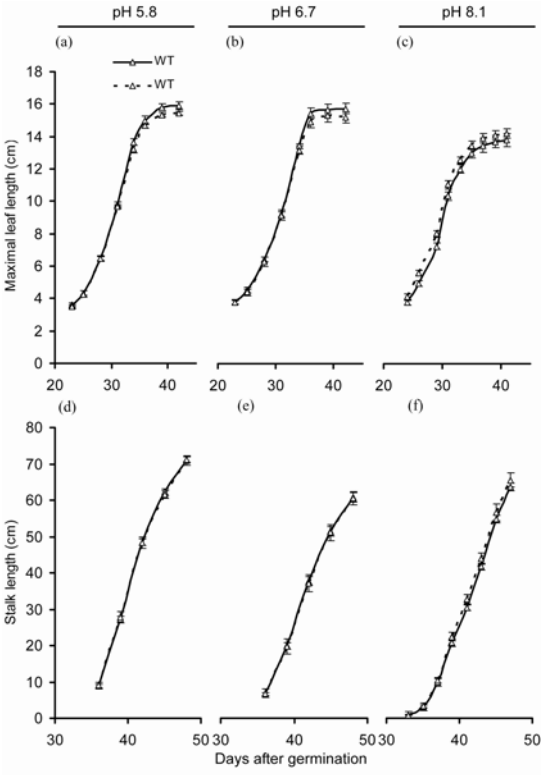

Figure S5. Leaf size and stalk length of WT plants were the same when they competed against each other in the glasshouse.

Mean (± SEM) maximal leaf lengths and stalk lengths were measured in 12 replicate pairs of size-matched WT and WT plants grown in soil buffered to pH 5.8 (**a** and **d**), pH 6.7 (**b** and **e**), pH 8.1 (**c** and **f**).

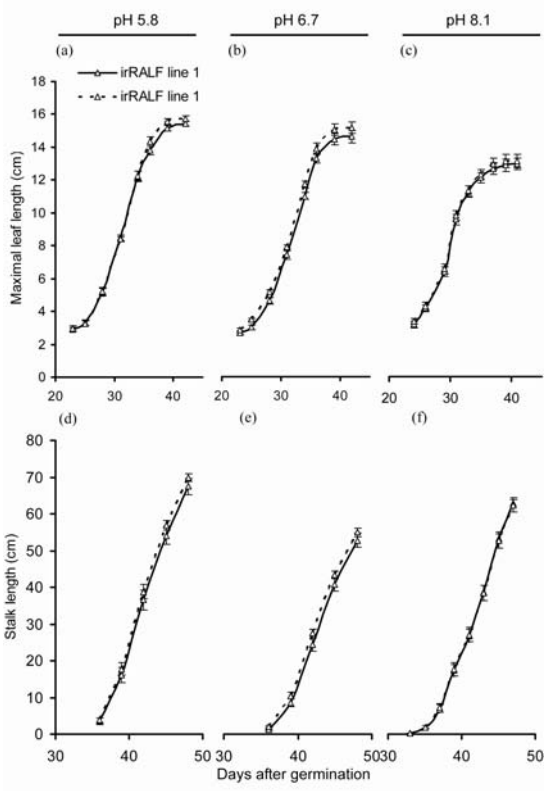

Figure S6. Leaf size and stalk length of ir*RALF* line 1 plants were the same when they competed against each other in the glasshouse.

Mean (± SEM) maximal leaf lengths and stalk lengths were measured in 12 replicate pairs of size-matched ir*RALF* line 1 and ir*RALF* line 1 plants grown in soil buffered to pH 5.8 (**a** and **d**), pH 6.7 (**b** and **e**), pH 8.1 (**c** and **f**).

Manuscript II

The Planta (2008)

Running title: MeJA is de-esterifed to JA in defense elicitation

Methyl jasmonate (MeJA)-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA?

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Abstract

Treatment with methyl jasmonate (MeJA) elicits herbivore resistance in many plant species and over-expression of JA carboxyl methyltransferase (JMT) constitutively increases JA-induced responses in *Arabidopsis*. When wild-type (WT) *Nicotiana attenuata* plants are treated with MeJA, a rapid transient endogenous JA burst is elicited, which in turn increases levels of nicotine and trypsin proteinase inhibitors (TPIs) and resistance to larvae of the specialist herbivore, *Manduca sexta*. All of these responses are impaired in plants silenced in lipoxygenase 3 expression (as*LOX3*) but are restored to WT levels by MeJA treatment. Whether these MeJA-induced responses are directly elicited by MeJA or by its cleavage product, JA, is unknown. Using virus-induced gene silencing (VIGS), we silenced MeJA-esterase (*NaMJE*) expression and found this gene responsible for most of the MeJA-cleaving

activity in *N. attenuata* protein extracts. Silencing *NaMJE* in as*LOX3,* but not in WT plants significantly reduced MeJA-induced nicotine and JA-Ile levels, and resistance to *M. sexta*, but not TPI levels. MeJA-induced transcript levels of threonine deaminase (*NaTD*) and phenylalanine ammonia lyase (*NaPAL1*), were also decreased in VIGS *MJE* (as*LOX3*) plants. Finally the performance of *M. sexta* larvae that fed on plants treated with JA or MeJA demonstrated that silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance in as*LOX3* plants. From these results, we conclude that the resistance elicited by MeJA treatment is not directly elicited by MeJA but by its de-methylated product, JA, either alone or after conjugation with Ile.

Key Words: MeJA esterase (*NaMJE*), methyl jasmonate (MeJA), jasmonate (JA), JA-Ile, *Nicotiana attenuata*, *Manduca sexta*

Introduction

Methyl jasmonate (MeJA), jasmonic acid (JA) and its amino acid conjugates, collectively referred to as jasmonates, are important cellular regulators mediating diverse developmental processes including root growth, pollen production, and plant resistance to insects and pathogens (Creelman and Mullet 1997; Kessler and Baldwin 2002). Jasmonates are synthesized in plants via the octadecanoid pathway (Creelman and Mullet 1997). Briefly, linolenic acid is oxygenated by lipoxigenase (LOX) and then converted to 12-oxo-phytodienoic acid (12-oxo-PDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). JA is synthesized from 12-oxo-PDA through reduction and three steps of β-oxidation, and then catabolized further to form its volatile counterpart, MeJA (Seo et al. 2001), and numerous conjugates including JA-isoleucine (JA-Ile) (Staswick and Tiryaki 2004; Kang et al. 2006; Wang et al. 2007a). MeJA is one of the JA metabolites proposed to play an important role in inter- and intra-plant signaling (Farmer and Ryan 1990; Seo et al. 2001; Karban et al. 2000; Kessler et al. 2006; Baldwin et al. 2006). When plants are exposed to a certain amount of MeJA, it will quickly elicit a series JA mediated defense responses and MeJA treatment is the most commonly used means of eliciting herbivore resistance in many different plant species (McConn et al. 1997; Baldwin 1998; Li et al. 2002). However, it is still not known how plants elicit herbivore resistance traits in response to MeJA exposure.

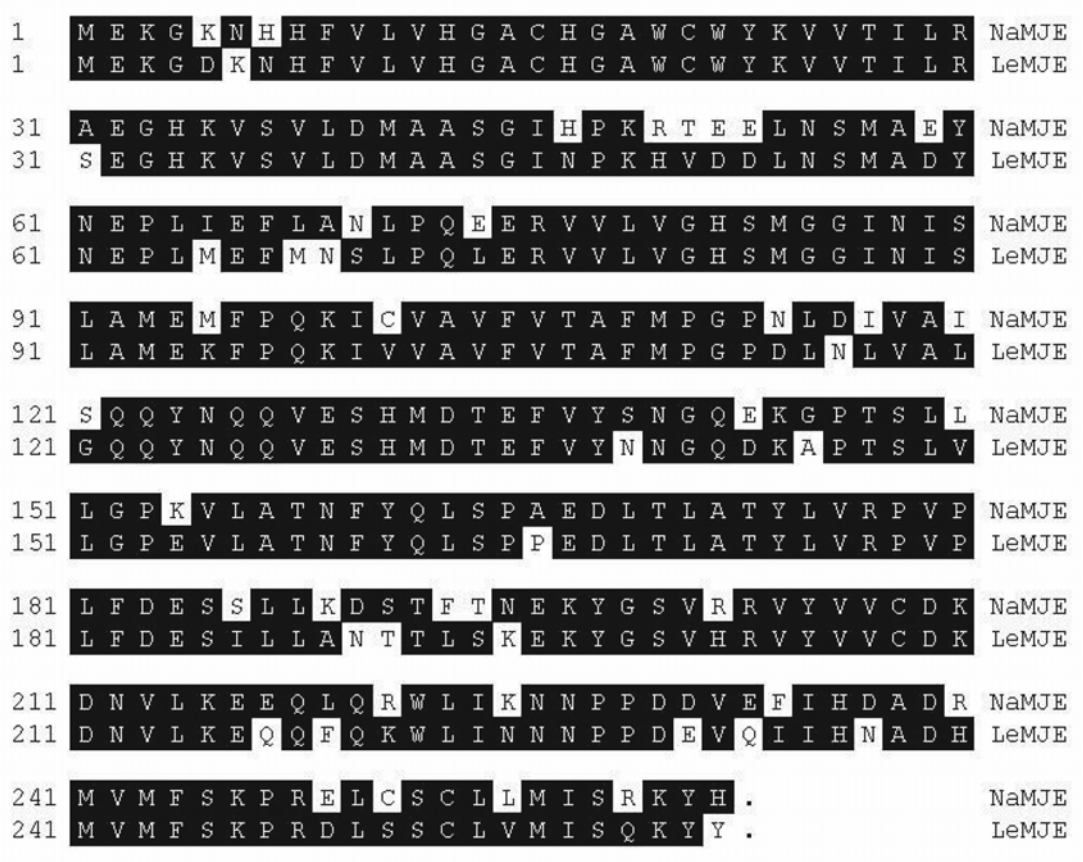

Figure 1. The deduced amino acid sequence of Na*MJE* (EU196055) compared with Le*MJE* (AY455313).
NaMJE has 80% sequence identity with LeMJE. The consensus amino acids are in black.

When *Nicotiana attenuata* is attacked by herbivores, it produces both volatiles to recruit the herbivores' natural enemies (Kessler and Baldwin 2001; Mattiacci et al. 1995) and secondary metabolites that function as direct defenses, such as the neurotoxin, nicotine (Baldwin 1999; Steppuhn et al. 2004), and trypsin proteinase inhibitors (TPIs) (Zavala et al. 2004). JA signaling plays a central role in these responses. Silencing a key gene involved in supplying fatty acid hydroperoxides for JA biosynthesis, lipoxygenase 3 (*NaLOX3*), reduces the wound- and herbivore-induced accumulation of JA. This JA deficiency inhibits the elicitation of direct (TPIs and nicotine) and indirect (volatiles) defenses and reduces *N. attenuata*'s resistance to attack by larvae of the specialist herbivore of *N. attenuata, Manduca sexta* (Halitschke and Baldwin 2003), and makes plants susceptible to two new herbivores, the leaf-chewing beetle *Diabrotica undecimpunctata* and the piercing-sucking leafhopper *Empoasca spp.* (Kessler et al. 2004). Interestingly, when *NaLOX3*-silenced plants are treated with MeJA, their ability to produce nicotine and herbivore-

resistance are fully restored (Halitschke and Baldwin 2003), suggesting that the exogenous MeJA treatment is sufficient to elicit most JA responses. However, these results raise an important question: does the exogenously supplied MeJA function directly as a signal or must it first be hydrolyzed to JA? Both JA and MeJA are elicitors of defense responses when applied exogenously, but the nature of the endogenous signal remains unclear as both are rapidly interconvertable. Over-expression of JA carboxyl methyltransferase (JMT) in *Arabidopsis* increases endogenous MeJA levels 3-fold without altering JA levels and results in the constitutive expression of JA-responsive genes, including VSP and PDF1.2 (Seo et al. 2001). These results suggest that MeJA rather than JA elicits systemically transmitted defense responses. Similarly, a long-standing debate whether salicylic acid (SA) or its methyl ester, MeSA, was the elicitor of systemic acquired resistance to pathogen attack was recently resolved by the ingenious combination of grafting together combinations of plants altered in their expression of either the MeSA-esterase and SA-methyl transferases (Park et al. 2007).

The discovery of MeJA-esterase (MJE) (Stuhlfelder et al. 2002; Stuhlfelder et al. 2004), which hydrolyzes MeJA to JA provides a means of determining whether MeJA is the elicitor of MeJA-elicited herbivore resistance. Since MeJA and JA treatment of plants is known to elicit endogenous JA production (Ziegler et al., 2001; Miersch and Wasternack 2000; Pluskota et al. 2007), the hypothesis is best tested in plants reduced in their endogenous JA production. We tested the hypothesis that MeJA-elicited herbivore resistance is actually elicited after de-esterification to JA and subsequent conjugation to JA-Ile. We used virus-induced gene silencing (VIGS) to silence MeJA esterase (*NaMJE*) transcripts in wild-type (WT) and *asLOX3 N. attenuata* plants (Halitschke and Baldwin, 2003) with a *tobacco rattle virus*-based system that had been optimized for *N. attenuata* (Saedler and Baldwin, 2004) and measured defense responses, transcripts and herbivore performance in plants elicited with MeJA or JA treatments.

Results

Silencing *NaMJE* dramatically reduces the hydrolysis of MeJA in protein extracts

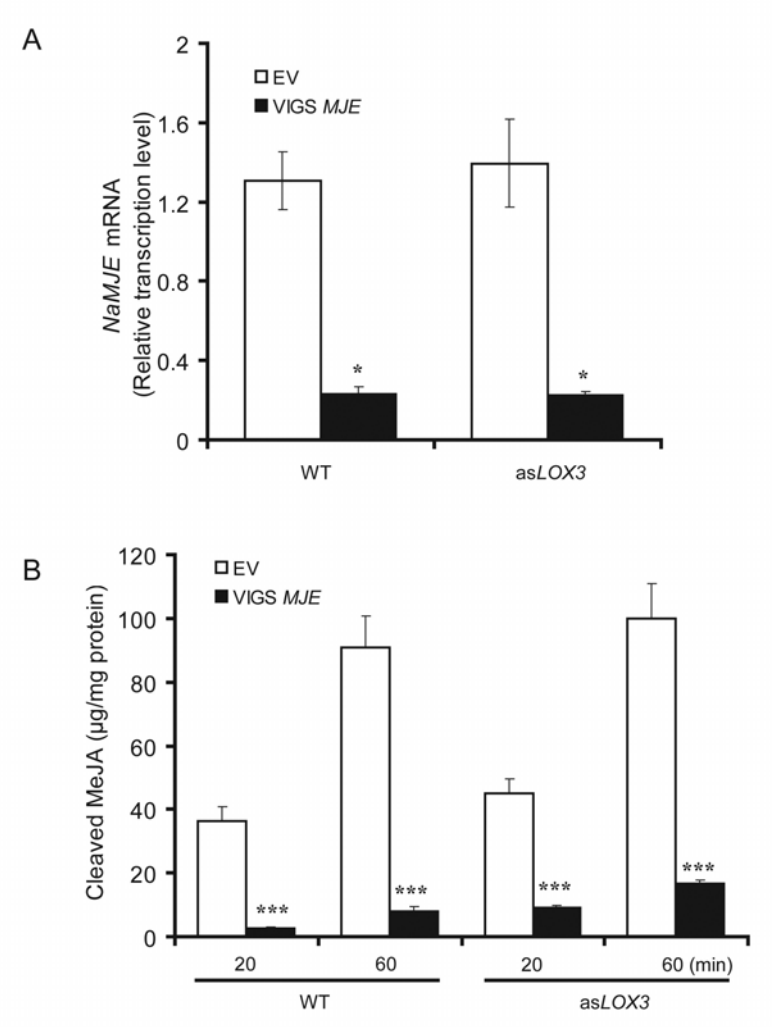

Figure 2. Silencing *NaMJE* reduces the MeJA-cleaving activity

WT and as*LOX3* plants were inoculated with *Agrobacterium*-harboring TRV constructs, which contained an empty vector (EV) or a 312-bp *NaMJE* fragment (VIGS *MJE*). After 14 days, four replicate source-sink transition leaves were harvested.

A: Mean (± SE) *NaMJE* transcript accumulation in source-sink transition leaves of EV and VIGS *MJE* plants from both WT and as*LOX3* backgrounds as analyzed with qRT-PCR normalized to levels of *NaActin*.

B: Mean (± SE) amount of MeJA hydrolyzed to free JA in 4 replicate total crude protein samples prepared from 4 individual EV and VIGS *MJE*-innoculated plants (WT and as*LOX3* background) after incubation with MeJA for 20 and 60 minutes.

Asterisks indicate the level of significant difference between EV and VIGS *MJE* plants (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$; ***, $P<0.005$).

The methyl jasmonate esterase (*NaMJE*) cDNA was cloned by RT-PCR using primers designed from *MJE* tomato (*LeMJE*). The predicted amino acid sequence of *NaMJE* shares 80% sequence identity with LeMJE (AY455313) (Figure 1). We used the VIGS system to silence *NaMJE* mRNA in WT and as*LOX3* plants. Plants were inoculated with *Agrobacterium*-harboring TRV constructs that contained an empty vector (EV) or a 312-bp *NaMJE* fragment (VIGS *MJE*). After 14 days, when the

visible bleaching of green tissues had been observed for 3 days in plants inoculated with phytoene desaturase (PDS), a gene required for the biosynthesis of xanthophylls of the antenna pigments, *NaMJE* transcripts were analyzed with qRT-PCR in 4 replicate source-sink transition leaves of EV and VIGS *MJE* plants and normalized to levels of *NaActin*. The results show that VIGS *MJE* plants had only 16% of the *NaMJE* transcripts that EV plants had in both WT and asLOX3 backgrounds (Figure 2A).

To determine if the *NaMJE* we silenced was responsible for hydrolyzing MeJA to free JA, we measured the MeJA-cleaving activity of proteins extracted from leaves of EV and VIGS *MJE* plants (Figure 2B). In proteins extracted from EV as*LOX3* plants, 45 µg MeJA was cleaved to free JA by 1 mg EV proteins after 20 minutes under standard conditions, while only 9 µg MeJA was cleaved by 1 mg of proteins extracted from VIGS *MJE* as*LOX3* plants (unpaired *t*-test, p=0.0002); after 60 minutes, the MeJA cleaved by VIGS *MJE* was only 16 % of the amount that EV proteins had cleaved (unpaired *t*-test, p=0.0003). Dramatically reduced MeJA-cleaving activity was also found in VIGS *MJE* proteins in WT background (Figure 2B). These results demonstrate that *NaMJE* is largely responsible for the MeJA-cleaving activity in *N. attenuata* leaves.

Silencing *NaMJE* inhibits MeJA-induced resistance to *M. sexta* in as*LOX3* plants but not in WT plants.

To determine if silencing *NaMJE* influenced MeJA-induced resistance against *M. sexta* larvae, the source-sink transition leaves of EV and VIGS *MJE* plants were treated with lanolin paste (lanolin) or 75 µg MeJA in lanolin (MeJA). After 4 days, *M. sexta* larvae were placed on these leaves and allowed to feed for 12 days. As expected, larvae feeding on lanolin treated as*LOX3* plants gained significantly more mass than those on lanolin treated WT plants and MeJA treatment dramatically reduced the *M. sexta* larval mass on EV plants: larvae that fed on MeJA-treated plants gained only 60.5 and 54% of the mass of those that fed on lanolin-treated WT and as*LOX3* plants, respectively (Figure 3). Larvae performed equally well on EV and VIGS *MJE* plants treated with lanolin in either WT or as*LOX3* genetic backgrounds (Figure 3). However, silencing *NaMJE* completely inhibited MeJA-induced resistance in as*LOX3* plants: larvae that fed on MeJA-treated VIGS *MJE* plants gained the same mass as those that fed on plants treated with lanolin (Figure 3), but gained 50% more mass

than those that fed on EV plants treated with MeJA (Figure 2; unpaired *t*-test, p=0.04). These results demonstrate that in *asLOX3* plants silencing *NaMJE* is sufficient to block MeJA-induced resistance.

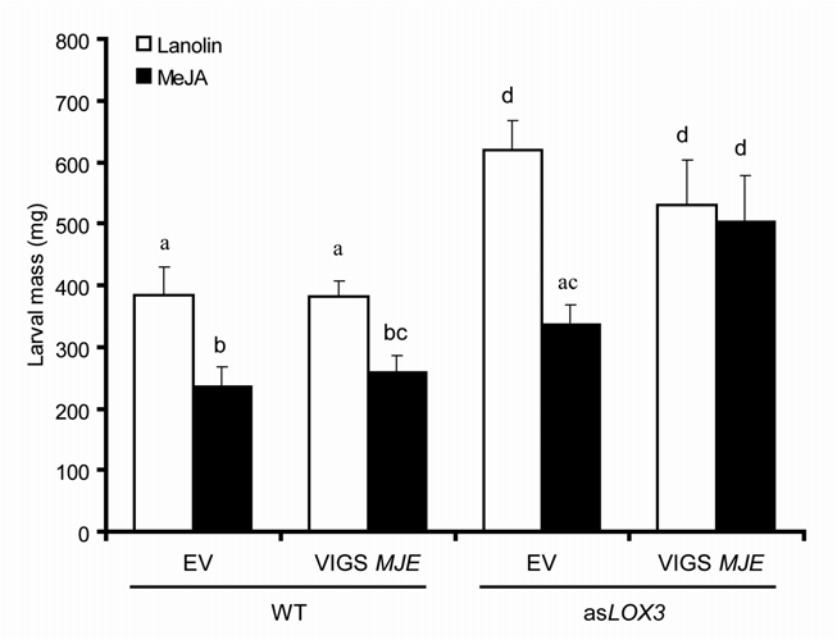

Figure 3. Silencing *NaMJE* inhibits MeJA-induced resistance to *M. sexta* in as*LOX3* plants but not WT plants.

Mean (± SE) mass of 18 replicate *M. sexta* larvae after 12 days of feeding individually on EV and VIGS *MJE* WT and as*LOX3* plants. The source-sink transition leaves of EV and VIGS *MJE* plants were treated with lanolin or 75 µg MeJA in lanolin. Four days later, *M. sexta* larvae were placed on these leaves. Different letters indicate statistically significant differences between treatments (Fisher's PLSD test; $P<0.05$).

Silencing *NaMJE* impairs MeJA-induced nicotine and *NaPAL1* transcripts but not TPI responses in as*LOX3* plants

To understand why silencing *NaMJE* transcripts had such a strong effect on caterpillar performance in MeJA-induced *asLOX3* but not WT plants, we measured MeJA-induced nicotine and TPI levels. After being treated with MeJA for 3 days, all EV plants had significantly increased nicotine levels (Figure 4A). In WT plants, VIGS *MJE* plants accumulated the same amount of nicotine as EV plants had after MeJA treatment. However, nicotine levels of MeJA-treated VIGS *MJE* as*LOX3* plants were only 66% of those of MeJA-treated EV plants (Figure 4A; unpaired *t*-test, p=0.01).

MeJA treatment elicited the same amount of TPI activity in EV and VIGS *MJE* plants in both WT and as*LOX3* backgrounds (Figure 4B), suggesting that silencing *NaMJE* had no effect on the elicitation of TPIs by MeJA. Measurements of

TPI transcripts in as*LOX3* plants 8 h after MeJA treatment also revealed no significant differences between EV and VIGS *MJE* plants (unpaired *t*-test, p=0.5).

To better understand why silencing *NaMJE* in as*LOX3* plants had such a large effect on MeJA-induced herbivore resistance, we compared transcript levels of phenylalanine ammonia lyase (*PAL1*), a well-known gene marker of phenolic-based defense responses, in the source-sink transition leaves of EV and VIGS *MJE* plants after MeJA treatment. *PAL1* was dramatically induced in EV plants. Four h after MeJA treatment, levels of *PAL1* transcripts in VIGS *MJE* plants were only 21% of those in EV plants (Figure 5; unpaired *t*-test, p=0.005); after 8 h, levels were 25% of those in EV plants (Figure 5; unpaired *t*-test, p=0.05).

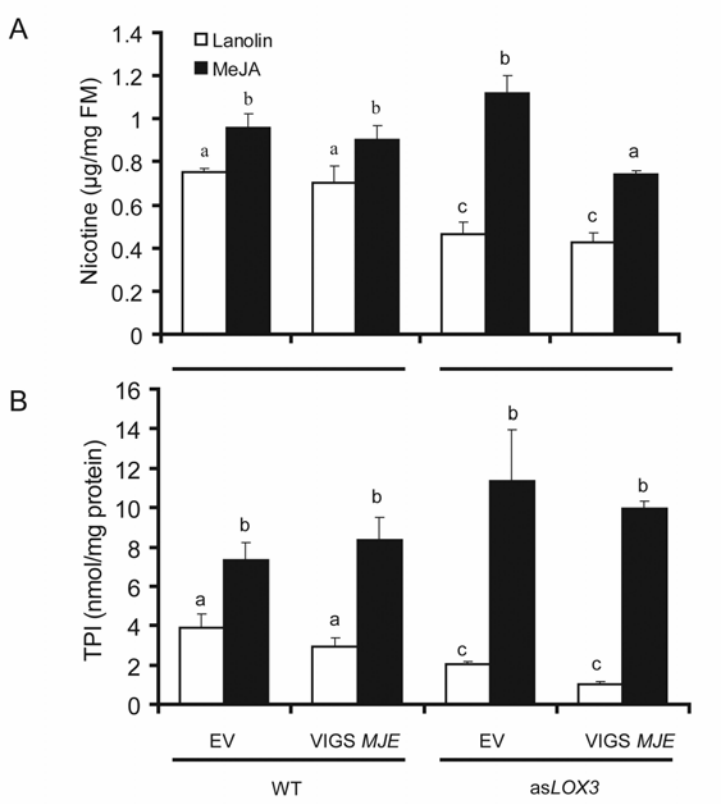

Figure 4. Effects of silencing *NaMJE* on MeJA-induced levels of nicotine and TPI in WT and as*LOX3* plants

The source-sink transition leaves of EV and VIGS *MJE* plants were treated with lanolin or 75 µg MeJA in lanolin. Three days later, 4 replicate leaf samples per treatment were harvested and analyzed for: **A** Mean (± SE) nicotine levels and **B** Mean (± SE) TPI levels. Different letters indicate statistically significant differences among treatments (Fisher's PLSD test; $P<0.05$).

Silencing *NaMJE* impairs MeJA-induced endogenous JA-Ile levels in *asLOX3* plants

The changes in endogenous levels of JA and JA-Ile elicited by MeJA treatment were measured in EV and VIGS *MJE* as*LOX3* plants (Figure 6) to better understand the loss of MeJA-induced resistance in *NaMJE* as*LOX3* plants. After 2 h both JA and JA-Ile levels tended to be lower in VIGS *MJE* plants, but the differences were not significant (unpaired *t*-test, p=0.21, 0.15, respectfully). While JA levels remained unchanged after 4 h, levels of JA-Ile were significantly lower in *NaMJE*-silenced plants than in EV plants (unpaired *t*-test, p=0.05). Threonine deaminase (*TD*) catalyzes the conversion of Thr to a-keto butyrate in Ile biosynthesis, and is strongly elicited in MeJA elicited leaves in *N. attenuata* where it supplies the Ile required for JA-Ile production (Kang et al., 2006). MeJA treatments dramatically increased *NaTD* transcripts in EV plants; however, in VIGS *MJE* plants *NaTD* transcript levels were only 9% of those in EV plants 8 h after MeJA treatment (Figure 6; unpaired *t*-test, p=0.03). Since JA-Ile is known to elicit nicotine production (Wang et al. 2007b), these results suggest that the de-esterification of MeJA supplies the JA required for JA-Ile production, nicotine elicitation and herbivore resistance.

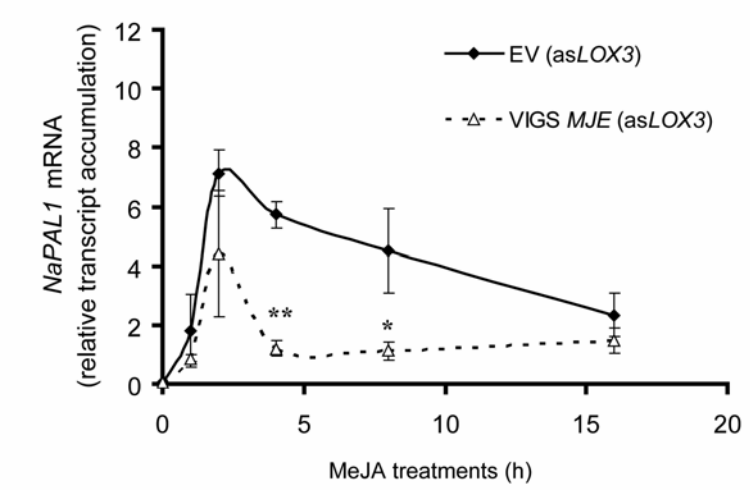

Figure 5. Silencing *NaMJE* inhibited MeJA-induced *NaPAL1* transcripts in as*LOX3* plants

Four replicate source-sink transition leaves of EV and VIGS *MJE* plants were treated with 75 µg MeJA in lanolin and harvested after 0, 1, 2, 4, 8, and 16 h. Mean (± SE) *NaPAL1* transcription levels were analyzed with qRT-PCR normalized to levels of *NaActin*. Asterisks indicate the level of significant difference between EV and VIGS *MJE* plants, both treated with MeJA (unpaired *t*-test: *, $P<0.05$; **, $P<0.005$).

Silencing *NaMJE* inhibits MeJA-induced but not JA-induced resistance to *M. sexta* in as*LOX3* plants

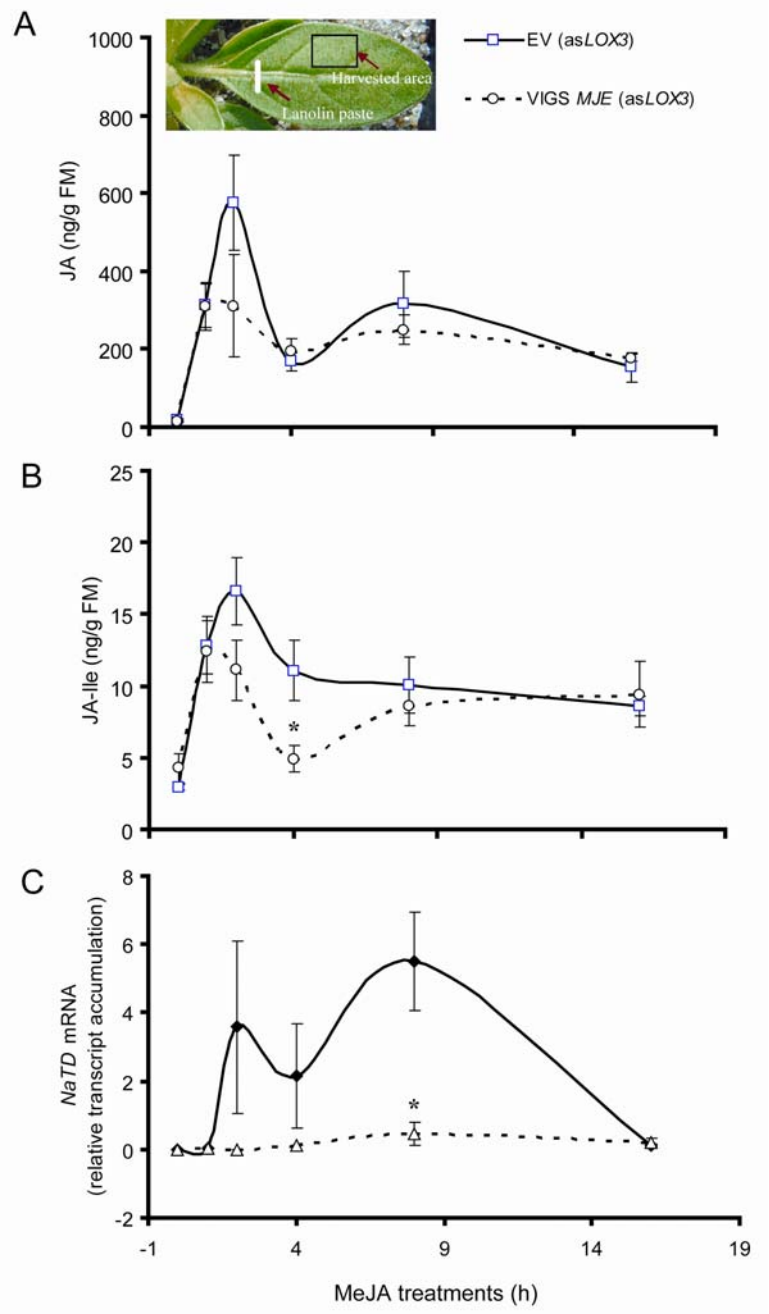

Figure 6. The effect of MeJA treatments on endogenous JA and JA-Ile and TD transcript levels in *asLOX3* plants

Four replicate source-sink transition leaves of EV and VIGS *MJE* plants were treated with 75 µg MeJA in lanolin and harvested after 0, 1, 2, 4, 8, and 16 h and analyzed for: (**A**): mean (± SE) JA levels, (**B**) mean (± SE) JA-Ile levels, and (**C**) mean (± SE) *NaTD* transcripts. **Inset**: the black box indicates the portion of the leaf lamina that was harvested for JA and JA-Ile analysis, and the white bar, the location of the MeJA-lanonlin paste application. Asterisks indicate the level of significant difference between EV and VIGS *MJE* plants, both treated with MeJA (unpaired *t*-test: *, $P<0.05$**, $P<0.005$).

If it is true that MeJA functions as a signal prior to being hydrolyzed to JA, silencing *NaMJE* should inhibit MeJA-induced resistance but have no effect on JA-induced resistance. Due to its polarity, JA does not elicit resistance when added to plants in a lipophilic lanolin paste. Therefore, aqueous solutions of JA and MeJA were added to standardized puncture wounds in leaves of EV and VIGS *MJE* as*LOX3* plants and we tested this hypothesis by measuring the performance of larvae. As expected, the mass of caterpillars that fed on EV plants treated with both JA and MeJA was dramatically reduced compared to the mass of those that fed on EV plants treated with water (Figure 7). Larvae that fed on JA-treated VIGS *MJE* plants gained the same mass as those that fed on EV plants treated with JA; however, larvae gained significantly more mass when they fed on VIGS *MJE* plants than on EV plants treated with MeJA (Figure 7).

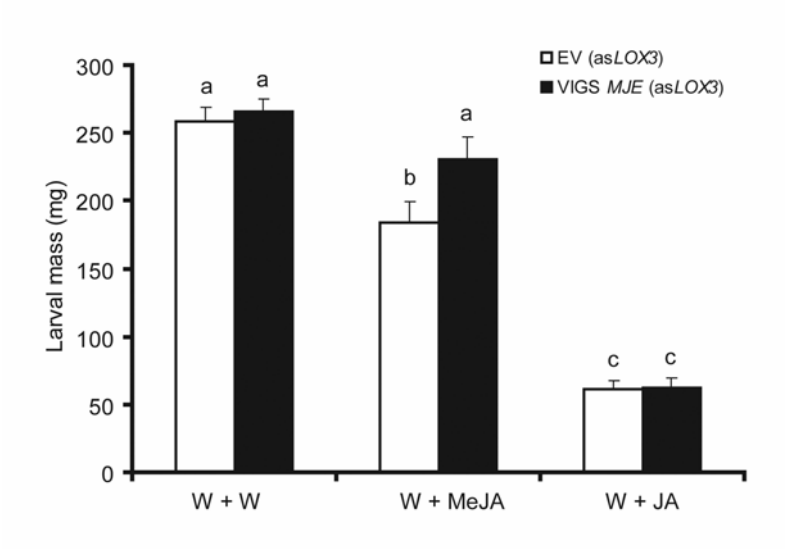

Figure 7. Silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance to *M. sexta* in as*LOX3* plants.

The source-sink transition leaves of EV and VIGS *MJE* plants were wounded with a fabric pattern wheel; water (12.5% ethanol), or 0.25 µmol JA, or 0.25 µmol MeJA were immediately applied to the puncture wounds. Two days later, 18 *M. sexta* larvae were placed individually on the source-sink transition leaves and weighed 15 days after the start of feeding. Asterisks indicate the level of significant difference among the masses of caterpillars that fed on EV and VIGS *MJE* plants, both treated with MeJA (unpaired *t*-test: *, $P<0.05$).

Discussion

Staswick's pioneering work on MeJA-insensitive *jar1* mutant in *Arabidopsis* suggests that exogenously applied MeJA is first demethylated and then conjugated to Ile before it becomes active in the inhibition of root growth (Staswick et al. 1992; Staswick and Tiryaki 2004). Indeed, MeJA hydrolyzing enzyme activity occurs in all

the plant species so far tested (Stuhlfelder et al. 2002). It is also reported that MeJA was rapidly hydrolyzed to JA and further metabolized like JA when tobacco BY-2 cells were treated with MeJA (Swiatek et al. 2004). In *N. attenuata*, a model plant with extensively studied herbivore-induced responses, we also detected high levels of MeJA-cleaving activity (Fig. 2b). Based on the sequence similarity with tomato *LeMJE* (Stuhlfelder et al. 2004), we cloned *NaMJE*, a MeJA esterase gene that shared very high sequence similarity with *LeMJE* (Fig. 1) and by silencing the expression of this gene, we demonstrated that *NaMJE* is largely responsible for the MeJA-cleaving activity of *N. attenuata* leaves (Fig. 2b).

MeJA treatment is the most commonly used means of eliciting herbivore resistance in many different plant species (McConn et al. 1997; Baldwin 1998; Li et al. 2002). However, it is still not known how herbivore resistance traits are elicited by MeJA treatment. We determine whether MeJA functions as a signal prior to being hydrolyzed to JA by investigating MeJA-induced defense responses in both WT and *asLOX3* plants with reduced MeJA-cleaving activity. Silencing *NaMJE* transcripts was sufficient to block most MeJA-induced responses in as*LOX3* but not WT plants, including the production of nicotine (Fig. 4); transcripts of *NaPAL1* and *NaTD* (Fig. 5 and 6); and the JA-Ile burst (Fig. 6). Importantly, the dramatic decreases in mass that are usually observed in larvae that feed on MeJA-treated EV plants disappeared when *NaMJE* transcripts were silenced (Fig. 3). Furthermore, silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance in as*LOX3* plants (Fig. 7). Why is silencing *NaMJE* sufficient to block most MeJA-induced responses in as*LOX* plants but not in WT plants? MeJA and JA treatment of plants is known to elicit endogenous JA production (Ziegler et al., 2001; Miersch and Wasternack 2000) and this is inhibited in as*LOX3* plants (Halitschke and Baldwin 2003). Based on these results, we conclude that most exogenous MeJA-induced herbivore responses are actually elicited by JA, either alone or after conjugation with Ile.

Kang et al. (2006) have suggested that JA-Ile plays an important role in the nicotine production. It was further confirmed in plants silenced both *NaJAR4* and *NaJAR6* (Wang et al. 2007b). *NaJAR4/6* encodes enzymes that conjugate isoleucine (Ile) to JA and in the production of JA-Ile (Wang et al. 2007a). When *NaJAR4* and *NaJAR6* are simultaneously silenced, the nicotine level elicited by JA treatment is only half of that in WT plants receiving the same treatment, but after JA-Ile treatments, WT plants and *NaJAR4*/*6*-silenced plants accumulated the same levels of

nicotine, demonstrating that JA-Ile is central player in the elicitation of nicotine production (Wang et al. 2007b). Hence, the reduction of MeJA-induced nicotine level in *NaMJE*-silenced as*LOX3* plants is likely caused by the attenuated JA-Ile levels, and this lower JA-Ile level is correlated with lower *NaTD* transcription level (Fig. 6). Exactly how the nicotine production is regulated by JA-Ile is still unclear. Recently, JA-Ile was shown to be the most active jasmonate-derivatives promoting physical interaction between COI1 and jasmonate ZIM-domain (JAZ) protein, which leads to the degradation of JAZ1, the repressor of the transcription of jasmonate-responsive genes (Thine et al. 2007; Chini et al. 2007). Although the function of JAZ proteins is not demonstrated in *N. attenuata*, it is likely that nicotine production is regulated through JAZ and COI1 dependent pathway.

Trypsin proteinase inhibitors (TPIs) have also been demonstrated to have a direct defensive role against herbivores in *N. attenuata* (Zavala et al. 2004). The activities and transcripts of TPIs can be dramatically induced by MeJA treatments (Fig. 4 and 5). Preston et al. (2004) showed that application of 5 μg MeJA was sufficient to elicit significant increase in TPI activity, which suggested that TPIs are very sensitive to MeJA treatments, which may explain why even a reduction of 84% of the MeJA-cleaving activity was not sufficient to inhibit the MeJA-induced TPI response in as*LOX3* plants. Therefore from these results, we can not determine whether the accumulations of TPIs were elicited by MeJA directly or by its cleaved product, JA.

Silencing *NaMJE* completely blocked the MeJA-induced resistance to *M. sexta* larvae in as*LOX3* plants (Fig. 3). Although the levels of MeJA-induced nicotine were significantly lower in VIGS *MJE* plants than in EV plants, the reduction in the nicotine response are not likely sufficiently large to account for the increase in herbivore performance (Steppuhn et al. 2004). Three additional possible explanations are suggested by the results: i) the reduction in *NaPAL1* transcripts, could reflect reductions in an unknown phenolic-based defense; ii) reduced *NaTD* transcripts, may reflect decreased TD activity, which may function post-ingestively as an antinutritive defense that limits the supply of Thr needed for herbivore growth (Chen et al., 2005); iii) the attenuated JA-Ile levels may reflect reductions in another unknown herbivore defense.

In summary, our results demonstrate that most herbivore-resistant responses are elicited by JA, either alone or after conjugation to Ile, when plants were treated with exogenously applied MeJA.

Material and Methods

Plant growth: We used seeds of the 21st generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts (synonymous with *Nicotiana torreyana*: Solanaceae) for transformation. Seed germination and plant growth were conducted as described by Krügel et al. (2002). In brief, seeds were sterilized and germinated on agar with Gamborg B5 (Duchefa, http://www.duchefa.com) after soaking in a 1:50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NY, USA) and 1 mM of gibberellic acid (GA_3). After 10 days, seedlings were planted into soil in Teku pots. Once established, plants were transferred to 1-liter pots in soil and grown in a growth chamber at 22°C, under 16 h of light supplemented with Philips Sun-T Agro 400 Na lights (Philips, http://www.philips.com) or not.

Isolating *NaMJE*: A 312 bp fragment of the *NaMJE* cDNA sequence was cloned with primers (MJE30_for: 5'- GCTAGTTCATGGAGCTTGTC -3' and MJE341_rev: 5'- TTAGGACCAGGCATGAAAGC -3') the design of which was based on the sequence similarity of EST (DB679695) and LeMJE (AY455313). After the first round of PCR with AP primer (5'-GCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT -3') and primer MJE30_for, 3 terminal cDNA was amplified with primer MJE 49_for (5'-CACGGTGCATGGTGTTGGTA -3') and primer NAP (5'-GCCACGCGTCGACTAGTAC -3'). The 5 terminal cDNA was obtained with primer MJE0_for (5'- AGATGACATGGAAAAGGGT -3') and primer MJE341_rev. All cDNA fragments were cloned into a pGEM-T EASY vector (Promega, http://www.promega.com) and sequenced.

Generation of VIGS plants: A 312 bp fragment of the *NaMJE* cDNA sequence, which was amplified by primers MJE30_for and MJE341_rev, was cloned into the pTV00. The pTV00 vector is a 5.5-kb plasmid with an origin of replication for *Escherichia coli* and *A. tumefaciens* and a gene for kanamycin resistance (Ratcliff et al. 2001). The *A. tumefaciens* (strain GV3101)–mediated transformation procedure was described previously (Saedler and Baldwin, 2004). To monitor the progress of VIGS, we silenced phytoene desaturase, a gene that oxidizes and cyclizes phytoene to

α- and β-carotene. These compounds are subsequently converted into the xanthophylls of the antenna pigments of the photosystems of plants, resulting in the visible bleaching of green tissues (Saedler and Baldwin 2004). When the leaves of phytoene desaturase-silenced plants began to bleach (5 weeks after germination), leaves of *NaMJE*-silenced (VIGS *MJE*) and empty vector-inoculated (EV) plants from both WT and *asLOX3* plants were used.

Caterpillar performance: Eggs of *Manduca sexta* were acquired from North Carolina State University (http://www.ncsu.edu) and kept in a growth chamber (Snijders Scientific, http://www.snijders-tilburg.nl) at 26℃ 16h light, 24℃ 8h darkness, until the larvae hatched. Freshly hatched neonates were placed directly on the source-sink transition leaves 3 days after MeJA treatment. In each treatment, 20 larvae were weighed after the indicated times.

Analysis of nicotine levels and TPI activity: Nicotine was extracted and quantified by HPLC as described in (Keinanen et al. 2001). Trypsin proteinase (TPI) activity was analyzed by radial diffusion activity as described in (van Dam et al. 2001).

JA and JA-Ile measurements: JA and JA-Ile were extracted and quantified by LC/MS as described in (Wang et al. 2007a). In brief, about 200 mg of leaf tissues from each sample was homogenized on a FastPrep homogenizer (http://www.thermo.com) with 1 mL of ethyl acetate spiked with 200 ng of 1,2-^{13}C-JA, D_4-SA and p-coumaric acid (PCA) in FastPrep tubes. After being centrifuged, the supernatants were transferred to fresh 2 mL tubes and evaporated on a vacuum concentrator. The residue was resuspended in 0.5 mL of 70% methanol (v/v) and centrifuged at maximum speed for 5 min. The supernatants were analyzed for JA, JA-Ile, and SA with a 1200L LC/MS/MS system (Varian, http://www.varianinc.com).

SYBR green real-time PCR assay (qPCR): Total RNA was extracted with TRI Reagent (Sigma, http://www.sigmaaldrich.com) according to the manufacturer's instructions, and cDNA was prepared from 500 ng total RNA with multiScribeTM reverse transcriptase (Applied Biosystems, http://www.appliedbiosys.com). The primers for *NaMJE* mRNA expression detection by qPCR were as follows:

NaMJE forward primer: 5'- GCTTTCATGCCTGGTCCTAA -3',

NaMJE reverse primer: 5'- GACCTTTCTCCTGTCCGTTG -3',

NaTPI forward primer: 5'- TCAGGAGATAGTAAATATGGCTGTTCA -3',

NaTPI reverse primer: 5'- ATCTGCATGTTCCACATTGCTTA -3',
NaTD forward primer: 5'- TAAGGCATTTGATGGGAGGC -3',
NaTD reverse primer: 5'- TCTCCCTGTTCACGATAATGGAA -3',
NaPAL1 forward primer: 5'- TTTGCATACGCTGATGACGC -3',
NaPAL1 reverse primer: 5'- TGGAAGATAGAGCTGTTCGCG -3',
NaActin forward primer: 5'- GGTCGTACCACCGGTATTGTG -3',
NaActin reverse primer: 5'- GTCAAGACGGAGAATGGCATG -3',
Real-time PCR was performed on an ABI PRISM 7700 Sequence Detection System (qPCR Core Kit, Eurogentec, http://www.eurogentec.com) with *NaActin* for normalization and according to the manufacturer's instructions under the following cycle conditions: 10 min 95°C; 40 cycles: 30 sec 95°C, 30 sec 55°C.

MeJA esterase activity assay: Around 200 mg leaf sample was ground in liquid nitrogen, and total proteins were extracted with buffer (100 mM Tris, 5% polyvinyl polypyrrolidone, 0.2% phenylthio urea, and 0.5% diethyl dithio carbamate, pH 7.6). A standard enzyme incubation mixture (total volume 50 µl) contained 10 µl total protein (2 µg), 2 µl MeJA (2.2 µg in 10% ethanol), and 38 µl 100 mM Tris (pH 7.6). Reactions were performed at 40°C as suggested as the optimum temperature by Stuhlfelder et al. (2002) for 0, 20, and 60 minutes and terminated by chilling on ice. 1ml ethyl acetate (spiked with internal JA standard $^{13}C_2$-JA 200 ng) was quickly added. The product JA from cleaved MeJA was quantified by LC/MS/MS as described in (Wang et al., 2007a). From the concentration of JA produced, we obtained the amount of cleaved-MeJA. We also performed reactions for negative controls with only 10 µl total proteins of EV and VIGS *MJE* plants individually or only MeJA in Tris buffer at the same condition for 60 minutes, the results showed that the amount of JA produced by these reactions is under the detection limit of our LC/MS/MS.

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Manuscript III

Running title: ROS in plant defense responses

Narboh D, a respiratory burst oxidase homolog in *Nicotiana attenuata*, is essential for resisting attack from both herbivores and pathogens

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Summary

Levels of ROS (reactive oxygen species) increase at wound sites when the specialist herbivore *Manduca sexta* attacks *Nicotiana attenuata*, as are transcripts of the respiratory burst oxidase homolog-*Narboh D*, which are also rapidly and transiently elicited by wounding and amplified when *M. sexta* oral secretions (OS) are added to the wounds. Silencing *Narboh D* significantly reduced ROS levels after OS elicitation, demonstrating that *Narboh D* is largely responsible for herbivore-elicited ROS production. In *Narboh D*-silenced plants, neither OS-elicited JA and JA-Ile bursts nor early transcripts (*NaJAR4* and *NaPAL1*) were influenced; however, the OS-elicited levels of trypsin proteinase inhibitors (TPIs), as well as of defense genes such

as *polyphenol oxidase, TPI* and *Thionin*, and of herbivore resistance, especially to generalist *Spodoptera littoralis*, were dramatically reduced, demonstrating the importance of ROS derived from *Narboh D* in herbivore-elicited late responses. *Narboh D* transcripts are also strongly elicited by the challenges from *Trichoderma sp*. and *Pseudomonas syringae* (DC3000). Plants silenced in *Narboh D*, but not *COI1* (coronatine insensitive1) and *NPR1* (non-expressor of PR1), are highly susceptible to *Trichoderma sp.*, a very mild pathogen widely used as biological control agent. We also observed that *P. syringae* performed better in *Narboh D*-silenced plants; this increased susceptibility, which is SA-independent, is a result of the lower accumulation of defense proteins such as NaThionin. Based on these results, we conclude that ROS derived from *Narboh D* are essential in both herbivore- and pathogen-elicited defense.

Key Words: *Nicotiana attenuata*, reactive oxygen species (ROS), *Narboh D*, *Manduca sexta*, *Spodoptera littoralis*, *Pseudomonas syringae*, *Trichoderma sp*.

Introduction

Plants are equipped with an array of defense mechanisms to protect themselves against insect herbivores and microbial pathogens. Generally, jasmonic acid (JA) plays a central role in herbivore-induced resistance (Halitschke and Baldwin, 2003a; Paschold *et al*., 2007), while pathogen-induced resistance depends largely on salicylic acid (SA) and reactive oxygen species (ROS, largely O_2^- and/or its dismutation product H_2O_2) (Lamb and Dixon 1997; Katagiri *et al.,* 2002). Recently, several lines of evidence indicate that ROS play an important role not only in pathogen-induced resistance but also in herbivore-elicited defense responses.

The rapid production of ROS is a hallmark of defense responses to pathogens, especially in incompatible interactions between resistant plants and avirulent pathogens (Lamb and Dixon 1997). Several reports also show that ROS levels are increased at the attack sites where insect herbivores consume soybean (Bi and Felton 1995), lima bean (Maffei *et al.,* 2006) and *Medicago truncatula* (Leitner *et al.,* 2005) leaves. ROS can be produced by chloroplasts, mitochondria, and peroxisomes, or by apoplastic cell-wall peroxidases, germin-like oxalate oxidases, and amino oxidases (Apel and Hirt 2004; Mittler *et al.,* 2004). However, pharmacological and molecular data indicate that the enzymes responsible for most of the ROS generated during

biotic interactions and in response to abiotic stresses are similar to the superoxide (O_2^-)-generating NADPH oxidases, originally characterized in mammalian phagocytes (Simon-Plas *et al.,* 2002; Torres *et al.,* 2002; Torres *et al.,* 2005; Yoshioka *et al.,* 2003; Sagi *et al.,* 2004). Interestingly, NADPH oxidase-dependent H_2O_2 is also proposed to function as a second messenger mediating the systemic expression of various defense-related genes in tomato plants (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas *et al.,* 2001; Sagi *et al.,* 2004). These results suggest that ROS are likely produced by an unknown NADPH oxidase after herbivory, which may play a central role in mediating responses in both plant-herbivore and -pathogen interactions. This inference raises several important questions: Do the ROS derived from herbivore-elicited NADPH oxidase directly influence JA signaling and thereby the outcome of plant-herbivore interactions? Are the same ROS also involved in plant-pathogen interaction? If so, what are the effects of these ROS on SA signaling? We explore these questions in the model plant, *Nicotiana attenuata*.

The herbivore-induced responses of *N. attenuata*, a post-fire annual native of the Great Basin Desert of California, Nevada, Idaho, and Utah, have been extensively studied with ecological, chemical, and molecular approaches (Baldwin 2001; Kessler and Baldwin 2002). Herbivory by the larvae of *N. attenuata*'s specialist *Manduca sexta* or elicitation by the application of larval oral secretions (OS) and regurgitant to puncture wounds activates a rapid burst of JA and JA-Ile, which in turn induces the accumulation of defense metabolites such as nicotine (Baldwin 1999; Kang *et al.,* 2006; Wang *et al.,* 2007; Wang *et al.,* 2008), diterpenoid glycosides (Jassbi *et al.,* 2008), and the anti-digestive proteins, trypsin proteinase inhibitors (TPIs) and threonine deaminase (Kang *et al.,* 2006; van Dam *et al*., 2001; Zavala *et al.,* 2004). Reducing nicotine, diterpenoid glycosides, and TPI production in transformed plants has demonstrated the importance of these compounds as herbivore defenses (Steppuhn *et al*., 2004; Zavala *et al*., 2004; Jassbi *et al*., 2008). JA signaling clearly plays a central role in these responses: blocking JA perception by silencing *COI1* in *N. attenuata* (ir*COI1* plants) abolishes most of the herbivore-elicited responses (Paschold *et al*., 2007). Therefore, *N. attenuata* is an ideal system for studying the role of the NADPH oxidase homolog in the entire herbivore-induced defense response.

Trichoderma sp., a widespread soil fungus, is the most commonly used biological control agent against plant pathogens (Harman *et al*., 2004). However, the mechanisms underlying how plants prevent *Trichoderma sp*. itself from becoming

pathogenic are unclear. When melon cotyledons are treated with cellulase produced by *Trichoderma logibrachiatum*, a striking increase in O_2^-, which is thought to be generated by an unknown NADPH oxidase, has been reported (Martinez *et al*., 2001). Here we examine whether the ROS derived from the NADPH oxidase that mediates herbivore-induced resistance also mediates resistance to *Trichoderma sp*.

Pseudomonas syringae pv tomato DC3000, which is virulent and causes disease in *Arabidopsis*, is also pathogenic to *N. attenuata*. After being challenged by this pathogen, *N. attenuata* increases SA and transcripts of at least two PR genes, *NaPR-1* and *NaThionin*. *NaThionin,* but not *NaPR-1,* was shown to mediate pathogen resistance and to be clearly independent of SA (Rayapuram *et al*., 2008). Here we ask whether NADPH-oxidase produced ROS are essential for eliciting *NaThionin* after pathogen challenge.

We report that *Narboh D* (*Nicotiana attenuata* respiratory burst oxidase homologue D), whose transcripts are rapidly and transiently wound- and OS-elicited, is the main source of ROS after herbivory, plays an essential role in both herbivore- and pathogen-induced defense responses in *N. attenuata*. Silencing *Narboh D* makes plants susceptible to the generalist insect herbivore, *Spodoptera littoralis,* and to two pathogens, *Trichoderma sp.* and *P. syringae*. We demonstrate that resistance to *Trichoderma sp.* is specifically mediated by *Narboh D,* but independent of both JA and SA pathways, which is essential for inducing defense genes such as *NaThionin* after *P. syringae* challenge.

Results

OS elicitation increases ROS levels

To investigate the production of ROS following simulated herbivore attack in *N. attenuata*, leaves were allowed to imbibe 1 mg/ml solution of DAB (3,3-diaminobenzidine) for 5 h, before being wounded with a fabric pattern wheel and treated with water or *M. sexta* OS. DAB polymerizes and turns deep brown in the presence of ROS, and the intensity of the brown color around wounding sites can be qualitatively assessed and photographed. As expected, treatment with wounding and water is sufficient to elicit the production of ROS (Figure 1a); however, ROS levels were enhanced by supplying OS to the wounding sites (Figure 1a). To confirm that OS can enhance wounding-induced ROS, we also measured the H_2O_2 levels after

elicitation. The results revealed that OS treatments elicited significantly higher levels of H_2O_2 than did wounding treatments (Figure 1a), suggesting that herbivore attack can increase ROS production.

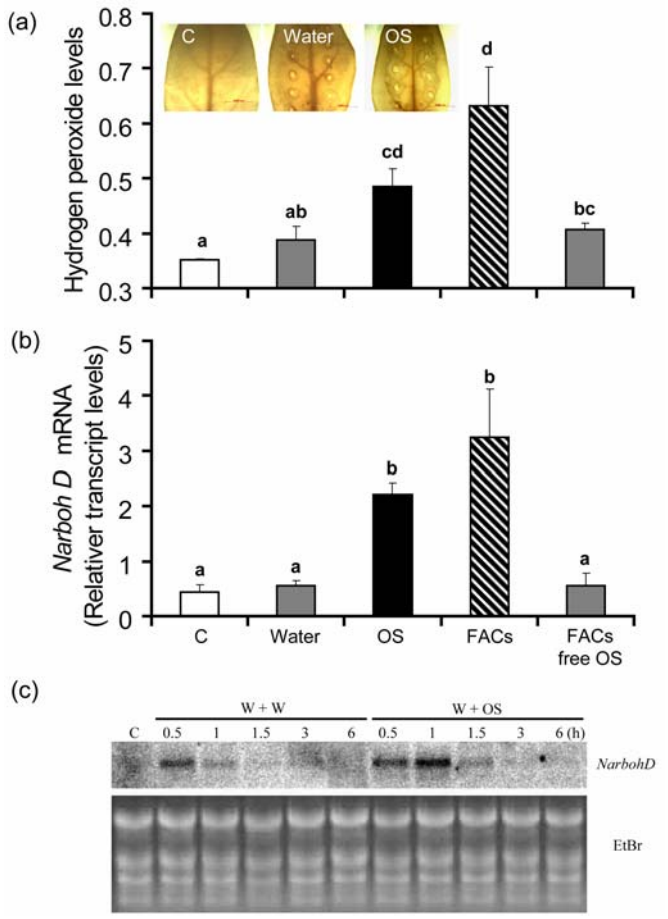

Figure 1. The elicitation of ROS and *Narboh D* transcripts.

(a) Mean (± SE) H_2O_2 levels were measured in leaf disks excised from 4 replicate source-sink transition leaves of rosette-stage plants after different treatments: wounding plus water (Water), *M. sexta* oral secretions (OS), FACs, and FAC-free OS. Inset: DAB staining of WT *N. attenuata* leaves 4 h after treatments of wounding plus water or OS.

(b) Mean (± SE) *Narboh D* transcripts were measured by real-time PCR in leaf disks excised from 4 replicate source-sink transition leaves after different treatments: wounding plus water (Water), *M. sexta* oral secretions (OS), FACs, and FAC-free OS. Different letters indicate statistically significant differences among treatments (Fisher's PLSD test; $P < 0.05$).

(c) *Narboh D* transcripts were measured by northern analysis in individual single leaves treated by wounding and applying water (W + W) or OS (W + OS); total leaf RNA (10 µg) was hybridized with specific probes. Control plants remained untreated. An ethidium bromide (EtBr) stained gel served as a loading control.

Because NADPH oxidase-dependent H_2O_2 is suggested to function as a second messenger mediating the systemic expression of various defense-related genes in tomato plants (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas *et al*., 2001; Sagi *et al*., 2004), we hypothesize that a specific NADPH oxidase is the source of ROS after herbivory. In order to distinguish the effects of OS on the production of

ROS from the effects of wounding itself, we searched for NADPH oxidase candidate genes which could be specifically or more strongly induced by OS than by wounding alone. The transcripts of one of the NADPH oxidase genes we cloned were elicited in just half an hour by wounding plus water treatments (Figure 1c), and they disappeared almost as quickly at 1 h; however, transcript accumulations were more strongly induced by wounding plus OS treatments, as the 1 h-induction peak in Figure 1c shows. Because this NADPH oxidase gene displays a high amino acid sequence similarity to *Ntrboh D* (97% identity, Figure S1) and occurs as a single-copy gene (Figure S2), we referred to it as *Narboh D* (accession number EU104741).

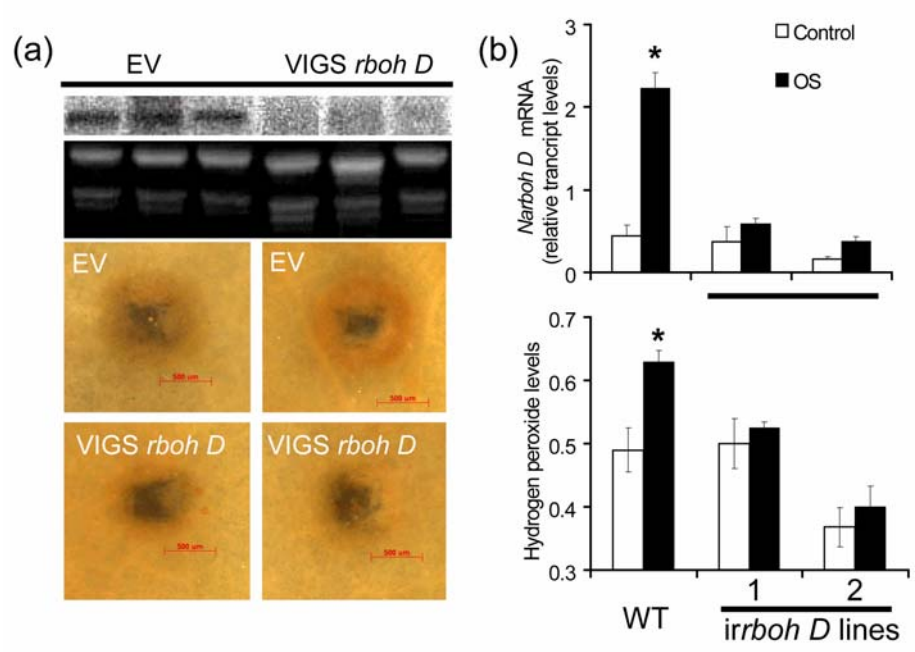

Figure 2. Reduced OS-elicited ROS levels in *Narboh D*-silenced plants.

(a) WT plants were inoculated with *Agrobacterium*-harboring TRV constructs, which contained either an empty vector (EV) or a 353 bp *Narboh D* fragment (VIGS *Narboh D*). Upper panel: *Narboh D* transcripts were measured by northern analysis in 3 replicate leaves of EV and VIGS *Narboh D* plants 1 h after OS-elicitation. An ethidium bromide (EtBr) stained gel served as a loading control. Lower panel: DAB staining of two replicate leaves of EV and VIGS *Narboh D* plants treated with wounding plus OS for 4 h.

(b) *Narboh D* was stably silenced in *N. attenuata* by *Agrobacterium*-mediated transformation. Upper panel: Mean (± SE) *Narboh D* transcripts were measured by real-time PCR in leaf disks excised from 4 replicate leaves 1 h after wounding plus OS treatments.

Lower panel: Mean (± SE) H_2O_2 levels were measured in leaf disks excised from 4 replicate leaves treated with wounding plus OS describe in method. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P<0.05$).

Microarray and biochemical analyses indicated that the fatty-acid-amino-acid-conjugates (FACs) in *M. sexta* OS are responsible for eliciting OS-specific MAPK, JA, VOCs, and transcriptional responses in *N. attenuata* (Wu *et al*., 2007a; Halitschke *et al*., 2001, 2003b). To determine if FACs activate *Narboh D*-mediated ROS

production in *N. attenuata*, synthetic FACs were applied to wounded *N. attenuata* leaves. Our results show that the levels of both *Narboh D* transcripts and H_2O_2 are more highly elicited by FACs than by wounding alone (Figure 1b). Furthermore, removing FACs by ion-exchange chromatography made the OS no more able than water to elicit both *Narboh D* transcripts and H_2O_2 levels (Figure 1b).

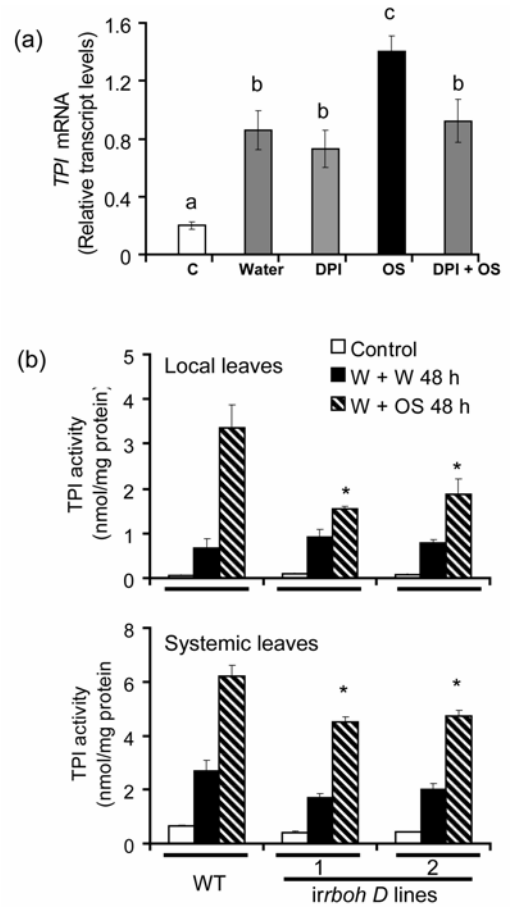

Figure 3. Reduction of ROS production decreases OS-elicited but not wounding-elicited TPI transcripts and activities.

(a) Mean (± SE) *NaTPI* transcripts were measured by real-time PCR in leaf disks excised from 4 replicate source-sink transition leaves after different treatments: wounding plus water (water), or DPI, or OS, or DPI and OS. Different letters indicate statistically significant differences among treatments (Fisher's PLSD test; $P < 0.05$).

(b) Mean (± SE) TPI activity of 5 replicate WT, ir*rboh D* line 1 and ir*rboh D* line 2 plants 48 h after wounding plus water or OS treatments. The treated leaves were harvested as local leaves and undamaged (-3) younger leaves were harvested as systemic leaves. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P<0.05$; **, $P<0.001$).

Silencing *Narboh D* reduces ROS production after herbivory

To further investigate whether *Narboh D* is the source of ROS production in response to herbivore attack, we used the VIGS system optimized for *N. attenuata* (Saedler and Baldwin 2004) to silence *Narboh D* mRNA in WT plants. Because when leaves are OS elicited, transcripts of *Narboh D* are highly induced after 1 h (Figure 1c), a northern blot was performed with total RNA from leaves inoculated with empty vector (EV) constructs and from constructs harboring a fragment of *Narboh D* (VIGS *Narboh D*) and harvested 1 h after OS elicitation. Transcripts of *Narboh D,* which were strongly OS-elicited in EV plants, were completely silenced in VIGS *Narboh D* plants (Figure 2a). EV and VIGS *Narboh D* leaves treated with wounding plus OS

were stained with DAB. In the tissues surrounding the wound sites, ROS levels were visibly lower in VIGS *Narboh D* plants than in EV plants (Figure 2a).

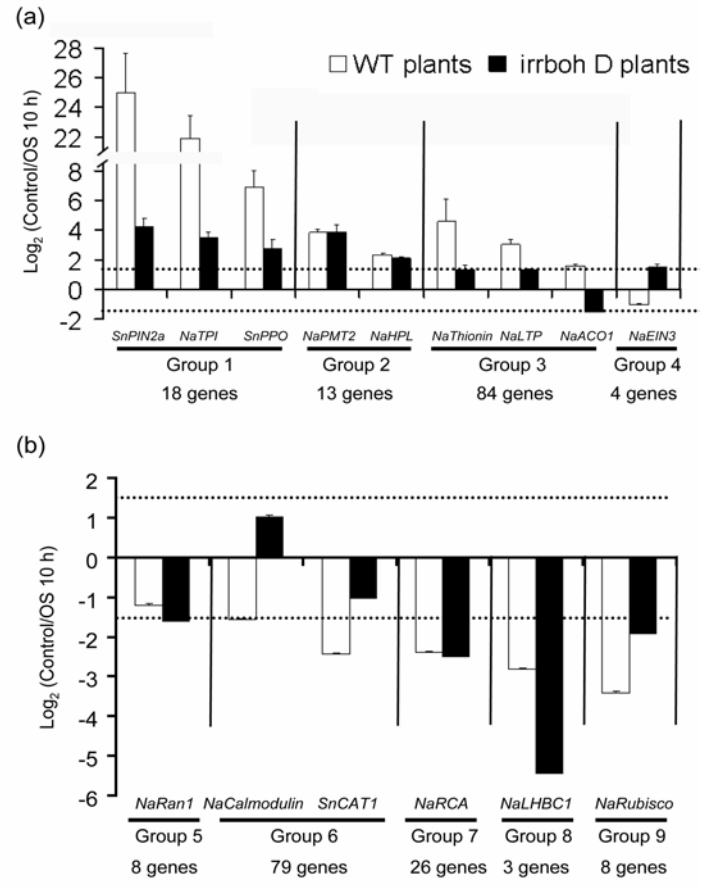

Figure 4. Silencing *Narboh D* greatly influences OS-elicited late transcript levels.

cDNA samples from untreated source-sink transition leaves were labeled with fluorescent dye Cy5, and samples of 10 h after OS-elicitation were labeled with Cy3. For each treatment, 3 pools of WT samples and 3 pools of ir*rboh D* samples were made, each consisting of 3 biological replicates. Each microarray was hybridized with one pool of untreated and OS-elicited samples of labeled cDNA from WT or ir*rboh D* plants. Three microarrays per treatment were hybridized and statistically analyzed. Genes are considered significantly ($P<0.05$) up-regulated when $\log_2$ (Cy3/Cy5) > 1.5 and down-regulated when $\log_2$ (Cy3/Cy5) < -1.5. The details of regulated gene name and accession number are shown in Supplemental table 1 and 2.

(a) Mean (± SE) value of $\log_2$Cy3/Cy5 of representative genes up-regulated in WT or ir*rboh D* plants 10 h after OS-elicitation. All genes are divided into four groups: up-regulated in both WT and ir*rboh D* plants, but the level is lower in ir*rboh D* plants (Group 1, 18 genes); up-regulated to the same levels in both WT and ir*rboh D* plants (Group 2, 13 genes); up-regulated only in WT plants (Group 3, 84 genes); up-regulated only in ir*rboh D* plants (Group 4, 4 genes).

(b) Mean (± SE) value of $\log_2$Cy3/Cy5 of representative genes down-regulated in WT or ir*rboh D* plants 10 h after OS-elicitation: down-regulated only in ir*rboh D* plants (Group 5, 8 genes); down-regulated only in WT plants (Group 6, 79 genes); down-regulated to the same levels in both WT and ir*rboh D* plants (Group 7, 26 genes); down-regulated in both WT and ir*rboh D* plants, but the level is lower in ir*rboh D* plants (Group 8, 3 genes); down-regulated in both WT and ir*rboh D* plants, but the level is lower in WT plants (Group 9, 8 genes).

We also silenced *Narboh D* in *N. attenuata* by *Agrobacterium*-mediated transformation (Krügel *et al*., 2002) using a pRESC5 transformation vector (Wu *et al*.,

2007b) containing a 353-bp *Narboh D* fragment in an invert-repeat orientation. Two independently transformed F2 lines, each harboring a single insertion as confirmed by Southern blot analysis (Figure S2), were selected for all further experiments (ir*boh D* lines 1 and 2). The silencing of endogenous *Narboh D* gene expression was also confirmed by qRT-PCR, which revealed that levels of *Narboh D* transcripts were five times the levels in control leaves 1h after OS elicitation in WT plants but remained unchanged in both ir*boh D* lines after elicitation (Figure 2b). At the same time, neither line had increased H_2O_2 levels after OS elicitation (Figure 2b). These results suggest that *Narboh D* is the main source of ROS that is elicited during herbivory.

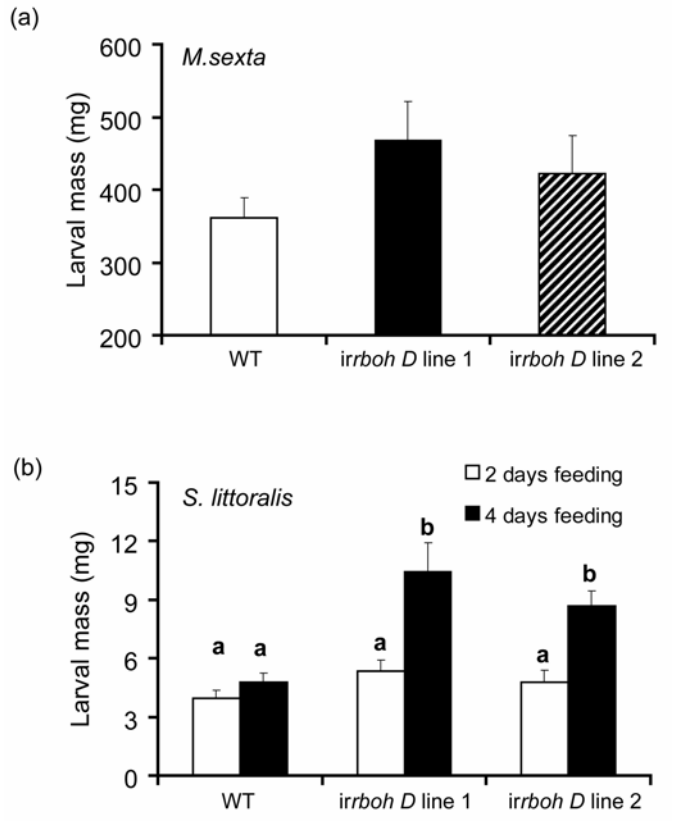

Figure 5. Silencing *Narboh D* decreases herbivore resistance.
(a) Mean (± SE) mass of *M. sexta* larvae 10 days after feeding on 20 replicates each WT and ir*boh D* plants.
(b) Mean (± SE) mass of *S. littoralis* larvae 2 and 4 days after feeding on 10 replicate leaves of WT and ir*boh D* plants. Leaves were harvested after treated with OS for 2 days, and then fed 3-day-old larvae for 2 and 4 days. Different letters indicate statistically significant differences among treatments (Fisher's PLSD test; $P < 0.05$).

Narboh D and herbivore-elicited responses

After herbivory, *N. attenuata* produces rapid and transient endogenous JA and JA-Ile bursts, which in turn increase levels of nicotine and TPI and resistance to

larvae of *M. sexta* (Wang *et al.,* 2008). Whether OS-elicited ROS influence the JA and JA-Ile bursts is unknown. Our investigation revealed that silencing *Narboh D* had little influence on these OS-elicited bursts which attain maximum values at 1h (Figure S3). Similarly, the OS-elicited early transcripts, *NaJAR4* (the enzyme conjugating JA and Ile to form JA-Ile) and phenylalanine ammonia lyase (*NaPAL1*), are increased to the same levels in both WT and ir*rboh D* plants (Figure S3). These results demonstrate that the ROS produced by *Narboh D* are not involved in early herbivore-induced responses.

However, 8 h after OS elicitation, *TPI* transcripts were significantly reduced when diphenylene iodonium (DPI, an NADPH oxidase inhibitor) was added to wounds before OS application, while wound-elicited *TPI* transcripts were unchanged after DPI treatments (Figure 3a). These results suggest that ROS signals derived from *Narboh D* are involved in OS-elicited, but not wounding-induced, late responses. Indeed, the TPI activity of OS-elicited ir*rboh D* line 1 is only 46.5% of WT levels after 48 h in local leaves and 73% of that in systemic leaves; similar trends were also observed in ir*rboh D* line 2 plants (Figure 3b). Meanwhile, wounding-elicited TPI levels do not differ in both local and systemic leaves of WT and ir*rboh D* lines (Figure 3b).

To further investigate the role of ROS in OS-elicited late responses, we used microarrays enriched in herbivore-elicited genes (a total of 1404 genes) to compare transcriptional changes in WT and ir*rboh D* plants 10 h after OS elicitation. Among the 115 up-regulated genes in WT plants after OS elicitation, 16% of these genes (Group 1; 18 genes), which are mostly involved in direct defense, including *SnPIN2a*, *NaTPI* and *SnPPO*, were also up-regulated in ir*rboh D* plants but at much lower levels than those of WT plants (Figure 4a and Supplemental Table 1); whereas 73% (Group 3; 84 genes) were not significantly up-regulated in ir*rboh D* plants, which included, for example, *NaThionin* (secondary metabolism) and *NaACO1* (ethylene signaling). Only 11% of the up-regulated genes (Group 2; 13 genes) were induced at the same levels in ir*rboh D* plants as in WT plants. These results revealed that ROS signals are essential for inducing most OS-elicited late response genes, especially *NaTPI* and *NaThionin*.

ROS signals are also required for the induction of most of the OS-elicited down-regulated genes (Figure 4b and Supplemental Table 1). Among the 116 down-regulated genes in WT plants after OS treatments, 68% (Group 6; 79 genes) were not

significantly down-regulated in ir*rboh D* plants, for example, *Calmodulin* and *SnCAT1*; meanwhile, only 22% (Group 7; 26 genes) were elicited at the same levels in ir*rboh D* plants as in WT plants, such as *NaRCA*; another 7% (Group 9; 8 genes) were also significantly down-regulated in ir*rboh D* plants but at much reduced levels than those of WT plants.

Since ROS signals derived from *Narboh D* are required for the accumulation of most OS-elicited late transcripts and TPI levels, we hypothesized that insect larvae would perform better on ir*rboh D* plants. After 10 days, larvae of the specialist herbivore, *Manduca sexta,* that fed on ir*rboh D* line 1 plants gained slightly but not significantly more mass than those that fed on WT plants (Figure 5a; unpaired *t*-test, p=0.066). However, when larvae of the generalist herbivore, *Spodoptera littoralis*, which are not specifically adapted to *N. attenuata*, fed on leaves from ir*rboh D* plants whose defense responses had been OS elicited 2 days prior, gained significantly more mass after 4 days than those that fed on similarly treated leaves from WT plants (Figure 5b; unpaired *t*-test, p=0.004).

Narboh D and pathogen-elicited responses

In *Arabidopsis*, *Atrboh D* is proposed to be the main source of extra-cellular ROS after pathogen attack (Torres *et al*., 2002), and silencing *Ntrboh D* prevents tobacco cells from producing ROS after the elicitation of cryptogein (Simon-plas *et al*., 2002), a low-molecular-weight protein secreted by oomycete *Phytophthora*; these results suggest *Narboh D* may have an important role in pathogen defense.

We noted that most ir*rboh D* plants, but not WT plants, exhibited severe root rot symptoms after being transferred to potting soil B410. Around 60-80% of ir*rboh D* plants wilted and eventually died, whereas adjacent pots of WT plants remained healthy and free of disease symptoms (Figure 6a and 6b). When planted into soil pasteurized with moist heat at 60 ºC for 40 min, ir*rboh D* plants remained as healthy as WT plants, suggesting that a soil-borne pathogen(s) was responsible. These results suggest that *Narboh D* plays a role in protecting roots against pathogens, which is consistent with its high transcript level in roots (Figure 6c).

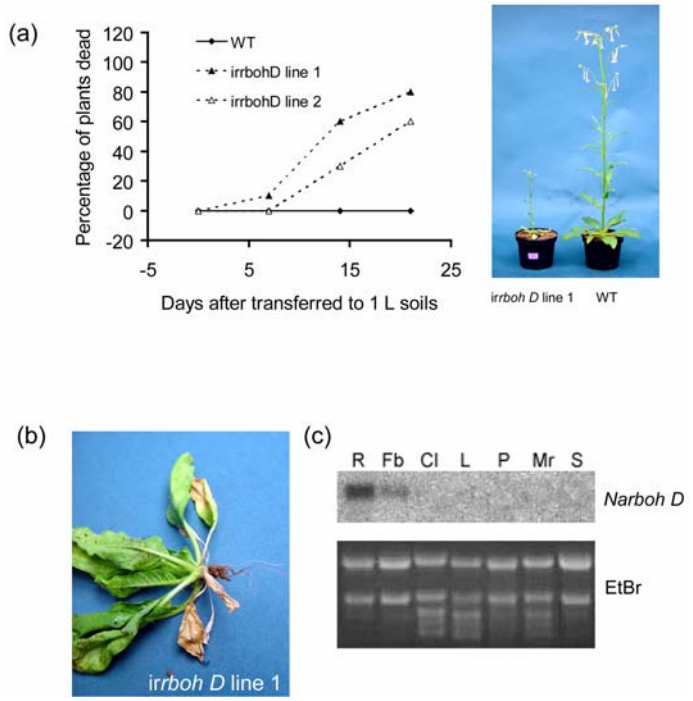

Figure 6. Silencing *Narboh D* decreases pathogen resistance in roots, which is consistent with the high expression level of *Narboh D* in roots.

(a) Left panel: Percentage of dead plants in 10 replicate WT and ir*rboh D* plants after being transferred to 1 L soil in greenhouse. Plants with more than 3 wilted leaves were recorded as dead. Right panel: typical 45-day-old ir*rboh D* line 1 and WT plants.

(b) A typical ir*rboh D* plant after being transferred to 1 L soil after 15 days, which showed severe symptoms of root rot.

(c) Transcript accumulation of *Narboh D* in different tissues of *N. attenuata*. Total RNA (10 µg) was extracted from roots (R), flower buds (Fb), cauline leaves (Cl), rosette-stage leaves (L), petioles (P), leaf midribs (Mr), and stems (S) of 37-day-old WT plants and analyzed by northern blotting. An ethidium bromide (EtBr)-stained gel served as a loading control.

From the infested roots of ir*rboh D* plants, we isolated three different fungi (data not shown), one of which causes severe root rot disease symptoms in ir*rboh D* seedlings; WT seedlings, however, were unaffected 12 days after inoculation in axenic GB5 medium (Figure 7a; Figure S6). This fungus was identified as *Trichoderma sp*. by using DNA sequence analysis of the internal transcribed spacer (ITS) region within the ribosomal RNA gene cluster (Figure S5). Interestingly, *Trichoderma sp*., a very widespread mild pathogen often used as a biological control agent, was never observed to cause any disease symptoms among WT plants in the glasshouse.

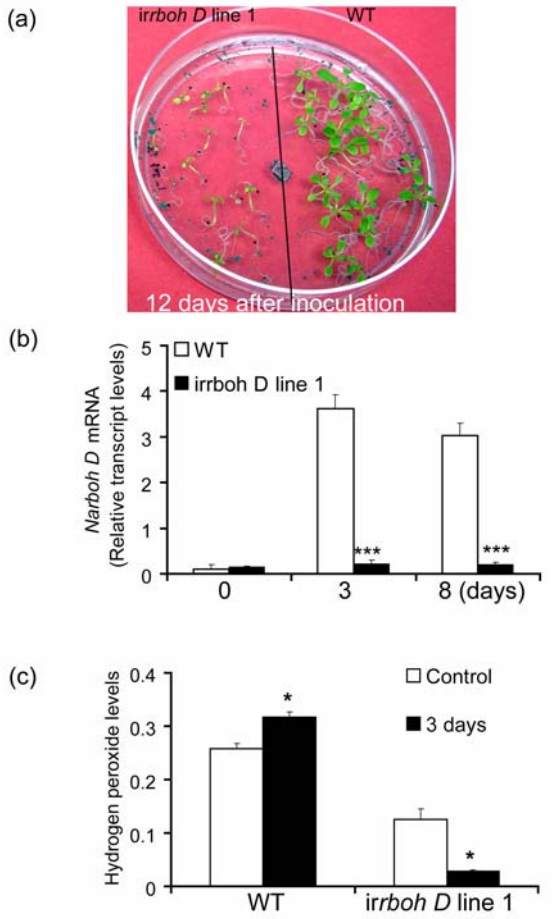

Figure 7. Silencing *Narboh D* decreases the resistance to *Trichoderma sp.* in roots.
(a) The growth of WT and ir*rboh D* line 1 seedlings 12 days after inoculation of *Trichoderma sp*.
(b) Mean (± SE) *Narboh D* transcripts were measured by real-time PCR in 4 replicate roots of WT and ir*rboh D* line 1 plants after inoculation with *Trichoderma sp*. 0, 3 and 8 days.
(c) Mean (± SE) H_2O_2 levels were measured in 5 replicate roots of WT and ir*rboh D* line 1 plants after inoculation with *Trichoderma sp*. 0, and 3 days. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P<0.05$).

To further examine the role of *Narboh D* in defense against *Trichoderma sp*., we used qRT-PCR to measure *Narboh D* transcript levels 3 and 8 days after inoculation with *Trichoderma sp*.. Results revealed that *Narboh D* transcripts in the roots were highly induced in WT but not in ir*rboh D* plants (Figure 7b), which is consistent with the higher levels of H_2O_2 levels in WT roots after inoculation compared to ir*rboh D* roots (Figure 7c). These results indicate that silencing *Narboh D* makes plants susceptible even to a beneficial fungus such as *Trichoderma sp*.

We also tested whether JA and SA pathways were involved in the *Trichoderma*-elicited defense responses, as both phytohormones are known to be elicited after inoculation (Martinez *et al*., 2001; Segarra *et al*., 2007). Although blocking JA perception by silencing *COI1* in *N. attenuata* (ir*COI1* plants) greatly reduces herbivore-elicited resistance (Paschold *et al*., 2007), neither WT nor ir*COI1* plants showed any disease symptoms after *Trichoderma sp*. inoculation (Figure S6). Moreover, we never observed any disease symptoms caused by *Trichoderma sp*. in as*LOX* plants [those in which a key enzyme involved in JA biosynthesis,

lipoxygenase 3 (*NaLOX3*)], is silenced (Halitschke and Baldwin 2003a) (data are not shown). Taking both results together, we conclude that JA does not play a critical role in the resistance to *Trichoderma sp*. in *N. attenuata*. Surprisingly, plants silenced in *NPR1* (nonexpressor of pathogenesis-related genes) by RNAi (ir*NPR1* plants), which are susceptible to many herbivores and pathogens when plants are planted in the Great Basin Desert (Rayapuram and Baldwin 2007), performed as well as WT plants after *Trichoderma sp*. inoculation (Figure S6), indicating that the SA pathway is also not important for the resistance to *Trichoderma sp*.

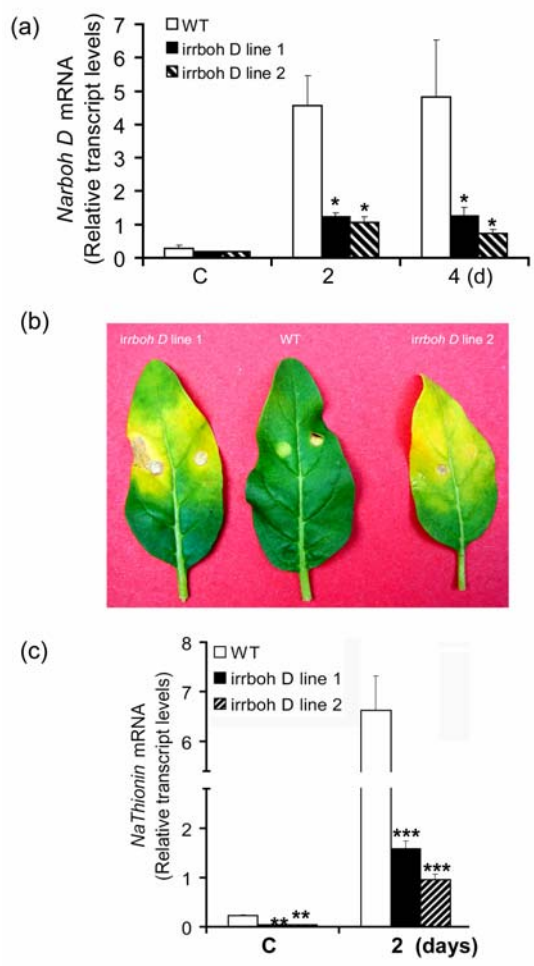

Figure 8. Silencing *Narboh D* decreases the resistance to *P. syringae* in leaves.
(a) Mean (± SE) *Narboh D* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants after inoculation with *P. syringae* 0, 2 and 4 days.
(b) Typical disease symptoms of WT and ir*rboh D* plants 2 weeks after *P. syringae* inoculation.
(c) Mean (± SE) *NaThionin* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants after inoculation with *P. syringae* 0, and 2 days.

In leaves, *Narboh D* transcripts can also be induced by the challenge of *Pseudomonas syringae* (Figure 8a). To determine the role of *Narboh D* in *P. syringae*-induced responses in *N. attenuata*, we infiltrated source-sink transition leaves of 5 replicate WT and ir*rboh D* plants with 300 µl *P. syringae* (re-suspended in sterile water with OD_{600}=0.001) per leaf. After two days, we found that ir*rboh D* plants contained more *P. syringae* than did WT (Figure S7a). After two weeks, three

of the five leaves from ir*rboh D* plants treated with *P. syringae* showed strong disease symptoms, whereas the five similarly treated WT leaves showed only slight symptoms (Figure 8b).

To determine whether the susceptibility of leaves from ir*rboh D* lines to *P. syringae* is due to impaired SA signaling, we analyzed free SA and *PR1* transcripts. Although both SA and *PR1* transcript levels were dramatically elicited by *P. syringae* inoculation, differences between WT and ir*rboh D* plants were slight (Figure S7b). Recently Rayapuram *et al*., (2008) have shown that when *NaPR1*- and *NaThionin*-silenced plants were transplanted into the plants' native habitats in the Great Basin Desert of Utah, opportunistic *Pseudomonas sp*. performed better on *NaThionin*-silenced plants than on *NaPR1*-silenced and WT plants, suggesting the defense role of *NaThionin* again *P. syringae*. These results lead us hypothesize that the susceptibility of ir*rboh D* plants to *P. syringae* is enhanced by lower levels of *NaThionin*, whose elicitation may be ROS dependent. Although *NaThionin* transcripts were highly elicited in WT, their level in ir*rboh D* line 1 plants was only 24% that of WT plants (unpaired *t*-test, p=0.0004) 2 days after *P. syringae* challenge (Figure 8). These results demonstrate that ROS produced by *Narboh D* are signals essential for eliciting *NaThionin* in resistance to *P. syringae*.

Discussion

Consistent with what has been found in lima bean (Maffei *et al*., 2006) and *Medicago truncatula* (Leitner *et al*., 2005), OS-elicitation enhanced wound-induced ROS levels in *N. attenuata* (Figure 1). *Narboh D*, a homolog of *Ntrboh D* and *Atrboh D*, is rapidly and transiently wound- and herbivore-induced (Figure 1). Silencing *Narboh D* almost abolishes OS-elicited ROS production (Figure 2), demonstrating that most ROS produced after herbivory results from the activity of the NADPH oxidase, Narboh D.

Like *Atrboh D* mutants in *Arabidopsis* (Torres *et al*., 2002), ir*rboh D* plants are morphologically normal; they grow as well as WT plants do in their seedling stages, 11 and 17 days after germination on GB_5 medium (Figure S2), but are significantly smaller than WT plants at rosette stages before bolting in soil (Figure S2). In contrast, silencing the *Rboh* homolog in tomato (*Solanum lycopersicon*) by antisense technology leads to dramatic changes in plant morphology: enhanced branching, curling leaflets, indeterminate inflorescences, and fasciated reproductive

organs (Sagi *et al*., 2004), suggesting that the *Rboh* in tomato may function differently from *Narboh D* in *N. attenuata* and *Atrboh D* in *Arabidopsis*. The role that this NADPH oxidase plays in defense signaling also seems to differ between *N. attenuata* and tomato.

We did not observe a reduction of wounding-elicited *TPI* transcripts in *N. attenuata* leaves of either DPI-treated WT plants (Figure 3a) or ir*rboh D* plants (Figure S4), although NAPDH oxidase-dependent H_2O_2 has been proposed to function as a second messenger mediating the wound-induced systemic expression of various defense-related genes in tomato plants (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas *et al*., 2001; Sagi *et al*., 2004). Furthermore, the levels of TPI activities elicited by wounding are also the same in WT and ir*rboh D* plants (Figure 3b). These results indicate that ROS from *Narboh D* are not involved in wound-elicited TPI responses in *N. attenuata*, unlike what has been observed in tomato. Similarly, the active systemin was found only in tomato, but not in tobacco. Even in the closely related *Solanum nigrum*, the systemin homolog of tomato does not play a role in defense responses (Schmidt and Baldwin, 2006).

However, silencing *Narboh D* does have a large effect on OS-elicited late responses. Most of defense genes, including *SnPIN2a*, *NaTPI*, *SnPPO*, *NaThionin*, *NaACO1*, and *NaLTP*, were elicited to much lower levels in ir*rboh D* plants than in WT plants (Figure 4). In addition, the levels of TPI activities 48 h after OS elicitation were also significantly reduced in ir*rboh D* plants in both local and systemic leaves (Figure 3). These changes in ir*rboh D* plants are likely responsible for the increased larvae performance (Figure 5), especially of the generalist herbivore, *S. littoralis*.

The fact that OS-elicited levels of JA/JA-Ile and early transcripts (*NaJAR4* and *NaPAL1*) are the same in WT and ir*rboh D* plants (Figure S3) is not consistent with the hypothesis that ROS directly interact with JA signaling. Thus, ROS may function independently of JA (or downstream of JA signaling). FACs are known to be responsible for eliciting OS-specific MAPK, JA, VOCs, and transcriptional responses in *N. attenuata* (Wu *et al*., 2007a; Halitschke *et al*., 2001, 2003). Levels of both *Narboh D* transcripts and H_2O_2 were more highly elicited by FACs than wounding alone, and removing FACs from OS by ion-exchange chromatography made the OS no more able than water to elicit, suggesting that FACs in *M. sexta* OS are the elicitor of both *Narboh D* transcripts and H_2O_2 (Figure 1). It is still not clear how NADPH oxidase is activated by FACs. Recently, it was proposed that the activity of NADPH

oxidase is modulated by Ca^{2+}, CDPK and Rac GTPase in rice (Kobayashi *et al*., 2007; Wong *et al*., 2007), which provide clues for how NADPH oxidase may be regulated by FACs.

As defense responses are costly, plants are usually thought to tailor energy-consuming defense responses to specific insults (e.g., insects vs. microbial pathogens), which suggests that Narboh D is not involved in pathogen defense since it can be specifically elicited by FACs. However, we found that Narboh D also plays an essential role in pathogen resistance, which contradicts the expectation, that pathogen and herbivore-specific responses are separately elicited.

Trichoderma sp., a widespread soil fungus, is the most commonly used biological control agents against plant pathogens (Harman *et al*., 2004). The mechanism by which *Trichoderma sp*. is able to control pathogen populations is thought to involve the production of antibiotics and hydrolytic enzymes which lead to mycoparasitism and competition for nutrient in the rhizosphere. However, more recent reports reveal that *Trichoderma sp*. may activate defense responses in host plants to control disease (Harman *et al*., 2004). JA, SA, and ROS are three major classes of signaling molecules activated by *Trichoderma sp*. in host plants (Martinez *et al*., 2001; Segarra *et al*., 2007) that may play essential roles in plant resistance to *Trichoderma sp* itself.

When melon cotyledons are treated with the cellulase produced by *Trichoderma logibrachiatum* (Martinez *et al*., 2001), a striking accumulation of O_2^- is observed, which is thought to be generated by an unknown NADPH oxidase. Consistent with a protective role for NADPH oxidase in *Trichoderma*-elicited roots is the observation that levels of *Narboh D* transcripts are low in the root of WT seedlings but increase after the inoculation of *Trichoderma sp*. (Figure 7a). Indeed, silencing *Narboh D* makes plants susceptible to *Trichoderma sp.* Around 60-80% of all ir*rboh D* plants wilted and eventually died from root rot when grown in non-sterile greenhouse soil, whereas adjacent pots of WT, ir*COI1*, and as*LOX3* plants remained healthy and free of disease symptoms (Figure 6). Later, *Trichoderma sp*. was isolated from the infested roots of ir*rboh D* plants. When we inoculated WT and ir*rboh D* plants with *Trichoderma sp*. in the seedling stage, most ir*rboh D* seedlings showed severe symptoms of root rot. Many even died, in contrast to WT seedlings, whose growth was promoted (Figure 7a; Figure S6). The same outcome occurred when we inoculated autoclaved potting soil with *Trichoderma sp*.: three of the five inoculated

ir*rboh D* line 1 plants died while WT plants were unaffected. Thus, irrespective of the precise mechanism whereby ROS produced by *Narboh D* regulates defense responses, our results establish the importance role of *Narboh D* in preventing *Trichoderma sp*. which is normally a beneficial fungus to becoming a devastating root rot pathogen.

Although an oversimplification, it is generally accepted that SA plays a major role in activating defenses against biotropic pathogens, whereas JA and ethylene are more usually associated with defense against insect herbivores and necrotrophic pathogens (Koornneef and Pieterse *et al*., 2008). NPR1 is the central regulator of SA-mediated responses (Mou *et al*., 2003), while JA induced-responses are largely dependent on COI1 (Xie *et al*., 1998). Blocking JA perception by silencing *COI1* (Paschold *et al*., 2007) and inhibiting the SA pathway by silencing *NPR1* (Rayapuram and Baldwin, 2007), does not influence a plant's resistance to *Trichoderma sp.* (Figure S6). These results revealed that the resistance to *Trichoderma sp.* is specifically regulated by *Narboh D*-derived ROS but not by JA or SA, suggesting that these ROS function independently of the JA and SA pathways. We observed similar results in the leaves: the same levels of both SA and *NaPR1* transcripts are elicited in both ir*rboh D* and WT plants after *P. syringae* inoculation, and the OS-elicited JA burst was the same in WT and ir*rboh D* plants.

It's still not clear how ROS prevents *Trichoderma sp*. from becoming a devastating root rot pathogen. One of the remarkable behaviors of *Trichoderma sp*. is that they penetrate roots of their host plants but limit their growth mainly to the epidermis and outer cortex (Yedidia *et al*., 1999), suggesting that hyphal growth is tightly regulated by its host plant. More lactophenol blue staining was observed in ir*rboh D* plants than in WT plants 6 days after inoculation of *Trichoderma sp*. (Figure S7), suggesting that ROS derived from *Narboh D* is involved as a signal, but how ROS trigger host defense responses remains to be discovered.

Narboh D transcripts were also highly induced by the challenge of *P. syringae* (Figure 8a), which caused more severe disease symptoms in ir*rboh D* plants than in WT plants. Although an SA-dependent pathway is proposed to be crucial for general resistance to *P. syringae* (Katagiri *et al*., 2002), our results revealed that both SA and *NaPR1* transcript levels are elicited to the same levels in both ir*rboh D* and WT plants (Figure S7), indicating that the susceptibility to *P. syringae* in ir*rboh D* plants is SA independent, which is consistent with what has been found in roots.

In the Great Basin Desert of Utah, opportunistic *Pseudomonas sp*. performed better on *NaThionin*-silenced plants compared to WT plants, however, the native herbivore community of *N. attenuata* attacked *NaThionin*-silenced plants to the same degree as it did in WT plants, indicating NaThionin protein provides resistance against pathogens but not against insect herbivores (Rayapuram *et al*., 2008). The transcripts of *NaThionin* were highly elicited in WT plants but much less so in ir*rboh D* plants after *P. syringae* inoculation (Figure 8c) and OS-elicitation (Figure 4, and Supplemental Table 1), demonstrating that ROS signals are essential for both OS- and pathogen-elicited *NaThionin* accumulation, and the overlap between herbivore- and pathogen-induced signaling pathways. The reason why plants accumulate NaThionin, which does not provide resistance to insect herbivores after OS-elicitation, is not clear; it might be a mechanism that plants use to prepare against future pathogen attack as the wounds left by insect feeding can be readily colonized by pathogens.

In summary, our results demonstrate that *Narboh D* is essential for resistance to both insect herbivores and pathogens in *N. attenuata*. After herbivory, when plants perceive FACs in the OS, they activate both ROS- and JA-signaling pathways. ROS and JA pathways collaborate, triggering late transcriptional changes and the accumulation of TPIs, which lower the performance of insect larvae. The defense role that *Narboh D* plays against *Trichoderma sp.* in roots is clearly not dependent on JA or SA pathways, as ir*COI1*, ir*NPR1*, and WT plants are resistant to *Trichoderma sp*. Finally, ROS from *Narboh D* are essential endogenous signals for the eliciting *NaThionin* in defending against *P. syringae* in leaves.

Materials and Methods

Plant growth: We used seeds of the 21st generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts (synonymous with *Nicotiana torreyana*: Solanaceae) for transformation in all experiments. Seed germination and plant growth were conducted as described by Krügel *et al*., (2002). All the soils were pasteurized with moist heat at 60 ºC for 40 min unless otherwise noted.

Isolating *Narboh D*: We obtained the cDNA of *Narboh D* by an RT-PCR with primers designed from sequences of *Ntrboh D* (accession number AJ309006) and *Nbrobh B* (accession number AB079499) (forward primer *Narboh D* 155 and reverse primer *Narboh D* 3235). The 3 kb cDNA product (accession number

EU104741), which contained the entire open reading frame, was cloned into a pGEM-T EASY vector (Promega, http://www.promega.com) and sequenced.

Generating VIGS and stably silenced plants: PCR was used to generate *Narboh D* fragments from *N. attenuata* in antisense orientations with the following primer pairs: forward primer; 5'- ATTGGTGGGTCTTGGAAT -3', reverse primer; 5'- AACGAGCATCACCTTCTTCA -3'. The *A. tumefaciens* (strain GV3101)-mediated VIGS procedure was previously described (Saedler and Baldwin 2004).

We also generated stable silencing plants by *Agrobacterium*-mediated transformation described in Krügel *et al*., (2002). The same *Narboh D* fragment used for VIGS was inserted into pRESC5 transformation vector (Wu *et al*., 2007b) in an inverted-repeat orientation. Two single-insertion lines (ir*rboh D* lines 1 and 2) were identified, bred to homozygosity and used in most of the experiments.

Analysis of TPI, JA, and JA-Ile: Trypsin proteinase inhibitor (TPI) activity was analyzed by radial diffusion as described in van Dam *et al*., (2001). JA and JA-Ile were extracted and quantified by LC-MS as described by Wu *et al*., (2008).

Nucleic acid analysis and Real-Time PCR assay (Supplemental methods)

Microarray analysis (Supplemental methods)

DAB staining and H_2O_2 measurements (Supplemental methods)

Isolation and identification of pathogen: the pathogen was isolated from the roots of diseased ir*rboh D* plants in unpasteurized soil B410. Root fragments were harvested and surface sterilized, and then plated on water agar plates (1.5%). Transfers were made from the edges of fungal colonies growing out of the roots onto potato dextrose agar medium (1/2 PDA). One of the isolates caused disease symptoms in ir*rboh D* plants 8 days after inoculation, while WT seedlings were unaffected. This fungus was identified as *Trichoderma sp*. based on morphological characteristics and DNA sequence analysis of the internal transcribed spacer (ITS) region within the ribosomal RNA gene cluster. Cultures of *Trichoderma sp*. were maintained (sub-cultured every 30 days) on 1/5 strength PDA.

Caterpillar performance: The experiment was performed according to Rayapuram and Baldwin (2007).

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Supplemental Material

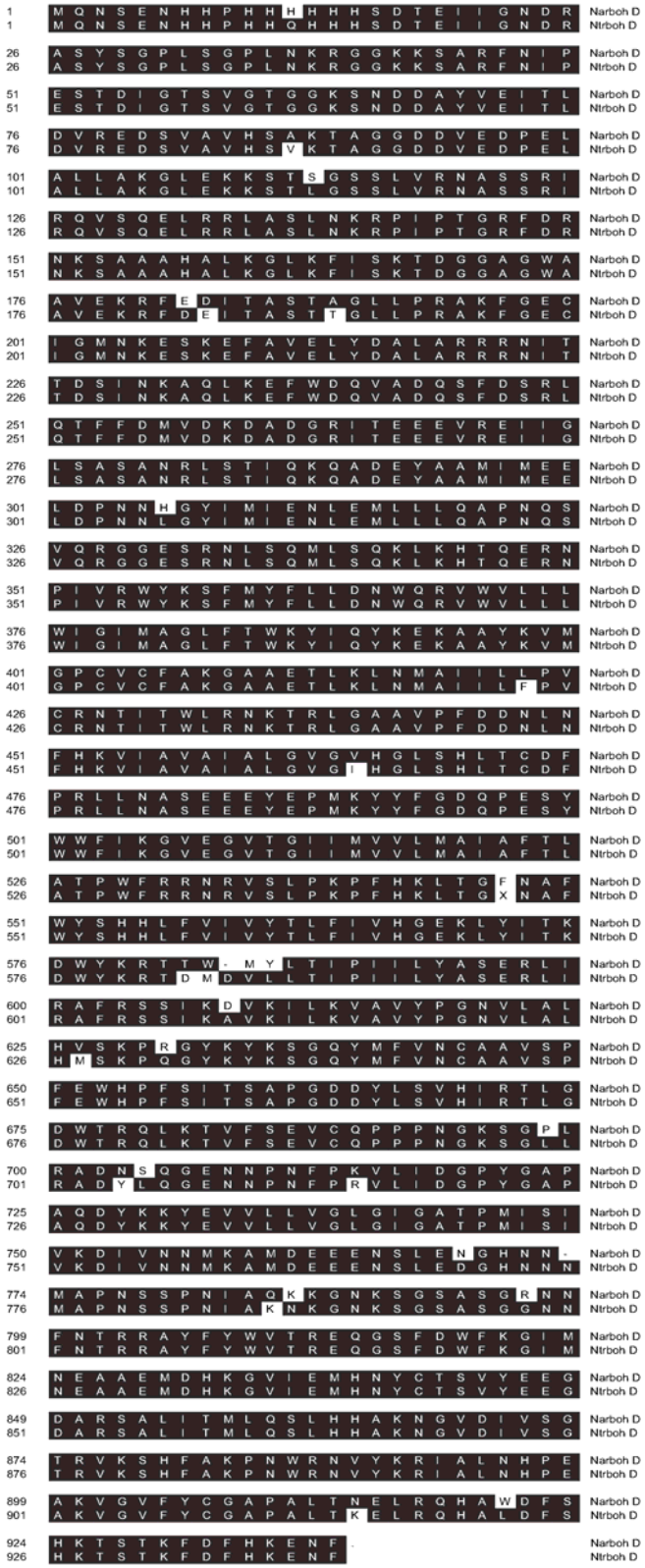

Figure S1. The amino acid sequence of Narboh D (EU104741) are compared with tobacco Ntrboh D (AJ309006; Simon-plas *et al*., 2002); the identical sequence are shaded with black.

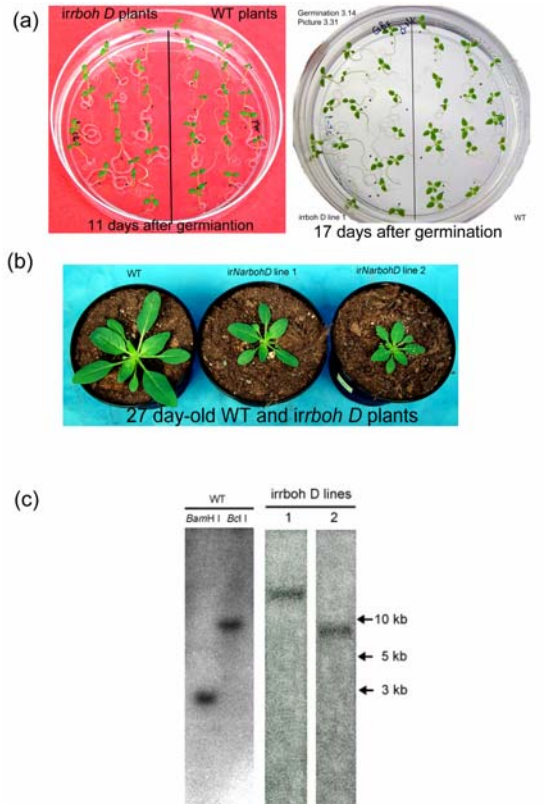

Figure S2.

(a) The growth of WT and ir*rboh D* line 1 seedlings 11 and 17 days after germination.

(b) 27-day-old WT and ir*rboh D* plants in soils.

(c) Southern blot analysis shows that only one copy of *Narboh D* is present in the *N. attenuata* genome (left). Two stably transformed lines with single-insertion are confirmed with Southern blot analysis with *Eco*R I and probed with *hpt II* gene (right).

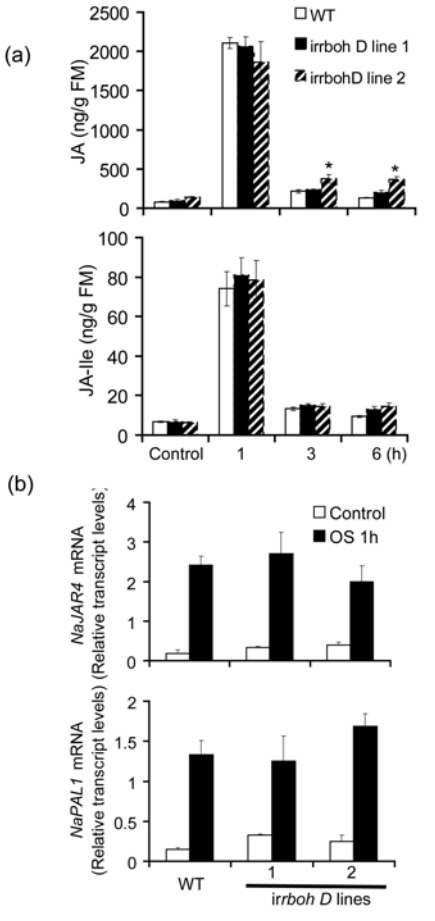

Figure S3.

(a) Mean (± SE) JA and JA-Ile levels were measured in 5 replicate source-sink transition leaves of WT and ir*rboh D* plants after being treated with wounding plus OS 0, 1, 3, and 6 h. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P<0.05$).

(b) Mean (± SE) *NaJAR4* and *NaPAL1* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants 1 h after OS-elicitation.

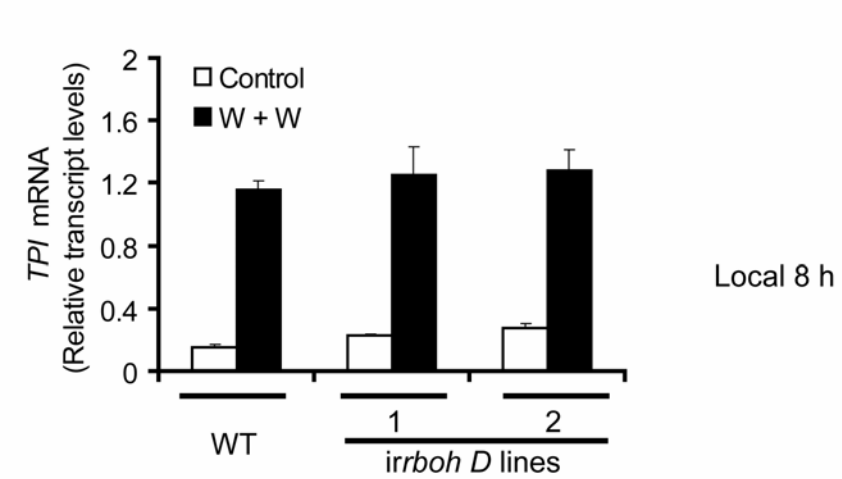

Figure S4. Mean (± SE) *NaTPI* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants 8 h after OS-elicitation.

ITS (internal transcribed spacer) sequence, which shows high sequence similarity to AY514867 (*Trichoderma sp.)*

TCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACT
CCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGG
ATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCC
GCCGGAGGACCAACCTAAAACTCTTATTGTATACCCCCTCGCGG
GTTTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGT
TTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
CGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAA
CCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCT
CCCTTAGCGGGTGGCCGTCTCCGAAATACAGTGGCGGTCTCGC
CGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAG
CGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAATGTT
GACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA**GCATATCA**
ATAAGCGGAGGA

Figure S5. The cloned internal transcribed spacer (ITS) sequence shows high sequence similarity to AY514867 of *Trichoderma sp*.

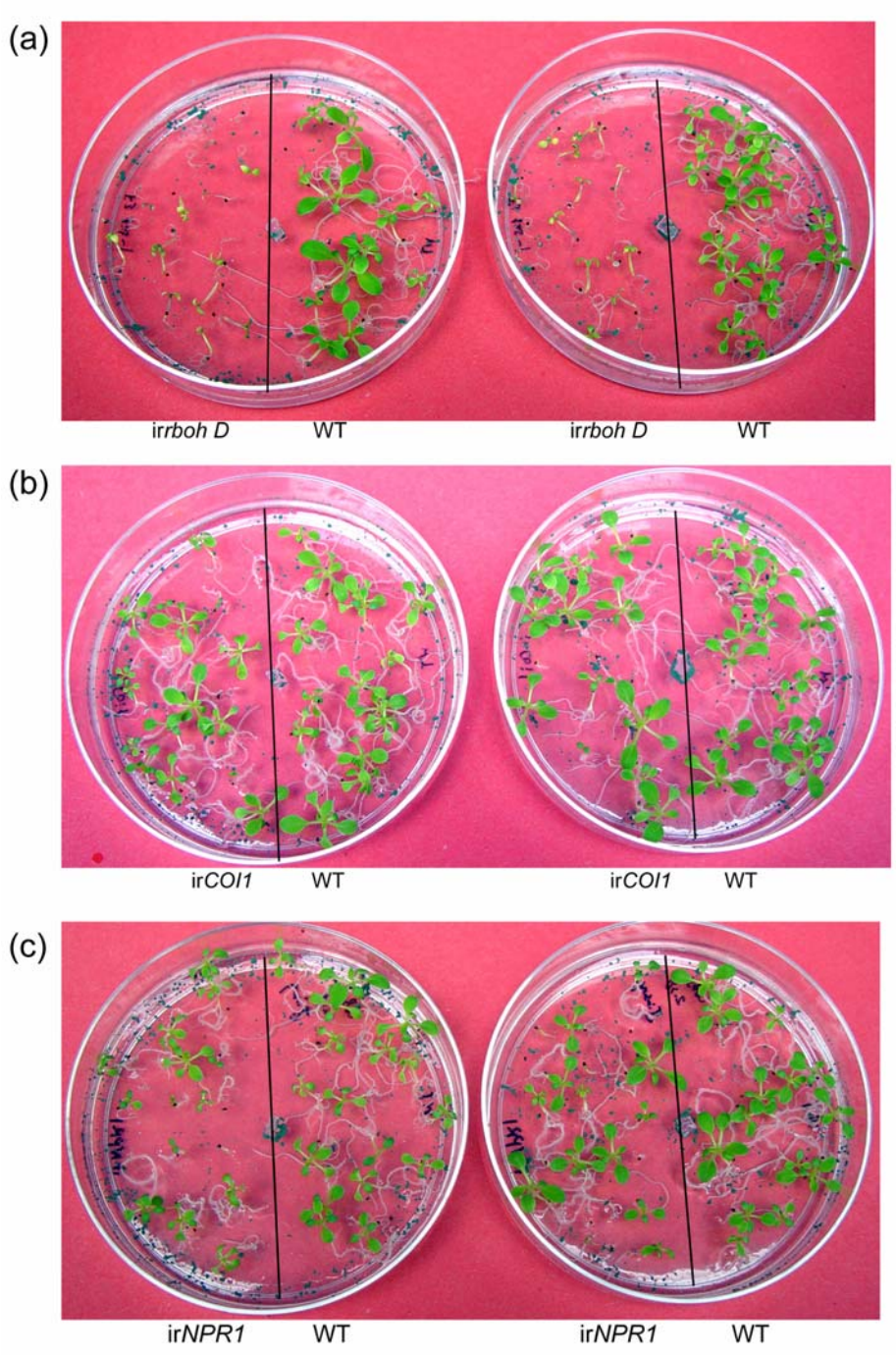

Figure S6. The growth of WT and ir*rboh D* line 1 **(a)**, WT and ir*COI1* **(b)**, and WT and ir*NPR1* **(c)** seedlings 12 days after *Trichoderma sp.* inoculation.

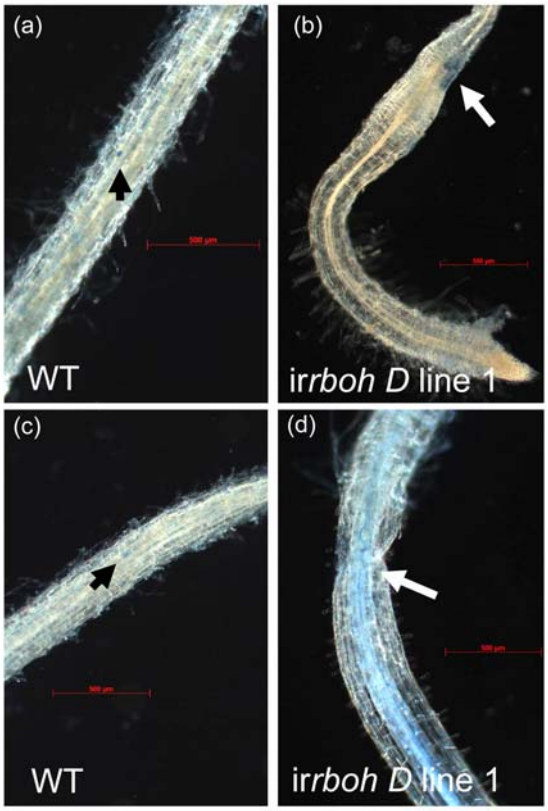

Figure S7. The roots of WT and ir*rboh D line 1* plants 6 days after *Trichoderma sp.* inoculation were stained with lactophenol cotton blue. The arrows point to tissues stained dark blue.

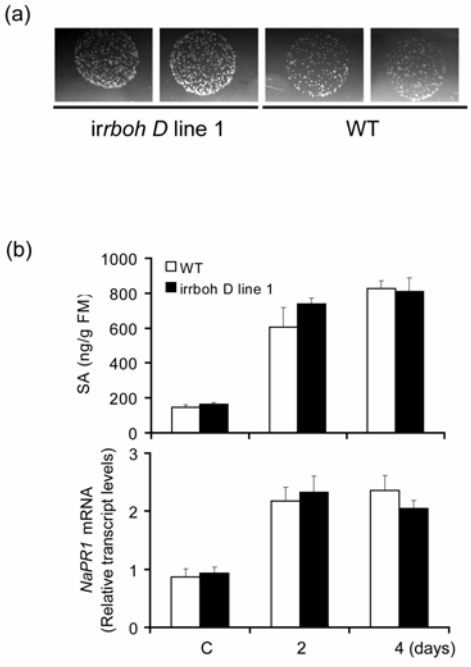

Figure S8.

(a) Surface-sterilized 2 replicate leaf discs (1 cm^2) from 2-day-old *P. syringae*-inoculated leaves or WT and ir*rboh D* line 1 plants were ground in 1 ml sterile water diluted 50 times; 15 µl of the diluted supernatant was plated on LB agar. The picture was taken 14 h after incubation at 28°C.

(b) Mean (± SE) SA levels and *NaPR1* transcripts were measured in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants 0, 2, and 4 days after inoculation with *P. syringae*.

Supplemental Table 1

Up-regulated or down-regulated genes 10 h after OS-elicitation in WT and ir*rboh D* plants.

Supplemental Table 2

Gene specific primers, probes, gene name and gene accession number.

Supplemental methods

Discussion

Root hair apoplastic pH regulation by NaRALF

Although the accumulation of *NaRALF* transcripts is rapidly increased by wounding and OS elicitation, since *M. sexta* larvae do not perform differently on ir*RALF* plants than on WT plants, we conclude that *NaRALF* does not play a central role in eliciting anti-herbivore defense responses. This result supports previous data showing that RALF peptides are more likely involved in plant development than in defense (Ryan *et al.*, 2002; Haruta and Constabel, 2003; Olsen *et al.,* 2002; Germain *et al.,* 2004). We also observed that *NaRALF* transcripts increased rapidly after exposure to UV-B radiation, but WT and *NaRALF*-silenced plants did not perform differently after UV-B exposure, suggesting that NaRALF does not play an important role in UV-B responses.

The ubiquity of RALF suggests it plays a fundamental physiological role. Previous work has shown that exogenous application of RALF can arrest root growth (Pearce *et al.,* 2001), suggesting that RALF may act as an endogenous negative regulator of root growth. Our characterization of the root phenotypes of ir*RALF* plants – longer root lengths, rapid growth rates, and longer root elongation zones, are consistent with this idea.

It's still unclear how root growth is regulated by NaRALF. Recently, it has been suggested that H_2O_2 is involved in root growth restriction and root hair formation (Dunand *et al.,* 2007). Arabidopsis seedlings had longer roots when grown in the presence of KI, an efficient scavenger of H_2O_2; however, the elongation of roots was inhibited by exogenously supplied H_2O_2. These results provide a possible mechanism for NaRALF's effects on root growth that less ROS accumulation is the reason of longer roots in ir*RALF* plants. Another explanation for the effect of silencing *NaRALF* could be that roots compensate for the loss of absorbing surface resulting from the absence of hairs by increasing their length.

Although the pH of the soil has long been proposed to strongly influence root hair formation (Ewens and Leigh, 1985), the reason has not been clear. More recently, the importance of the localized acidification of the cell wall during root hair initiation and tip growth has been demonstrated (Bibikova *et al.,* 1998), but how the process was regulated has until now remained unclear. Our results provide evidence that NaRALF is involved in regulating the apoplastic pH required for root hair formation.

Silencing *NaRALF* led to a slower rate of oscillation in extracelluar pH, and in Arabidopsis such pH oscillations have been linked to the regulation of wall properties directly related to the control of tip growth (Monshausen *et al*., in review), suggesting possible reasons why ir*RALF* lines have disrupted root hair formation. Root hairs in the ir*RALF* lines often rupture, and the fact that a large increase in alkalinization levels always preceded a root hair rupture led us to hypothesize that the disrupted regulation of apoplastic pH at the root hair tips was one cause of the root hair phenotype of ir*RALF* lines. This hypothesis was confirmed by the partial recovery of the root hair phenotype (root hair length and number) when ir*RALF* lines were grown in low-pH buffered medium; moreover, WT plants produced a root hair phenotype similar to that of ir*RALF* lines when WT plants were grown in medium with the same pH (pH 6.8), but strongly buffered with Tris (or HEPES) buffer. Thus, irrespective of the precise mechanism whereby NaRALF is regulating the root hair growth, these results suggest an important element of NaRALF's action is its effects on proton fluxes associated with growth. Additional evidence came from the competition experiments: WT and ir*RALF* plants grew similarly in low pH soils; however, in high pH soil, where plants need more energy to generate a proton gradient across their cell walls for nutrient uptake, ir*RALF* plants were strongly out-competed by WT plants.

Silencing *NaRALF* in *N. attenuata* results in altered dynamics of pH oscillations and increased maximal pH at the tips of root hairs. This result may seem to contradict the fact that the RALF peptide elicits a rapid alkalinization of the medium of tobacco suspension-cultured cells. Cell cultures are expected to differ from intact plants, particularly in their cell-cell communication and given that *NaRALF* is highly expressed in roots, studies of intact roots are likely to provide a more accurate analysis of NaRALF's function.

The apoplastic localization of RALF (Escobar *et al*., 2003), together with the discovery of 120 kDa and 25 kDa cell membrane proteins, which can specifically bind to RALF (Scheer *et al*., 2005), suggest RALF may exert its biological activity through a specific interaction with a cell membrane receptor. We do not yet have direct evidence that NaRALF regulates H^+-ATPase activity; however, high levels of H^+-ATPase genes are expressed in developing root hairs in *Nicotiana plumbaginifolia* (Moriau *et al.,* 1999), which suggests the possibility that they are regulated by NaRALF.

ROS may be one of the down-stream signals regulated by NaRALF. Previous work with the *rhd 2* mutant demonstrated that the ROS produced by NADPH oxidase in the trichoblast is important for forming tip-focused calcium gradients required for tip growth. We also observed a decreased accumulation of ROS in the root hair initiation zone of the ir*RALF* lines, suggesting that NaRALF may also affect root hair development through this ROS-dependent series of events.

Root hairs are thought to be important for nutrient uptake. For example, a high-affinity P transport gene *LePT1* was found to be highly expressed in root hairs (Daram *et al.,* 1998) and the presence of root hairs significantly increased whole plant P uptake under P-limiting conditions (Bates and Lynch, 2000). Thus, alterations in root hair development should translate into changes in plant fitness. Indeed, the fitness of the Arabidopsis mutant *act2-1*, which produces root hairs that are only 10 to 70% as long as those of WT plants, is reduced (Gilliland *et al.,* 2002). When grown in pH 5.8 soils in the glasshouse, ir*RALF* lines showed leaf sizes and stalk lengths similar to those of WT plants. Low soil pH facilitates the active transport of nutrients, and the longer roots of ir*RALF* plants might compensate for the loss of nutrients that are normally absorbed by root hairs. When plants were grown in the basic soils in which *N. attenuata* plants are commonly found in nature, the fitness of ir*RALF* plants was severely reduced. Two factors may be responsible: either (a) the ir*RALF* lines have fewer and shorter root hairs, or (b) the trichoblasts have lost the ability to regulate cell wall pH dynamics. Additional work will be required to determine which if any of these mechanisms are responsible for the reduction in plant growth.

In summary, our results demonstrate that NaRALF is a peptide signal needed for regulating root growth and the apoplastic pH of the tip of trichoblasts. NaRALF is activated by unknown developmental stimuli, affects the levels of ROS accumulation and possibly the activity of H^+-ATPase in plasma, and subsequently influences the periodicity of pH oscillations, and maintains an appropriate cell wall pH environment. When *NaRALF* is silenced, trichoblasts become “deaf” to developmental and environmental stimuli; the cell wall pH oscillates more slowly and attains higher pH values; these changes in pH profile cause the normally highly controlled expansion of the very apex of the hair to become disrupted, leading to “bulging”, until the cells finally rupture.

MeJA is de-esterifed to JA in defense elicitation

Staswick's pioneering work on JAR1 in *Arabidopsis* (Staswick et al. 1992; Staswick and Tiryaki 2004) suggests that exogenously applied MeJA first needs to be demethylated into JA to be activated. Indeed, MeJA hydrolyzing enzyme activity occurs in all the plant species so far tested (Stuhlfelder et al. 2002). It is also reported that MeJA was rapidly hydrolyzed to JA and further metabolized like JA when tobacco BY-2 cells were treated with MeJA (Swiatek et al. 2004). In *N. attenuata*, a model plant with extensively studied herbivore-induced responses, we also detected high levels of MeJA-cleaving activity (Figure 2B). Based on the sequence similarity with tomato *LeMJE* (Stuhlfelder et al. 2004), we cloned *NaMJE*, a MeJA esterase gene that shared very high sequence similarity with *LeMJE* (Figure 1) and by silencing the expression of this gene, we demonstrated that *NaMJE* is largely responsible for the MeJA cleaving-activity of *N. attenuata* leaves (Figure 2B).

MeJA treatment is the most commonly used means of eliciting herbivore resistance in many different plant species (McConn et al. 1997; Baldwin 1998; Li et al. 2002). However, it is still not known how plants elicit herbivore resistance traits in response to MeJA treatment. We determine whether MeJA functions as a signal prior to being hydrolyzed to JA by investigating MeJA-induced defense responses in both WT and *asLOX3* plants with reduced MeJA-cleaving activity. Silencing *NaMJE* transcripts was sufficient to block most MeJA-induced responses in as*LOX3* but not WT plants, including the production of nicotine (Figure 4); transcripts of *NaPAL1* and *NaTD* (Figures 5 and 6); and the JA-Ile burst (Figure 6). Importantly, the dramatic decreases in mass that are usually observed in larvae that feed on MeJA-treated EV plants disappeared when *NaMJE* transcripts were silenced (Figure 3). Furthermore, silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance in as*LOX3* plants (Figure 7). Why is silencing *NaMJE* sufficient to block most MeJA-induced responses in as*LOX* plants but not in WT plants? MeJA and JA treatment of plants is known to elicit endogenous JA production (Ziegler et al., 2001; Miersch and Wasternack 2000; Pluskota et al. 2007) and this is inhibited in as*LOX3* plants (Halitschke and Baldwin 2003). Based on these results, we conclude that most exogenous MeJA-induced herbivore responses are actually elicited by JA, either alone or after conjugation with Ile.

Kang et al. (2006) have suggested that JA-Ile plays an important role in the nicotine production. It was further confirmed in plants silenced both *NaJAR4* and *NaJAR6* (Wang et al. 2007b). *NaJAR4/6* encodes enzymes mainly for conjugating JA

and isoleucine in the production of JA-Ile (Wang et al. 2007a). When *NaJAR4* and *NaJAR6* were simultaneously silenced, the induction of nicotine level by JA treatment was only half of that in WT plants receiving the same treatment, but after JA-Ile treatments, WT plants and *NaJAR4* and *NaJAR6* -- both silenced plants -- accumulated the same levels of nicotine, suggesting that JA-Ile is a crucial signal for the induction of nicotine (Wang et al. 2007b). Therefore, the reduction of MeJA-induced nicotine level in *NaMJE*-silenced as*LOX3* plants is likely caused by attenuated JA-Ile level, and this lower JA-Ile is correlated with lower *NaTD* transcripts (Figure 6). Exactly how the nicotine level is regulated by JA-Ile, however, is still unclear. Recently, JA-Ile is demonstrated to be the most active jasmonate-derivatives promoting physical interaction between COI1 and jasmonate ZIM-domain (JAZ) protein, which lead to the degradation of JAZ1, the repressor of the transcription of jasmonate-responsive genes (Thine et al. 2007; Chini et al. 2007). Although the function of JAZ proteins is not demonstrated in *N. attenuate*, it is likely that nicotine production is regulated JA-Ile through JAZ and COI1 dependent pathway.

Trypsin proteinase inhibitors (TPIs) have also been demonstrated to have a direct defensive role against herbivores in *N. attenuata* (Zavala et al. 2004). The activities and transcripts of TPIs can be dramatically induced by MeJA treatments (Figures 4 and 5). Preston et al. (2004) showed that application of 5 µg MeJA was sufficient to elicit significant increase in TPI activity, which suggested that TPIs are very sensitive to MeJA treatments, which may explain why even a reduction of 84% of the MeJA-cleaving activity was not sufficient to inhibit the MeJA-induced TPI response in as*LOX3* plants. Therefore from these results, we can not determine whether the accumulations of TPIs were elicited by MeJA directly or by its cleaved product, JA.

Silencing *NaMJE* completely blocked the MeJA-induced resistance to *M. sexta* larvae in as*LOX3* plants (Figure 3). Although the levels of MeJA-induced nicotine were significantly lower in VIGS *MJE* plants than in EV plants, the reduction in the nicotine response are not likely sufficiently large to account for the increase in herbivore performance (Steppuhn et al. 2004). Three additional possible explanations are suggested by the results: i) the reduction in *NaPAL1* transcripts, could reflect reductions in some unknown phenolic-based defense; ii) reduced *NaTD* transcripts, may reflect reduced TD activity, which may function post-ingestively as an

antinutritive defense that limits the supply of Thr needed for herbivore growth (Chen et al., 2005); iii) the attenuated JA-Ile levels may reflect reductions in another unknown herbivore defense.

In summary, our results demonstrate that MeJA functions as a signal prior to being hydrolyzed to JA, which either alone or after its conjugation to Ile, elicits herbivore defense responses.

ROS in defense responses

Consistent with what has been found in lima bean (Maffei *et al*., 2006) and *Medicago truncatula* (Leitner *et al*., 2005), OS-elicitation enhanced wound-induced ROS levels in *N. attenuata* (Figure 1). *Narboh D*, a homolog of *Ntrboh D* and *Atrboh D*, is rapidly and transiently wound- and herbivore-induced (Figure 1). Silencing *Narboh D* almost abolishes OS-elicited ROS production (Figure 2), demonstrating that most ROS produced after herbivory results from the activity of the NADPH oxidase, Narboh D.

Like *Atrboh D* mutants in *Arabidopsis* (Torres *et al*., 2002), ir*rboh D* plants are morphologically normal; they grow as well as WT plants do in their seedling stages, 11 and 17 days after germination on GB_5 medium (Figure S2), but are significantly smaller than WT plants at rosette stages before bolting in soil (Figure S2). In contrast, silencing the *Rboh* homolog in tomato (*Solanum lycopersicon*) by antisense technology leads to dramatic changes in plant morphology: enhanced branching, curling leaflets, indeterminate inflorescences, and fasciated reproductive organs (Sagi *et al*., 2004), suggesting that the *Rboh* in tomato may function differently from *Narboh D* in *N. attenuata* and *Atrboh D* in *Arabidopsis*. The role that this NADPH oxidase plays in defense signaling also seems to differ between *N. attenuata* and tomato.

We did not observe a reduction of wounding-elicited *TPI* transcripts in *N. attenuata* leaves of either DPI-treated WT plants (Figure 3a) or ir*rboh D* plants (Figure S4), although NAPDH oxidase-dependent H_2O_2 has been proposed to function as a second messenger mediating the wound-induced systemic expression of various defense-related genes in tomato plants (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas *et al*., 2001; Sagi *et al*., 2004). Furthermore, the levels of TPI activities elicited by wounding are also the same in WT and ir*rboh D* plants (Figure 3b). These results indicate that ROS from *Narboh D* are not involved in wound-

elicited TPI responses in *N. attenuata*, unlike what has been observed in tomato. Similarly, the active systemin was found only in tomato, but not in tobacco. Even in the closely related *Solanum nigrum*, the systemin homolog of tomato does not play a role in defense responses (Schmidt and Baldwin, 2006).

However, silencing *Narboh D* does have a large effect on OS-elicited late responses. Most of defense genes, including *SnPIN2a*, *NaTPI*, *SnPPO*, *NaThionin*, *NaACO1*, and *NaLTP*, were elicited to much lower levels in ir*rboh D* plants than in WT plants (Figure 4). In addition, the levels of TPI activities 48 h after OS elicitation were also significantly reduced in ir*rboh D* plants in both local and systemic leaves (Figure 3). These changes in ir*rboh D* plants are likely responsible for the increased larvae performance (Figure 5), especially of the generalist herbivore, *S. littoralis*.

The fact that OS-elicited levels of JA/JA-Ile and early transcripts (*NaJAR4* and *NaPAL1*) are the same in WT and ir*rboh D* plants (Figure S3) is not consistent with the hypothesis that ROS directly interact with JA signaling. Thus, ROS may function independently of JA (or downstream of JA signaling). FACs are known to be responsible for eliciting OS-specific MAPK, JA, VOCs, and transcriptional responses in *N. attenuata* (Wu *et al*., 2007a; Halitschke *et al*., 2001, 2003). Levels of both *Narboh D* transcripts and H_2O_2 were more highly elicited by FACs than wounding alone, and removing FACs from OS by ion-exchange chromatography made the OS no more able than water to elicit, suggesting that FACs in *M. sexta* OS are the elicitor of both *Narboh D* transcripts and H_2O_2 (Figure 1). It is still not clear how NADPH oxidase is activated by FACs. Recently, it was proposed that the activity of NADPH oxidase is modulated by Ca^{2+}, CDPK and Rac GTPase in rice (Kobayashi *et al*., 2007; Wong *et al*., 2007), which provide clues for how NADPH oxidase may be regulated by FACs.

As defense responses are costly, plants are usually thought to tailor energy-consuming defense responses to specific insults (e.g., insects vs. microbial pathogens), which suggests that Narboh D is not involved in pathogen defense since it can be specifically elicited by FACs. However, we found that Narboh D also plays an essential role in pathogen resistance, which contradicts the expectation, that pathogen and herbivore-specific responses are separately elicited.

Trichoderma sp., a widespread soil fungus, is the most commonly used biological control agents against plant pathogens (Harman *et al*., 2004). The mechanism by which *Trichoderma sp*. is able to control pathogen populations is

thought to involve the production of antibiotics and hydrolytic enzymes which lead to mycoparasitism and competition for nutrient in the rhizosphere. However, more recent reports reveal that *Trichoderma sp*. may activate defense responses in host plants to control disease (Harman *et al*., 2004). JA, SA, and ROS are three major classes of signaling molecules activated by *Trichoderma sp*. in host plants (Martinez *et al*., 2001; Segarra *et al*., 2007) that may play essential roles in plant resistance to *Trichoderma sp* itself.

When melon cotyledons are treated with the cellulase produced by *Trichoderma logibrachiatum* (Martinez *et al*., 2001), a striking accumulation of O_2^- is observed, which is thought to be generated by an unknown NADPH oxidase. Consistent with a protective role for NADPH oxidase in *Trichoderma*-elicited roots is the observation that levels of *Narboh D* transcripts are low in the root of WT seedlings but increase after the inoculation of *Trichoderma sp*. (Figure 7a). Indeed, silencing *Narboh D* makes plants susceptible to *Trichoderma sp.* Around 60-80% of all ir*rboh D* plants wilted and eventually died from root rot when grown in non-sterile greenhouse soil, whereas adjacent pots of WT, ir*COI1*, and as*LOX3* plants remained healthy and free of disease symptoms (Figure 6). Later, *Trichoderma sp*. was isolated from the infested roots of ir*rboh D* plants. When we inoculated WT and ir*rboh D* plants with *Trichoderma sp*. in the seedling stage, most ir*rboh D* seedlings showed severe symptoms of root rot. Many even died, in contrast to WT seedlings, whose growth was promoted (Figure 7a; Figure S6). The same outcome occurred when we inoculated autoclaved potting soil with *Trichoderma sp*.: three of the five inoculated ir*rboh D* line 1 plants died while WT plants were unaffected. Thus, irrespective of the precise mechanism whereby ROS produced by *Narboh D* regulates defense responses, our results establish the importance role of *Narboh D* in preventing *Trichoderma sp*. which is normally a beneficial fungus to becoming a devastating root rot pathogen.

Although an oversimplification, it is generally accepted that SA plays a major role in activating defenses against biotropic pathogens, whereas JA and ethylene are more usually associated with defense against insect herbivores and necrotrophic pathogens (Koornneef and Pieterse *et al*., 2008). NPR1 is the central regulator of SA-mediated responses (Mou *et al*., 2003), while JA induced-responses are largely dependent on COI1 (Xie *et al*., 1998). Blocking JA perception by silencing *COI1* (Paschold *et al*., 2007) and inhibiting the SA pathway by silencing *NPR1* (Rayapuram and Baldwin, 2007), does not influence a plant's resistance to *Trichoderma sp.*

(Figure S6). These results revealed that the resistance to *Trichoderma sp.* is specifically regulated by *Narboh D*-derived ROS but not by JA or SA, suggesting that these ROS function independently of the JA and SA pathways. We observed similar results in the leaves: the same levels of both SA and *NaPR1* transcripts are elicited in both ir*rboh D* and WT plants after *P. syringae* inoculation, and the OS-elicited JA burst was the same in WT and ir*rboh D* plants.

It's still not clear how ROS prevents *Trichoderma sp*. from becoming a devastating root rot pathogen. One of the remarkable behaviors of *Trichoderma sp*. is that they penetrate roots of their host plants but limit their growth mainly to the epidermis and outer cortex (Yedidia *et al*., 1999), suggesting that hyphal growth is tightly regulated by its host plant. More lactophenol blue staining was observed in ir*rboh D* plants than in WT plants 6 days after inoculation of *Trichoderma sp*. (Figure S7), suggesting that ROS derived from *Narboh D* is involved as a signal, but how ROS trigger host defense responses remains to be discovered.

Narboh D transcripts were also highly induced by the challenge of *P. syringae* (Figure 8a), which caused more severe disease symptoms in ir*rboh D* plants than in WT plants. Although an SA-dependent pathway is proposed to be crucial for general resistance to *P. syringae* (Katagiri *et al*., 2002), our results revealed that both SA and *NaPR1* transcript levels are elicited to the same levels in both ir*rboh D* and WT plants (Figure S7), indicating that the susceptibility to *P. syringae* in ir*rboh D* plants is SA independent, which is consistent with what has been found in roots.

In the Great Basin Desert of Utah, opportunistic *Pseudomonas sp*. performed better on *NaThionin*-silenced plants compared to WT plants, however, the native herbivore community of *N. attenuata* attacked *NaThionin*-silenced plants to the same degree as it did in WT plants, indicating NaThionin protein provides resistance against pathogens but not against insect herbivores (Rayapuram *et al*., 2008). The transcripts of *NaThionin* were highly elicited in WT plants but much less so in ir*rboh D* plants after *P. syringae* inoculation (Figure 8c) and OS-elicitation (Figure 4, and Supplemental Table 1), demonstrating that ROS signals are essential for both OS- and pathogen-elicited *NaThionin* accumulation, and the overlap between herbivore- and pathogen-induced signaling pathways. The reason why plants accumulate NaThionin, which does not provide resistance to insect herbivores after OS-elicitation, is not clear; it might be a mechanism that plants use to prepare against future pathogen attack as the wounds left by insect feeding can be readily colonized by pathogens.

Conclusion

Root hair apoplastic pH regulation by NaRALF

NaRALF, a 49-amino-acid peptide is activated by unknown developmental stimuli, affects the levels of ROS accumulation and possibly the activity of H^+-ATPase in plasma, and subsequently influences the periodicity of pH oscillations, and maintains an appropriate cell wall pH environment which is needed for root hair development and plant growth in native soils.

MeJA is de-esterifed to JA in defense elicitation

MeJA functions as a signal prior to being hydrolyzed to JA, which either alone or after its conjugation to Ile, elicits herbivore defense responses.

ROS in defense responses

Narboh D is essential for resistance to both insect herbivores and pathogens in *N. attenuata*.

After herbivory, when plants perceive FACs in the OS, they activate both ROS- and JA-signaling pathways.

ROS and JA pathways collaborate, triggering late transcriptional changes and the accumulation of TPIs, which lower the performance of insect larvae.

The defense role that *Narboh D* plays against *Trichoderma sp.* in roots is clearly not dependent on JA or SA pathways, as ir*COI1*, ir*NPR1*, and WT plants are resistant to *Trichoderma sp*.

Finally, ROS from *Narboh D* are essential endogenous signals for the eliciting *NaThionin* in defending against *P. syringae* in leaves.

Zusammenfassung

Apoplastische pH-Regulation in Haarwurzeln durch NaRALF

Rapid alkalinization factor (RALF) ist ein Peptid mit 49 Aminosäuren das schnell Tabakzellkulturen alkalisiert. Das Gen *NaRALF* liegt im wilden Tabak *Nicotiana attenuata* als einzelne Kopie vor und wird in den Wurzeln und Blattstielen stark exprimiert.

Das Ausschalten der *NaRALF*-Transkription führte zu Pflanzen (ir*RALF*) mit wildtyp (WT) -ähnlichem Phänotyp des oberirdischen Pflanzenteils während die Wurzeln länger waren und Trichoplasten produzierten welche anormale Wurzelhaare ausbildeten. Aus den meisten Trichoplasten entwickelte sich eine lokale Wölbung ohne das jedoch ein Wurzelhaarspitzenwachstum erfolgte. Des Weiteren bildeten sich weniger Trichoplasten die auch nur 10% so lang waren als solche von WT-Pflanzen. Dieser Wurzelhaarphänotyp war assoziiert mit verlangsamten pH-Oszillationen, erhöhtem pH an der Spitze der Trichoplasten und einer verringerten Akkumulation von reaktiven Sauerstoffspezies (ROS) in der Wurzelhaarinitiationszone.

Der Wurzelhaarphänotyp wurde partiell wieder hergestellt wenn ir*RALF*-Pflanzen in niedrig-pH gepufferten Substrat wuchsen. WT-Pflanzen hatten einen ähnlichen Phänotyp wie ir*RALF* wenn sie in hoch-pH gepufferten Substrat aufgezogen wurden.

Wenn ir*RALF*-Pflanzen zusammen mit WT-Pflanzen in pH 5,6; 6,7 und 8,1 gepufferter Erde im Gewächshaus kultiviert wurden hatten die WT-Pflanzen einen Fitnessvorteil im alkalischen Substrat, jedoch nicht in saurem. Wurden ir*RALF*- und WT-Pflanzen in den alkalischen Boden des natürlichen Habitats von *N. attenuata* in der Great Basin Desert gepflanzt, so hatten die ir*RALF*-Pflanzen kleinere Blätter, kürzere Stängel und weniger Blüten und Samenkapseln als WT-Pflanzen.

Wir folgern daraus das NaRALF benötigt wird für die extrazelluläre pH-Regulation um die Wurzelhaare, den Übergang von Wurzelhaarinitiation zu Spitzenwachstum und Pflanzenwachstum in alkalischen Böden.

Esterhydrolyse von MeJA zu JA während der Auslösung der pflanzlichen Abwehr

Mittels virus-induced gene silencing (VIGS) hemmten wir die Expression von MeJA-Esterase (*NaMJE*) und entdeckten das dieses Gen für den größten Anteil der

MeJA Esterhydrolyse in *N. attenuata* Proteinextrakten verantwortlich ist. Im Gegensatz zu WT-Pflanzen reduzierte die Inhibierung von *NaMJE* in as*LOX3*-Pflanzen signifikant MeJA induzierte Nikotin- und JA-Ile-Konzentrationen und die Resistenz gegen *Manduca sexta*. Die TPI-Konzentration wird nicht reduziert. MeJa-induzierte Transkription von Threonindeaminase (*NaTD*) und Phenylalaninammoniumlyase (*NaPAL1*) war ebenfalls reduziert in VIGS *MJE* (as*LOX3*)-Pflanzen. Letztendlich konnte mit Hilfe der Performanz an JA- oder MeJA-behandelten Pflanzen von *M. sexta* gezeigt werden dass das Ausschalten von *NaMJE* MeJA-induzierte aber nicht JA-induzierte Abwehrmechanismen in as*LOX3*-Pflanzen inhibiert. Anhand dieser Ergebnisse kann man schlussfolgern das die Resistenz, ausgelöst durch MeJA-Behandlung, nicht direkt durch MeJA aktiviert wird sondern durch das demethylierte Produkt JA oder dessen Konjugationsprodukt JA-Ile.

ROS im der Verteidigung

NarbohD ist für die Resistenz gegen herbivore Insekten sowie gegen Pathogene in *N. attenuata* erforderlich.

Wenn Pflanzen bei Herbivorenbefall die im Raupenspeichel (oral secretion, OS) enthaltenen FACs (fatty acid-amino acid conjugate) wahrnehmen, aktivieren sie sowohl ROS (reactive oxygen species)-abhängige als auch Jasmonsäure (JA)-abhängige Signalketten.

Beide Signalwege wirken zusammen und lösen dabei Änderungen im Transkriptionsmuster aus. Darüber hinaus führen sie zur Akkumulation von TPIs (Proteinaseinhibitoren), welche das Wachstum von Insektenlarven verlangsamen.

Die Rolle von *NarbohD* in der Verteidigung gegen *Trichoderma sp.* in den Wurzeln ist eindeutig nicht von JA oder SA (Salicylsäure) abhängig, da ir*COI*-, ir*NPR1*- und Wildtyp-Pflanzen gegen *Trichoderma sp*. resistent sind.

Desweiteren sind von *NarbohD* gebildete reaktive Sauerstoffformen (ROS) ein wichtiges endogenes Signal für die Bildung von *NaThionin* in der Abwehr gegen *P. syringae* in den Blättern.

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Oral presentations

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Selbständigkeitserklärung

Entsprechend der Promotionsordung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Personen, die an der Durchführung und Auswertung des Materials und bei der Herstellung der Manuskripte beteiligt waren, sind am Beginn der Arbeit ("Manuscript Overview") und jedes Manuskriptes angegeben.

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Die vorgelegte Arbeit wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

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