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

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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 (irRALF) 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 irRALF lines were grown in a low-pH buffered medium and reproduced in WT plants grown in a high-pH buffered medium. When irRALF 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 irRALF lines were planted into the basic soils of *N. attenuata*’s native habitat in the Great Basin Desert, irRALF 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 irRALF line 2. G. M. Hummel measured the root growth velocity of WT and irRALF line 1 plants. I performed the remaining work.
Manuscript overview

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 (asLOX3) 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 asLOX3, 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* (asLOX3) 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 asLOX3 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 *COII* (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.

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.
1. Introduction

Introduction

_Nicotiana attenuata_ and the challenges it faces in nature

![Figure 1. N. attenuata and its natural habitat. (Photo courtesy by D. Kessler)](image)

(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 America. 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.
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**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 alkalization 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 *lipoxigenase3 (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 asLOX3 plants.

Reactive oxygen species (ROS) and Narbod D

The early production of reactive oxygen intermediates (ROS, largely O$_2^-$ and/or its dismutation product H$_2$O$_2$) 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,
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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$_2$O$_2$ 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.
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 (irRALF) 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 irRALF lines were grown in a low-pH-buffered medium and reproduced in WT plants grown in a high-pH-buffered medium. When irRALF 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 irRALF lines were planted into the basic soils of *N. attenuata*’s native habitat in the Great Basin Desert, irRALF 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).
2.1 Manuscript I: Root hair apoplastic pH regulation by NaRALF

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 RALFs 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 alkanization 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.
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.

**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 irRALF 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 irRALF 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 irRALF lines.

Figure 3. Longer root lengths of *irRALF* lines result from faster growth.

The mean (± SEM) maximal root tip velocity (a), maximal relative elemental growth rate (REGR\text{max}) (b), and length of root elongation zone (c) were measured in 5 replicated WT and *irRALF* 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 *irRALF* 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
2.1 Manuscript I: Root hair apoplastic pH regulation by NaRALF

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 (irRALF 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 irRALF lines. Four days after germination, the root length in WT and irRALF lines did not differ. However, by day 6, roots of both irRALF lines were significantly longer than in WT
2.1 Manuscript I: Root hair apoplastic pH regulation by NaRALF

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$_{\text{max}}$), and the length of the elongation zone in WT and irRALF line 1 plants. Because root growth correlates positively with temperature (Walter et al., 2002; Pahlavanian and Silk 1988), root tip velocity, REGR$_{\text{max}}$, 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 irRALF 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$_{\text{max}}$, which characterizes the maximal expansion rate of cells, was also significantly higher in irRALF 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 irRALF plants.

![Figure 5. Reduced root hair number of irRALF lines result from broken trichoblasts.](image)

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 irR alf 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 irRALF 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 irRALF 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 irRALF 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 irRALF line 2 plants.
Figure 7. Root hairs of WT plants display the root hair phenotypes of irRALF 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 irRALF lines grown on Gamborg B5 unbuffered media (pH 6.8).

Figure 8. WT root hair phenotype is partially restored in irRALF 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 irRALF 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 irRALF 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 irRALF lines 1 and 2 (unpaired t-test: *, P<0.05; **, P<0.001; ***, P<0.0001).
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**Apoplastic pH and root hair development in irRALF plants**

Clearly irRALF 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 irRALF plants have such root hair phenotypes, we grew WT and irRALF 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 irRALF 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 extracellular 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 irRALF plants with a pH-sensitive dye fluorescein-dextran. As the dye is dextran-conjugated, it is excluded from the cell and reports extracellular 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 irRALF line 2, the length of the period was twice as long as that of oscillations in WT plants (Figure 6b; irRALF 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 irRALF plants was higher than that of WT plants (pH in WT plants increased to 5.79 ± 0.24 and in irRALF line 2 to 5.94 ± 0.37; unpaired t-test, \( P = 0.02 \), n=6 separate roots). Furthermore, a large increase in alkanization 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 extracellular pH oscillations, which are required for tip growth during root hair development. We propose that in irRALF 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 irRALF 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 irRALF 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 irRALF 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 irRALF plants. When grown on a low-pH-buffered medium, root hair length in irRALF line 1 increased from 67 to 292 μm, and in irRALF line 2, from 41 to 228μm (Figures 8a, d). Root hair density was also partially recovered; for example, the number of root hairs in irRALF 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 irRALF 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 irRALF 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 irRALF 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 irRALF line 1; the intensity of ROS accumulation was defined as relative amounts in comparison to the irRALF 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 irRALF 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 irRALF plants. 3,3’-diaminobenzidine (DAB) staining of the root tip revealed less ROS accumulation in the root hair initiation zone of irRALF line 1 compared to in the zone of WT plants (Figure 9). Measurement of ROS
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accumulation in root segments made as described previously in (Shaw and Long, 2003) revealed that the roots of irRALF line 1 contained significantly less ROS relative to the roots of WT plants (Figure 9).

Figure 10. Leaf size and stalk length of irRALF 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 irRALF 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 irRALF line 1-irRALF line 1 pairs; see Supplemental Figure S5, 6) and similar results were obtained in WT-irRALF line 2 competitions (see supplemental Figure S4). The asterisks indicate the level of significant differences between WT and irRALF line 1 (paired t-test: *, P<0.05).
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irRALF 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 irRALF lines led us to hypothesize that irRALF 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 irRALF plants grown in three different pH soils in the glasshouse, and in addition transplanted these WT-irRALF pairs into a native population in Utah.

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 irRALF line 1 with 13.0 ± 1.1; unpaired t-test, P = 0.1) were found between WT and irRALF plants. When plant pairs were grown in soil amended with 5% limestone, which
increased the soil pH to 6.7, the growth of irRALF 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 (irRALF 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 irRALF 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, irRALF line 1 produced 9.4 ± 0.7 capsules and irRALF 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.

<table>
<thead>
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<th>Genotype</th>
<th>buds</th>
<th>flowers</th>
<th>capsules</th>
</tr>
</thead>
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<tr>
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<td>7.7 ± 1.5</td>
<td>6.4 ± 2</td>
<td>4 ± 1.5</td>
</tr>
<tr>
<td>irRALF line 1</td>
<td>4.2 ± 1</td>
<td>*</td>
<td>1 ± 0.5</td>
</tr>
<tr>
<td>irRALF line 2</td>
<td>0.8 ± 0.5</td>
<td>**</td>
<td>0.1 ± 0.1</td>
</tr>
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</table>

Table 1. irRALF 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, irRALF plants of ten matched triplicates after 55 days of growth. The asterisks indicate the level of significant differences between WT and irRALF 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 irRALF 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 irRALF line 1 plants (5.8 ± 0.4, 6.9 ± 0.6, 12 ± 0.8 and 12.7 ± 1 cm), and irRALF line 2 (4 ± 0.7, 4.4 ± 0.8, 7.5 ± 1.3, and 7.5 ± 1.4 cm).
The stalks of WT plants were taller than those of irRALF lines (Figure 11a): 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 irRALF line 1 and 4.9 ± 2.1 cm (unpaired *t*-test, *P* < 0.0001) in irRALF 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 irRALF 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 irRALF 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 irRALF 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$_2$O$_2$ 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$_2$O$_2$; however, the elongation of roots was inhibited by exogenously supplied H$_2$O$_2$. These results provide a possible mechanism for NaRALF’s effects on root growth that less ROS accumulation is the reason of longer roots in irRALF plants. Another explanation for the effect of
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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 extracellular 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 irRALF lines have disrupted root hair formation. Root hairs in the irRALF 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 irRALF lines. This hypothesis was confirmed by the partial recovery of the root hair phenotype (root hair length and number) when irRALF lines were grown in low-pH buffered medium; moreover, WT plants produced a root hair phenotype similar to that of irRALF 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 irRALF 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, irRALF 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 *irRALF* 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, *irRALF* 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 *irRALF* 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 *irRALF* plants was severely reduced. Two factors may be responsible: either (a) the *irRALF* 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
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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 GA3. After 10 days, seedlings were planted into soil in Teku pots (Pöppelmann, http://www.poepelmann.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-irRALF line 1; (2) WT-irRALF line 2; (3) WT-WT (as a control); (4) irRALF line 1-irRALF line 1 (as a control); (5) irRALF line 2-irRALF 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% CaCO₃, 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).
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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 FeSO₄·7H₂O and 3.93 g of Titriplex in 1L distilled H₂O, diluted 100 fold) after 7 days. After 3-4 weeks, WT and irRALF 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 irRALF 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 mm². 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<sub>max</sub>), 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<sub>max</sub> and root tip
velocity were averaged over 3 different time periods to compare WT plants and irRALF 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 irRALF plants to acidify their rhizosphere, three 7-day-old seedlings per genotype were pressed onto pH indicator gel (1 mM CaSO₄, 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 irRALF 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 irRALF 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 to 10 kDa dextran (Sigma, http://www.sigmaaldrich.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: ATGGGAGTTCTTCTAGGTTT, 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 (irRALF line 1 and 2; Figure S2c) were identified, bred to homozygosity, 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°C 16h light, 24°C 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 *EcoRI*, *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.
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**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’- TTCAATGCGACCCTG-3’,
NaRALF reverse primer; 5’- TACTCCGTGATCCCTCCT-3’,
ECI forward primer; 5’-AGAACTGCAGGGTACTGTTGG-3’,
ECI reverse primer; 5’-CAAGGAGGTATAACTGTTGCCC-3’,
FAM labeled NaRALF probe; 5’- GATTGGGTGATGCCGGAGA-3’,
VIC labeled ECI probe; 5’-CGTCAAAATTCTCCACTTGGTCTGCAACTGT-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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References


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Supplementary Data

Video Clip S2. Time-lapse movie of apoplastic 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 32P 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 (irRALF 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 irRALF lines 1 and 2 harbor only one T-DNA as determined with an hptII-specific probe. 5 μg of genomic DNA were digested with Hind III and Dra I.
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Figure S3. Caterpillar performance and morphologies of irRALF and WT plants after UV-B treatments.
(a): Mean (± SEM) mass of *M. sexta* larvae after 10 days of growth on WT and irRALF rosette-stage plants. A single freshly hatched larva was placed on a fully developed leaf of 12 replicates of WT and irRALF plants.
(b): Morphologies of irRALF line 1 and WT plants after UV-B treatment. irRALF 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 irRALF 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 irRALF 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 irRALF line 2 (paired t-test: *, P<0.05).
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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 irRALF 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 irRALF line 1 and irRALF 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).
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 (asLOX3) 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
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activity in *N. attenuata* protein extracts. Silencing *NaMJE* in asLOX3, 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* (asLOX3) 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 asLOX3 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.
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
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 *asLOX3* 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 asLOX3 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 asLOX3 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 asLOX3 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 asLOX3 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 asLOX3 plants, respectively (Figure 3). Larvae performed equally well on EV and VIGS MJE plants treated with lanolin in either WT or asLOX3 genetic backgrounds (Figure 3). However, silencing NaMJE completely inhibited MeJA-induced resistance in asLOX3 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.

Silencing NaMJE impairs MeJA-induced nicotine and NaPAL1 transcripts but not TPI responses in asLOX3 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 asLOX3 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 asLOX3 backgrounds (Figure 4B), suggesting that silencing NaMJE had no effect on the elicitation of TPIs by MeJA. Measurements of
TPI transcripts in asLOX3 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 asLOX3 plants had such a large effect on MeJA-induced herbivore resistance, we compared transcript levels of phenylalanine ammonia lyase (PALI), 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. PALI was dramatically induced in EV plants. Four h after MeJA treatment, levels of PALI 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 asLOX3 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**
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The changes in endogenous levels of JA and JA-Ile elicited by MeJA treatment were measured in EV and VIGS MJE asLOX3 plants (Figure 6) to better understand the loss of MeJA-induced resistance in NaMJE asLOX3 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.

Silencing NaMJE inhibits MeJA-induced but not JA-induced resistance to M. sexta in asLOX3 plants

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**Figure 5. Silencing NaMJE inhibited MeJA-induced NaPAL1 transcripts 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. 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).
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).
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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 asLOX3 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).

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 *asLOX3* 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 *asLOX3* plants (Fig. 7). Why is silencing *NaMJE* sufficient to block most MeJA-induced responses in *asLOX* 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 *asLOX3* 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 asLOX3 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 asLOX3 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 asLOX3 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 NaPALI 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 (GA3). 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’- GCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT -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’- AGATGACATGGAAAAGGTT -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
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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 NaMJEl-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°C 16h light, 24°C 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 1,2-13C-JA, D4-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’- GCTTTTCATGCCTGGTCCTAA -3’,
NaMJE reverse primer: 5’- GACCTTTCTCCTGTCCGTTG -3’,
NaTPI forward primer: 5’- TCAGGAGATAGTAAATATGGCTTTCA -3’,

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*NaTPI* reverse primer: 5’- ATCTGCATGGTTCCACATTGCTTA -3’,
*NaTD* forward primer: 5’- TAAGGCATTGGATGGGAGGC -3’,
*NaTD* reverse primer: 5’- TCTCCCTGTTCACGATAATGGAA -3’,
*NaPAL1* forward primer: 5’- TTTGCATACGCTGATGACGC -3’,
*NaPAL1* reverse primer: 5’- TGGAAGATAGAGCTGTTCGCG -3’,
*NaActin* forward primer: 5’- GTCAAGACGGAGAATGGCATG -3’,
*NaActin* reverse primer: 5’- GTCAAGACGGAGAATGGGCATG -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.

**Literature**

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc Natl Acad Sci USA 95: 8113-8118


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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 COII (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$_2$O$_2$) (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
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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$_2$O$_2$ 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 (irCOI1 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$_2$O$_2$ levels after
elicitation. The results revealed that OS treatments elicited significantly higher levels of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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).

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$_2$O$_2$ 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$_2$O$_2$ levels (Figure 1b).

**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

**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, *irrboh D* line 1 and *irrboh 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 *irrboh D* plants (unpaired *t*-test: *, *P*<0.05; **, *P*<0.001).
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).

![Graph](image)

**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 *irboh 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 *irboh D* plants. Three microarrays per treatment were hybridized and statistically analyzed. Genes are considered significantly (P<0.05) up-regulated when \( \log_2 (\text{Cy3}/\text{Cy5}) > 1.5 \) and down-regulated when \( \log_2 (\text{Cy3}/\text{Cy5}) < -1.5 \). The details of regulated gene name and accession number are shown in Supplemental table 1 and 2.

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

(b) Mean (± SE) value of \( \log_2 \text{Cy3}/\text{Cy5} \) of representative genes down-regulated in WT or *irboh D* plants 10 h after OS-elicitation: down-regulated only in *irboh 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 *irboh D* plants (Group 7, 26 genes); down-regulated in both WT and *irboh D* plants, but the level is lower in *irboh D* plants (Group 8, 3 genes); down-regulated only in both WT and *irboh 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.*, 2002).
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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 (irrboh 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 irrboh D lines after elicitation (Figure 2b). At the same time, neither line had increased H2O2 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 irrboh D plants.

(b) Mean (± SE) mass of *S. littoralis* larvae 2 and 4 days after feeding on 10 replicate leaves of WT and irrboh 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
larrvae 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 *irrboh 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 *irrboh 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 *irrboh D* line 2 plants (Figure 3b). Meanwhile, wounding-elicited TPI levels do not differ in both local and systemic leaves of WT and *irrboh 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 *irrboh 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 *irrboh 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 *irrboh 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 *irrboh 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 *irrboh D* plants, for example, *Calmodulin* and *SnCAT1*; meanwhile, only 22% (Group 7; 26 genes) were elicited at the same levels in *irrboh D* plants as in WT plants, such as *NaRCA*; another 7% (Group 9; 8 genes) were also significantly down-regulated in *irrboh 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 *irrboh D* plants. After 10 days, larvae of the specialist herbivore, *Manduca sexta*, that fed on *irrboh 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 *irrboh 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 *irrboh D* plants, but not WT plants, exhibited severe root rot symptoms after being transferred to potting soil B410. Around 60-80% of *irrboh 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, *irrboh 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).
From the infested roots of \textit{irrboh} \textit{D} plants, we isolated three different fungi (data not shown), one of which causes severe root rot disease symptoms in \textit{irrboh} \textit{D} seedlings; WT seedlings, however, were unaffected 12 days after inoculation in axenic GB5 medium (Figure 7a; Figure S6). This fungus was identified as \textit{Trichoderma sp.} by using DNA sequence analysis of the internal transcribed spacer (ITS) region within the ribosomal RNA gene cluster (Figure S5). Interestingly, \textit{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.
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 *irrboh D* plants (Figure 7b), which is consistent with the higher levels of H$_2$O$_2$ levels in WT roots after inoculation compared to *irrboh 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* (*irCOI1* plants) greatly reduces herbivore-elicited resistance (Paschold et al., 2007), neither WT nor *irrCOI1* plants showed any disease symptoms after *Trichoderma sp.* inoculation (Figure S6). Moreover, we never observed any disease symptoms caused by *Trichoderma sp.* in *asLOX* 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 (irNPR1 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.*

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 irrboh D plants with 300 µl *P. syringae* (re-suspended in sterile water with OD<sub>600</sub>=0.001) per leaf. After two days, we found that irrboh D plants contained more *P. syringae* than did WT (Figure S7a). After two weeks, three
of the five leaves from irrboh 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 irrboh D lines to *P. syringae* is due to impaired SA signaling, we analyzed free SA and *PRI* transcripts. Although both SA and *PRI* transcript levels were dramatically elicited by *P. syringae* inoculation, differences between WT and irrboh D plants were slight (Figure S7b). Recently Rayapuram *et al.*, (2008) have shown that when *NaPRI1*- 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 *NaPRI1*-silenced and WT plants, suggesting the defense role of *NaThionin* again *P. syringae*. These results lead us hypothesize that the susceptibility of irrboh 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 irrboh 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), irrboh D plants are morphologically normal; they grow as well as WT plants do in their seedling stages, 11 and 17 days after germination on GB5 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
organ (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 irrboh D plants (Figure S4), although NAPDH oxidase-dependent H$_2$O$_2$ 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 irrboh 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 irrboh 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 irrboh D plants in both local and systemic leaves (Figure 3). These changes in irrboh 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 irrboh 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$_2$O$_2$ 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$_2$O$_2$ (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 irrboh D plants wilted and eventually died from root rot when grown in non-sterile greenhouse soil, whereas adjacent pots of WT, irrCOI1, and asLOX3 plants remained healthy and free of disease symptoms (Figure 6). Later, *Trichoderma sp.* was isolated from the infested roots of irrboh D plants. When we inoculated WT and irrboh D plants with *Trichoderma sp.* in the seedling stage, most irrboh 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
irrboh 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 irrboh D and WT plants after P. syringae inoculation, and the OS-elicited JA burst was the same in WT and irrboh 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 irrboh 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 irrboh 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 irrboh D and WT plants (Figure S7), indicating that the susceptibility to P. syringae in irrboh 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 *irrboh 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 *irCOI1*, *irNPR1*, 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 °C 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
which contained the entire open reading frame, was cloned into a pGEM-T EASY vector (Promega, [http://www.promega.com](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 (*irboh 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 H₂O₂ measurements (Supplemental methods)**

**Isolation and identification of pathogen:** the pathogen was isolated from the roots of diseased *irboh 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 *irboh 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 (subcultured every 30 days) on 1/5 strength PDA.

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

**References**
2.3 Manuscript III: ROS in defense responses


Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N., 


2.3 Manuscript III: ROS in defense responses


Winz, R.A., Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. IV. Insect-Induced
2.3 Manuscript III: ROS in defense responses


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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.
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Figure S2.
(a) The growth of WT and irrboh D line 1 seedlings 11 and 17 days after germination.
(b) 27-day-old WT and irrboh 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 EcoR 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 irrboh 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 irrboh 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 irrboh D plants 1 h after OS-elicitation.
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Figure S4. Mean (± SE) NaTPI transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and irrboh D plants 8 h after OS-elicitation.

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

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

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TCCGTAGGTGAAACCTGCGGAGGGATCTTACCGAGTTTACAACCTCCAAACCAATGTGACGTATTACAAAACACTGTGCTCCGCGGAG
ATCTCTGGCCCGGCTCGAGCCCAGCCGCAGCCGACACAGCGCCGCCC
GCCGGGAGGACCAACCTAAACTCTTAATTGTATACCCCTCCTGGG
GTTTTTTTTTTATAATCGAGCTTTCGCTGCGGCCTCTCTGCTAGGGCTT
TTCTGAAAATGAATCAAATAACCTTAAACACAAGGTATCTTGGTTGCT
GCATGCGATGAGAACGACGGAAATGCAATAAATGTAAGTT
GCAGAATCTAGAGAACATCGAATCTAAGACGACAACTTGGAAAGC
CCGCTAGTTTCTGCCGGCGATGGCTCTGCAGCGCTATTCAAA
CCCTGAAACCCTCCTGGGGGTTGGCGCTGGGATCGGACCCCT
CCCTGAGCTTGCCGGCGCTCTGGAAATCTGGGCGGTCGGC
CGAGCCCTCTCCCTGCACTGTGGACACACTGCTCGAGGAG
CGGGGCGGGGCTCCACGGCGTTAAACAGCCCAACTTGGTTAAG
GACCTCGGAGTCAGTAACTACCCGCTGAACTTAAGCGTATCG
ATAAGCGGAGGAG
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Figure S6. The growth of WT and irboh D line 1 (a), WT and irCOI1 (b), and WT and irNPR1 (c) seedlings 12 days after Trichoderma sp. inoculation.
Figure S7. The roots of WT and irrboh 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 cm²) from 2-day-old *P. syringae*-inoculated leaves or WT and irrboh 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 *irrboh 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 \textit{irrboh D} plants.

Supplemental Table 2
Gene specific primers, probes, gene name and gene accession number.

Supplemental methods
3. Discussion

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 irRALF 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 irRALF 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 H₂O₂ 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 H₂O₂; however, the elongation of roots was inhibited by exogenously supplied H₂O₂. These results provide a possible mechanism for NaRALF’s effects on root growth that less ROS accumulation is the reason of longer roots in irRALF 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.
3. Discussion

Silencing *NaRALF* led to a slower rate of oscillation in extracellular 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 *irRALF* lines have disrupted root hair formation. Root hairs in the *irRALF* 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 *irRALF* lines. This hypothesis was confirmed by the partial recovery of the root hair phenotype (root hair length and number) when *irRALF* lines were grown in low-pH buffered medium; moreover, WT plants produced a root hair phenotype similar to that of *irRALF* 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 *irRALF* 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, *irRALF* 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 irRALF 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, irRALF 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 irRALF 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 irRALF plants was severely reduced. Two factors may be responsible: either (a) the irRALF 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-esterified 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 *asLOX3* 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 *asLOX3* plants (Figure 7). Why is silencing *NaMJE* sufficient to block most MeJA-induced responses in *asLOX* 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 *asLOX3* 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 asLOX3 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 asLOX3 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 asLOX3 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), irrboh D plants are morphologically normal; they grow as well as WT plants do in their seedling stages, 11 and 17 days after germination on GB5 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 irrboh D plants (Figure S4), although NAPDH oxidase-dependent H2O2 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 irrboh 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 *irrboh 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 *irrboh D* plants in both local and systemic leaves (Figure 3). These changes in *irrboh 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 *irrboh 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$_2$O$_2$ 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$_2$O$_2$ (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 irrboh D plants wilted and eventually died from root rot when grown in non-sterile greenhouse soil, whereas adjacent pots of WT, irCOI1, and asLOX3 plants remained healthy and free of disease symptoms (Figure 6). Later, Trichoderma sp. was isolated from the infested roots of irrboh D plants. When we inoculated WT and irrboh D plants with Trichoderma sp. in the seedling stage, most irrboh 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 irrboh 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 biotrophic 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.
3. Discussion

(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 *NaPRI* transcripts are elicited in both *irrboh D* and WT plants after *P. syringae* inoculation, and the OS-elicited JA burst was the same in WT and *irrboh 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 *irrboh 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 *irrboh 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 *NaPRI* transcript levels are elicited to the same levels in both *irrboh D* and WT plants (Figure S7), indicating that the susceptibility to *P. syringae* in *irrboh 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 *irrboh 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.
4. Conclusion

**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 *irCOI1*, *irNPR1*, 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 alkalization 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 (irRALF) 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 irRALF-Pflanzen in niedrig-pH gepufferten Substrat wuchsen. WT-Pflanzen hatten einen ähnlichen Phänotyp wie irRALF wenn sie in hoch-pH gepufferten Substrat aufgezogen wurden.

Wenn irRALF-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 irRALF- und WT-Pflanzen in den alkalischen Boden des natürlichen Habitats von N. attenuata in der Great Basin Desert gepflanzt, so hatten die irRALF-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
5. Zusammenfassung


**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.
6. Literature

Literature Cited


Bubner B, Gase K, Baldwin IT (2004) Two-fold differences are the detection limit for determining
6. Literature

transgene copy numbers in plants by real-time PCR. BMC Biotechnology 4: 1-11


Halitschke R, Gase K, Hui D, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana*
attenuata. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiol 131: 1894-902.


6. Literature


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6. Literature


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Publications:
1. Wu J, Wang L, Baldwin IT (2007) Narboh D, a respiratory burst oxidase homolog in Nicotiana attenuata, is essential for resisting attack from both herbivores and pathogens. (Submitted)
8. Curriculum Vitae


Oral presentations

Wu Jinsong (April 2007): Communication between plants: induced resistance in wild tobacco plants following of neighbouring sagebrush is mediated by NaMJE. ViBi-Meeting at GSF-Biochemical Plant Pathology 2007 (Virtuelles Institut für Biotische Interaktionen), Neuherberg, Germany.

Wu Jinsong (April 2007): Silencing NaRALF, a root hair tip apoplastic pH regulator required for root hair development, impairs growth in the plant’s native soils; ICE Seminar, Jena, Germany

Wu Jinsong (March 2007): NaRALF, a root hair apoplastic pH regulator, is required for the transition from root hair initiation to tip growth; 6th Biannual IMPRS Symposium, Dornburg, Germany.

Wu Jinsong (March 2006): RNA interference identifies a RALF peptide homolog involving in root hair development and nutrients uptake; 4th Biannual IMPRS Symposium / MPI for Chemical Ecology, Jena, Germany.

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Selbständigkeitserklärung

Entsprechend der Promotionsordnung 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.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen.

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

Jinsong Wu
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