Growth dynamics of *Nicotiana attenuata* after simulated herbivory

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1. Introduction

1.1 Plant growth and development in a changing environment

Plant growth and development occur at the cellular level through the processes of cell division, cell expansion and cell differentiation. Growth is an irreversible change in dry mass or plant size and is a resource-demanding process. The general growth pattern of plant organs is genetically fixed, but the appropriate regulation of plant growth in response to environmental cues has obvious adaptive significance. An environmental stimulus must be integrated and converted into a decision about growth and development. This allows the immobile plant to place structures in the optimal position with respect to water, nutrients and light and to adjust their shape to changing conditions. Accordingly, plants have evolved precise mechanisms for such regulation in order to acclimate to myriads of fluctuating biotic and abiotic factors. For instance temperature (Walter *et al.* 2002), mechanical constraints such as soil compaction (Bengough & Young 1993), water availability (van der Weele *et al.* 2000), or nutrient availability (Zhang & Forde 1998; Walter *et al.* 2003) and light (Nagel *et al.* 2006) all interact with growth. Biotic interactions similarly affect growth, directly via herbivory for instance or indirectly via competition and the associated change in resource availability. These factors are not displayed homogeneously, but are rather changing permanently in space and time: Hence plants require a precise internal control system of growth to respond adequately and dynamically in order to optimize their resource acquisition (Walter & Schurr 2005). Within the framework of their genetically determined response options that vary throughout ontogeny, they react towards stress situations by maximizing protection against stress while minimizing deviations from optimal growth and development.

1.2 Herbivory and plant defense

Herbivory is probably the prime biotic stress factor for a wide range of plant species. Especially in seedlings, herbivory represents the major cause of mortality (Hanley & May 2006). However, plants are not defenseless against herbivores and have evolved a wide

array of chemical, physiological and morphological defense traits. Direct defenses are any plant traits that act directly against further attack or reduce herbivore performance, such as thorns, silica, trichomes, secondary metabolites such as nicotine, as well as defense-related proteins such as protease inhibitors (Kudo 1996; Traw & Dawson 2002; Steppuhn *et al.* 2004; Zavala *et al.* 2004). Indirect defense is based on attracting the plant's 'enemies': Volatile chemicals such as cis-α-bergamotene are emitted from leaves into the airspace: (E)-β-caryophyllene is released from roots into soil, extrafloral nectar attracts specific predators and parasitoids of herbivores (De Moraes *et al.* 1998; Kessler & Baldwin 2001; Rasmann *et al.* 2005). These defense traits are usually displayed constitutively at low levels and are dramatically increased when needed, namely when attacked by herbivores (induced defense) (Karban & Baldwin 1997). Although defense traits might benefit plants in the presence of herbivores, plant resistance can be costly in the absence of plant enemies (Baldwin 1998; Strauss *et al.* 2002). Hence it is suggested that plants have evolved the ability to induce defense and thus forego fitness costs when defense is not needed (Baldwin 2001). The fitness costs of defense can arise from internal processes to the plant, such as when limiting resources are allocated to defense that cannot be rapidly reallocated to growth or reproduction (Herms & Mattson 1992). However, the costs of a particular defense trait are dependent upon the environment in which it is measured, including resources available to the plant and the ecological interactions of the community. Bergelson & Purrington (1996) suggested that costs of defense should increase in stressful environments because of resource limitations. Moreover, allocation costs also depend on whether the plant can revoke an allocation 'decision'. The investment of limited resources into a specific defense trait is associated with higher costs, when it cannot be re-utilized for another plant function like growth or reproduction. For instance in *Nicotiana attenuata* up to 6% of the plant's nitrogen content is in the toxin nicotine alone, which is not accessible for the plant again (Baldwin *et al.* 1998). Moreover, costs may arise from autotoxicity, which results from negative effects of defense a trait on the plants own metabolism (autotoxicity costs) or ecological interaction that occurs when defense expression results in a reduced pollination, attracts enemies or impairs the expression of other defense traits (ecological costs) (Heil & Baldwin 2002; Heil 2002; Wittstock & Gershenzon 2002). Induced defense responses are ideal systems with which to measure the putative trade-offs because by inducing the defenses, one can alter the allocation to defense within the same genetic back ground of the plant.

Some studies have demonstrated resource-based trade-offs between growth and induced defense on large time scales (Zangerl *et al.* 1997; Zavala *et al.* 2004; Walls *et al.* 2005); but the short-term dynamics of growth immediately following herbivory are completely unknown. The first events in plant-herbivore interaction occur within seconds after herbivore attack and large metabolic changes proceed within hours (Maffei *et al.* 2007). Hence, large constraints on growth are evident in the short-term. Yet, it is unclear how quickly a trade-off-linked reorganization of plant metabolism can occur. This ignorance stems partly from the lack of appropriate tools for the study of growth at high temporal resolution. Recently, development of growth imaging methods (Digital Image Sequence Processing: DISP) has allowed the study of short-term growth responses of above- and belowground sink organs (Walter *et al.* 2002; Walter *et al.* 2005; Nagel *et al.* 2006).

The objective of this PhD project was to use imaging methods to monitor growth dynamics after simulated herbivore attack at high temporal and spatial resolution to compare growth with defense and hormonal dynamics and to elucidate mechanisms of endogenous growth control. To study those biotic-interactions *N. attenuata* Torr. Ex Wats. (Solanaceae) and the natural specialist lepidopteran herbivore *Manduca sexta* were selected as it has become a model system in which the signals activating herbivore defense responses are known (Baldwin 2001).

2. Induced defense in seedlings

The development of growth imaging methods has allowed the study of short-term growth responses of above- and belowground sink organs towards fluctuations of environmental factors. Clear responses are often seen best in young seedlings as they are characterised by highest relative growth rates throughout plant ontogeny. Hummel *et al* (2007 A1, A2) grew plants sterile in Petri dishes filled with translucent plant agar with full Gamborg as medium. In this cultivation system primary root growth can be monitored relatively easy (Walter & Schurr 2005; Nagel *et al.* 2006), while growth of the small leaves is somewhat difficult to analyze since leaves need to be forced mechanically into the focal plane of a camera for image acquisition (Schmundt *et al.* 1998; Walter & Schurr 2005). In this cultivation system different wounding treatments were performed, in order to mimic herbivory. Methyl jasmonate (MeJA) was dissolved in lanolin paste and applied directly to the primary leaf of 13 day-old seedlings. Methyl jasmonate is an endogenous wound signalling molecule which increases in plants after herbivore attack. When applied externally, it can stimulate plant defense without the destruction of plant tissue (Zhang & Baldwin 1997a). Moreover a second method to simulate herbivory was applied by Hummel *et al.* (2007 A1, A2) and Walter & Hummel (2008 A4): Primary leaves were squeezed with tweezers, to produce small puncture holes. By this procedure, a maximum of 13% of the total leaf area (including the cotyledons) was damaged. Immediately after wounding, 1 µL H_2O (buffered) or 1µL *Manduca sexta* larval oral secretions and regurgitants (diluted 1:5 with buffer; OS) was applied to the wounds.

Only a few studies have been shown the existence of induced defense in seedlings (Bodnaryk 1992; Cipollini & Bergelson 2000), hence Hummel *et al.* (2007 A1) first studied the existence and dynamics of defense traits in seedlings of *N. attenuata.*

Defense traits are constrained by plant ontogeny, as has been described for the induction of nicotine (Ohnmeiss & Baldwin 2000), volatile emissions (Halitschke *et al.* 2000) and protease inhibitors (van Dam *et al.* 2001). *N. attenuata* is thus spatially and temporally limited in its ability to deploy certain defenses against herbivores. However, these studies have been assessed with plants at later developmental stages (rosette plants, bolting plants, flowering plants) and did not include plants at seedling stage, apart from van Dam *et al.* (2001) who reported that seedlings do not contain any protease inhibitors. Hummel *et al.* (2007 A1) demonstrated in 10-day old *N. attenuata* seedlings that the simulation of herbivory via the application of MeJA dissolved in lanolin paste or wounding of the

primary leaves led to increased nicotine concentrations and trichome density and trichome length within 72 h. Other studies have revealed induced formation of PIs and glucosinolates after mechanical wounding of cotyledons or first true leaves in 1 week old *Brassica napus* (Bodnaryk 1992; Cipollini & Bergelson 2000). This increase in defense compounds of seedlings was shown in field studies to be negatively correlated with rates of herbivoral attack (Hanley & Lamont 2001). Hence, a rapid induction of defense systems can avert an attack through herbivores already in seedlings. The induced formation of hypocotyl trichome density may play an important role for repelling herbivores from seedlings, as here the hypocotyl still represents a relatively high fraction of the plant tissue. So far increasing trichome densities have only been shown on a larger timescale for leaves emerging after herbivore attack (Agrawal 1999; Traw & Dawson 2002; Traw & Bergelson 2003). Leaves that are already differentiated by the time of herbivore attack are not capable of strengthening their physical defense systems as this can only be performed in developing and non-mature tissues (Mayers & Bazely 1991; Nagata *et al.* 1999). Obviously the hypocotyl epidermis cells are still able to differentiate into trichomes, as this tissue is displaying secondary growth. To conclude, induced defense occurs already in seedlings of *N. attenuata* and large amounts of resources are diverted from growth processes into defense. This fact is remarkable, as here roots and shoots are extremely limited in size and one can assume that growth should be also of great priority to position the organs within their surrounding in an optimal way to assure nutrient uptake and light interception.

3. Growth dynamics after simulated herbivore attack

Leaves and roots both acclimate to environmental conditions. While roots generally respond strongly and directly to fluctuating environments, leaves display a more ‘conservative’ growth control which integrates over environmental fluctuations like temperature or light regimes (Walter & Schurr 2005). Walter and Schurr (2005) suggest that the endogenous control mechanisms buffer leaf growth from external factors which fluctuate strongly and would negatively affect leaf growth processes and leaf shape. In contrast, roots are exposed to much more stable temperature conditions and might not require the same buffering mechanisms as leaves. However, the distribution of nutrients in soil is extremely heterogeneous in space and time and roots need to react rapidly and adequately in order to optimize the efficient uptake of nutrients and water. Hence, roots

require an endogenous control mechanism which allows a rapid and direct transduction between environmental stimuli and the growth machinery. This control mechanism might also be used or affected via signals originating from distal organs like the shoot. Hummel *et al.* (2007 A1) demonstrated that wounding of the primary leaves leads to an immediate and transient reduction in root growth; leaf growth in turn was only marginally affected. The application of oral secretions and regurgitants of *Manduca sexta* to wounds led to a more pronounced reduction of growth than if a mere mechanical wounding was imposed. Again roots were more strongly affected than leaves, emphasizing that roots respond more susceptibly towards herbivory, even when the stress was imposed to the shoot. In these experiments a strong diel variation in growth was observed for roots and shoots. Leaves displayed an endogenous diel growth rhythm as has been previously demonstrated in many other species (Matsubara & Walter 2006). The highest growth rates were reached in the early morning (when temperature was low) and growth rate decreased throughout the afternoon, reaching zero growth at night (Hummel *et al.* 2007 A1). In contrast, roots show a strong and highly significant correlation with the temperature of the plant agar in which they were growing (Walter & Hummel 2008 A4). To uncouple the reduction by wounding from temperature-induced growth reductions, the experiment was repeated under continuous light and temperature, leading to a comparable result (Walter & Hummel 2008 A4). In the control plants no diel (throughout 24 h) growth rhythm for roots was found supporting the results from Walter *et al.* (2002). Hence, the observed correlation between root growth and temperature could be used to rectify the damage-induced reduction of root growth from the superimposed diel temperature effect.

The kinetics of the root growth depression observed by Hummel *et al.* (2007 A1, A2) point to a superposition of several physiological effects: During the first hour after wounding, root growth decreases similarly in control and OS-treated plants, indicating a mere hydraulic response (however an electrical response is conceivable as well): subsequently a second drop is observed, which might rather be explained by a hormonal response.

4. Kinetics and signaling of root growth depression after simulated herbivory

Wounding or herbivory-events induce a wide range of different signals, such as damage induced ion imbalances causing variations in membrane potential or changes in hydraulic properties, production of reactive oxygen species, kinase activities, phytohormones or signaling peptides. Those events are responsible for recognition and triggering of signal transduction pathways, eventually leading to direct or indirect plant responses. In this chapter mechanisms and signaling is discussed, which might be responsible for the observed reduction in root growth after simulated herbivory.

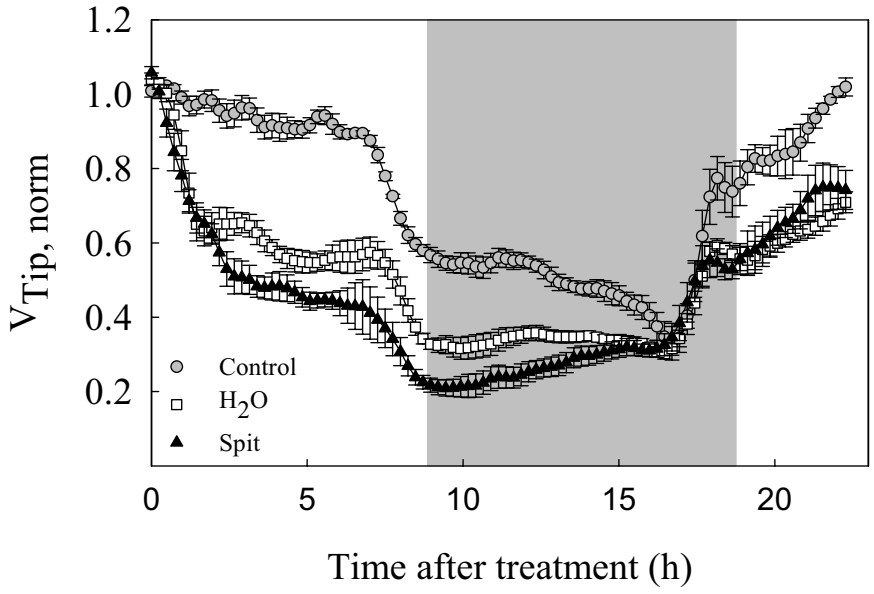


Figure 1: Time series of high-resolution root growth analysis. Light was switched on at 6 am and switched off at 8 pm (night period gray shaped). Treatments were performed at 1300 h. Normalized values of root growth velocities (V_{Tip}) with high resolution after wounding and the application of water (H_2O) or 1 µl oral secretions of *Manduca sexta* (Spit) diluted 1:5 (mean values ± SE; n=5) (Hummel *et al.* 2007 A1).

4.1 Resource-based trade-off between growth and defense

The use of allocation models in which plants divide limited resources among growth, reproduction and defense, is one of the theoretical approaches used to study phenotypic costs (Herms & Mattson 1992; Stamp 2003). In particular, seedlings are limited in their resource acquisition due to the limited size of their organs, hence they provide a suitable system to study resource-based trade-offs. Herms and Mattson (1992) postulated that one mechanism by which seedlings overcome resource-based constraints on chemical defense is by drawing on previously acquired stored reserves. A trade-off-linked reorganization of plant metabolism after wounding can occur within hours, diverting resources away from growth or development towards defense (Hummel *et al.* 2007 A1). Resource-based trade-offs balance the diversion of resources for the build-up of the particular defense trait with metabolic demands needed for the biosynthesis of defense traits. For instance, Zangerl *et al.* (1997) demonstrated in wild parsnip that most of the large increases in respiration that occur after wounding are due to the metabolic demands of induced furanocoumarin synthesis.

4.2 Carbon signaling

Root growth is highly dependent on carbon availability (Aguirrezabal *et al.* 1994; Muller *et al.* 1998; Freixes *et al.* 2002). Decreasing carbohydrate import to roots can lead to an immediate and strong reduction of root growth (Nagel *et al.* 2006). Hence, the observed reduction in root growth upon herbivory (Hummel *et al.* 2007 A1) is the consequence of a decrease in carbon import to roots due to the partial destruction of photosynthetic active tissue (13% of total leaf area). This direct effect might be responsible for lower root growth observed by Hummel *et al.* (2007 A1) two and three days after the wounding treatments compared to control plants. However, the destruction of photosynthetically active tissue can only partly explain the reduction in root growth, as the excision of further leaf area (approximately 40% of total leaf area) was not associated with a further reduction in root growth (Hummel *et al.* 2007 A1). This clearly indicates that other signals are involved, mediating the reduction of root growth. Increased export of carbohydrates in *N. attenuata* and *Populus tremuloides* from shoots to roots within two hours following simulated herbivore attack or the application of JA to leaves was reported recently in two studies

utilizing the short-lived isotope ^{11}C to monitor carbon flux within the plant (Babst *et al.* 2005; Schwachtje *et al.* 2006). In *N. attenuata* the increased carbon import to roots after a herbivore attack is regulated independently from the JA-pathway by a β-subunit of a SnRK1 (SNF1-related kinase) protein kinase (GAL83). After herbivory, transcripts of GAL83 are rapidly down-regulated in source leaves and assimilate transport to roots is increased (Schwachtje *et al.* 2006). Silenced GAL83 plants show higher carbon import to roots compared to wild type plants which is not associated with an increase in root growth (Hummel unpublished data). Thus one can assume that (i) the carbohydrates are stored in roots or (ii) the carbohydrates are utilized to fuel the defense machinery, namely the nicotine *denovo* biosynthesis, which occurs only in the roots. Hence, the herbivore-induced increase in carbon import to roots must be accompanied by further signals, which regulate root growth. A change in source-sink relations could in itself act as a signal, as changes in carbohydrate concentrations are known to affect gene regulation (Smeekens 1998), but phytohormones are involved as well. A re-allocation or storage of carbohydrates in the root would ecologically make sense, as carbohydrates would then be retrieved from leaf-consuming herbivores and could be used for regrowth and/or reproduction after the threat has passed by. This response to herbivore attack is termed a tolerance reaction.

Recently Smith and Stitt (2007) suggested in a review that the trade-off between defense and growth should not be viewed as a straight competition for resources, in which a reduction in growth is due to direct carbohydrate limitation. They proposed that a reduction in carbon availability via a rapid induction of defense will trigger an acclimatory response, resulting in a down-regulation of growth to a level that can be sustained at the new level of carbon availability. In short: growth is adjusted via signals to the available amount of carbon, allowing an optimization for sustained growth; plants respond to low carbon by increasing storage and decreasing growth and acclimatory mechanisms are triggered before acute carbon depletion occurs. This hypothesis also implies that plants grow somewhat more slowly than they would in a given condition without defensive processes operating. Hummel *et al.* (2007 A2) provided some evidence for this hypothesis. By blocking the ethylene perception with the ethylene action inhibitor 1-methycylopropen (1-MCP) growth was dramatically increased in roots and slightly in shoots. This demonstrates that ethylene might generally suppress growth. However, Smith and Stitt (2007) suggest that the acclimatory response is rather mediated via sugar signaling.

4.3 Hydraulic response

Wounding of the primary leaves immediately and steeply reduces root growth throughout 65 minutes, but growth then stabilizes until a second slight decrease occurs approximately 2 hours after wounding (Fig. 1; Hummel *et al.* 2007 A1, A2; Walter & Hummel 2008 A4). This first steep decrease points to a hydraulic response of the plant towards wounding. Some studies have revealed a direct relationship between xylem pressure and growth (Okamoto *et al.* 1984; Okamoto *et al.* 1989; Stahlberg & Cosgrove 1995). A sudden change of the xylem pressure can occur in single or multiple xylem vessels through wounding or after an insect bite and is associated with a decrease in turgor pressure which immediately reduces root growth (Stahlberg & Cosgrove 1995; Nagel *et al.* 2006). Stahlberg & Cosgrove (1998) demonstrated in cucumber seedlings that wound-induced release in xylem pressure was responsible for the immediate but transient reduction in hypocotyl growth. Approximately 30 minutes after wounding growth recovered to similar rates as before wounding. The study revealed that hydraulics can affect growth even over long distances; hence it displays a mechanism by which fast growth responses can be mediated across the plant. However, apart from a rapid change in the hydraulic properties, an electric signal could also account for a decrease in growth (Fromm & Lautner 2007).

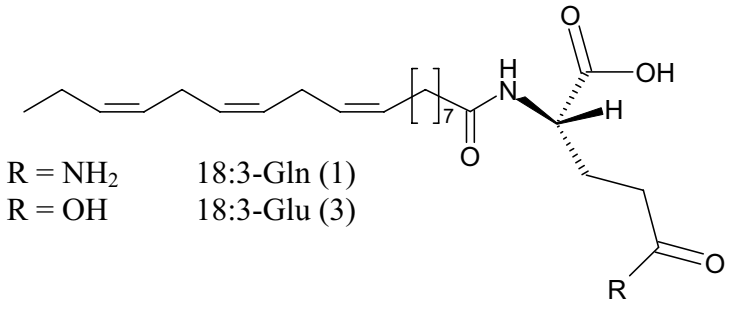
4.4 Electrical signaling

The earliest cellular responses towards biotic and abiotic stresses are changes in the plasma transmembrane potential or modulation of ion fluxes at the plasma membrane level (Ebel & Mithöfer 1998; Maischak *et al.* 2007). Herbivory-induced changes in plasma membrane potentials are followed by a fast electrical signal that travels through the entire plant from the point of origin of the perceived input (Fromm & Lautner 2007). Herbivory induces action potentials, which are a momentary change in electrical potential on the surface of a cell that propagates up to 40 m sec^{-1}. Changes in transmembrane potentials are smaller and travel approximately 1-2 cm min^{-1} similar to the speed of chemical signals moving in xylem or phloem (Maffei *et al.* 2007). Hence, the immediate reduction in root growth observed by Hummel *et al.* (2007 A1, A2) upon leaf wounding could be mediated via electrical signaling. Shiina and Tazawa (1986) reported a reduction in growth elongation of the stem after the generation of a single action potential in *Luffa cylindrica*. However, nearly nothing

is known about growth control via electrical signaling, presumably due to the lack of accurate methods to monitor growth at sufficiently high temporal resolution to detect such fast acting signals.

4.5 Hormonal signaling

When herbivores attack plants, they cause wounding, but the response of plants to herbivores cannot be mimicked by mechanical wounding. Several types of elicitors in the oral secretions and regurgitants of herbivorous insects have been reported to alter the response of a plant to wounding. For example, a β-glucosidase in the oral secretions and regurgitants of *Pieris brassica* larvae elicits the release of volatile organic compounds that function as indirect defense (Mattiacci *et al.* 1995). N-(17-Hydroxylinolenoyl)-L-Gln (volicitin) was the first fatty acid-amino acid conjugate (FAC) that showed biological activity by inducing volatile emissions in *Zea mays* (Alborn *et al.* 1997). Many of the defense transcriptional responses elicited by *M. sexta* attack can be mimicked by applying oral secretions and regurgitants of *M. sexta* to mechanically produced puncture wounds and FACs (*N*-linolenoyl-L-Gln and *N*-linolenoyl-L-Glu, Fig. 2) in *M. sexta* have been shown to be necessary and sufficient to elicit an endogenous jasmonic acid burst and an ethylene burst (Halitschke *et al.* 2001; Winz & Baldwin 2001). Both plant hormones are rapidly induced in *N. attenuata* when leaves are mechanically wounded; however, JA and ethylene accumulation is dramatically amplified by the application of OS of *M. sexta* into wounds via FACs (Kahl *et al.* 2000; Schittko *et al.* 2000).



R = NH_2 18:3-Gln (1)
R = OH 18:3-Glu (3)

Figure 2: Fatty acid-amino acid conjugates (FAC): (1) *N*-linolenoyl-L-Gln; (2) *N*-linolenoyl-L-Glu.

This increase in plant hormones mediates the specific defense responses in *N. attenutata* (Baldwin 1996; Halitschke *et al.* 2000; Zavala *et al.* 2004; Hummel *et al.* 2007 A1). In most plants, jasmonates (jasmonic acid and other oxylipin derivates) play a major role in mediating induced defense responses to herbivore attack. Jasmonates are involved not only in plant defense against pathogens and herbivores but also in plant development processes such as root growth, fruit ripening, tendril coiling, tuberization, reproductive development and senescence (Creelman & Mullet 1997; Lorenzo & Solano 2005)

JA and its derivates are known to be potent root growth inhibitors and many studies have used this property to isolate mutants deficient in their JA signaling, such as *jar1* (Staswick *et al.* 1992), *jin1* and *jin4* (Berger *et al.* 1996), and *coi1* (Feys *et al.* 1994). Ethylene is involved in processes like ripening and senescence, and it has also been shown to be a potent root growth inhibitor (Abeles *et al.* 1992; Tholen *et al.* 2007). Hummel *et al.* (2007 A1, A2) demonstrated, in *N. attenuata*, that the application of the oral secretions of *M. sexta* to the leaves led to a transient decrease in root growth that was more pronounced than if a mere mechanical wounding was imposed (Fig. 1). When FACs were applied to wounds, root growth reduction occurred in the same intensity as when oral secrete was applied, indicating that the growth response is specifically triggered by this herbivore-specific cue. With mutant lines of *N. attenuata* silenced in the biosynthesis and the perception of JA (as*LOX3* and ir*COI1*-plants, respectively) and treatments with 1-MCP, Hummel *et al.* (2007 A2) demonstrated that the reduction in root growth upon simulated herbivory is mediated via wound induced JA and not ethylene. NaLOX3 (Lipoxygenase) is expressed in roots and shoots of *N. attenuata* and transcript levels are rapidly induced after herbivore attack (Halitschke & Baldwin 2003).

The antisense expression of as*LOX3* in *N. attenuata* reduces the production of JA by as much as 50%. COI1 (coronatin insensitive 1), an F-box protein degrades the dominant repressor protein JAZ (jasmonate ZIM-domain) and thus activates the key transcriptional activator MYC2 (Chini *et al.* 2007; Thines *et al.* 2007). The transformation of an inverted repeat construct (ir*COI1*) into *N. attenuata* reduced its JA perception remarkably. Accordingly, Hummel *et al.* (2007 A2) demonstrated that ir*COI1* plants were less affected in terms of root growth upon simulated herbivore attack than as*LOX3* plants.

Baldwin *et al.* (1997) demonstrated that JA pools in roots of *Nicotiana sylvestris* are increased (3.5-fold) within 180 minutes after mechanical leaf wounding and Zhang & Baldwin (1997a) showed via the exogenous application of 2-^{14}C-labeled JA to leaves, that JA is directly transported from leaves to roots and could account for the systemic increase

in root JA pools after leaf wounding. Moreover Thorpe *et al.* (2007a) reported a rapid transport of ^{11}C-labelled methyl jasmonate (MeJA) from leaves to roots in *Nicotiana tabacum* within 60 minutes. Hence, wound-induced JA in leaves could be imported to the roots and mediate there the reduction in root growth, while triggering the defense machinery. The OS amplified increase in JA would explain the stronger reduction in root growth compared to mechanical wounding alone, which induces smaller amounts of JA. Further experiments with the application of dissolved MeJA in lanolin paste to leaves led similarly to a reduction in root growth, which coincides remarkably well with the 'decreasing' root growth dynamics of wounded plants. This provides further evidence that JA mediates the second decrease in root growth after simulated herbivore attack. However, the mechanism of root growth inhibition is not known. Swiatek *et al.* (2002) demonstrated that JA inhibits growth rather by the disruption of the meristem activity than by decreasing cell expansion. In summary, the results provide more evidence that endogenous JA acts as a distress signal, slowing vegetative growth during defense responses.

4.6 Peptide Signaling

Within the past decade, it has been discovered that plants have several peptide hormones that play important roles in diverse physiological processes, including inducible defense processes (Pearce *et al.* 1991), cell division and differentiation (Matsubayashi & Sakagami 1996), growth and development (Brand *et al.* 2000; Pearce *et al.* 2001) and reproduction. Systemin is an 18-amino acid protein that is proteolytically cleaved from a 200-amino acid precursor (Pearce *et al.* 1991; Mcgurl *et al.* 1992). This protein is able to induce the production of protease inhibitors in tomato. Recently Holton *et al.* (2007) demonstrated in *Solanum pimpinellifolium* that systemin also reduces root growth and seems to have a role in plant development. A further recent study by Berger & Baldwin (2007) reported that systemin does not play a central role in the anti-herbivore defense responses in *N. attenuata*, reflecting that systemin acts differently among species.

RALF (rapid alkalization factor), a 49-amino-acid peptide, was isolated by Pearce *et al.* (2001) while searching for peptides (systemin) that regulate wound response of cultivated tobacco. It elicits MAP kinase activity and when added to tomato or *Arabidopsis* seedlings root growth is immediately arrested (Pearce *et al.* 2001). Transcripts of RALF are rapidly accumulated in *N. attenuata* after mechanical wounding or after abiotic stress like increased

UV-irradiation or wind stress. High levels of transcript can be found in the roots and petioles, but low levels in leaves, flower buds, and mid-ribs (Wu *et al.* 2007 A3). As RALF is not mediating an increase in defense (nicotine, protease inhibitors) in *N. attenuata*, a growing body of evidence is suggesting that RALF is required for growth and development (Haruta & Constabel 2003). Wu *et al.* (2007 A3) silenced RALF in *N. attenuata* and demonstrated that root growth and the size of the root elongation zone were increased, suggesting that *Na*RALF is needed for regulating root growth. However, it is not clear whether RALF could mediate a reduction in root growth as a wound derived signal upon biotic or abiotic stress.

5. Synopsis and outlook

This PhD thesis gives an insight into the growth dynamics of root and shoot growth after simulated herbivore attack and demonstrates that wound-induced jasmonic acid mediate the observed reduction in root growth after wounding the shoot. However, the wounding was simulated in a single event, and does not reflect the natural behavior of feeding larvae, which consume leaves by continuously clipping off and ingesting small pieces of tissue. This process can be considered as a series of mechanical wounds, and is often accompanied by simultaneous introduction of oral secretions and regurgitants. Even the feeding pattern is highly dynamic in space and time as the larvae migrate over the plant and feed on different sites, or moults and thereby disrupts feeding for a period of time. For instance Mithöfer *et al.* (2005) demonstrated in *Phaseolus lunatus* that the continuous damage of leaves via an (artificial) mechanical caterpillar (MecWorm), induced the release of a different blend of volatiles that resembled herbivory more than a single wounding event. Hence, it would be of great interest to study growth and defense dynamics during continuous wounding and to elucidate the plant response. The persistent induction of wound-signals like JA might be integrated in a different manner and could trigger different growth responses.

Furthermore, the PhD thesis demonstrates that hormonal signaling and resource-based trade-offs has intertwined effects on root growth, which are difficult to disentangle. While defense gene expression and JA biosynthesis have received much recent attention, the mechanism of JA-mediated growth inhibition is largely unknown. Hence, the increase in knowledge concerning the mechanism of how JA arrests root growth would provide the possibility to use molecular techniques to uncouple the growth machinery from the defense

machinery. This would allow deeper insights into the regulation of allocation trade-offs and costs of defense after wounding.

Finally this PhD thesis reveals that there is an immediate reduction in root growth following a shoot-wounding event. The observed immediate effect might be explained via hydraulics but an electrical impulse is also conceivable. Interestingly, nearly nothing is known about the effects of electric signals on plant growth, presumably because no adequate methods are available to monitor plant growth at sufficiently high temporal resolution in order to detect the growth response to such rapid signaling.

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7. Root growth dynamics of *Nicotiana attenuata* seedlings are affected by simulated herbivore attack

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Root growth dynamics of *Nicotiana attenuata* seedlings are affected by simulated herbivore attack

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ABSTRACT

Many studies demonstrate resource-based trade-offs between growth and defence on a large timescale. Yet, the short-term dynamics of this growth reaction are still completely unclear, making it difficult to explain growth-defence trade-offs mechanistically. In this study, image-based non-destructive methods were used to quantify root growth reactions happening within hours following simulated herbivore attack. The induction of wound reactions in *Nicotiana attenuata* in the seedling stage led to transiently decreased root growth rates. Application of the oral secretion of the specialist herbivore *Manduca sexta* to the leaves led to a transient decrease in root growth that was more pronounced than if a mere mechanical wounding was imposed. Root growth reduction was more pronounced than leaf growth reduction. When fatty acid–amino acid conjugates (FACs) were applied to wounds, root growth reduction occurred in the same intensity as when oral secretion was applied. Timing of the transient growth reduction coincided with endogenous bursts of jasmonate (JA) and ethylene emissions reported in literature. Simulation of a wound response by applying methyl jasmonate (MeJA) led to more prolonged negative effects on root growth. Increased nicotine concentrations, trichome lengths and densities were observed within 72 h in seedlings that were treated with MeJA or that were mechanically wounded. Overall, these reactions indicate that even in a very early developmental stage, the diversion of plant metabolism from primary (growth-sustaining) to secondary (defence-related) metabolism can cause profound alterations of plant growth performance.

Key-words: *Manduca sexta*; fatty acid–amino acid conjugates (FACs); image analysis; nicotine; plant defence; plant–insect interactions; trichomes.

INTRODUCTION

Throughout their entire life, plants need to acclimate to a suite of fluctuating biotic and abiotic factors. Within the framework of their genetically determined response options that vary throughout ontogeny, they react towards stress situations by maximizing protection against stress while minimizing deviations from optimal growth and development. Herbivore attack is probably the prime biotic stress factor for a wide range of plants. It has been well investigated that acclimation occurs both on the biochemical and morphological level, for example, by increasing defence compounds (Karban & Baldwin 1997), by synthesizing defence-related proteins such as protease inhibitors (PI), by emitting volatiles to attract predators and parasites of herbivores (De Moraes *et al.* 1998), and by altering plant morphology via increased formation of trichomes, thorns or scleromorphy (Kudo 1996; Traw & Dawson 2002; Dalin & Bjorkman 2003). Those defence reactions are necessarily associated with ecological and genetic costs or allocation trade-offs (Heil & Baldwin 2002).

Many studies have demonstrated resource-based trade-offs between growth and defence on a large timescale (Ohnmeiss & Baldwin 1994; Zangerl, Arntz & Berenbaum 1997; Collantes, Gianoli & Niemeyer 1998; Smith & Schowalter 2001; Zavala *et al.* 2004; Walls *et al.* 2005). Yet, it is unclear how fast a trade-off-linked reorganization of plant metabolism, diverting resources away from growth or development towards defence, can occur. In recent years, development of growth imaging methods (Walter & Schurr 2005) has allowed the study of short-term growth responses of above- and belowground sink organs towards fluctuations of environmental factors, such as alterations of atmospheric CO_2 (Walter *et al.* 2005) or light climate (Lai *et al.* 2005; Nagel, Schurr & Walter 2006). Clear responses are often seen best in young seedlings as they are characterized by highest relative growth rates (RGRs) throughout plant ontogeny. There, primary root growth can be monitored relatively easy by cultivation in translucent agar-filled Petri dishes (Walter & Schurr 2005; Nagel *et al.* 2006), while growth of the small leaves is somewhat difficult to analyse because leaves need to be forced mechanically into the focal plane of a camera for image acquisition (Schmundt *et al.* 1998; Walter & Schurr 2005).

In seedlings, defence reactions such as induced formation of PIs and glucosinolates after mechanical wounding of cotyledons or first true leaves in 1-week-old *Brassica napus* plants are reported (Bodnaryk 1992; Cipollini & Bergelson 2000). Yet, for PIs, it has been reported that in *Nicotiana attenuata*, defence-related induction is only possible in the rosette, but not in the seedling or flowering stage (van Dam *et al.* 2001). Hanley & Felton (2007) recently reported that cotyledon removal in seedling stage strongly affected

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growth and flowering in mature plants; however, the short-term growth dynamics following wounding remains still unclear. As *N. attenuata* has become a model system for studying herbivory-induced defence reactions, it would be interesting to elucidate its defence system in the seedling stage in more detail.

When *N. attenuata* is attacked by the specialist lepidopteran herbivore *Manduca sexta*, a diverse set of plant hormones like jasmonate (JA), methyl jasmonate (MeJA) and ethylene are rapidly induced immediately following wounding or herbivore attack (McCloud & Baldwin 1997; Zhang & Baldwin 1997; von Dahl & Baldwin 2004). JA is known to reduce root growth (Staswick, Su & Howell 1992; Uppalapati *et al.* 2005), albeit root growth-promoting effects at low JA and MeJA concentrations have also been reported in literature (Tung *et al.* 1996; Toro, Martin-Closas & Pelacho 2003). However, in theses studies, JA has been directly applied to roots, which does not reflect the situation of herbivory, because JA bursts occur in the wounded leaf tissue and JA is subsequently transported downwards to the root system (Zhang & Baldwin 1997). Ethylene concentrations and emissions have also been reported to increase strongly when *M. sexta* is feeding on *N. attenuata* (Kahl *et al.* 2000), and growth-inhibiting effects of ethylene have been reported in numerous studies (Abeles 1972). Yet, apart from a hormonal-induced decrease of growth activity, an increase of root growth as immediate reaction towards herbivore attack in *N. attenuata* is conceivable as well. Increased export of carbohydrates from shoots to roots within 2 h following simulated herbivore attack or the application of JA to leaves has been reported recently in two studies utilizing the short-lived istotope ^{11}C to monitor carbon flux within the plant (Babst *et al.* 2005; Schwachtje *et al.* 2006). Such a re-allocation of carbohydrates to the root would ecologically make sense, as carbohydrates would then be retrieved from leaf-consuming herbivores and could be used for regrowth and/or reproduction after the threat has passed by. Increasing carbohydrate import to the root can lead to immediate and strong induction of root growth (Aguirrezabal, Deleens & Tardieu 1994; Nagel *et al.* 2006).

Hence, the aims of our study were (1) to monitor whether young seedlings of *N. attenuata* were able to induce defence-related reactions within a short time upon a simulated herbivore attack and (2) to assess the dynamics of root growth reaction following such an attack to provide more insight into the temporal coordination of resource allocation trade-offs during defence reactions.

MATERIAL AND METHODS

Plants and cultivation systems

Seeds of *Nicotiana attenuata* Torr. ex Wats. (Solanaceae) from an inbred line originating from a natural population in Utah were smoke treated and sterilized as described by Krügel *et al.* (2002). Plants were raised in square Petri dishes (120 × 120 × 17 mm). Five seeds were placed in line in the Petri dishes containing 125 mL sterile 1% Phytagel (w/v) with full-strength Gamborg B5 Medium (Duchefa, Haarlem, the Netherlands). Seeds were pushed approximately 1 mm into the agar to ensure that roots were growing within the agar. The Petri dishes were sealed with fabric tape (Micropore; 3M Health Care, Neuss, Germany) to facilitate gas exchange. They were set almost horizontal until germination (5 d after sowing). Afterwards, the Petri dishes were put almost vertical (85°) to guarantee that the roots grew along the lid of the Petri dish. The shoots grew in the air-filled volume of the Petri dish. In this system, treatments were applied as specified further below. The plants were grown in cultivation rooms under 26 °C during the light phase and under 22 °C during the dark phase with a photoperiod of 14 h. They were exposed to a photon flux density of 85 μmol m^{-2} s^{-1}. Light was switched on at 0600 h and switched off at 2000 h.

A slightly different cultivation system than the conventional one described earlier was used for application of treatments in high-resolution growth monitoring experiments (Fig. 1a) and for application of MeJA. In this 'microrhizotron' set-up, which is described in more detail elsewhere (Nagel *et al.* 2006), shoots grew outside the Petri dish, which was almost completely filled with Phytagel (200 mL) (Duchefa). After the Phytagel (Duchefa) had cooled down, three small holes were melted into the side of the Petri dish using a glowing bolt to guarantee sterility.

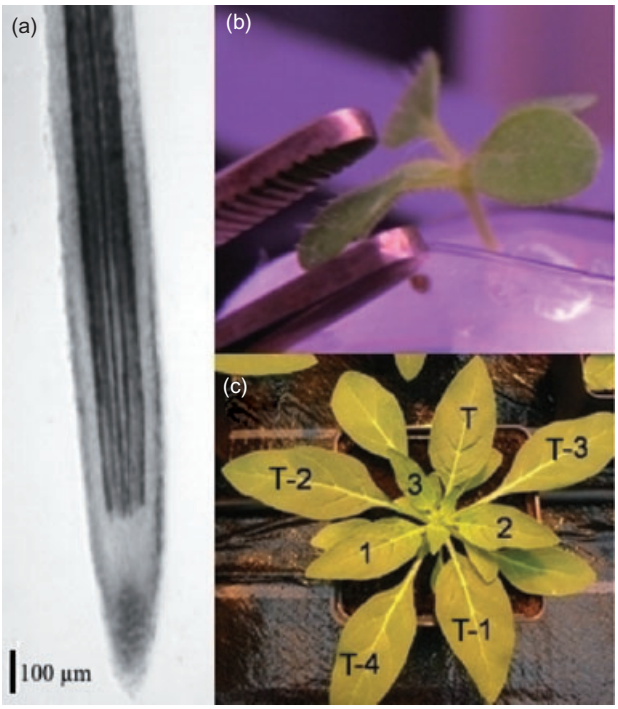

Figure 1. Experimental set-up. (a) Original image of a root tip acquired by a CCD camera and used for digital image sequence processing. (b) Wounding procedure with sterile tweezers. (c) *Nicotiana attenuata* at rosette stage before wounding treatments were applied. Numbers depict successive leaf developmental stages. T, transition leaf (see text for explanation).

Immediately after piercing, 2-day-old sterile seedlings from the conventional system were transplanted into each hole. The holes were sealed with sterilized silicon fat to prevent contamination (Baysilone-Paste; Bayer, Leverkusen, Germany). The Petri dishes were then set almost vertical and were exposed to the same experimental conditions as described earlier.

Wounding treatments

The inducibility of wound reactions in seedlings was tested by applying the wound-signalling substance MeJA (experiments 1 and 3). MeJA was dissolved in 1 μL lanolin paste (Sigma-Aldrich, Steinheim, Germany). This solution was applied directly on the primary leaf (the first true leaf appearing after cotyledons) of *N. attenuata* 16 d after germination. Control plants were treated with lanolin paste only. In this developmental stage, the third leaf just emerged and shoots were about 15 mm high. For these experiments, shoots were grown outside of the Petri dishes (as described previously) to avoid a direct effect of volatile MeJA on the roots. In experiment 1, plants treated with 500 ng MeJA were harvested 4 d after application for nicotine and trichome analysis. In experiment 3, plants were treated with 50, 500 or 5000 ng MeJA, respectively.

For the other wounding experiments of seedlings (experiments 2, 4–6), approximately one-third of the primary leaf tissue was squeezed 16 d after germination using sterile tweezers to simulate herbivore attack (Fig. 1b). By this procedure, 13 ± 3% of the total leaf area (including the cotyledons) was damaged. Immediately after wounding, one of the following substances was applied: (1) 1 μL H_2O; (2) 20 μL oral secretions and regurgitants of the natural herbivore *M. sexta* (diluted 1:5 with phosphate buffer; spit); (3) fatty acid–amino acid conjugates [FACs, *N*-linolenoyl-L-Gln (50 ng μL^{-1}, 0.12 mM) and *N*-linolenoyl-L-Glu (138 ng μL^{-1}, 0.34 mM)], which are known to be the main elicitors of plant defence responses in *M. sexta* regurgitant. To monitor the basic reaction of root growth (experiments 4 and 5), the plants were grown inside Petri dishes; therefore, the treatments were performed within sterile clean benches to avoid any contamination. For monitoring high-resolution effects on root growth, shoots were grown outside the Petri dish and treatments were applied in the microrhizotron set-up (experiment 6). All treatments were performed at 1300 h.

To test the effect of simulated herbivory on leaf growth (experiment 7), two leaves (developmental stages T-3 to T-1; see further below) were wounded and growth reaction was either monitored in one of those leaves or by characterizing growth of the entire leaf rosette area or by characterizing 'systemic' effects on younger, strongly expanding leaves (+1 or +2). Wounding was applied by a pattern wheel as described in Halitschke *et al.* (2000); the wounded area was treated with 20 μL of water (buffered to 7.8 pH by 50 mM phosphate buffer; H_2O) or 20 μL oral secretions and regurgitants of the natural herbivore *M. sexta* (diluted 1:5 with phosphate buffer; spit).

Trichome quantification

Glandular trichomes of *N. attenuata* contain high amounts of nicotine and are hence chemical and physical defence systems (Duffey 1986; Baldwin & Karb 1995). Trichomes emerge from predisposed epidermal cells usually in an early developmental stage but can also develop during secondary growth of hypocotyl and stem. For quantification of trichome length and number, complete hypocotyls were photographed using a binocular microscope immediately prior to the destructive harvests for nicotine analysis in experiments 1 and 2. The contrast of the images was improved with Corel Photo Paint (Corel Corporation, New York, NY, USA). The projection of trichomes was used to determine their length and their number per millimetre hypocotyl length. Their densities were therefore underestimated as trichomes pointing towards the microscope were not visible. Because all plants were treated equally, the determined 'quasi-lateral' trichome density was a good proxy for the total trichome density. Moreover, in this study, we were not interested in the absolute trichome density, but rather in the induction of trichome formation per se by wounding. The total lengths of hypocotyls were also determined to test whether the induced trichome density was due to reduction in growth or to an increase in absolute trichome number. However, no significant difference in hypocotyl length and diameter was found between treatments.

Nicotine analysis

The entire shoot was harvested in experiments 1 and 2, weighed and immediately shock frozen in liquid N_2. Plant material was ground frozen with a pestle in a 2 mL tube. The samples were extracted with 400 μL of 40% MeOH (v/v) containing 0.5% acetic acid (v/v) and were shaken at 23 °C for 1 h. After extraction, the samples were centrifuged (10 min, 9 300 g), filtered (0.45 μM) and transferred into a vial for the HPLC analysis. Nicotine was separated by a Merck–Hitachi HPLC system (Darmstadt, Germany) on a Multospher column (120 RP 18-HP 3 μm, 250 × 4 mm; CS-Chromatographie Service, Langerwehe, Germany) attached to a Multospher pre-column (Multospher 100 RP 18; 5 μm, CS-Chromatographie Service) and detected with a diode array detector at 254 nm. The mobile phases were the following: A: water (0.1% H_3PO_4); B: acetonitrile. The flow rate was 0.5 mL min^{-1}, and the following gradient programme was performed: 0–28 min, linear gradient 95–70% A; 28–38 min, isocratic 70% A; 38–60 min, linear gradient 70–40% A (remaining fraction for B, respectively).

Basic root growth monitoring

Growth velocity of the primary root tip (V_{Tip}) of each individual seedling was quantified using a ruler in experiments 1–6. Each day, the position of the root tip was marked with a pen on the rear side of the Petri dish. For root growth analysis, we included only plants with a root length between 35 and 50 mm at treatment day. All suitable plants were

treated and V_{Tip} was averaged within each Petri dish. The number of Petri dishes was taken as the replicate number for each treatment in experiments 3–5. Temperature of the Phytagel (Duchefa) of one representative dish was recorded once per minute using a thermocouple attached to a datalogger (Delta-t Devices Ltd, Cambridge, England).

Root growth data were normalized in Figs 4–7 by calculating the ratio between the population mean values of growth at the depicted point in time and growth at the time of treatment.

High-resolution root growth monitoring

Five days after germination, the Petri dishes were positioned into the microrhizotron set-up. Every 20 s, an image of the root growth zone was taken with a CCD camera (Sony XC-ST50; Sony, Köln, Germany; Fig. 1a) with a resolution of 700 × 480 pixels. Each pixel corresponded to a real area of 3.6 × 2.5 mm. Infrared illumination ($\lambda = 940$ nm) enabled image acquisition also during the dark phase. The camera was equipped with a low-pass infrared filter (RG; Schott, Mainz, Germany) to block visible irradiation. Each replicate was measured for at least 24 h. The root tips were followed via a tracking algorithm that controlled a set of x-y moving stages that repositioned the entire Petri dish and hence the root tip, when it approached the borderline of the image. The custom-made algorithms for root tracking and image sequence acquisition are based on a digital image sequence processing software package (Heurisko; Aeon, Hanau, Germany) (Schmundt *et al.* 1998; Walter *et al.* 2002; Walter, Feil & Schurr 2003). The image sequences were used to calculate the root tip velocity (V_{Tip}) and the distribution of relative elemental growth rate (REGR) along the root growth zone via the structure tensor method (Schmundt *et al.* 1998; Haußecker & Spies 1999). *Nicotiana attenuata* grew at this developmental stage with an average V_{Tip} of 550 ± 20 μm h^{-1} and 310 ± 20 μm h^{-1} during light and dark phases, respectively.

Leaf growth measurements

To assess whether the observed growth effects were specific for roots or whether they might have a more general impact on overall growth performance of the plant, leaf growth analyses were performed with *N. attenuata* plants in a developmental stage typical for herbivory experiments in this plant (experiment 7). It was not possible to analyse leaf growth in seedlings of the developmental stage used throughout the rest of this study, as leaves were too small. On the other hand, it was also impossible to monitor root growth in the 'usual stage' of herbivory experiments because of the extended root system of the plant in this developmental stage and because it is not possible to raise plants to this size in the Petri dish system.

For leaf growth analysis, the plants were raised in the greenhouse at 16 h/8 h light/dark cycles with 28 °C/22 °C, respectively. Mean midday irradiance [photosynthetically active radiation (PAR)] averaged 800 μmol m^{-2} s^{-1}, with maximal values of 1200 μmol m^{-2} s^{-1}. When sunlight was lower than 130 μmol m^{-2} s^{-1}, artificial illumination was provided by SON-T Agro 400 W (Philips, Köln, Germany) lamps. The plants were potted in soil and watered and provided with nutrient solution as needed. Approximately 4 weeks after germination, the plants were assigned to the wounding treatments (Fig. 1c). At this stage, leaves were characterized according to the terminology of Wait, Jones & Coleman (1998) and Halitschke *et al.* (2000), where the sink–source transition leaf T is defined as the youngest, completely unrolled leaf. All other leaves were numbered in chronological order of appearance relative to T (Fig. 1c).

Growth of the entire leaf rosette area (total leaf area) was recorded by measuring the length and width of all leaves each day with a ruler. Individual leaf area A is given by $A = 0.64 \times \text{length} \times \text{width}$. The allometric factor of 0.64 was determined empirically in a pre-experiment by sketching the outline of 50 leaves of different developmental stages on paper (density: 80 g m^{-2}), cutting and weighing the paper and relating its area to the product of length and width. Total leaf area was calculated by taking the sum of all individual leaf areas. RGR of the total leaf area was then calculated (unit: % d^{-1}) by $RGR = 100 \times \ln$ (area at day 2/area at day 1), assuming an exponential growth model.

The increase of individual leaf area was monitored in high temporal resolution by attaching a thread to the leaf tip, guiding it over a custom-made rotary displacement transducer (F. Gilmer, Jülich, Germany) and straining it with a weight of 12 g. The rotation of the displacement transducer was proportional to the increase in leaf length and was detected as the electrical signal of a variable resistor. The length increase was analysed with a temporal resolution of 10 min, and the RGR of the leaf length was calculated as described earlier (unit: % h^{-1}).

Statistical analysis

Data were analysed with Statistica, version 6.0 (StatSoft Inc., Tulsa, USA). Trichome density, trichome length and nicotine concentration following MeJA applications were each tested by *t*-tests. Two-way analyses of variance (ANOVAs) with treatments and harvest days as main effects were performed for the destructive wounding experiment. V_{Tip} data were normalized by dividing all values by V_{Tip} at treatment day and were analysed with repeated measures one-way ANOVA. The experiment with FACs was tested with one-way ANOVA. All ANOVAs were followed by Fisher's protected least significant difference (LSD) post hoc comparisons.

RESULTS

Trichome and nicotine analysis in seedlings

Plants treated with 500 ng MeJA (experiment 1) significantly increased trichome density 1.3-fold ($P = 0.003$, Fig. 2a) and trichome length 1.6-fold ($P < 0.001$, Fig. 2b) within 4 d. Moreover, nicotine concentrations increased 8.8-fold compared to the controls ($P < 0.001$) showing that both

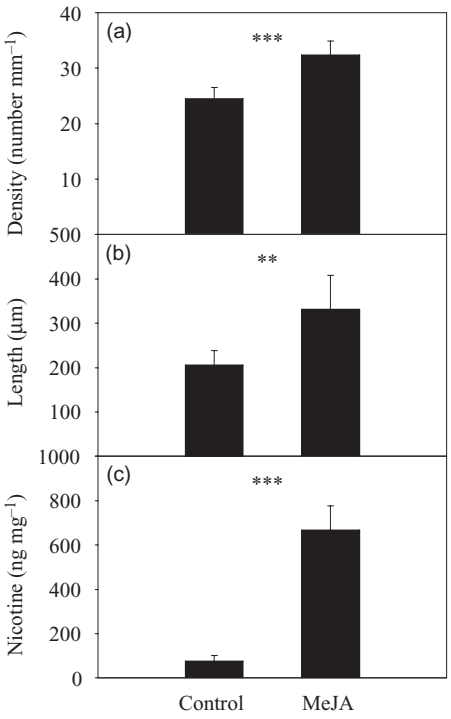

Figure 2. Defence properties of *Nicotiana attenuata* 4 d after application of 500 ng methyl jasmonate (MeJA) on primary leaf (experiment 1). (a) Trichome density (mean values ± SE; control, $n = 6$; MeJA, $n = 8$). (b) Trichome length (mean values ± SE; control, $n = 6$; MeJA, $n = 8$). (c) Nicotine concentration of fresh weight (mean values ± SE; control, $n = 4$; MeJA, $n = 5$). Asterisks indicate significant differences between control and MeJA (**$P < 0.01$, ***$P < 0.001$).

chemical and physical defence mechanisms were strongly induced (Fig. 2c). Wounding treatments (experiment 2) significantly affected trichome densities ($F_{2,39} = 5.633$, $P = 0.007$; Fig. 3a). The application of H_2O to wounds increased trichome density significantly within 72 h compared to the control plants ($P = 0.006$). Spit-treated plants did not significantly increase their trichome density within 72 h compared to control plants ($P = 0.066$); however, we found a significant increase in trichome densities when compared to the treatment day ($P = 0.001$). Whole plant nicotine contents were significantly increased by wounding treatments ($F_{2,39} = 19.672$, $P < 0.001$; Fig. 3b). The application of H_2O and spit to mechanically wounded leaves significantly increased nicotine concentrations within 24 h compared to control plants (1.7-fold, $P < 0.001$; 1.6-fold, $P < 0.001$, respectively). After 72 h, nicotine concentrations of H_2O- and spit-treated plants still remained significantly higher compared to controls, but no further increase was found (Fig. 3b). These results demonstrate clearly that *N. attenuata* is already inducible in very early developmental stages, and hence the seedling represents a convenient model to be used in the sterile Petri dish system for root growth analysis.

To test whether cultivation of *N. attenuata* seedlings in low light conditions necessary for root growth monitoring leads to erroneous nicotine concentrations, a side experiment with four Petri dishes in the greenhouse was performed. There, very similar nicotine concentrations were reached as in the growth room conditions (88 ± 7 ng mg^{-1}, $n = 4$; data not represented graphically).

Basic root growth analysis

Plants treated with MeJA (experiment 3) significantly reduced V_{Tip} in a dose–response relationship within 24 h ($F_{3,63} = 68.112$, $P < 0.001$; Fig. 4). Moreover, different wounding treatments (experiments 4 and 5) significantly affected V_{Tip}, and statistical differences were found among the factors: treatment, time and the interaction of both factors ($F_{3,36\ \text{Treatment}} = 15.334$, $P < 0.001$; $F_{2,72\ \text{Time}} = 17.248$, $P < 0.001$; $F_{6,72\ \text{Time×Treatment}} = 4.258$, $P = 0.001$; Fig. 5). Wounding of leaves with the application of water (H_2O) significantly reduced root growth by about 27% compared to the control plants ($P = 0.009$), but growth started to recover already during the second day (Fig. 5, experiment 4). To understand whether the reduction of root growth was only due to a reduction in photosynthetic active leaf area, we excised the complete primary leaf ('excised' treatment) representing more than three times the leaf area which was wounded in the H_2O treatment. No significant reduction of V_{Tip} was found compared to the H_2O treatments ($P = 0.425$, Fig. 5). These results demonstrate that the short-term reduction of root growth induced by wounding cannot exclusively be explained through the diminution of the photosynthetic leaf area, but other factors must play a role. Strongest reduction of root growth was observed for the

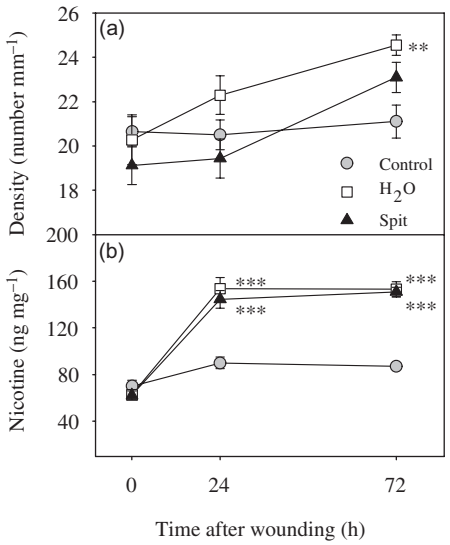

Figure 3. Defence dynamics of *Nicotiana attenuata* after wounding and application of 1 µL water (H_2O) or 1 µL oral secretions of *Manduca sexta* (spit) diluted 1:5 with water (experiment 2). (a) trichome densities and (b) nicotine concentration of fresh weight (mean values ± SE, $n = 5$). Asterisks indicate significant differences between treatments and control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

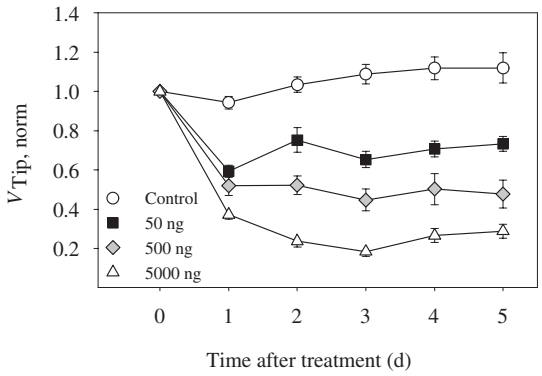

Figure 4. Normalized values of root tip growth (V_{Tip}) after application of different amounts of methyl jasmonate (MeJA) dissolved in lanolin paste (experiment 3, mean values ± SE, $n = 15$).

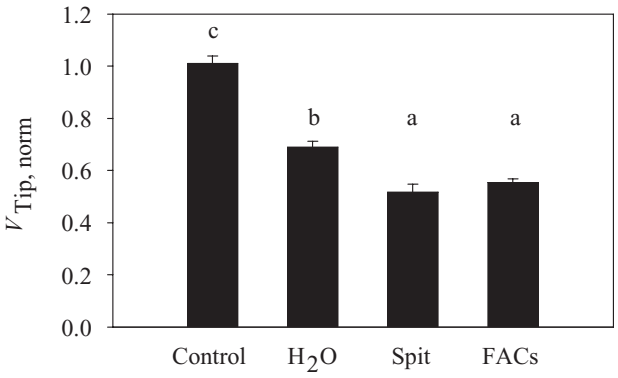

Figure 6. Normalized values of root growth (V_{Tip}) 24 h after wounding (experiment 5). Primary leaf was wounded with tweezers and immediately supplied with 1 μL phosphate buffer (H_2O) or oral secretions of *Manduca sexta* (spit) diluted 1:5 with phosphate buffer or fatty acid–amino acid conjugates (FACs) (mean values ± SE, $n = 10$). Different letters indicate significant differences at $P < 0.05$ level.

treatment with oral secretions and regurgitants of *M. sexta*. V_{Tip} decreased to less than 50% of the initial value and was significantly lower than V_{Tip} of H_2O-treated plants ($P = 0.006$, Fig. 5). However, the spit-treated plants increased V_{Tip} significantly over time and had significantly higher values during the second and the third day compared to the first day (second day, $P = 0.001$; third day, $P < 0.001$). These results demonstrate that the application of spit to wounds decreases root growth stronger than mechanical damage alone. Yet, the growth reduction induced by a single wounding event is only transient; the recovering phase is started during the second day after treatments.

The effect of spit could be reproduced when FACs occurring naturally in oral secretions of *M. sexta* were applied to the wounded leaves ($F_{3,36} = 80.354$, $P < 0.001$; Fig. 6; experiment 5). These results indicate that spit transiently reduces root growth for about 24 h and demonstrates that FACs are the elicitors of this transient reduction in root growth.

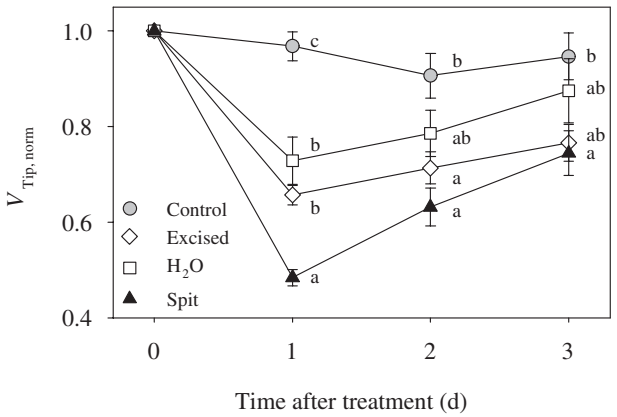

Figure 5. Normalized values of root tip growth (V_{Tip}) after wounding (experiment 4). Primary leaf was wounded with tweezers and immediately supplied with 1 μL water (H_2O) or oral secretions and regurgitant of *Manduca sexta* (spit) diluted 1:5 with phosphate buffer (mean values ± SE, $n = 10$). Different letters indicate significant differences at $P < 0.05$ level.

High-resolution root growth analysis

To elucidate root growth dynamics within the first 24 h after wounding, we analysed root growth by digital image sequence processing with high spatial and temporal resolution (experiment 6). Control plants displayed a diel variation of root growth activity (V_{Tip}) that was correlated to the temperature observed in the Phytagel (Duchefa) (Fig. 7a,b): from 1300 h to the end of the day, temperature and V_{Tip} of control plants remained largely constant. At night, V_{Tip} and temperature decreased monotonically, and from dawn until 1300 h, they rose again to their initial values. As temperature strongly affects root growth (Pahlavanian & Silk 1988; Walter *et al.* 2002), the diel fluctuations affecting root growth in all treatments can be considered to be caused by the prevailing temperature regime and will not be discussed later on. In contrast to this parallel behavior of temperature and root growth, wounded plants treated with H_2O showed an immediate decrease of V_{Tip} throughout 90 min following wounding at 1300 h. Spit-treated plants decreased growth for more than 150 min down to 50% of the initial value of V_{Tip} and remained 10% below the H_2O-treated plants. At night, V_{Tip} of plants from both treatments approached V_{Tip} of control plants gradually, which is best displayed by differences between control and treatment values (Fig. 7c). Seventeen hours after treatment, exactly when light was switched on again (Fig. 7b), all three populations showed the same V_{Tip}.

Two hours after wounding, both treatments showed reduced REGR distributions compared to control plants throughout the entire growth zone (Fig. 8a). The strongest difference between H_2O and spit treatment was present between 1.0 and 1.2 mm behind the root tip. Maximal REGR was decreased in both treatments to about 60% of that of control plants. In the middle of the night, maximal REGR was almost comparable to that of control plants again, but growth zone length was much shorter in both

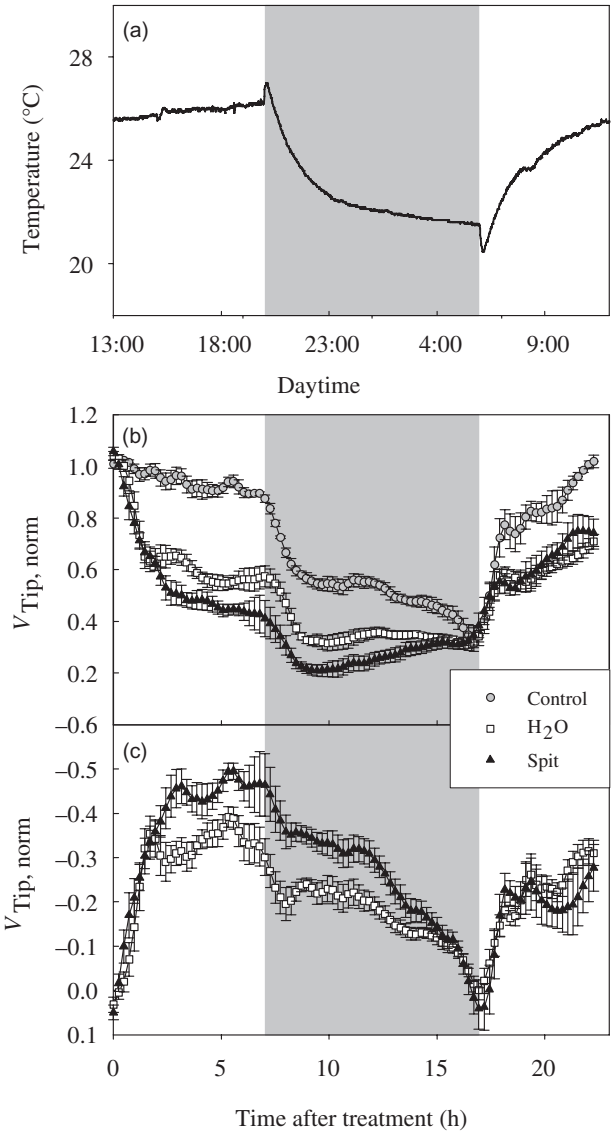

Figure 7. Time series of high-resolution root growth analysis (experiment 6). (a) Temperature course over 24 h during the experiments. Light was switched on at 0600 h and switched off at 2000 h (night period, gray shaped). Treatments were performed at 1300 h. (b) Normalized values of root growth velocities (V_{Tip}) with high resolution after wounding and the application of water (H_2O) or 1 μL oral secretions of *Manduca sexta* (spit) diluted 1:5 (mean values ± SE, $n = 5$). (c) For each specific time point, values of control and treatment were subtracted and differences are displayed.

treatments (Fig. 8b). Now, strongest differences between H_2O and spit-treated plants were present in the basal flank of the REGR distribution, while the apical flanks were practically identical.

Leaf growth analysis

For leaf growth, diel variation was even more pronounced than for root growth and was not directly related to the variation of temperature (Fig. 9a, experiment 7). Highest growth rates were reached in the early morning (when temperature was low; compare Fig. 7a) and growth rate decreased throughout the afternoon, reaching zero growth at night. Because of this peculiarity of the diel leaf growth cycle, wound treatments in the depicted experiments were applied at the beginning of the day. When treatments were performed in the afternoon or evening, no growth effect was observed (data not shown), probably because of the fact that the leaves were practically not growing at this time at all. Even when wounding was performed in the early morning, total leaf area growth was not decreased strongly (Fig. 9b). Only for the first 24 h period immediately following wound treatment, slight effects were visible with RGR of water- and spit-treated plants ranging about 10% below that of control plants (19.2 and 19.5% d^{-1} versus 21.4% d^{-1}, in water- and spit-treated plants versus control plants, respectively). Treated leaves showed a rapid initial decrease of RGR, with more pronounced diminution of growth in spit- versus water-treated leaves (Fig. 9c). Approximately 5–6 h after treatment, RGR of water- and spit-treated leaves was comparable again and did not differ strongly from RGR of control leaves anymore. Systemically affected leaves (Fig. 9d) showed an even weaker, but noticeable response, which also lasted for about 5–6 h. Leaf growth

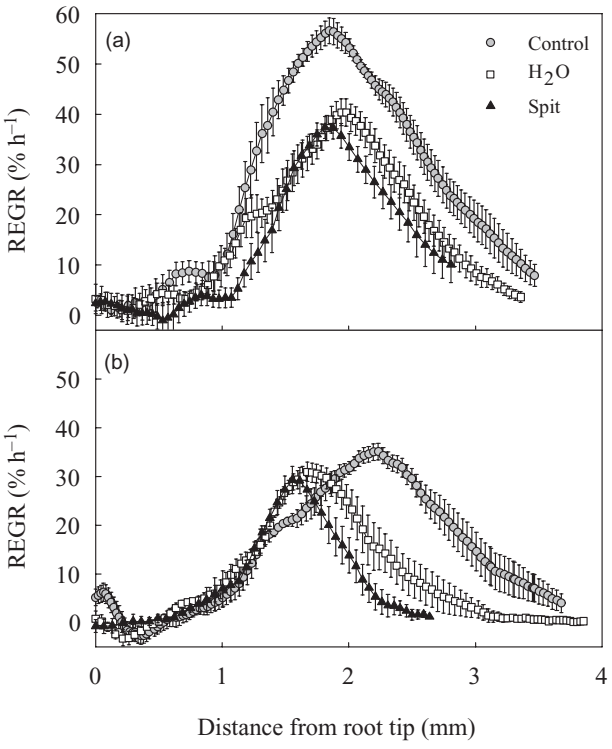

Figure 8. Distribution of relative elemental growth rate (REGR) along the root growth zone (a) 4 h (day) and (b) 11 h (night) after wounding of *Nicotiana attenuata* and application of water (H_2O) or 1 μL oral secretions of *Manduca sexta* (spit) diluted 1:5 (experiment 6, mean values ± SE, $n = 5$). REGR distributions were averaged over 1 h, from 3–4 h and 11–12 h after treatment, respectively.

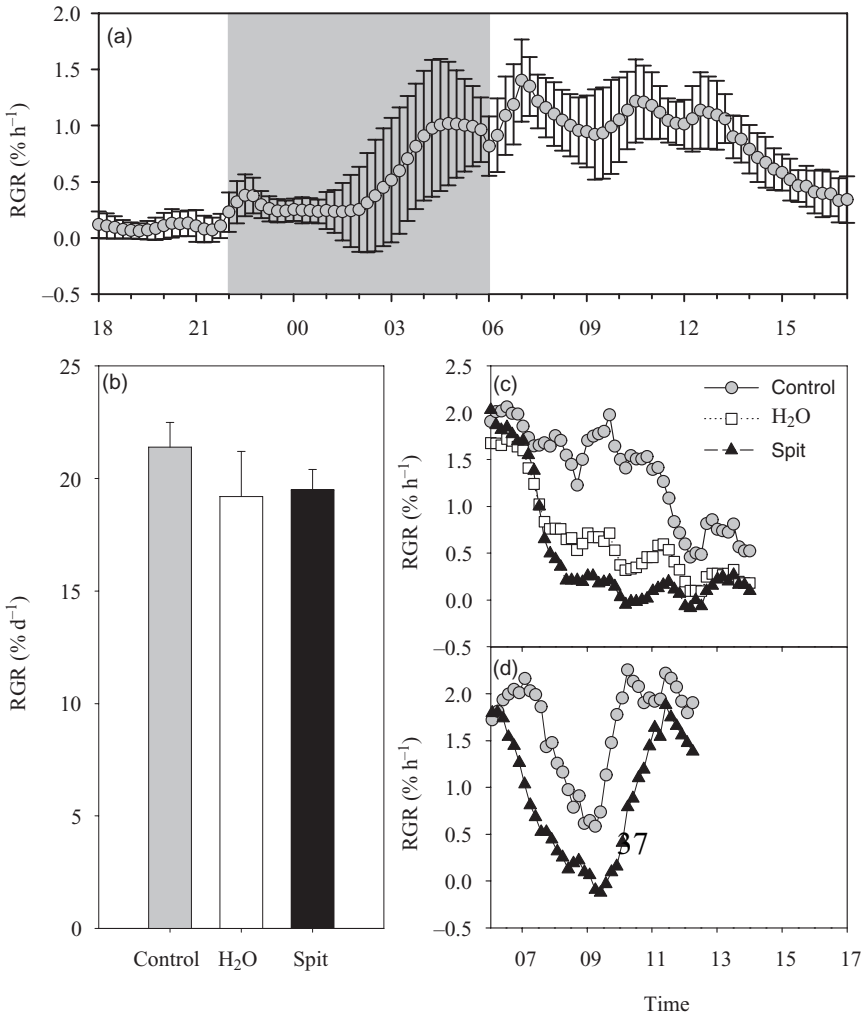

Figure 9. Leaf growth of *Nicotiana attenuata* after wounding and the application of 20 μL water (H_2O) or oral secretions of *Manduca sexta* (experiment 7, spit). Treatments were performed at 0600 h. (a) Time series of relative growth rate (RGR) throughout 24 h (mean values ± SD, $n = 3$). (b) RGR of total leaf area after wounding and the application of H_2O and spit (mean values ± SD, $n = 3$). (c) Time series of RGR of wounded leaf after the application of H_2O and spit. (d) Time series of RGR of systemically affected leaf after wounding and application of spit.

experiments have also been performed under laboratory conditions at lower light intensity. There, no detectable growth reduction following wound treatments was observed (data not shown).

DISCUSSION

The seedling system

The results demonstrate clearly that *N. attenuata* shows inducible defence in very early developmental stages. The application of MeJA induced strongly increased whole plant nicotine concentration, hypocotyl trichome length and trichome density within 4 d. It has been pointed out that PIs are not inducible in seedlings of *N. attenuata*, but only in later developmental stages (van Dam *et al.* 2001), indicating that not the entire suite of defence mechanisms is active in seedlings and is hence constrained by plant ontogeny. This fact is also supported by the finding that in *Nicotiana sylvestris*, nicotine induction after wounding only occurs during the rosette stage, but not during the elongation or flowering stages (Ohnmeiss & Baldwin 2000).

The induced increase of hypocotyl trichome length and density may play an important role for repelling herbivores from seedlings as here, the hypocotyl still represents a relatively high fraction of the plant tissue. Hitherto, increasing trichome densities have only been reported on a larger timescale for leaves emerging after herbivore attack (Agrawal 1999; Traw & Dawson 2002; Traw & Bergelson 2003). Leaves that are already differentiated by the time of herbivore attack are not capable to strengthen their physical defence systems as this can only be performed in developing and non-mature tissues (Mayers & Bazely 1991; Nagata *et al.* 1999). Clearly, the hypocotyl epidermis cells are still able to differentiate into trichomes, as this tissue is displaying secondary growth. Hence, hypocotyl trichome density may represent a powerful monitoring trait for non-invasive detection of defence induction in similar experiments.

The dynamics of nicotine and trichome induction were different for the two treatments investigated (Fig. 3). While the 'pure' wounding treatment (application of H_2O) induced both nicotine and trichome formation rapidly, the spit treatment also led to a rapid increase of nicotine content, but trichome density was only increased 3 d after

wounding. In addition to the root growth data, which will be discussed later, this may be an indication for the transiently reduced growth potential of the seedling upon herbivoral attack. If the application of spit specifically leads to a transient depletion of metabolites required for growth processes, a delayed elongation of trichomes would be a logical consequence.

It has to be pointed out that all experiments have been conducted at very low light intensity to allow high-resolution root growth monitoring. It is conceivable that at low light intensity, both growth and defence may be carbon limited, and hence it might be problematical to draw conclusions for a trade-off between growth and defence. It is well documented that seedling root growth is suboptimal under the light intensity reached in this cultivation system (Nagel *et al.* 2006). Yet, the fact that nicotine concentrations in greenhouse-grown seedlings and growth room-cultivated seedlings were comparable rather leads to the speculation that defence mechanisms are realized in a relatively 'normal' manner despite the limited availability of photosynthates. This may in turn be realized by the simple fact that the plant body itself is much smaller under low light conditions, and a low amount of nicotine or other defence-related substances consequently results in concentrations similar to those found in high-light-grown plants. A detailed analysis of the relation between light intensity and defence induction would thus surely be interesting in future studies.

Inhibition of root growth by application of MeJA

JA and MeJA inhibit root growth when applied directly to roots; however, growth-promoting effects at low concentrations are also reported in literature (Staswick *et al.* 1992; Tung *et al.* 1996; Creelman & Mullet 1997; Toro *et al.* 2003; Uppalapati *et al.* 2005). In this study, MeJA was applied to leaves and root growth inhibition was observed subsequently. In contrast to application of spit, no recovering of root growth was observed during the experiment, suggesting that MeJA was constantly diffusing from lanolin paste into the leaf and was thereby maintaining growth inhibition. Using radioactively labelled JA, Zhang & Baldwin (1997) demonstrated a rapid transport of JA from leaves to roots via the phloem in *N. sylvestris*. They suggest that wound-induced JA from leaves accounts for the systemic increase of JA in the roots peaking approximately 180 min after a herbivore attack and mediating de novo synthesis of nicotine. Moreover, Thorpe *et al.* (2007) reported a rapid transport of ^{11}C-labelled MeJA from leaves to roots in *Nicotiana tabacum* within 60 min.

Growth reaction after wounding treatments

An immediate root growth response was observed for the wounding treatments. The first rapid decrease clearly points to a hydraulic response of the plant as loosing water or turgor pressure is immediately reducing root growth (Nagel *et al.* 2006). Apart from the hydraulic response, there is a strong response towards the spit of *Manduca* larvae, which coincides with the time frame of about 2–3 h that was reported for the systemic increase of JA transported from shoot to root. Baldwin *et al.* (1997) reported that JA pools in roots are increased systemically (3.5-fold) within 180 min following mechanical wounding. This increase in JA pools of roots could account for the reduction in root growth. The 10-fold higher JA burst following herbivory of *M. sexta* larvae or the application of their spit to wounds (Kahl *et al.* 2000) could be responsible for a more pronounced inhibition of root growth. Seventeen hours after the spit treatment, root growth of spit- and H_2O-treated plants was comparable again and had even reached the same intensity as that of control plants. Hence, it is conceivable that JA pools or ethylene concentrations in seedling roots have decreased by this time again. However, the ethylene burst elicited by *Manduca* or their spit may mediate a reduction of root growth as well.

Recently, Schwachtje *et al.* (2006) reported that a β-subunit of an SNF1-related kinase, GAL83, which is induced via herbivore specific elicitors, regulates root–shoot partitioning of carbohydrates after herbivore attack independently of JA signalling. This change in source–sink relations could in itself act as a signal, as changes in carbohydrate concentrations are known to affect gene regulation (Koch 1996; Smeekens 1998). However, an increase in carbohydrate import to roots would rather result in an increase of root growth as reported by (Nagel *et al.* 2006), suggesting a down-regulation of root growth via wound signalling by ethylene or JA.

Decreased import of carbohydrates due to destruction of a relevant fraction of photosynthetic tissue can lead to decreased root growth (Aguirrezabal *et al.* 1994; Nagel *et al.* 2006). Hence, the permanent destruction of 13% of the source leaf tissue during the wounding treatment may be responsible for the slow recovery of root growth throughout several days (Fig. 5) and for lower growth of H_2O- and spit-treated plants compared to control plants in the morning after the treatment day (Fig. 7).

The strongest difference concerning the distribution of REGR between H_2O and spit treatment was initially observed about 1 mm behind the root tip in the beginning of the central elongation zone. Here, meristematic activity has practically ceased, which is indicated by increase of REGR. This means that spit-induced growth reduction might mainly be caused by reduction of growth during an early stage of cell expansion. Eight hours later, cells from this region have been displaced towards the end of the growth zone, where now strongest differences are observed. Hence, spit might mostly affect a cell file in an early stage of cell expansion, and the root zone recovers as new cells that are formed in the meristematic part of the root growth zone at the very tip are being displaced into the elongation zone.

Unfortunately, it was impossible to perform leaf and root growth monitoring in response to simulated herbivore attack in comparable stages of plant development. Nevertheless, the reaction of leaf growth was monitored in a later

stage of development to gain some insight whether growth reactions might be restricted to roots or whether they might be more pronounced in the aboveground plant part, where herbivore attacks occur and where hence a growth reduction would intuitively seem more meaningful. The results show clearly that the leaf rosette, at least in this developmental stage, shows a weaker reaction than the primary root. If there is any reduction at all towards a single wounding event, it is also of transient nature and seems to prevail for a shorter time than observed in the root. Yet, it has to be pointed out that because of the overall low leaf growth rate during large parts of the diel cycle and because of the high variability among individuals, leaf growth results should be interpreted carefully.

In summary, our results obtained on seedling plants in laboratory conditions clearly indicate that a single herbivore attack at a leaf, which is sensed via the presence of FACs, leads to an immediate growth response in the root. The growth reduction is transient and is regulated by wound-induced JA transported from leaves to roots. Following herbivore attack, the plant diverges its metabolism towards the production of defence substances and structures within hours, leaving a significant imprint in a reduction of growth. The results of Schwachtje *et al.* (2006) show that in a more realistic situation under higher light intensity, allocation of sugars to roots is increased upon herbivoral attack, leading not to stronger growth, but to increased C storage there. Overall, the picture emerges that not the leaf attacked aboveground, but the belowground root system turns down its spatial expansion and strengthens its function as a 'safe retreat' for carbon to produce reproductive growth eventually.

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8. Herbivore induced jasmonic acid bursts in leaves of *Nicotiana attenuata* mediate short-term reductions in root growth

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Abstract

Root growth in *Nicotiana attenuata* is transiently reduced after application of oral secretions (OS) of *Manduca sexta* larvae to wounds in leaves. Feeding *Manduca sexta* or OS-elicitation is known to results in jasmonic acid (JA) and ethylene bursts, and activates a suite of defense responses. Because both plant hormones are known to strongly reduce root growth, their activation might account for the observed reduction of root growth following herbivory. To test this hypothesis, we measured primary root growth with digital image sequence processing at high temporal resolution in as*LOX3* and ir*COI1* seedlings which are impaired in JA biosynthesis and perception, respectively and wild-type (WT) seedlings. Higher root growth rates in ir*COI1* compared to WT were observed after OS elicitation. The dynamics of wound-induced root growth reduction coincide with the dynamics of root growth reduction induced by external application of methyl JA. In an experiment with 1-methylcyclopropen (1-MCP), a potent ethylene-receptor blocker, no wounding-specific difference between growth of 1-MCP-treated plants and non-treated plants was observed, suggesting that wound-induced endogenous JA and not ethylene mediates the wounding-specific reduction in root growth. Yet, inhibiting the ethylene response by applying 1-MCP led to markedly increased root growth compared to that of control plants, indicating ethylene normally suppresses plant growth potential in *N. attenuata* seedlings.

Introduction

When the specialist lepidopteran herbivore *Manduca sexta* attacks *Nicotiana attenuata*, the immediate growth reductions that are observed are more severe in the root than in the shoot (Hummel *et al.* 2007). Merely wounding leaves mechanically also leads to transient root growth reduction, but more pronounced effects are found when oral secretion and regurgitants of *Manduca sexta* (OS) are applied to puncture wounds (OS-elicitation). The interaction between the insect and its natural host plant is well studied at different levels (Kessler & Baldwin 2002) and the central role played by jasmonic acid (JA) in the signaling cascade has been shown elegantly in recent years (Baldwin 1998; Halitschke & Baldwin 2003; Li *et al.* 2004). Yet, as a number of different signaling systems are induced upon herbivore attack and as growth is controlled by a network of factors, it is unclear what triggers the process that results in reduced growth.

In most plants, jasmonates (jasmonic acid and other oxylipin derivates) play a major role in mediating induced defense responses to herbivore attack. Jasmonates are involved not only in plant defense against pathogens and herbivores but also in plant development processes such as root growth, fruit ripening, tendril coiling, tuberization, reproductive development and senescence (Creelman & Mullet 1997; Lorenzo & Solano 2005). JA and its derivates are known to be potent root growth inhibitors and many studies have used this property to isolate mutants deficient in their JA signaling, such as *jar1* (Staswick *et al.* 1992), *jin1* and *jin4* (Berger *et al.* 1996), and *coi1* (Feys *et al.* 1994). Yet these findings resulted from experiments in which JAs were applied exogenously to roots, often at non-physiological concentrations (Tung *et al.* 1996; Toro *et al.* 2003; Uppalapati *et al.* 2005; Paschold *et al.* 2007). Whether the herbivory elicited reductions in root growth are mediated by changes in endogenous JA levels has not yet been shown. Zavala & Baldwin (2006) recently reported that a JA-deficient mutant (as*NaLOX3*) outperformed wild-type plants in terms of growth and fitness when plants were undamaged and grown under glasshouse conditions, suggesting growth is regulated via JA signaling.

Upon herbivore attack and OS-elictation, both JA- and ethylene-signaling are strongly activated and levels of both phytohormones accumulate above the levels that are elicited by mechanical wounding alone (Kahl *et al.* 2000). Hence, it is conceivable that a reduction of root growth is mediated by ethylene, which is know to be a potent root growth inhibitor (Abeles *et al.* 1992; Tholen *et al.* 2007). It is also conceivable that the immediate cause of growth reduction upon herbivore attack is related neither to JA nor to ethylene directly but,

rather, to the diversion of metabolites from primary (growth-sustaining) to secondary (defense-related) metabolism (Herms & Mattson 1992), which in turn may be mediated by hormonal signaling.

The aim of this study was to determine if either ethylene or JA signaling mediates the reduction in root growth that is associated with herbivore attack. To elucidate this question, we first analyzed the effect of 1-methylcyclopropen (1-MCP) on root growth in *N. attenuata.* 1-MCP is a noncompetitive inhibitor which persistently binds to ethylene receptors, inhibiting plants from reacting to ethylene (Sisler *et al.* 1996). In subsequent experiments, we tested whether root growth dynamics are altered in transgenic *N. attenuata* plants impaired in JA signaling. We used an antisense-*LOX3* line (as*LOX3*), in which the specific lipoxygenase that supplies the JA biosynthetic cascade with fatty acid hydroperoxides is silenced and has reduced JA levels after wounding and OS-elicitation compared to the wild-type (WT) plants (Halitschke & Baldwin 2003), and a COI1-silenced line (ir*COI1*), in which the F-box protein that mediates JA-perception is silenced by expression of an inverted repeat construct (Paschold *et al.* 2007).

Materials and Methods

Plants and cultivation system for 1-MCP experiments

We used seeds of an inbred line of *Nicotiana attenuata* Torr. Ex Watts (22 generation) as a WT genotype originating from a natural population in Utah. Seeds were treated with smoke and sterilized as described by Krügel *et al.* (2002). Five seeds were planted into square Petri dishes (120 x 120 x 17 mm) half-filled with sterile, solidified 1% Phytagel (w/v) with full-strength Gamborg B5 Medium (Duchefa, Haarlem, The Netherlands). Petri dishes were wrapped with one layer of fabric tape (Micropore, 3M Health Care, Neuss, Germany) to guarantee gas exchange and Petri dishes were placed at an 85° angle to ensure that roots grew along the bottom. The shoots grew in the air-filled volume of the Petri dish. Seedlings were treated as they grew in this cultivation system. Plants were grown in a cultivation room under 26°C during the light phase and 22°C during the dark phase. They were exposed to a photon flux density of 85 µmol m^{-2} s^{-1} between 0600 h and 2000 h.

Root growth

Growth velocity of the primary root tip (V_{Tip}) of each individual seedling was quantified using a ruler in the 1-MCP experiment. Each day at 1300 h the position of the root tip was marked with a pen on the rear side of the Petri dish. For our analysis of root growth we included only plants with a root length between 25 and 35 mm on the treatment day. All suitable plants were treated and V_{Tip} was averaged within each Petri dish. The number of Petri dishes was used as the replicate number for each treatment; the same holds true for other analyses unless otherwise specified.

1-MCP application

Two days before treatment with gaseous 1-methylcylopropene (1-MCP), five holes (Ø 0.5 cm) were melted with a soldering iron into the upper border of the Petri dishes to increase gas diffusion into the dishes. Subsequently they were transferred into a tightly sealed glass chamber (220 x 340 x 550 mm) with a total volume of 41.3 L. 1-MCP was obtained as SmartFresh (Agrofresh Inc./Rohm and Haas; Spring House; USA), containing 0.14% 1-MCP [w/w]. 1.32 g SmartFresh were put into a glass beaker that was placed into the glass chamber. After the chamber was tightly sealed, 100 ml water was added via a silicon tube and a syringe from the exterior of the glass chamber to prevent any loss of 1-MCP gas. 1-MCP gas was released through the reaction of SmartFresh powder with

water, yielding a concentration of 45 µL L^{-1} within the glass chamber (Ma *et al.* 2003). To guarantee a homogeneous distribution of the 1-MCP gas, we placed an 8 cm fan into the glass chamber. Every 24 h, the glass chamber was opened to mark the position of the primary root tips at the rear of each Petri dish. 1-MCP treatments were immediately renewed after each measurement. The same setup was utilized for the controls, although the 1-MCP was not placed into the glass beaker.

Plant genotypes and cultivation system for JA experiments

A slightly different plant cultivation and growth monitoring system was used for analyzing the effects of JA on root growth. Here, it was necessary to wound leaves and to monitor root growth with higher temporal resolution. In this so-called 'microrhizotron' setup, which is described in more detail elsewhere (Nagel *et al.* 2006; Hummel *et al.* 2007), shoots grew outside the Petri dish, which was almost completely filled with Phytagel. After the Phytagel had cooled, three small holes were melted into the upper border of the Petri dish; immediately after piercing, 2-day-old sterile seedlings were transplanted into each hole. Holes were sealed with sterilized silicon fat (Baysilone-Paste, Bayer, Leverkusen, Germany) to prevent contamination. Petri dishes were then placed at an 85°angle to ensure that roots grew along the bottom. Plants were grown in the same cultivation room as described above under 26°C during the light phase and 22°C during the dark phase. They were exposed to a photon flux density of 85 µmol m^{-2} s^{-1} between 0600 h and 2000 h. Petri dishes were not placed into the sealed glass chamber.

In addition to the WT plants described above, we used transgenic *N. attenuata* plants isogenic with the WT plants that were silenced in jasmonate biosynthesis and perception. One line was transformed with a construct carrying a fragment of the *N. attenuata* LOX3 (lipoxygenase 3) gene in an antisense (as) orientation (as*LOX3* plants) which accumulate about 50% of the JA that WT plants do after OS-elicitation (Halitschke & Baldwin 2003). Another line (ir*COI1*) was transformed to express a fragment of the *N. attenuata* gene coding for the F-box protein, COI1 (coronatin-insensitive 1), in an inverted repeat (ir) orientation and was dramatically inhibited in it's ability to perceive jasmonates (Paschold *et al.* 2007).

High-resolution root growth monitoring

Ten days after germination, experiments were performed with plants grown in the microrhizotron setup. Every 20 s an image of the primary root growth zone was taken with a CCD camera (Sony XC-ST50, Sony, Köln, Germany) having a resolution of 740 x 480 pixels, which corresponds to an absolute area of 4.4 x 2.9 mm. Infrared illumination (λ = 940 nm) enabled images to be acquired during the dark phase as well. The camera was equipped with a low-pass infrared filter (RG, Schott, Mainz, Germany) to block visible irradiation. Each replicate was measured for at least 24 h. The root tips were followed via a tracking algorithm; this algorithm controlled a set of x-y-moving stages that repositioned the entire Petri dish and hence the root tip, when it approached the border of the image field (Hummel *et al.* 2007). Root tip velocities (V_{Tip}) were calculated via image sequence processing algorithms described in more detail elsewhere (Schmundt *et al.* 1998; Walter *et al.* 2002; Walter *et al.* 2003). Root growth data was normalized in Figures 4 to 7 by dividing the V_{Tip} values with V_{Tip} at the time point of treatment (1300 h) to make treatments easier to compare. Temperature of the Phytagel of one representative dish was recorded once per minute using a thermocouple attached to a data logger (Delta-t Devices ltd, Cambridge, England).

Wounding and OS-elicitation treatments

To simulate herbivory, both primary leaves were squeezed with tweezers, to produce small puncture holes. By this procedure, a maximum of 2% of the total leaf area (including the cotyledons) was damaged. Immediately after wounding, 1 µL H_2O (buffered to 7.8 pH by 50 mM phosphate buffer; H_2O) or 1µL *Manduca sexta* larval OS (diluted 1:5 with phosphate buffer) was applied to the wounds. For the 1-MCP experiment the plants were grown inside opened the Petri dishes and the treatments were performed within sterile clean benches to avoid any contamination.

In MeJA experiments, 500 ng methyl jasmonate (MeJA, Sigma-Aldrich, Steinheim, Germany) dissolved in 1 µL lanolin paste (Sigma-Aldrich, Steinheim, Germany) were applied to the undamaged primary leaf. Control plants received the same amount of pure lanolin paste only. All treatments were performed at 1300 h.

JA analysis

Eight to ten seedlings (entire shoots) were pooled to produce approximately 150 mg samples per replicate. These pooled samples were immediately shock frozen in liquid N_2

after harvest. For JA extraction, plant material was transferred to FastPrep tubes containing 900 mg of FastPrep matrix (BIO 101, Vista, USA). One milliliter of ethyl acetate mixed with 100 ng of D_2-JA was used as an internal standard for JA analysis and was added to each sample. Samples were then homogenized on a FastPrep homogenizer (Savant Instruments, Holbrook, USA). After centrifugation at 12,100 g for 20 min at 4°C, extraction was repeated with 1 ml ethyl acetate. The supernatants were combined and then evaporated to dryness on a vacuum concentrator. The dried residue was dissolved in 300 µl 70% (v/v) methanol and subsequently centrifuged at 13000 rpm for 10 minutes.

Measurements were conducted on a 1200L liquid chromatography-triple quadrupole mass spectrometry system (Varian, Palo Alto, USA). At a flow rate of 0.1 ml/min, 15 µl of each sample was injected onto a ProntoSIL column (C18; 5 µm, 50 × 2 mm, Bischoff, Germany) attached to a precolumn (C18, 4 x 2 mm, Phenomenex, USA). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode for the separation. The mass spectrometer was operated in a negative electrospray ionization mode. The most abundant and characteristic fragment ion was chosen for quantification (Wu *et al.* 2007).

Nicotine analysis

Shoots from the 1-MCP treatment were harvested and immediately shock frozen in liquid N_2. Plant material was ground frozen with a pestle in a 1.5 ml tube. The samples were extracted with 400 µl of 40% MeOH (v/v) containing 0.5% acetic acid (v/v) and vigorously shaken at 23°C for 2 h. After extraction the samples were centrifuged (10 min, 12 100 g), filtered (0.45 µM), and transferred into a vial for HPLC analysis. Nicotine was separated by a Merck-Hitachi HPLC system (Darmstadt, Germany) on a Multosphere column (120 RP 18-HP 3 µm, 250 x 4 mm, CS-Chromatographie Service, Langerwehe, Germany) and detected with a diode array detector at 254 nm as described in Hummel *et al.* (2007).

Statistical analysis

Data was analyzed with Statistica, version 6.0 (StatSoft Inc., Tulsa, USA). A repeated measures two-factorial ANOVA was used to analyze V_{Tip} of the 1-MCP experiment, while a one-factorial ANOVA was used to detect the first significant difference in time between MeJA-treated and control plants. For the kinetics of the nicotine and JA concentrations a two-factorial ANOVA was performed. All ANOVAs were followed by Fisher's protected least-significance difference (LSD) test for post hoc comparisons.

Results

Root growth when perception of the OS-elicited ethylene is inhibited

Experiments were performed in which *M. sexta* OS was applied to leaf wounds and in which 1-MCP was applied to inhibit the plants' perception of the wound- and OS-induced ethylene bursts (Figs. 1, 2). Prior exposure of plants to 1-MCP led to an overall increase of root growth in all treatments (Fig. 1). OS-elicited 'control' plants (Fig. 1a) showed a reduction in root growth that was transiently more pronounced than in plants treated with H_2O. Exactly the same reaction pattern was observed in plants treated with 1-MCP (Fig. 1b): wounding led to a decrease of root growth compared to non-wounded control plants and this decrease was more pronounced for 2 d if OS rather than buffer were applied to the wounds and the differences are all highly significant ($P < 0.001$). This demonstrates that ethylene is not specifically involved in the decrease of growth after herbivore attack, but suggests that it might generally suppress growth in plants cultivated on agar-filled Petri dishes.

These observations of the elongation of the primary root are consistent with the responses observed in the analysis of total root length, root, and shoot fresh mass (Fig. 2): The significantly faster root growth we observed in treated 1-MCP plants compared to control plants led to significantly longer roots within 3 d ($F_{1,58} = 96.886$, $P < 0.001$; Fig. 2a). Non-wounded treated 1-MCP plants had significantly longer roots (26%) than did control plants ($P < 0.001$), and H_2O and OS treatments led to longer roots in plants pre-treated with 1-MCP compared to control plants (17%, $P < 0.001$; 16%, $P < 0.001$, respectively). Furthermore, 1-MCP treatments also significantly increased root fresh mass ($F_{1,42} = 5.693$, $P = 0.022$; Fig. 2b). The masses of non-damaged plants when pre-treated with 1-MCP (14%) were significantly higher that the masses of control plants ($P = 0.026$). No significant differences in shoot biomass were found between treatments (Fig. 2c), but the biomass of plants treated with1-MCP tended to be higher that the biomass of non-treated plants.

De novo nicotine biosynthesis was significantly increased in both wounding treatments compared to the control – with and without application of 1-MCP ($F_{2,25} = 42.393$, $P < 0.001$; Fig. 3). When OS were applied to wounds, more nicotine was found in plants treated with 1-MCP than in non-treated plants(1.17-fold difference; $P = 0.01$), consistent with the observation that OS-elicited ethylene attenuates the *de novo* biosynthesis of nicotine (Kahl et al. 2000; Winz and Baldwin, 2001).

Root growth responses to OS-elicitation in plants with inhibited JA responses

As shown above (Fig. 1a), a singular application of OS leads to a transient reduction of root growth in plants that are OS-elicited compared to plants that are wounded but only treated with buffer. This transient reduction, which is not mediated by ethylene (Fig. 1b) and lasts approximately 1 d, has already been shown in an earlier study (Hummel *et al.* 2007). Hence, we wondered if, at a high temporal resolution, JA affects root growth during the 24 h immediately following wound treatment, in the same way that wounding induced a JA burst that was dramatically amplified when OS was applied to wounds (Fig. 4). Root growth is strongly affected by temperature and closely tracks variation temperature (Fig. 5a). The time series analysis of WT, as*LOX3*, and ir*COI1* plants are similar (Fig. 5), with the strongest differences found between 1600 h and 2000 h when ir*COI1* plants grow more slowly than WT plants.

When WT plants were wounded they displayed the reaction pattern indicated in Fig. 1a; a significantly stronger reaction was observed when OS was applied to wounds compared to when H_2O was applied (Fig. 6a). Root growth in OS-treated WT plants was 33% that of control plants seven hours after wounding (just before dark phase; Fig. 6a). Root growth in H_2O-treated plants was 51% that of control plants seven hours after wounding (Fig. 6a). as*LOX3* plants, which display a 50% reduction in JA levels, show very similar reaction patterns (Fig 6b): Root growth is reduced stronger in OS-treated plants compared to H_2O-treated plants. In comparison to control plants, growth was reduced to 61% and 44% of that in H_2O-treated and OS-treated plants seven hours after wounding, respectively.

In ir*COI1* lines, wounded plants of both treatments reached V_{Tip}-values of 60% of control plants at the end of the day (Fig. 6c). Differences between H_2O- and OS-treated plants were not significant, indicating that JA perception is crucial for the specific root growth reduction observed in OS-treated plants to take place.

Independent of the genotype, an immediate decrease of V_{Tip} during 65 ± 4 minutes after wounding was observed with values of 64 ± 8% of the initial V_{Tip}, independent of the specific wounding treatment or the genotype (Fig. 6). This immediate ‘first’ decrease was followed by a phase of steady V_{Tip}-values and remained relatively stable for 57 ± 10 minutes when averaged over all treatments. Thereafter, a ‘second’ decrease in root growth was observed, lasting several hours until the end of the day. This second decrease is primarily responsible for the growth reductions described above.

High resolution root growth analysis after MeJA application

To elucidate MeJA-elicited changes in root growth, MeJA was applied to primary leaves and root growth was measured at high temporal resolution (Fig. 7a). MeJA dramatically reduced root growth and a first significant difference between V_{Tip}-values of control and treatment plants was observed approximately 97 minutes after MeJA application ($P = 0.028$). After this point in time, the V_{Tip} of MeJA-treated plants was significantly reduced throughout the entire measurement period, which is best displayed by depicting the ratio of V_{Tip} of MeJA-treated and V_{Tip} of control plants (Fig. 7b). This ratio decreased almost linearly from wounding until the beginning of the night, reaching values between 40% and 50% throughout the night.

Discussion

Inhibiting ethylene perception increases root growth but not the response to OS-elicitation

Mechanical wounding of *N. attenuata* leaves transiently increases endogenous JA and ethylene levels, and when the OS of the specialist lepidopteran larvae *M. sexta* is applied to the wounds, these transient increases increase dramatically (Kahl *et al.* 2000; Schittko *et al.* 2000). Ethylene is known to reduce root elongation considerably (Abeles *et al.* 1992; Tholen *et al.* 2004; Tholen *et al.* 2007). In Petri dish cultivation systems, it can occur in high concentrations and lead to strong root growth reduction (Buer *et al.* 2003). As our results show (Figs. 1, 2), 1-MCP alleviates this reduction significantly. These are consistent with results from *Arabidopsis* and *Epipremnum* (Muller *et al.* 1997; Ma *et al.* 2003), where it was shown previously that 1-MCP leads to an increase in root growth. Similar results were obtained in *Brassica napus* by using another ethylene receptor inhibitor (1-cyclopropenylmethyl; Saleh-Lakha *et al.* (2004)). The reduction of root growth due to OS (Hummel *et al.* 2007) was still observed when 1-MCP was applied, clearly indicating that the wound-induced ethylene burst is not inducing this reaction.

In *N. attenuata*, wounding or elicitation with MeJA induces *de novo* biosynthesis of nicotine used for defense (Steppuhn *et al.* 2004); however, other resistance traits are regulated by JAs, such as trypsin protease inhibitors, diterpene glycosides, and volatile emissions involved in indirect defense (Halitschke *et al.* 2000; Steppuhn *et al.* 2004). We tested whether ethylene alters nicotine concentrations of our seedling system after

wounding, as was previously demonstrated for *N. attenuata* at later developmental stages by Kahl *et al.* (2000). The results clearly demonstrate that ethylene attenuates the nicotine response when OS is applied to wounds, suggesting the mechanism reported by Kahl *et al.* (2000) and Winz & Baldwin (2001) is already active in the seedling stage of *N. attenuata*. The ecological interpretation of this attenuated nicotine response is reviewed in Kessler & Baldwin (2002). They hypothesize that the *M. sexta*-induced ethylene burst adaptively reduces nicotine production, as the larvae is alkaloid-tolerant and can use increased levels of nicotine for its own defense against parasitoids or predators.

Inhibition of JA-signaling alters the OS-elicited root growth response

JA up-regulates the expressions of many constitutively expressed defense genes, but down-regulates many genes involved in growth (Creelman & Mullet 1997; Halitschke *et al.* 2003). Hence we tested whether transgenic *N. attenuata* plants, impaired in their JA-signaling pathways, display altered root growth in response to OS-elicitation. We first monitored root growth without any damage treatment and found that *asLOX3* and wild-type plants showed no difference concerning the diurnal variation of root growth activity, which is strongly affected by temperature.

LOX3 is the specific wound and herbivory-induced isoform of the lipoxygenase gene family (*LOX*) in *N. attenuata*, which is involved in the JA biosynthesis. as*LOX3* plants are impaired in the wound-induced biosynthesis of JA but are unaltered in their unelicited constitutive levels (Halitschke & Baldwin 2003). Hence, unsurprisingly, root growth is comparable between wild-type and *asLOX3* seedlings without wounding (Fig. 5). This result contrasts the results of Zavala & Baldwin (2006), who reported that the growth and fitness of as*LOX3* plants was better than that of undamaged WT plants. However, their plants were glasshouse-grown in soil-filled pots. Environmental stresses (temperature, light) or biotic stresses (elicitation by soil microbes) are also mediated via the JA pathway, which might have down-regulated growth in WT plants (Creelman & Mullet 1997; Pozo *et al.* 2004; Yadav *et al.* 2005).

COI1, an F-box protein is required for the perception of JA, and several studies have demonstrated that root growth of COI1-deficient plants is relatively insensitive to MeJA treatments (Feys *et al.* 1994; Xie *et al.* 1998; Li *et al.* 2004; Paschold *et al.* 2007). Diurnal root growth in ir*COI1* plants was similar to that in WT and as*LOX3* plants; however, in the afternoon ir*COI1* plants grew more slowly than did wild-type plants, suggesting that COI1 might play a role in controlling root growth at least during the afternoon.

After wounding we observed in all genotypes an immediate and steep reduction of root growth for 65 minutes until growth temporarily stabilized again, as previously described in Hummel *et al.* (2007). This first rapid decrease points to a hydraulic response as a loss of water and turgor pressure immediately reduces root growth (Nagel *et al.* 2006); however, the immediate reduction in root growth could also have been mediated via electrical signaling (Fromm & Lautner 2007). A second negative growth response was observed approximately two hours after wounding, which coincides remarkably well with the 'decreasing' root growth dynamics of MeJA-treated plants (Fig 7a). This suggests that the second decrease in root growth might be mediated via wound-elicited JA, imported from the wound sites in leaves to the roots. Baldwin *et al.* (1997) reported that JA pools in roots of *Nicotiana sylvestris* increase 3.5-fold 180 minutes after mechanical leaf wounding, and Zhang & Baldwin (1997), by exogenously applying 2-^{14}C-labeled JA to leaves, demonstrated that JA is directly transported from leaves to roots and could account for the systemic increase in root JA pools after leaf wounding. Moreover Thorpe *et al.* (2007) reported a rapid transport of ^{11}C-labelled methyl jasmonate from leaves to roots in *N. tabacum* within 60 minutes. The intensity with which root growth is reduced the second time depends on the specific wounding treatment and the genotype. The root growth of WT plants was more strongly reduced when OS were applied to wounds than when seedlings were only mechanically wounded, confirming previous results of Hummel *et al.* (2007). The root growth of as*LOX3* plants was less reduced compared to that of WT plants in both wounding treatments; nevertheless the application of OS also led also to strong reductions in root growth. These results are consistent with the endogenous JA concentration levels of as*LOX3* plants after wounding, which are also amplified by the application of OS, although not to the same extent as in WT plants (Halitschke & Baldwin 2003).

In ir*COI1* lines, wounding leads to an immediate hydraulic response; however, the second response is clearly less intense compared to wild-type and as*LOX3* plants. Only negligible differences in root growth of OS- and H_2O-treted plants were observed, suggesting that ir*COI1* plants are insensitive to wound-induced JA changes in the plants. This confirms findings by Paschold *et al.* (2007), who direct applied different concentrations of MeJA to roots and showed that ir*COI1* plants are remarkably growth-insensitive to JA. These results provide further evidence that (i) the second decrease in root growth is mediated by JA and (ii) the more pronounced reduction of root growth after application of OS to wounds results from the higher OS elicited JA levels.

To conclude, our study demonstrates that JA signaling is involved in mediating root growth inhibition and demonstrates how the shoot may regulate reduction in root growth. We provide further evidence that endogenous JA acts as a distress signal, slowing vegetative growth during defense responses.

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Figures

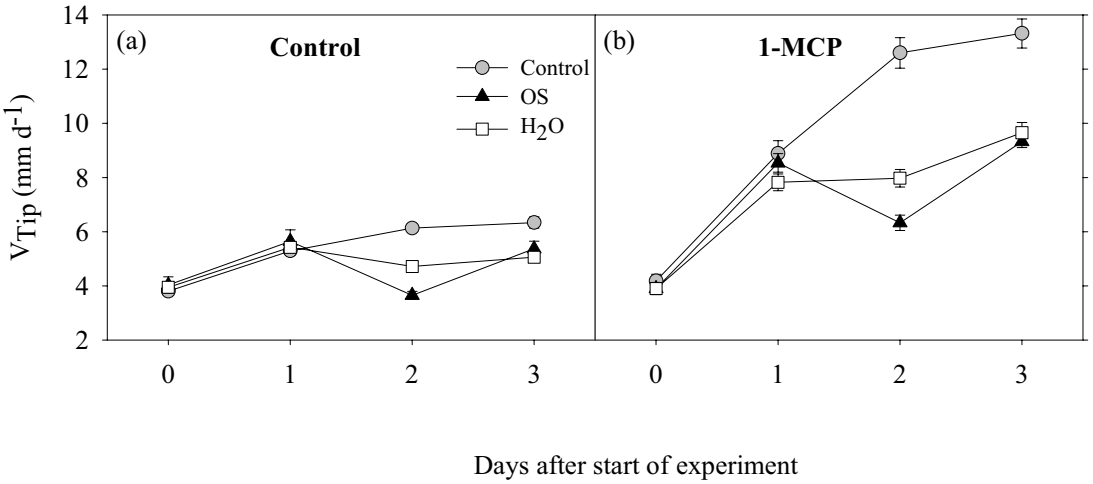

Figure 1. Root growth of *N. attenuata* after pretreatment with 1-MCP (day zero) and following simulated herbivory (OS-elicitation performed on day one). Primary leaves were wounded with tweezers and immediately supplied with 1 µl water (H_2O) or oral secretions and regurgitant of *Manduca sexta* (OS) diluted 1:5 with phosphate buffer. (a) Control plants (b) 1-MCP treated plants (mean values ± SE; n = 10).

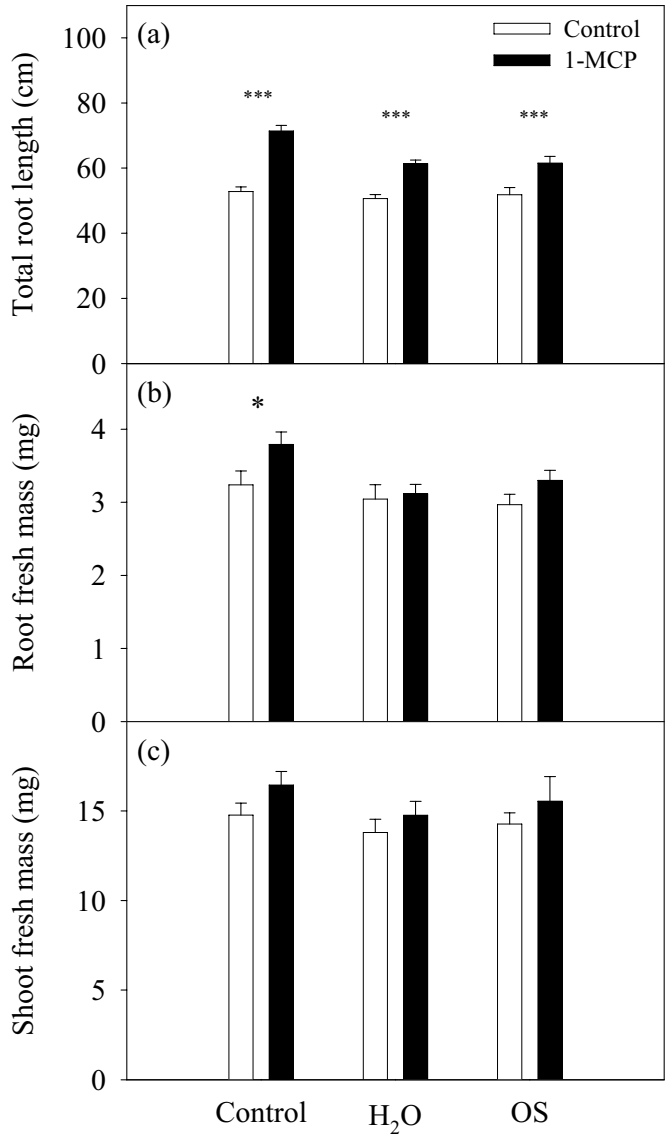

Figure 2. Fresh mass of *N. attenuata* after 1-MCP pretreatment and simulated herbivory (harvested 3 d after 1-MCP application). Primary leaves were wounded with tweezers and immediately supplied with 1 µl water (H_2O) or oral secretions and regurgitant of *Manduca sexta* (OS) diluted 1:5 with phosphate buffer. (a) Total root length (mean values ± SE, n = 9). (b) Root fresh mass (mean values ± SE, n = 7). (c) Shoot fresh mass (mean values ± SE, n = 7). Asterisks indicate significant differences between wounding treatments (* $P < 0.05$; *** $P < 0.001$)

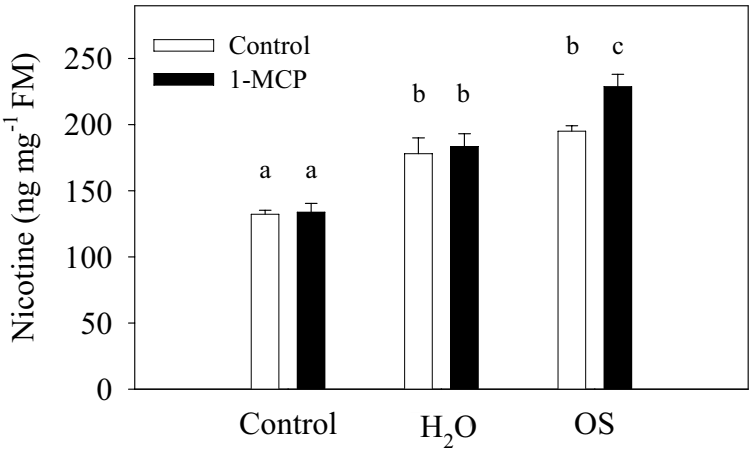

Figure 3. Nicotine concentrations of *N. attenuata* after 1-MCP pretreatment and simulated herbivory (harvested 48 h after wounding); (FM: fresh mass). Primary leaves were wounded with tweezers and immediately supplied with 1 µl water (H_2O) or oral secretions and regurgitant of *Manduca sexta* (OS) diluted 1:5 with phosphate buffer (mean values ± SE; n = 5). Different letters indicate significant differences at $P < 0.05$ level.

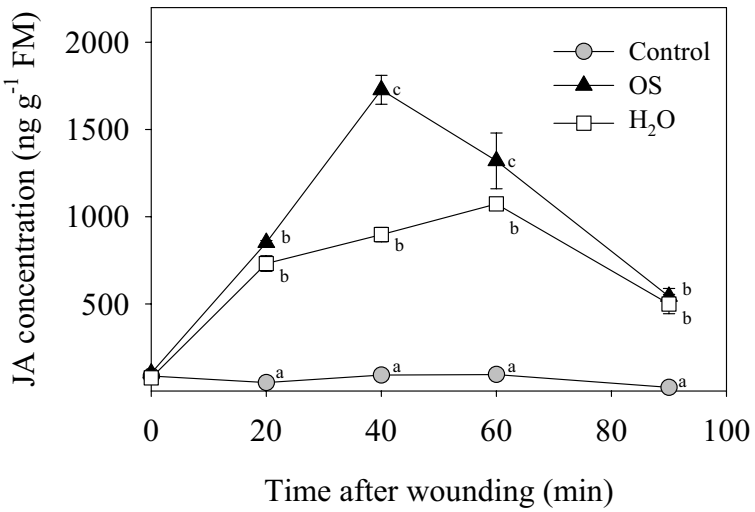

Figure 4. Jasmonic acid (JA) concentrations in seedlings of *N. attenuata* after simulated herbivory (FM: fresh mass). Primary leaves were wounded with tweezers and immediately supplied with 1 µl water (H_2O) or oral secretions and regurgitant of *Manduca sexta* (OS) diluted 1:5 with phosphate buffer and harvested from 0 to 90 minutes after wounding (mean values ± SE; n = 3-5). Different letters indicate significant differences at $P < 0.05$ level.

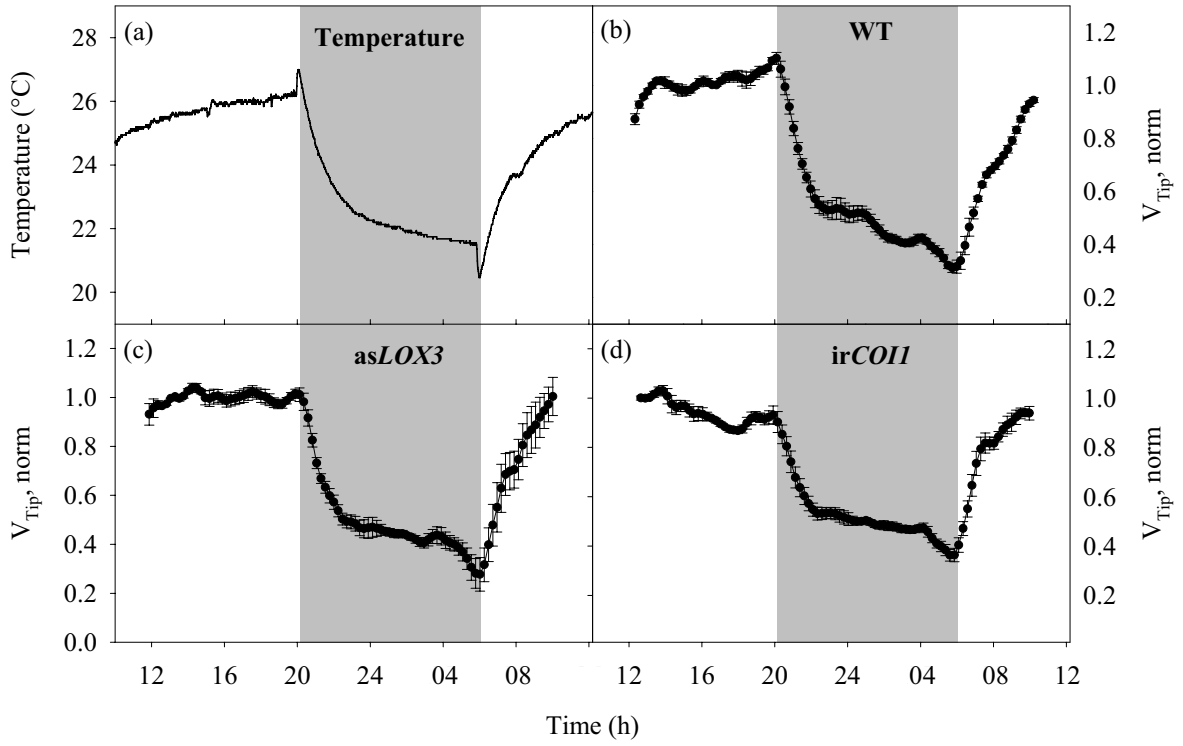

Figure 5. High resolution time series analyses of root growth of different JA-mutants of *N. attenuata*. (a) Temperature course of Phytagel over 24 h during the experiments. Light was switched on at 0600 h and switched off at 2000 h (night period gray shaped). (b) Normalized values of root growth velocities (V_{Tip}, norm) of wild type (WT) (c) as*LOX3* and (d) ir*COI1* (mean values ± SE, n = 4).

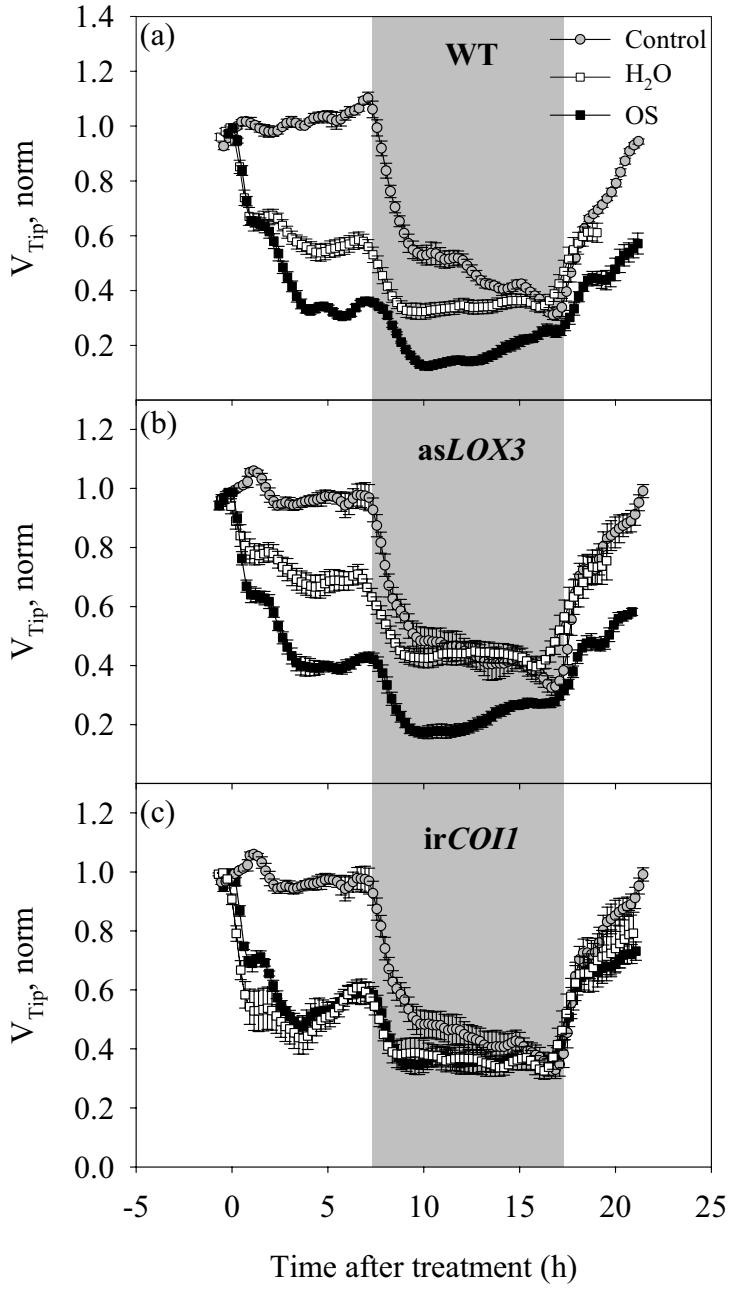

Figure 6. Normalized values of root growth velocities (V_{Tip}, norm) conducted with high resolution from different JA-mutants of *N. attenuata* after simulated herbivore attack. Primary leaves were wounded (W) with tweezers and immediately supplied with 1 µl water (H_2O) or oral secretions and regurgitant of *Manduca sexta* (OS) diluted 1:5 with phosphate buffer. (a) Wild type plants (WT) after W + H_2O (mean values ± SE, n = 15). (b) as*LOX3* plants after W + OS (mean values ± SE, n = 12). (c) ir*COI1* plants after W + H_2O (mean values ± SE, n = 5).

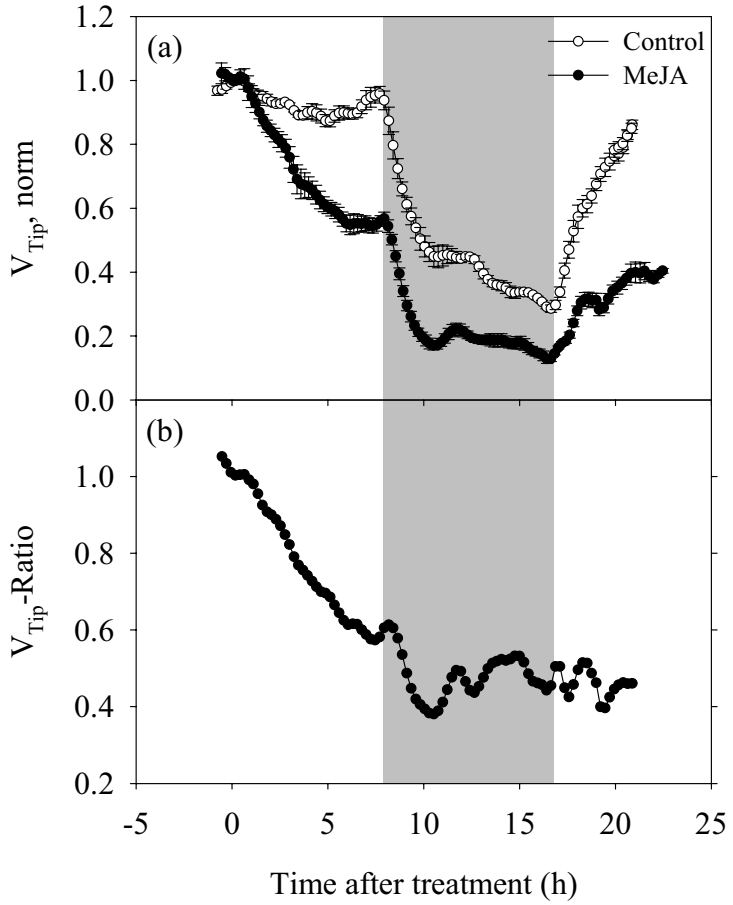

Figure 7. Normalized values of root growth velocities (V_{Tip}) of *N. attenuata* after treatment with 500 ng methyl jasmonate (MeJA) dissolved in 1 µL lanolin paste. (a) 24 h overview. Light was switched on at 0600 h and switched off at 2130 h (night period gray shaped). (b) V_{Tip}-Ratio: V_{Tip} of MeJA-treated plants divided by V_{Tip} of control plants (mean values ± SE, n = 4).

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9. *Na*RALF, 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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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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Summary

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

Keywords: ***NaRALF,*** **root hair, tip growth, extracellular pH oscillation, plant fitness,** ***Nicotiana attenuata*****.**

Introduction

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 mitogen-activated protein (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- 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: (i) unlike systemin, RALF does not elicit either the synthesis of tobacco trypsin inhibitors (Ryan *et al.*, 2002) or an increase in *phenylalanine ammonia lyase* (*PAL*) transcripts (Haruta and Constabel, 2003); (ii) transcript levels of seven *RALF*s are not altered in either *mpk4* (constitutive systemic acquired resistance) or *ctr1* (constitutive ethylene response) mutant backgrounds in Arabidopsis (Olsen *et al.*, 2002). These

results suggest that RALF may play roles that are not defense related.

In Arabidopsis, RALF belongs to a large family of 34 genes (Olsen *et al.*, 2002). At least five RALF-like genes have also been identified in *Solanum chacoense* (Germain *et al.*, 2004). The large size of the gene family adds to the difficulty of identifying the functions of RALF 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) that 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: (i) 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); (ii) a high-affinity P transport gene *LePT1* was found to be highly expressed in root hairs (Daram *et al.*, 1998); (iii) the fitness of Arabidopsis mutant *act2-1*, which produces root hairs that are only 10–70% 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* (Bohme *et al.*, 2004; Favery *et al.*, 2001; Foreman *et al.*, 2003; Parker *et al.*, 2000; Rigas *et al.*, 2001; Schiefelbein and Somerville, 1990). The *rhd2* mutant is unable to form the tip-focused calcium gradients required for hair growth. RHD2 has proven to be an NADPH oxidase, a protein that transfers electrons from NADPH to an electron acceptor, which leads in turn to the formation of reactive oxygen species (ROS) (Foreman *et al.*, 2003). The lesion in tip growth exhibited by *rhd2* strongly implicates ROS in the regulation of root hair development. The *TRH1* gene encodes a K^+ transporter (Rigas *et al.*, 2001), indicating that K^+ transport is required to cooperate with other localized transporters in driving the transition to tip growth. Indeed, ion fluxes have been intimately linked to the progression of root hair growth. It has been demonstrated that a tip-focused calcium gradient is essential for establishing tip growth (Bibikova *et al.*, 1997; Wymer *et al.*, 1997). Similarly, localized acidification of the cell wall and alkalinization of the cytoplasm are some of the first detectable indications of imminent root hair initiation (Bibikova *et al.*, 1998). The local apoplastic acidification of the cell wall can be prevented by treatment with high-pH buffers, which not only prevent the trichoblast from bulging but also arrest the elongation of root hairs that have started tip growth (Bibikova *et al.*, 1998). These results suggest that not only root hair initiation but also tip growth requires tight regulation of apoplastic pH. This pH change may, for example, activate expansin proteins that promote loosening of the cell wall, and so facilitate turgor-driven expansion (Baluska *et al.*, 2000; Bibikova *et al.*, 1998). 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 the ion exchange capacity of the cell 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 the 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 the natural habitat of *Nicotiana* 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 *Nicotiana 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 open reading frame (ORF) sequence as a probe. Instead of several gene copies, as in poplar (Haruta and Constabel, 2003), only one gene copy was found in the genome of *N. attenuata* (Figure 1b). Accordingly, we are unlikely to silence other homologous genes when plants are transformed with an RNAi construct composed of the same sequence.

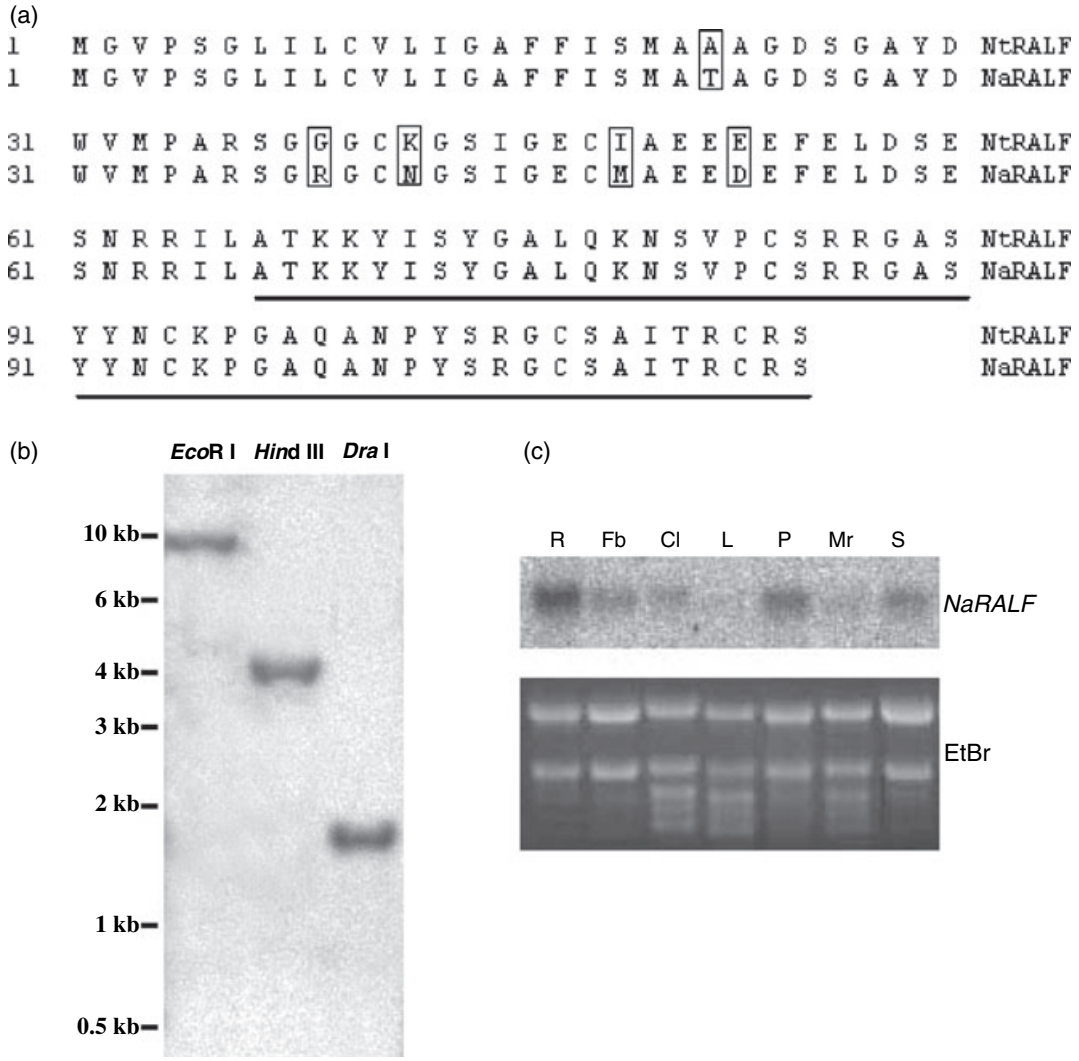

Figure 1. Isolation and characterization of *NaRALF* in wild-type *Nicotiana attenuata*.
(a) The amino acid sequence of NaRALF (AY456269) compared with tobacco RALF (NtRALF, AF407278). The mature peptide sequence of *NaRALF* is underlined, and the amino acids that differ are outlined by boxes.
(b) Southern blot analysis shows that only one copy of *NaRALF* is present in the *N. attenuata* genome. Genomic DNA (5 μg) was digested with *Eco*RI, *Hin*dIII and *Dra*I, and the open reading frame (ORF) sequence of *NaRALF* was labeled with [^{32}P]dCTP for the probe.
(c) Transcript accumulation of *NaRALF* in different tissues of *N. attenuata*. Total RNAs (10 μg) were extracted from roots (R), flower buds (Fb), cauline leaves (Cl), rosette-stage leaves (L), petioles (P), leaf midribs (Mr) and stems (S) of 37-day-old wild-type (WT) plants, and were analyzed by northern blotting, probed with a ^{32}P-labeled *NaRALF* ORF sequence. An ethidium bromide (EtBr) stained gel serves as a loading control.

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

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

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

inverted-repeat orientation (Figure S2b). Two independently transformed F2 lines, each harboring a single insertion as confirmed by Southern blot analysis (Figure S2c), were selected for all further experiments (ir*RALF* lines 1 and 2). The silencing of endogenous *NaRALF* gene expression was also confirmed by qRT-PCR, which revealed that *NaRALF* expression was reduced by 90% in the roots of these lines compared with WT plants.

Because treatment with exogenous RALF has been reported to arrest root elongation (Pearce *et al.*, 2001), we examined the rate of root growth in ir*RALF* lines. Four days after germination, the root length in WT and ir*RALF* lines did not differ. However, by day 6, roots of both ir*RALF* lines were significantly longer than in WT plants (Figure 2). To determine whether the longer roots resulted from higher root growth rates, we measured root tip growth velocity, maximal relative elemental growth rate ($REGR_{max}$), and the length of the elongation zone in WT and ir*RALF* line-1 plants. Because root growth correlates positively with temperature (Pahlavanian and Silk, 1988; Walter *et al.*, 2002), root tip velocity, $REGR_{max}$ and the elongation zone were measured during three time intervals, which bracketed the greatest changes in temperature: morning (from 6:00 to 13:00 h), afternoon (from 13:00 to 21:00 h) and night (from 21:00 to 6:00 h). The average root tip velocity in WT lines was 0.12 ± 0.01 mm h^{-1} in the morning and 0.17 ± 0.01 mm h^{-1} in the afternoon; however, the average root tip velocity in ir*RALF* line 1 was significantly greater both in the morning (142% of WT plants) and in the afternoon (171% of WT plants), but not in the evening (Figure 3a). The $REGR_{max}$, which characterizes the maximal expansion rate of cells, was also significantly higher in ir*RALF* plants during the afternoon measurements (Figure 3b) than in WT plants. The elongation zone of *irRALF* plants was significantly longer at all three times than in WT plants (Figure 3c). These results indicate that rapid root growth and a longer elongation zone can account for the longer roots of ir*RALF* plants.

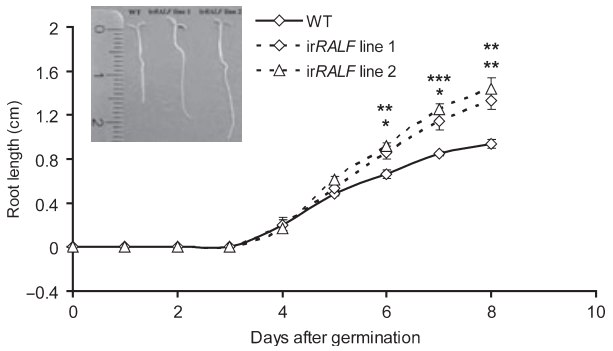

Figure 2. Root length of ir*RALF* and wild-type (WT) seedlings.
The root lengths (mean ± SEM) of 20 replicated WT and ir*RALF* seedlings were measured for 8 days after germination. All seedlings were grown vertically on the surface of Gamborg B5 medium (pH 6.8). Inset: photograph of 8-day-old WT and ir*RALF* lines. The asterisks indicate the level of significant differences between WT and ir*RALF* lines 1 or 2 (unpaired Student's *t*-test: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$).

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 (Figure 4b,d), grow at a rate of 0.58 ± 0.24 μm min^{-1} and are produced at 29 ± 2 hairs per 500 μm of root length under our standardized growth conditions (Figure 4c,d). However, in ir*RALF* plants around two-thirds of trichoblasts produced only a localized 'bulge' and never commenced tip growth (Figure 4c,e,f). The remaining third of these 'bulges' successfully completed the transition to tip growth (Figure 4c,e,f); however, their growth rate was only 64% that of WT root hairs (unpaired Student's *t*-test, $P < 0.001$), and their final root hair length was only 10% of that of WT root hairs (Figure 4b,e,f). Most of the 'bulges' we observed swelled until they ruptured (Figure 5; Video Clip S1).

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

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

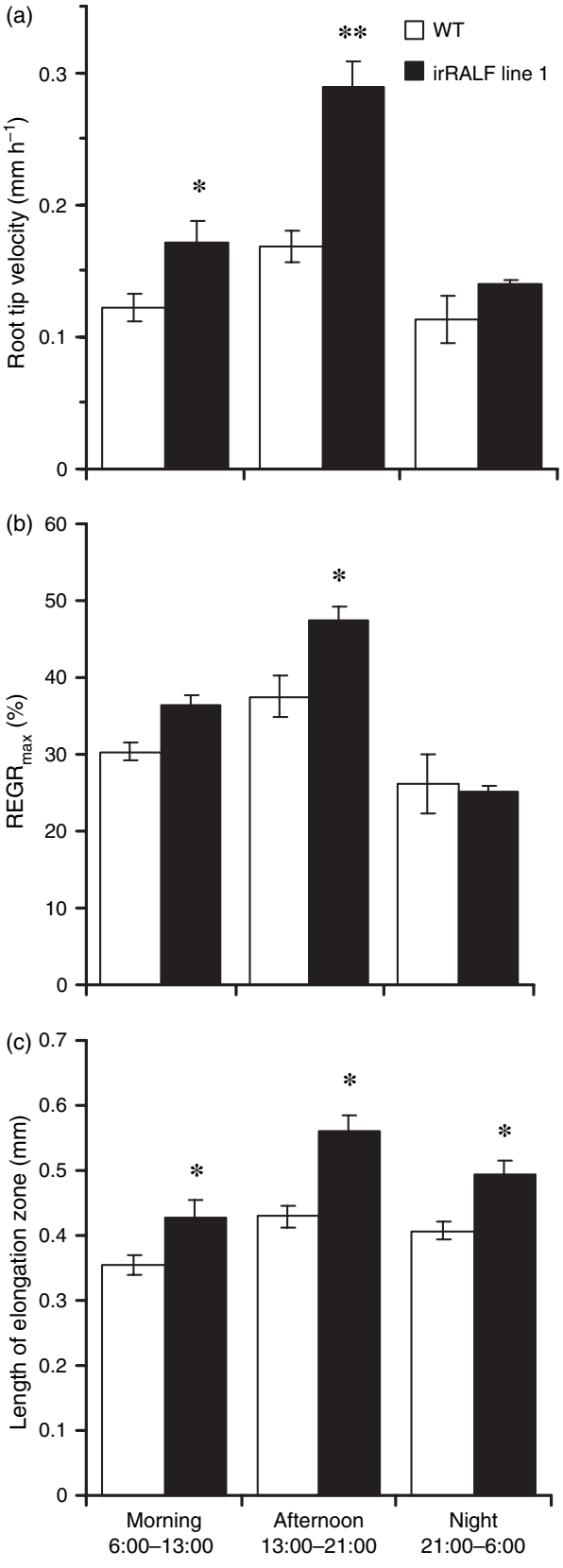

Figure 3. Longer root lengths of ir*RALF* lines result from faster growth.
The mean (±SEM) maximal root tip velocity (a), maximal relative elemental growth rate ($REGR_{max}$) (b) and length of root elongation zone (c) were measured in five replicated wild-type (WT) and ir*RALF* line-1 seedlings. The lights were switched on at 6:00 h in the morning and switched off at 20:00 h in the evening. The asterisks indicate the level of significant differences between WT and ir*RALF* line 1 (unpaired Student's *t*-test: $*P < 0.05$; $**P < 0.001$). All seedlings were grown vertically on the surface of Gamborg B5 medium (pH 6.8).

tip of the cell wall always preceded the rupturing of trichoblasts (Video Clip S2).

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

It is also possible that suppression of *NaRALF* expression leads to a range of growth effects on root hairs, and so the altered pH profiles in the ir*RALF* lines might reflect a consequence rather than cause of the disrupted cell expansion in these plants. In order to test whether the altered pH of the cell wall was primarily responsible for the altered growth seen in root hairs, we grew WT and ir*RALF* plants in media containing either a low-pH buffer [2-(*N*-morpholine)-ethanesulphonic acid (MES) or phosphate buffer, 20 mM, pH 5.5) or a high-pH buffer (Tris or HEPES buffer, 20 mM, pH 6.8) to determine if (i) growing ir*RALF* plants on low-pH-buffered media could restore a WT root hair phenotype, and (ii) growing WT plants on high-pH-buffered media could produce a root hair phenotype reminiscent of ir*RALF* plants. WT plants produced the same root hair phenotype as did ir*RALF* lines when they were grown in a high-pH-buffered medium (Figure 7): many trichoblasts produced only 'bubble' structures; although some were capable of normal tip growth, they produced only short root hairs. When grown on a low-pH-buffered medium, root hair length in ir*RALF* line 1 increased from 67 to 292 μm, and in ir*RALF* line 2, from 41 to 228 μm (Figure 8a,d). Root hair density was also partially recovered; for example, the number of root hairs in ir*RALF* line 1 increased from 10 to 14.8 (Figure 8b,d) per 500 μm root segment in the low-pH-buffered medium.

*ROS accumulation decreased in ir*RALF *line 1*

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

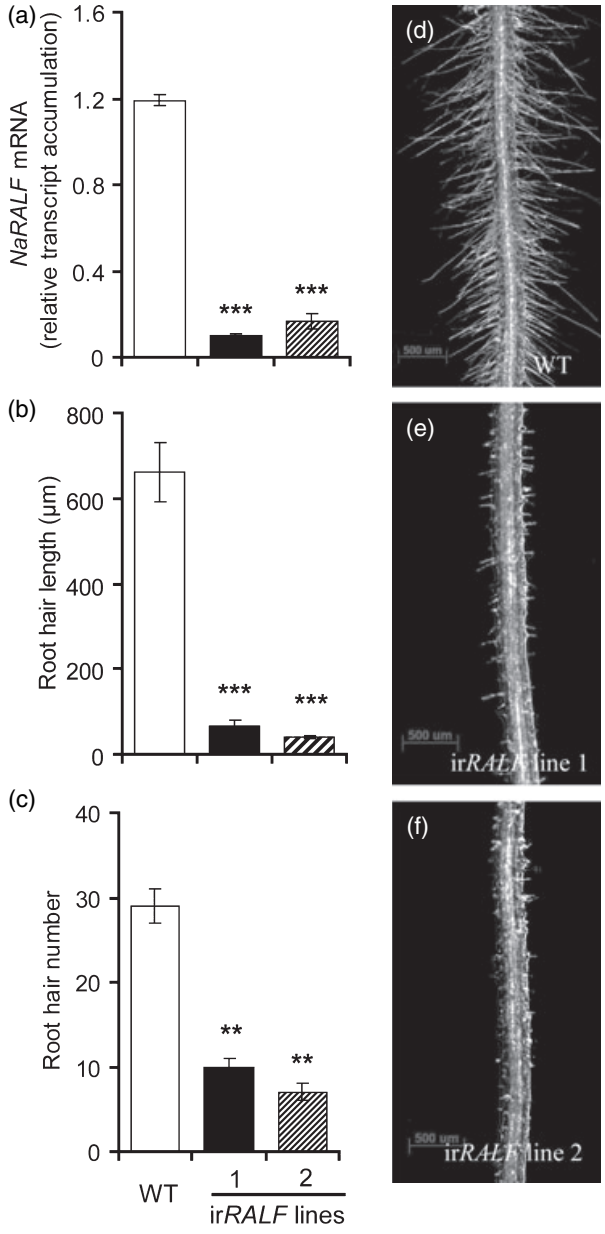

Figure 4. Relative *NaRALF* transcript accumulation, and root hair length and number in roots of wild-type (WT) and ir*RALF* seedlings.
Mean (±SEM) *NaRALF* transcript accumulation in roots as analyzed with qRT-PCR normalized to levels of a constitutively unregulated gene (sulfite reductase) (a), root hair length (b) and number (c) in 11 replicate 7-day-old WT (d) and ir*RALF* line 1 (e) and ir*RALF* line 2 (f) seedlings. The root hair number was counted per 500-μm root segment. The asterisks indicate the level of significant differences between WT and ir*RALF* lines 1 and 2 (unpaired Student's *t*-test: $^{**}P < 0.001$; $^{***}P < 0.0001$). All seedlings were grown vertically on the surface of Gamborg B5 medium (pH 6.8).

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

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

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

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 (Figure 10a,d) or capsule production (WT plants with 15.6 ± 0.9 and ir*RALF* line 1 with 13.0 ± 1.1; unpaired Student's *t*-test, $P = 0.1$) were found between WT and ir*RALF* plants. When plant pairs were grown in soil amended with 5% limestone, which increased the soil pH to 6.7, the growth of ir*RALF* lines was significantly reduced in comparison with WT plants (Figure 10b,e) in the later stages of growth. At 36 days after germination the maximal leaf length was significantly reduced from 15.7 ± 0.2 cm (WT) to 14.3 ± 0.3 cm (ir*RALF* line 1) (unpaired Student's *t*-test, $P = 0.0025$). When size-matched pairs were transplanted into potting soil amended with 20% limestone to produce a soil (pH 8.1) with a pH similar to the calcareous soils in which *N. attenuata* grows in Utah, the growth of ir*RALF* plants was severely reduced (Figure 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 Student's *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 Student's *t*-test, $P = 0.005$; Figure 10f). Reductions in leaf size and stalk length lowered the lifetime production of seed capsules: in soil of pH 8.1, ir*RALF* line 1 produced 9.4 ± 0.7 capsules and ir*RALF* line 2 produced 7.7 ± 0.8 capsules, about one-half of 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.

In the native habitat of *Nicotiana* in the Great Basin Desert, rosette leaf diameter, stalk length, and the production of flower buds, flowers, and seed capsules were recorded in WT plants and ir*RALF* lines 30, 33, 48 and 55 days after

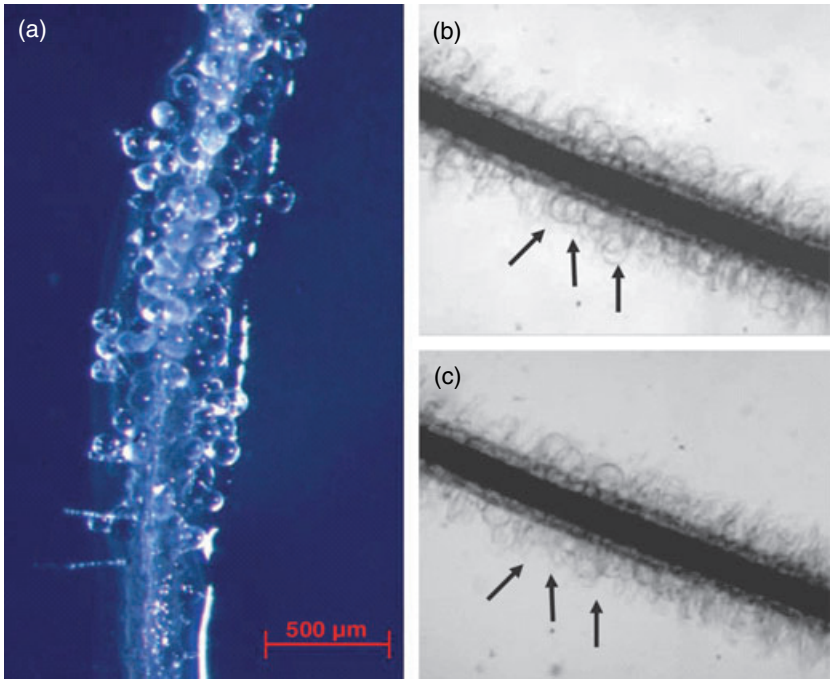

Figure 5. Reduced root hair number of ir*RALF* lines result from broken trichoblasts.
Root segments close to the root hair initiation zone of 5-day-old ir*RALF* line-1 seedlings, grown on the surface of Gamborg B5 medium (pH 6.8), are depicted. Most of the trichoblasts produced only a localized 'bulge'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

size-matched plants were planted in a recently burned juniper forest site near Santa Clara, UT, USA. The rosette leaf diameter of WT plants was 11.1 ± 0.9, 11.7 ± 0.8, 16.2 ± 1.1 and 16.7 ± 1.4 cm; this was significantly larger than that of both ir*RALF* line 1 plants (5.8 ± 0.4, 6.9 ± 0.6, 12 ± 0.8 and 12.7 ± 1 cm) and ir*RALF* line-2 plants (4 ± 0.7, 4.4 ± 0.8, 7.5 ± 1.3 and 7.5 ± 1.4 cm) (Figure 11a). The stalks of WT plants were taller than those of ir*RALF* lines (Figure 11b): 36.3 ± 4.7 cm high 55 days after plants were transplanted to the field compared with 15.4 ± 2.8 cm (unpaired Student's *t*-test, $P = 0.002$) in ir*RALF* line 1 and 4.9 ± 2.1 cm (unpaired Student's *t*-test, $P < 0.0001$) in ir*RALF* line 2. Most WT plants started producing flower buds 48 days after transplanting, and after 55 days WT plants had produced significantly more flower buds, flowers and seed capsules than had ir*RALF* lines (Table 1). These results are consistent with the hypothesis that *NaRALF* expression is essential for plant growth and reproductive performance in the basic soils of the native habitat of these plants.

Discussion

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

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

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

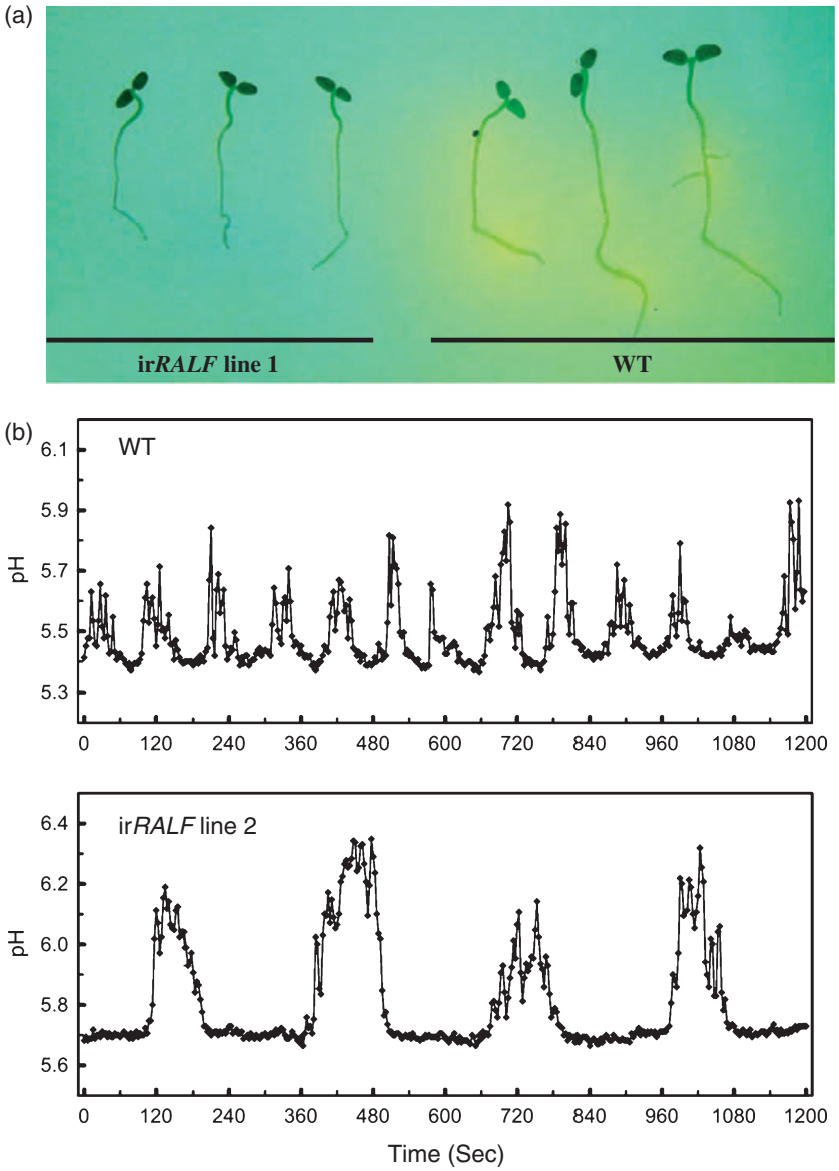

Figure 6. Extracellular pH of root hairs in *NaRALF*-silenced plants oscillates more slowly at higher pH value than do those of wild-type (WT) plants.
(a) *NaRALF*-silenced plants are unable to acidify the media. Seven-day-old seedlings of WT and ir*RALF* line 1 were pressed into pH indicator gel (pH 6.3), and images were taken after 48 h. The yellow color of the media surrounding the roots of WT seedlings illustrates their ability to acidify the media. Seedlings of neither ir*RALF* line 1 nor ir*RALF* line 2 (data not shown) were able to turn the media yellow.
(b) Typical extracellular pH oscillations at the tip of growing root hairs in WT and ir*RALF* plants. Upper panel: extracellular pH oscillations at the tip of one root hair in WT plants. Lower panel: extracellular pH oscillations at the tip of one root hair in ir*RALF* line 2 plants.

importance of the localized acidification of the cell wall during root hair initiation and tip growth has been demonstrated (Bibikova *et al.*, 1998), but how the process was regulated has until now remained unclear. Our results provide evidence that NaRALF is involved in regulating the apoplastic pH required for root hair formation. Silencing *NaRALF* led to a slower rate of oscillation in extracelluar pH, and in Arabidopsis such pH oscillations have been linked to the regulation of wall properties directly related to the control of tip growth (G. Monshausen *et al.*, unpublished data), suggesting possible reasons why ir*RALF* lines have disrupted root hair formation. Root hairs in the ir*RALF* lines often rupture, and the fact that a large increase in alkalinization levels always preceded a root hair rupture led us to hypothesize that the disrupted regulation of apoplastic pH at the root hair tips was one cause of the root hair phenotype of ir*RALF* lines. This hypothesis was confirmed by the partial recovery of the root hair phenotype (root hair length and number) when ir*RALF* lines were grown in low-pH-buffered medium; moreover, WT plants produced a root hair phenotype similar to that of ir*RALF* lines when WT plants were grown in medium with the same pH (pH 6.8), but strongly buffered with Tris (or HEPES) buffer. Thus, irrespective of the precise mechanism whereby NaRALF is regulating the root hair growth, these results suggest an important element of the action of NaRALF is the effect it has on proton fluxes associated with growth. Additional evidence came from the competition experiments: WT and ir*RALF* plants grew similarly in low-pH soils; however, in high-pH soil, where plants need more energy to

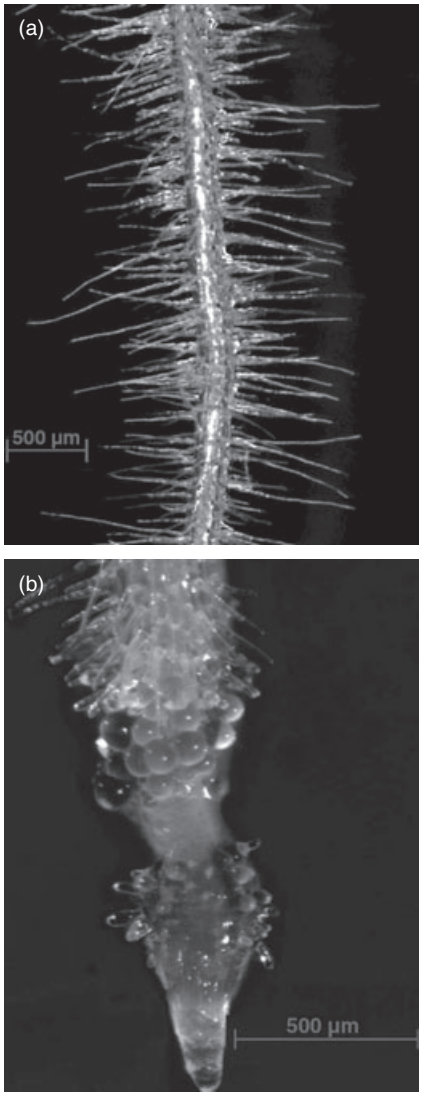

Figure 7. Root hairs of wild-type (WT) plants display the root hair phenotypes of ir*RALF* lines when grown in Tris (or HEPES) buffered medium (20 mM, pH 6.8).
WT seedlings grown in Gamborg B5 medium at pH 6.8 produce root hairs with an average length of 660 µm (a); however, when grown on the same medium but supplied with Tris or HEPES buffer (20 mM, pH 6.8), root hairs were short and some trichoblasts produced only a localized 'bulge' (b), reminiscent of ir*RALF* lines grown on Gamborg B5 unbuffered media (pH 6.8).

generate a proton gradient across their cell walls for nutrient uptake, ir*RALF* plants were strongly out-competed by WT plants.

Silencing *NaRALF* in *N. attenuata* results in altered dynamics of pH oscillations and in 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 the function of NaRALF.

The apoplastic localization of RALF (Escobar *et al.*, 2003), together with the discovery of 120- and 25-kDa cell membrane proteins, which can specifically bind to RALF (Scheer *et al.*, 2005), suggests 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 downstream signals regulated by NaRALF. Previous work with the *rhd2* mutant demonstrated that the ROS produced by NADPH oxidase in the trichoblast is important for forming the tip-focused calcium gradients required for tip growth. We also observed a decreased accumulation of ROS in the root hair initiation zone of the ir*RALF* lines, suggesting that NaRALF may also affect root

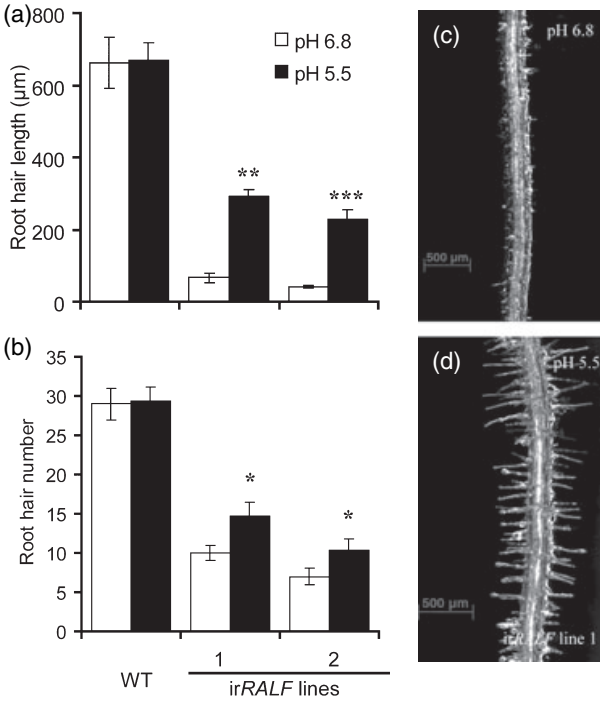

Figure 8. The wild-type (WT) root hair phenotype is partially restored in ir*RALF* lines when grown on low-pH-buffered medium.
Mean (±SEM) root hair length (a) and number (b) of 11 replicate 7-day-old WT and ir*RALF* plants measured in Gamborg B5 medium (pH 6.8) either with or without MES buffer (or phosphate buffer) (20 mM, pH 5.5). The number of root hairs was counted per 500-µm root segment. Examples of a root from a 7-day-old ir*RALF* line 1 plant grown in Gamborg B5 medium at pH 6.8 (c), and in medium supplied with 20 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES)/phosphate buffer at pH 5.5 (d). The asterisks indicate the level of significant differences between WT and ir*RALF* lines 1 and 2 (unpaired Student's *t*-test: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$).

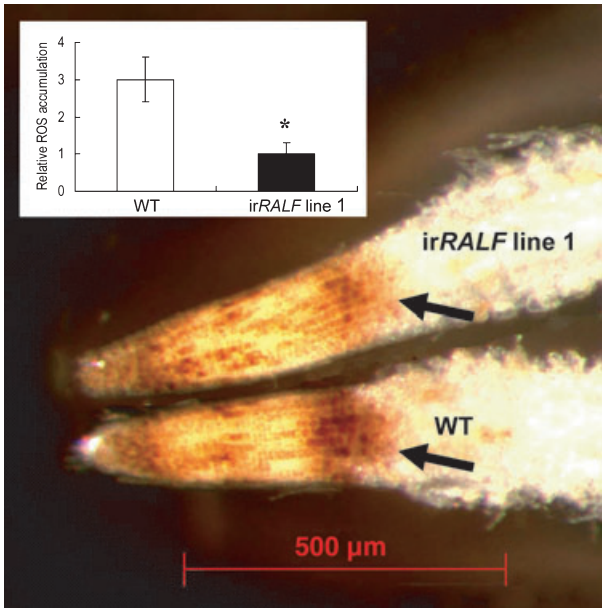

Figure 9. ROS accumulation decreased in roots of ir*RALF* lines.
Five-day-old seedlings were stained with 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB) for 2 h. The root hair initiation zone of WT plants showed more intense DAB staining (as indicated by the arrows) than did that of ir*RALF* line 1. Inset (representative of $n > 5$ roots): mean reactive oxygen species (ROS) levels measured using the Amplex red hydrogen peroxide/peroxidase assay kit in 11 replicate 1-cm-long root tips of WT and ir*RALF* line 1; the intensity of ROS accumulation was defined as relative levels in comparison with the ir*RALF* line 1 value, which was set as 1.0. The asterisk indicates the level of significant difference (unpaired Student's *t*-test: $*P < 0.05$).

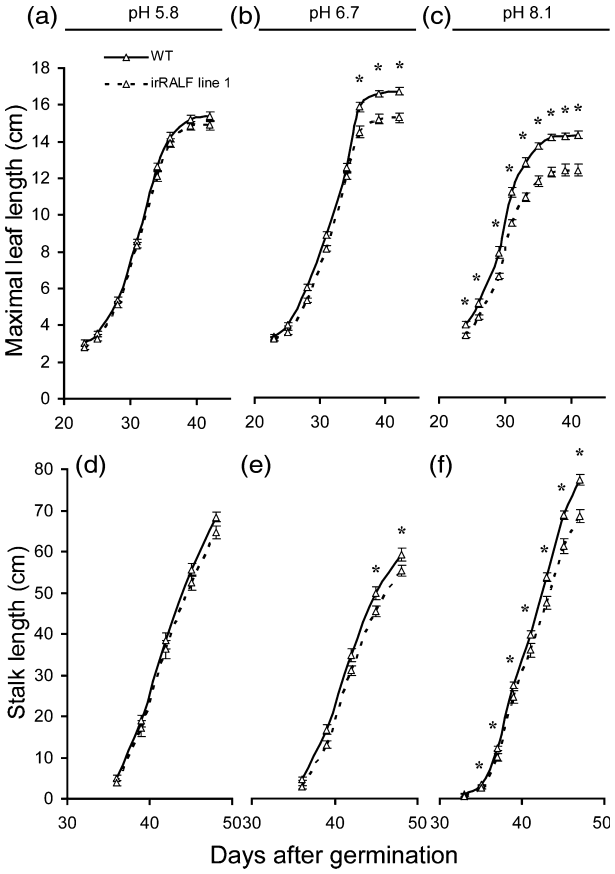

Figure 10. Leaf size and stalk length of ir*RALF* lines were reduced when they competed against wild-type (WT) plants in the glasshouse.
Mean (±SEM) maximal leaf length and stalk length were measured in 12 replicate pairs of size-matched WT and ir*RALF* line-1 plants grown in soil buffered to pH 5.8 (a and d), pH 6.7 (b and e) and pH 8.1 (c and f). No differences were observed in symmetrical competition pairs (WT–WT or ir*RALF* line 1–ir*RALF* line 1 pairs; see Figures S5 and S6), and similar results were obtained in WT–ir*RALF* line 2 competitions (see Figure S4). The asterisks indicate the level of significant differences between WT and ir*RALF* line 1 (paired Student's *t*-test: $*P < 0.05$).

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 10–70% as long as those of WT plants, is reduced (Gilliland *et al.*, 2002). When grown in pH-5.8 soils in the glasshouse, ir*RALF* lines showed leaf sizes and stalk lengths similar to those of WT plants. Low soil pH facilitates the active transport of nutrients, and the longer roots of ir*RALF* plants might compensate for the loss of nutrients that are normally absorbed by root hairs. When plants were grown in the basic soils in which *N. attenuata* plants are commonly found in nature, the fitness of ir*RALF* plants was severely reduced. Two factors may be responsible: either (a) the ir*RALF* lines have fewer and shorter root hairs, or (b) the trichoblasts have lost the ability to regulate cell wall pH dynamics. Additional work will be required to determine which if any of these mechanisms are responsible for the reduction in plant growth.

In summary, our results demonstrate that NaRALF is a peptide signal needed for regulating root growth and the apoplastic pH of the tip of trichoblasts. NaRALF is activated by unknown developmental stimuli, affects the levels of ROS accumulation, and possibly the activity of H^+-ATPase in plasma, and subsequently influences the periodicity of pH oscillations and maintains an appropriate cell wall pH environment. When *NaRALF* is silenced, trichoblasts become 'deaf' to developmental and environmental stimuli, the cell wall pH oscillates more slowly and attains higher pH values and 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.

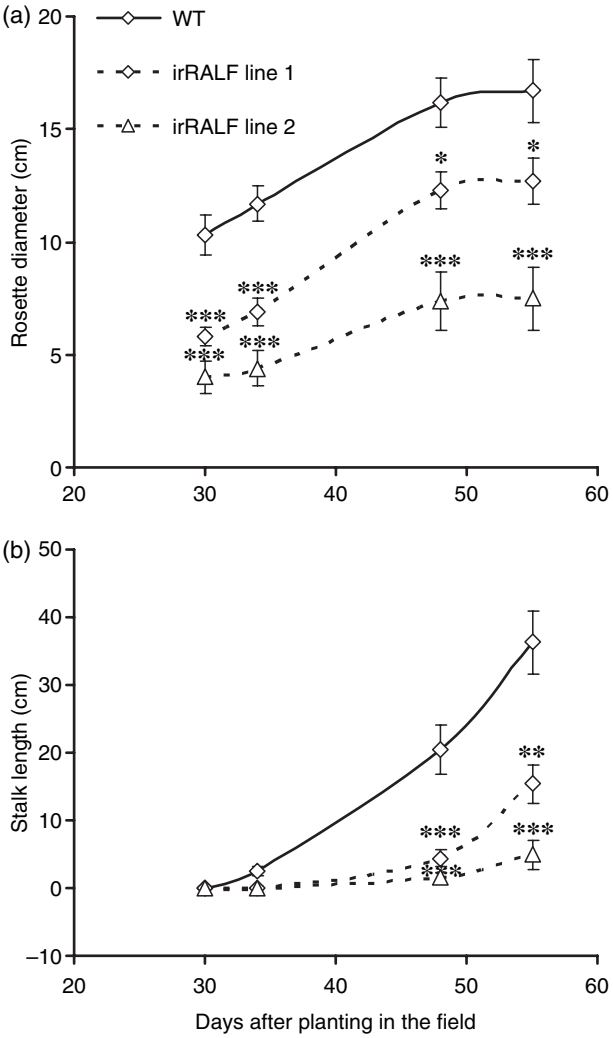

Figure 11. When grown in the native habitat of the Great Basin Desert, ir*RALF* plants attain smaller rosette sizes and shorter stalk lengths than wild-type (WT) plants.
Mean (±SEM) rosette diameters (a) and stalk lengths (b) of 10 replicate WT and ir*RALF* line-1 and -2 plants measured 30, 34, 48 and 55 days after planting in a native population in Utah. The asterisks indicate the level of significant differences between WT and ir*RALF* lines (unpaired Student's *t*-test: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$).

Table 1 ir*RALF* lines produced fewer buds, flowers and capsules than did wild-type (WT) plants in the native habitat

Genotype	Buds	Flowers	Capsules
WT	7.7 ± 1.5	6.4 ± 2	4 ± 1.5
ir*RALF* line 1	4.2 ± 1*	1 ± 0.5**	0
ir*RALF* line 2	0.8 ± 0.5**	0.1 ± 0.1***	0

Mean (±SEM) number of buds, flowers and capsules of WT and ir*RALF* plants of 10 matched triplicates after 55 days of growth. The asterisks indicate the level of significant differences between WT and ir*RALF* lines (unpaired Student's *t*-test: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$).

Experimental procedures

Plant growth

We used seeds of the 21st generation of an inbred line of *N. 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 gibberellic acid (GA_3). After 10 days, seedlings were planted into soil in Teku pots (Pöppelmann, http://www.poeppelmann.com).

For the competition experiments in the glasshouse, after 10 days in Teku pots, two seedlings of similar size and appearance were transplanted 7 cm apart in 2-l pots under the conditions as described by Zavala *et al.* (2004). A total of 180 pairs were assigned to the following five groups: (i) WT–ir*RALF* line 1; (ii) WT–ir*RALF* line 2; (iii) WT–WT (as a control); (iv) ir*RALF* line 1–ir*RALF* line 1 (as a control); (v) ir*RALF* line 2–ir*RALF* line 2 (as a control). The five groups were grown in three kinds of soil: potting soil B410 (Stender; http://www.stender.de); B410 soil augmented with 5% limestone (95% $CaCO_3$, Trollius; 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 the natural habitat of *Nicotiana*. Additional fertilizers were supplied after the plants were transferred to soil: 2 g of Borax (Nic.Sosef International B.V., http://www.sosef.nl) was added to each pot on the day after transplantation; 1.25 g Borax and 35 g of Peters General Purpose (Scotts International, http://www.scottsinternational.com) were supplied after 1 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 soils of pH 5.8 and pH 8.1).

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

To measure the velocity of root tip growth, 5-day-old seedlings were placed into a micro-rhizotron as described by Nagel *et al.* (2006) under a photoperiod of 14 h at 27°C in light and at 22°C in the dark phase. Every 30 sec an image was taken of the root tip with a CCD camera (Sony XC-ST50; Sony, http://www.sony.net). Infrared illumination (λ = 940 nm) allowed images to be acquired during the dark phase. The acquired images had a resolution of 700 × 480 pixels, which corresponds to an area of 3.6 × 2.5 mm^2. The camera was equipped with a low-pass infrared filter (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, http://www.aeon.de; Schmundt *et al.*, 1998). The image sequences were used to calculate root tip velocity and $REGR_{max}$, a process which is described in detail in Walter *et al.* (2002, 2003). Because root growth correlated positively with temperature (Pahlavanian and Silk, 1988; Walter *et al.*, 2002), $REGR_{max}$ and root tip velocity were averaged over three different time periods to compare WT plants and ir*RALF* line 1 (morning, from 6:00 to 13:00 h; afternoon, from 13:00 to 21:00 h; night, from 21:00 to 6:00 h).

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

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

Analysis of the extracellular pH of trichoblasts by the pH-sensitive dye fluorescein-dextran

Surface pH measurements were conducted as described by G. Monshausen *et al.* (unpublished data). Five-day-old seedlings of WT and ir*RALF* line 2 were transferred to cuvettes and covered with 0.7% (w/v) low-temperature gelling agarose containing 10% (w/v) Gamborg B5 salts, 1% (w/v) sucrose and 150 μg ml^{-1} pH-sensitive dye fluorescein conjugated to10-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 (Zeiss, http://www.zeiss.com) 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 sec, with each individual image scan lasting 2.2 sec. 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 *Nicotiana tabacum RALF* cDNA sequence (forward primer, ATGGGAGTTCCTTCAGGTTT; reverse primer, TTAACTCCTGCAACGAGTGA). The ORF fragment was cloned into a pGEM-T EASY vector (Promega, http://www.promega.com) and sequenced.

Generation of the transgenic plants

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

Caterpillar performance

Eggs of *M. sexta* were acquired from North Carolina State University (http://www.ncsu.edu) and were kept in a growth chamber (Snijders Scientific, http://www.snijders-tilburg.nl) at 26°C in 16-h light, and 24°C in 8-h 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). DNA (5 μg) was digested with *Eco*RI, *Hind*III and *Dra*I, and then blotted onto a nylon membrane. A probe was prepared by labeling the *NaRALF* ORF with ^{32}P using a random prime labeling kit (RediPrime II; Amersham, 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* OS, we exposed rosette-stage plants to UV-B produced by one TL40W bulb (Philips, http://www.philips.com) for 0, 2 and 6 h (each with three replicates). For OS elicitation, leaves on three replicate plants were wounded with a fabric pattern wheel, water or *M. sexta* OS were immediately applied to the puncture wounds, and plants were harvested 0. 0.5, 1, 3 and 6 h after elicitation as described in Zavala *et al.* (2004). Leaf samples were harvested and immediately frozen in liquid nitrogen.

Real-time PCR assay (qPCR)

Total RNA was extracted with TRI Reagent (Sigma) according to the manufacturer's instructions, and cDNA was prepared from 500 ng total RNA with multiScribe™ 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′-TTCAATGGCGAC-

CGCTG-3′, NaRALF reverse primer, 5′-TACTCCCGTTGCATCCCCT-3′; ECl forward primer, 5′-AGAAACTGCAGGGTACTGTTGG-3′, ECl reverse primer, 5′-CAAGGAGGTATAACTGGTGCCC-3′; FAM-labeled NaRALF probe, 5′-GATTGGGTGATGCCGGCGAGA-3′; VIC-labeled ECl probe, 5′-CGTCAAAATTCTCCACTTGTTTCAACTGT-3′. The assays using a double dye-labeled probe were performed on an ABI PRISM® 7700 Sequence Detection System (qPCR™ Core Kit; Eurogentec, http://www.eurogentec.be) with *N. attenuata sulfite reductase* (ECl) for normalization and, according to the manufacturer's instructions, with the following cycle conditions: 10 min at 95°C; 40 cycles of 30 sec at 95°C, and 30 sec at 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 1 mg ml^{-1} DAB for 2 h, and were photographed with a stereomicroscope.

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Supplementary Material

The following supplementary material is available for this article online:

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

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

Figure S3. Caterpillar performance and morphologies of ir*RALF* and wild-type (WT) plants after UV-B treatments.

Figure S4. Leaf size and stalk length of ir*RALF* line 2 were reduced when they competed against wild-type (WT) plants in the glasshouse.

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

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

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

Video Clip S2. Time-lapse movie of apoplatic pH increasing before a trichoblast bursts.

This material is available as part of the online article from http://www.blackwell-synergy.com

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Supplementary data

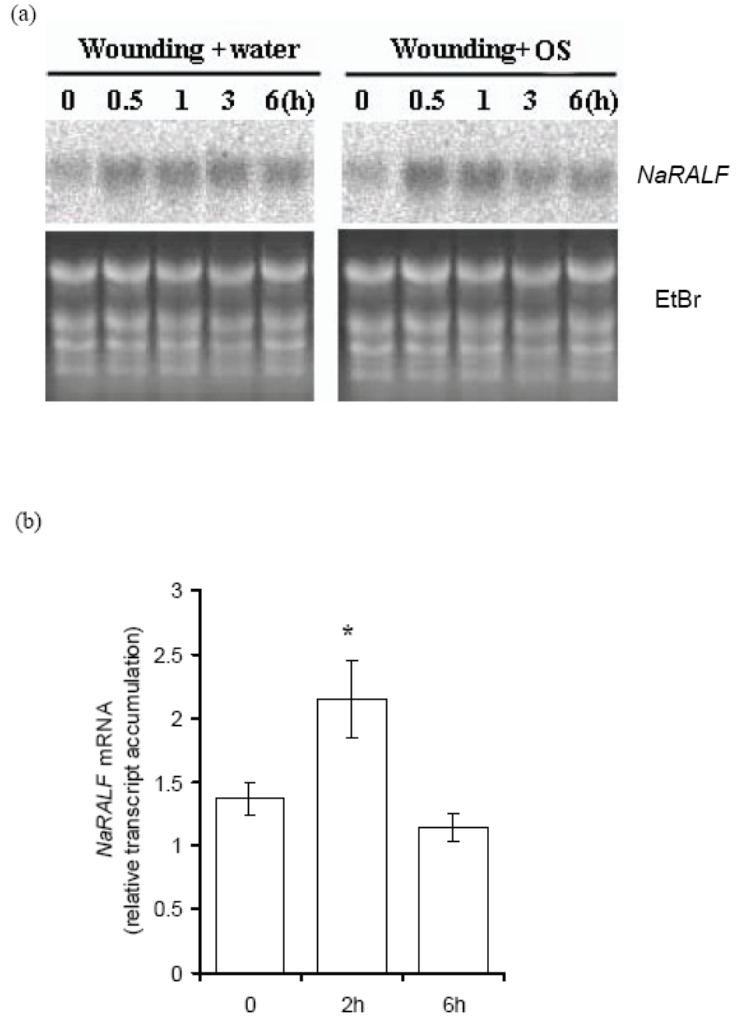
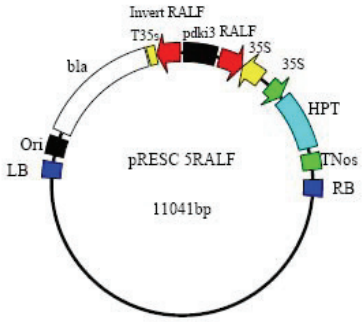


Figure S1. Transcript accumulation of *NaRALF* in response to different stresses. (a) Northern blot analysis of *NaRALF* in response to wounding and water or OS (oral secretions of *M. sexta*). Samples were collected from rosette-stage leaves elicitated by puncture wounds and treated with water (wounding+water) or *M. sexta* oral secretions (wounding+OS) after 0, 0.5, 1, 3, and 6 h. Total RNAs (10µg) were loaded, probed with a ^{32}P labeled *NaRALF* ORF sequence. An ethidium bromide (EtBr) stained gel serves as a loading control. (b) Mean (± SEM) *NaRALF* transcript accumulation in response to UV-B irradiation as analyzed by qRT-PCR normalized to levels of sulfite reductase transcripts. Three replicate rosette-stage plants were exposed to UV-B for 0, 2 and 6 h for each treatment time. The asterisk indicates the level of significant differences between control and treated plants (unpaired *t*-test: *, $P<0.05$).

(a)



(b)

GGGCCTACGATTGGGTGATGCCGGCGAGATCTGGTAGGGGATGCAACGGGAGTATCGGAGA

GTGCATGGCTGAAGAAGATGAGTTTGAGCTGGACAGTGAGTCAAACAGGCGTATTTTAGCT

ACCAAAAAGTACATCAGCTATGGTGCGCTGCAGAAGAACAGTGTACCTTGTTCTCGCCGTG

GAGCTTCGTATTACAACTGCAAACCTGGTGCTCAGGCTAATCCTTACTCTCGTGGTTGCAGT

GCTATCACTCGTTGCAGG

(c)

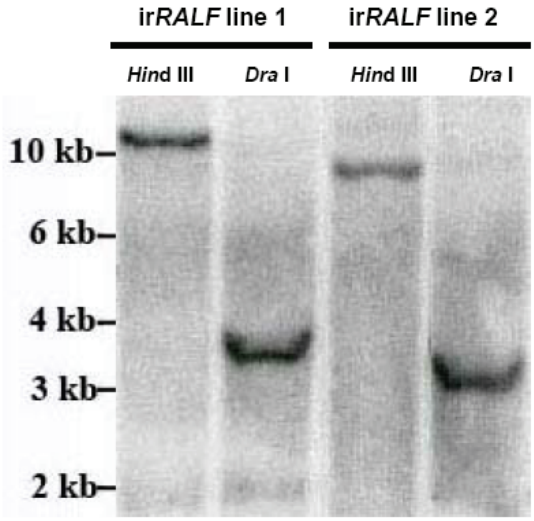


Figure S2. Generation of invert-repeat transgenic plants (ir*RALF* lines). Transformation vector pRESC5 (a), described in Bubner *et al*. (2006), used to transform *N. attenuata* plants to silence *NaRALF* expression by transferring a 263bp-fragment (b) of *NaRALF* inserted twice in opposite directions and resulting in an inverted repeat construct. (c) Southern blot analysis reveals that both ir*RALF* lines 1 and 2 harbor only one T-DNA as determined with an *hptII*-specific probe. 5 µg of genomic DNA were digested with *Hin*d III and *Dra* I.

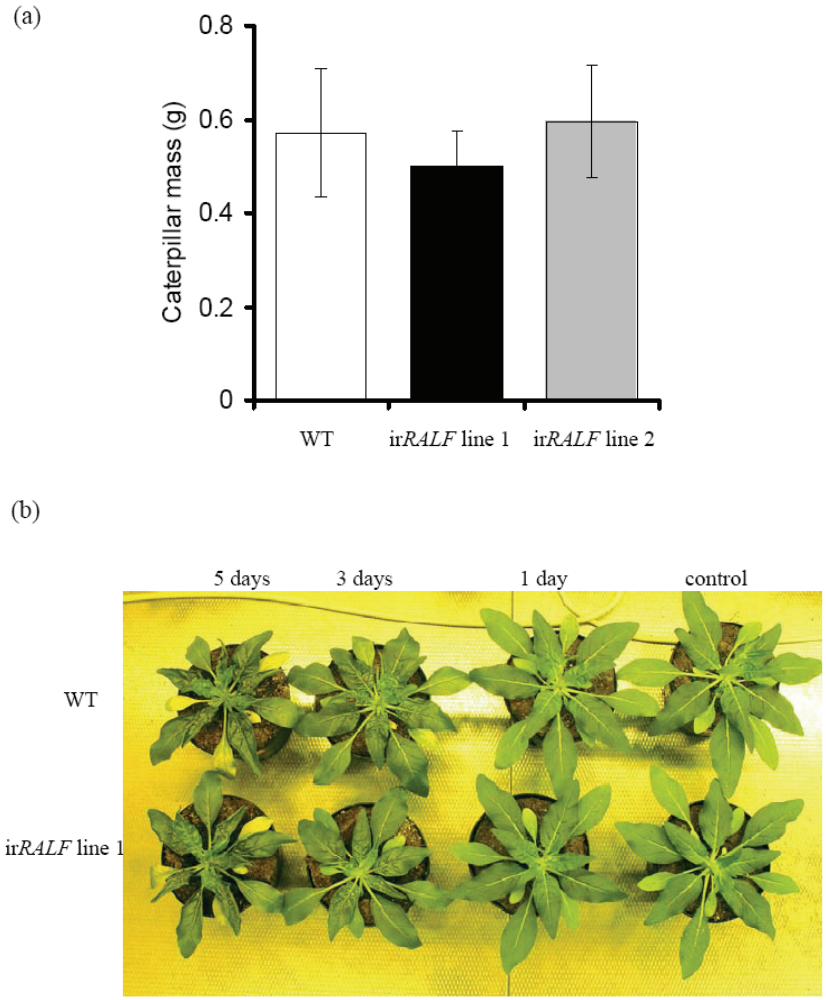


Figure S3. Caterpillar performance and morphologies of ir*RALF* and WT plants after UV-B treatments. (a) Mean (± SE) mass of *M. sexta* larvae after 10 days of growth on WT and ir*RALF* rosette-stage plants. A single freshly hatched larva was placed on a fully developed leaf of 12 replicates of WT and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

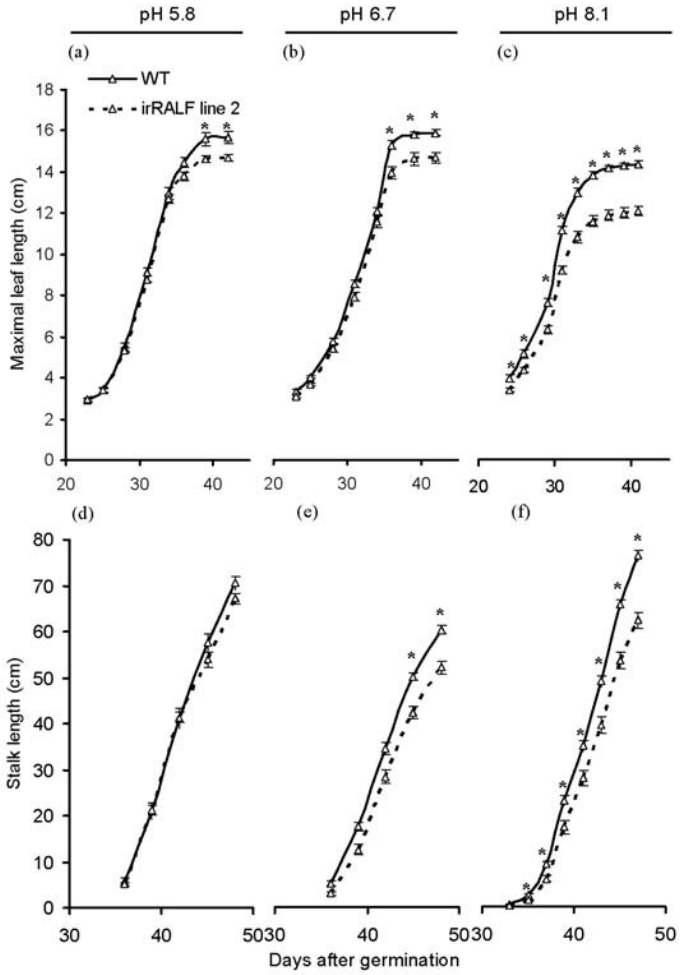


Figure S4. Leaf size and stalk length of ir*RALF* line 2 were reduced when they competed against WT plants in the glasshouse. Mean (± SE) maximal leaf lengths and stalk lengths were measured in 12 replicate pairs of size-matched WT and ir*RALF* line 2 plants grown in soil buffered to pH 5.8 (**a** and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

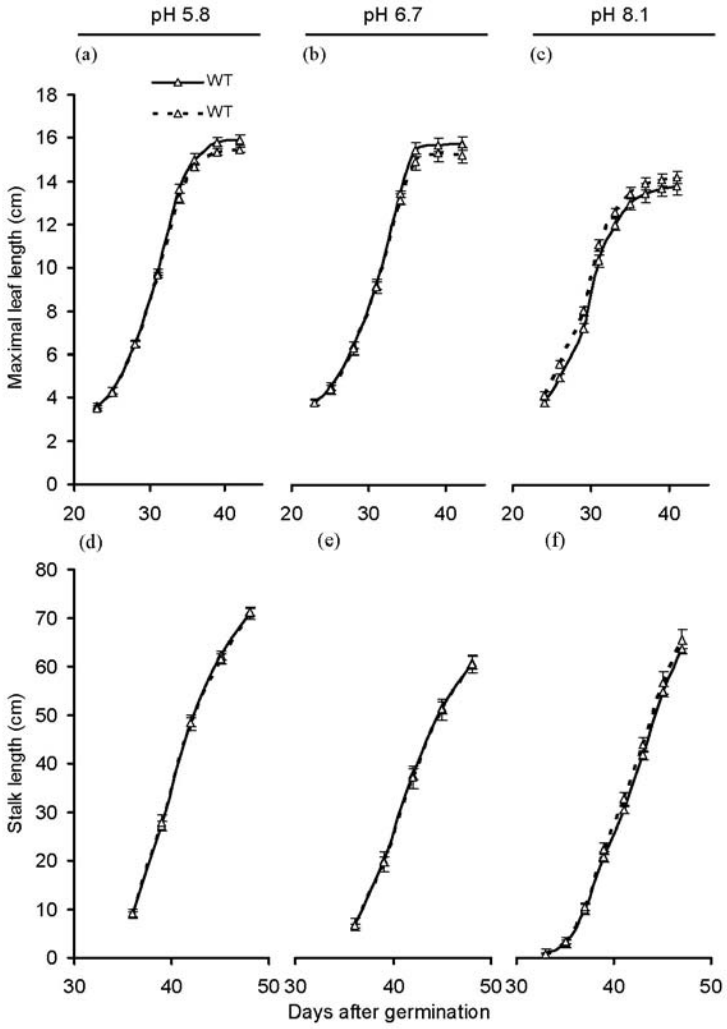


Figure S5. Leaf size and stalk length of WT plants were the same when they competed against each other in the glasshouse. Mean (± SE) maximal leaf lengths and stalk lengths were measured in 12 replicate pairs of size-matched WT and WT plants grown in soil buffered to pH 5.8 (a and d), pH 6.7 (b and e), pH 8.1 (c and f).

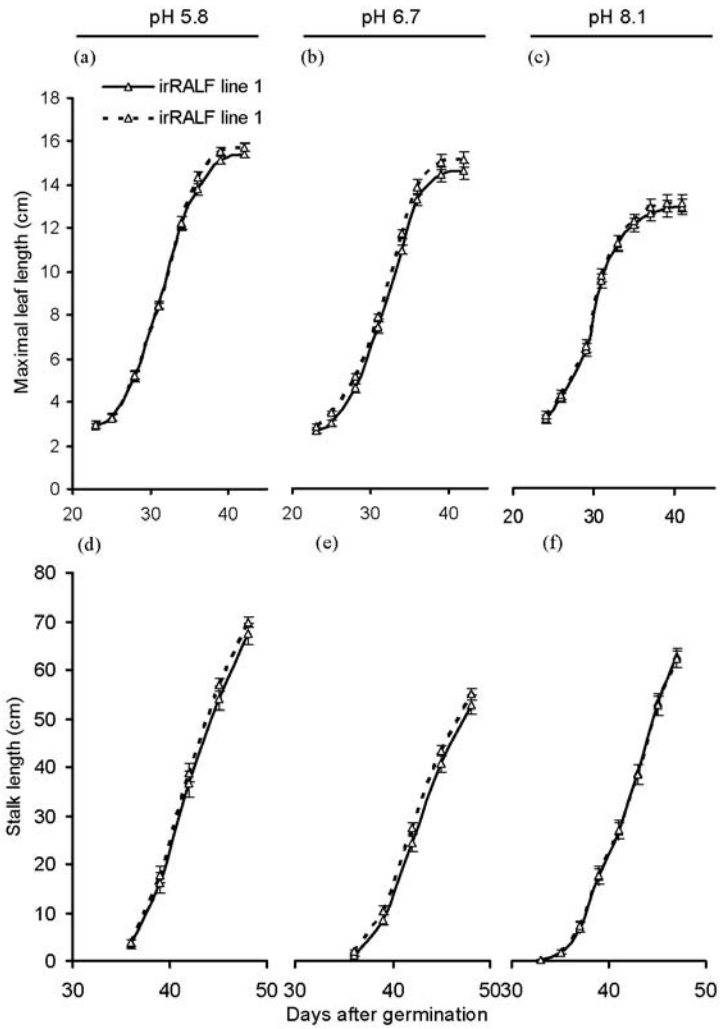


Figure S6. Leaf size and stalk length of ir*RALF* line 1 plants were the same when they competed against each other in the glasshouse. Mean (± SE) maximal leaf lengths and stalk lengths were measured in 12 replicate pairs of size-matched ir*RALF* line 1 and ir*RALF* line 1 plants grown in soil buffered to pH 5.8 (a and d), pH 6.7 (b and e), pH 8.1 (c and f).

10. Root growth of *Nicotiana attenuata* is decreased immediately after simulated leaf herbivore attack

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Abstract

Image-based non-destructive methods were used to quantify root growth reactions happening within hours following simulated leaf herbivore attack (1). The induction of wound reactions in leaves of seedlings of *Nicotiana attenuata* led to transiently decreased root growth rates: Upon application of the oral secretions and regurgitants of the specialist herbivore *Manduca sexta*, a transient decrease in root growth was observed that was more pronounced than if a mere mechanical wounding was imposed. Root growth reduction was more severe than leaf growth reduction and the timing of the transient growth reduction coincided with endogenous bursts of jasmonate (JA) and ethylene emissions reported in literature. The reaction of root growth was superimposed by a strong diel variation of root growth, which was caused by the fluctuating temperature to which the plants were exposed. Apart from the observed root growth reaction, other defense-related traits such as increased nicotine concentration, trichome length and density were activated within 72 h after wounding. Further experiments indicated that the response was elicited by fatty acid-amino acid conjugates that are contained in the oral secretions and that JA signalling is crucial for root-shoot communication here.

Main text

Plants constantly need to acclimate to a suite of fluctuating biotic and abiotic factors. Within the framework of their genetically determined response options, they react towards stress situations by maximizing protection against stress while minimizing deviations from optimal growth and development. Feeding of the larvae of the specialist lepidopteran herbivore *Manduca sexta* on *N. attenuata* has become a model case scenario for studying biotic stress (2). It has been shown in great detail there, that a diverse set of plant hormones like JA, methyl jasmonate and ethylene are rapidly induced immediately following wounding or herbivore attack (3,4,5)(McCloud & Baldwin 1997; Zhang & Baldwin 1997b; von Dahl & Baldwin 2004). Acclimation occurs both on the biochemical and morphological level, e.g. by increasing defence compounds (6), by synthesizing defence related proteins, by emitting volatiles to attract predators and parasites of herbivores (7) and by altering plant morphology via increased formation of trichomes, thorns or scleromorphy (8,9). Many studies demonstrated resource based trade-offs between growth and defence on a large time scale (e.g. 10, 11). Yet, it is unclear how fast a trade-off-linked reorganization

of plant metabolism, diverting resources away from growth or development towards defence, can occur.

Recently, development of growth imaging methods has allowed to study short-term growth responses of above- and belowground sink organs towards fluctuations of environmental factors, such as alterations of light climate (12) or responses towards gravitropic stimuli (13,14,15). Clear responses are often seen best in young seedlings as they grow fast and as they can be cultivated in agar-filled, translucent Petri dishes allowing live imaging. Hence, it was the aim of our study to monitor defence reactions in the *N. attenuata* seedling system while at the same time assessing the dynamics of root growth reaction following such an attack.

Herbivory-induced wound reactions were simulated by applying different substances on the mechanically wounded primary leaf of the seedling plant 16 d after germination. Investigated substances were: i) oral secretions and regurgitants of *M. sexta* larvae (OS), ii) methyl jasmonate, iii) fatty acid-amino acid conjugates, iv) control solutions for the different test substances (water, buffer, lanolin). All wounding treatments were performed at the same time of the day to make sure that the reaction was not masked by fluctuating responses of the plant to temperature changes (plants were grown in a 14 h / 10 h light/ dark regime with temperatures of 26°C and 22°C at day and night, respectively).

Root growth was affected strongly by temperature (Fig. 1a) and increased linearly with temperature in the analyzed range. Root growth decreased markedly, if OS was added to the wounds. OS-treated and wounded control plants differed in growth reduction from about 2 h after wounding onwards. To uncouple this reduction from temperature-induced growth reductions, the experiment was repeated under continuous light and temperature, leading to a comparable result (Fig. 1b). When OS was added to wounds, nicotine concentration and trichome length and density increased within 72 h after the wounding reaction. Root growth reaction was comparable between OS-treatment and treatments, in which methyl jasmonate or fatty-acid amino acid conjugates were supplied to the wounds. The strongest difference concerning the distribution of relative elemental growth rate along the root growth zone between control and OS treatment was observed about 1 mm behind the root tip in the beginning of the central elongation zone. Leaf growth was monitored in an additional set of experiments. There, a transient decrease of growth was seen as well, but it was not as pronounced as for root growth.

The kinetics of the root growth depression point on a superposition of two physiological effects: During the first hour after wounding, root growth decreases similarly in control and

OS-treated plants, indicating a mere hydraulic response due to the loss of water and turgor pressure (12). Thereafter, a specific response towards OS is observed, which coincides with the time frame of about 2-3 h that was reported for the systemic increase of JA transported from shoot to root. Baldwin *et al.* (16) reported that JA pools in roots are increased systemically (3.5-fold) within 180 minutes following mechanical wounding. Hence, JA might mediate the reduction in root growth and induce the defense machinery, which diverts resources from growth to defense.

Figures

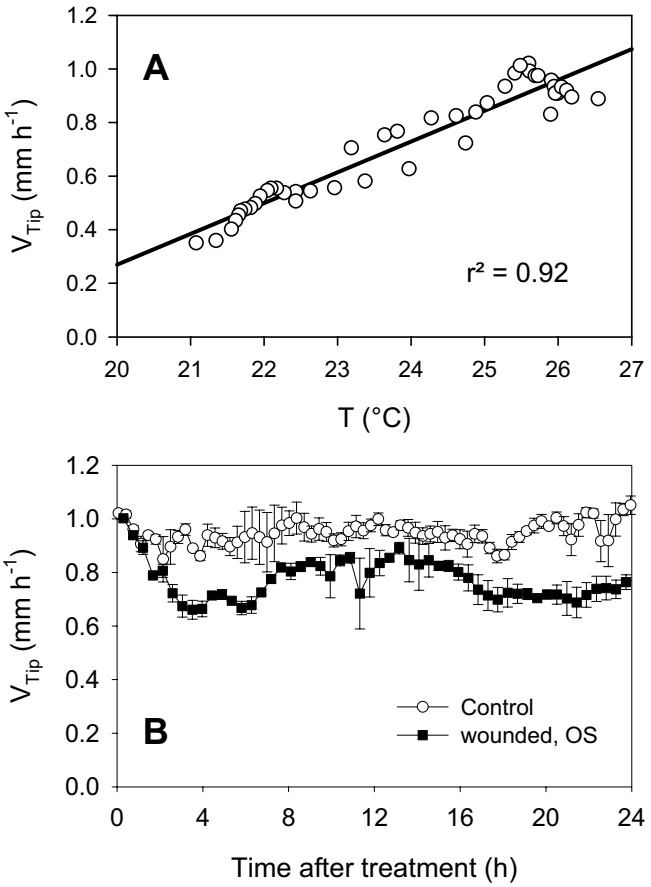

Figure 1: Root growth in *Nicotiana attenuata*, grown on agar-filled Petri dishes. A) Velocity of the root tip (V_{Tip}) versus temperature of the agar medium. B) Velocity of the root tip of control plants and of plants with leaves that were wounded at t = 0 and treated with oral secretions and regurgitants (OS) of *Manduca sexta*. Plants were grown in continuous light at T = 26°C (n = 3, mean values and SE).

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11. Abstract

The appropriate regulation of plant growth in response to environmental cues has adaptive significance. Accordingly, plants have evolved precise mechanisms for such regulation in order to acclimate to myriads of fluctuating biotic and abiotic factors. Herbivory attack displays a prime biotic stress factor and induces physiological and morphological defense traits diverting limited resources from primary (growth-sustaining) to secondary (defense-related) metabolism.

The aim of the present PhD thesis was to use imaging methods to monitor growth dynamics after simulated herbivore attack at high temporal and spatial scale to compare growth with defense and hormonal dynamics and to elucidate mechanisms of endogenous growth control. *Nicotiana attenuata* and the natural specialist lepidopteran herbivore *Manduca sexta* was used as a system to study these biotic interactions, as the signals activating herbivore defense responses are known.

The PhD demonstrated that simulated herbivory reduces root growth transiently and affects roots stronger than shoots. The reaction of root growth was superimposed by a strong diel variation of root growth, which was caused by the fluctuating temperature to which the plants were exposed. A strong positive correlation of *N. attenuata* root growth with temperature was found. Apart from the observed root growth reaction, defense traits such as increased nicotine concentration, trichome length and density were activated within 72 h after wounding.The kinetics of the root growth depression point on a superposition of two physiological effects: The immediate decrease in root growth might be explained by a hydraulic response due to the loss of water and turgor pressure. Thereafter, a specific response towards the oral secretions of *M. sexta*, which coincides with the time frame for the systemic increase of jasmonic acid (JA) transported from shoot to root. Further experiments indicated that the response was elicited by fatty acid-amino acid conjugates that are contained in the oral secretions. With mutant lines of *N. attenuata* silenced in the biosynthesis and the perception of JA (as*LOX3* and ir*COI1*-plants, respectively) and treatments with 1-methylcyclopropene (ethylene activation blocker) it was demonstrated that the reduction in root growth upon simulated herbivory is mediated via wound induced JA and not ethylene. Furthermore a strong induction of the oligopeptide RALF was found in roots after wounding, and experiments with plants silenced in their expression of RALF

show increased root growth, suggesting that RALF is needed for regulating and development of root growth.

To conclude JA signaling is involved in mediating root growth inhibition and demonstrates how the shoot may regulate reduction in root growth. This PhD thesis provides further evidence that endogenous JA acts as a distress signal, slowing vegetative growth during defense responses.

12. Zusammenfassung

Pflanzen verfügen über die Möglichkeit, ihr Wachstum flexibel an sich permanent ändernde Umweltbedingungen anzupassen. Innerhalb ihrer genetischen Möglichkeiten können sie sich an biotischen und abiotischen Umweltstress kurzfristig akklimatisieren und längerfristig adaptieren. Herbivorie stellt einen der größten biotischen Stressfaktoren dar. Insektenfraß induziert den Aufbau von physiologischen und morphologischen Verteidigungseigenschaften, welche hohe Investitionen limitierter Ressourcen und Energie erfordern, die dem Wachstum und der Reproduktion nicht mehr zur Verfügung stehen. Es entsteht eine Konkurrenz (trade-off) um Nährstoffe zwischen dem primären und sekundären Metabolismus.

Ziel der vorliegenden Dissertation war es, mit Hilfe von zeitlich und räumlich hochauflösender Bildsequenzverarbeitung die Wachstumsdynamik nach simulierter Herbivorie zu untersuchen. Der Vergleich hormoneller Dynamiken mit Wachstumsdynamiken soll hierbei genutzt werden, um mögliche intrinsische Mechanismen der Wachstumskontrollen oder Wachstumssignale aufzuklären. Um die Interaktion zwischen Wachstum und Verteidigung zu untersuchen, wurden *Nicotiana attenuata* und der spezialisierte Schwärmer *Manduca sexta* als Modellsystem ausgewählt.

Die vorliegende Arbeit zeigt, dass eine simulierte Herbivorie Attacke des Primärblattes das Wachstum temporär reduziert. Die Wurzel ist stärker betroffen als der Spross. Die Verwundungsreaktion der Wurzel unterliegt einem starken Tagesgang, der durch die Temperaturschwankung des Substrates herbeigeführt wird. Es konnte eine starke positive Korrelation zwischen dem Wurzelwachstum und der Temperatur gezeigt werden. Die genauere Betrachtung der Wachstumskinetik ergibt, dass zwei Effekte an der Reduktion des Wurzelwachstums beteiligt sind. Der erste starke Rückgang des Wurzelwachstums ist mit einem hydraulischen Effekt erklärbar: die Verletzung des Blattgewebes ist mit einer Reduktion des Tugors verknüpft, welche eine sofortige Verringerung des Wachstums nach sich zieht. Ein zweiter verzögerter Effekt ist zu erkennen, der spezifisch durch die Applikation von oralen Sekreten der Larve verstärkt wird und mit der Dynamik der durch Verwundung ausgelösten Jasmonsäureproduktion (JS) übereinstimmt. Diese Reaktion konnte mit der Applikation von Fettsäure-Aminosäure-Konjugaten, welche im oralen Sekret des Larvenspeichels enthalten sind reproduziert werden. Des Weiteren wurden Mutanten von *N. attenuata* herangezogen, deren JS-Produktion (*Na*as*LOX3*) und JS-

Wahrnehmung (*Na*ir*COI1*) stark reduziert ist. Versuche zeigten, dass JS für den zweiten Abfall des Wurzelwachstums verantwortlich ist. Das ebenfalls bei Herbivorie induzierte Ethylen hingegen hat keinen Einfluss auf das Wurzelwachstum, was mit Hilfe von 1-Methylcyclopropen (Ethylen-Blocker) ermittelt werden konnte. Weiterhin konnte gezeigt werden, dass das Oligopeptid RALF nach Verwundung in der Wurzel verstärkt exprimiert wird. Mutanten deren RALF Biosynthese stark reduziert wurde, erreichten ein höheres Wurzelwachstum als der Wildtyp.

Zusammenfassend wurde gezeigt, dass endogene JS-Produktion das Wachstum beeinflußt und eine Möglichkeit darstellt, wie der Sproß nach Herbivorie über Signale das Wurzelwachstum reduziert. JS induziert demnach nicht nur die Verteidigungsmechanismen, sondern verringert gleichzeitig das vegetative Wachstum.

13. List of abbreviations

°C: Degree Celsius

as: antisense

cm: Centimeter

COI: Corotantine insensitive

d: Day

DISP: Digital image sequence processing

et al.: et alii

FAC: Fatty acid-amino acid conjugates

Fig.: Figure

GAL: Glycogen-binding domain

Gln: Glutamin

Glu: Glutamic acid

h: hour

ir: inverted repeat

JA: Jasmonic acid

jar: Jasmonate resistant

jin: Jasmonate insensitive

JS: Jasmonsäure

LOX: Lipoxygenase

MAP: Mitogen-activated protein

MCP: methylcyclopropen

MeJA: Methyl jasmomic acid

µL: Micro liter

µm: Micro meter

mg : Milligramm

min: Minute

ml: Milliliter

mm: Millimeter

Na: *Nicotiana attenuata*

Norm: Normalized

OS: Oral secretions and regurgitants

RALF: Rapid alkalization factor

REGR: Relative elemental growth rate

RGR: Relative growth rate

SD : Standard deviation

SE : Standard error

sec: Second

SNF: Sucrose NonFermentable

UV: Ultraviolet

$\mathbf{V_{Tip}}$: Velocity of root tip (absolute)

WT: Wild type

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine Promotionsversuche unternommen.

Jülich den 16.11.2007

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Grégoire Martin Hummel