Integrating below- and above-ground signaling in *Nicotiana attenuata*: root oxylipins systemically regulate leaf responses to attack and increase plant resistance

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Variluska Fragoso, M.S.
geboren am 06.08.1981
in Ponta-Porã, Brasilien

MAX-PLANCK-GESELLSCHAFT

Max-Planck-Institut für chemische Ökologie

Gutachter:

1. Prof. Dr. Ian T. Baldwin, Max Planck for Chemical Ecology – Jena, Germany.

2. Prof. Dr. Ralf Oelmüller, Friedrich-Schiller University – Jena, Germany.

3. Prof. Dr. John Jelesko, Virginia Polytechnic Institute and State University, Blacksburg, USA.

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1. Introduction

1.1. Roots, as part of whole plants and ecosystems

Between around 420 and 360 million years ago, in the Devonian period, plants underwent major adaptations in order to live on dry land; these adaptations allowed them to colonize the earth successfully above and below ground and, ultimately, to "green" the continents. Only 30 cm tall in the Early Devonian, plants in Late Devonian reached 30 m. Likewise, unicellular extensions of prostrate stems (i.e. rhizoids) of the Early Devonian period gradually developed below ground. As a result, deep root systems were able to provide the appropriate anchorage, nutrients and water needed by tall trees (Waisel, Eshel & Kafkafi, 2002). Other important traits also evolved in this period, such as vascularization, branching and true leaves (megaphylls). Interestingly, the first documented appearance of roots in the fossil records slightly precedes the appearance of leaves (Labandeira, 2007).

Despite their essential role in plant life, roots have been largely unstudied compared to above-ground tissues. Our knowledge of root traits, as well as of the interplay between leaf and root traits, is unbalanced and limited. Only recently have researchers begun to grasp the importance of plants' "hidden half". A recent study that investigated the evolutionary correlations between leaf and root functional traits revealed complex relationships between above- and below-ground traits (Kembel & Cahill, 2011). The authors conclude that, contrary to the prediction that traits of both leaves and roots evolved as parts of a single organism, in fact these organs evolved under fundamentally different selective pressures, and thus employ different strategies. Clearly, our knowledge of plant processes has much to gain by including the below-ground tissues.

Along with the differentiation of specialized tissues and organs, plants have also evolved refined signaling networks in order to regulate and coordinate their genome expression, metabolism and growth at the tissue and organ level (**Figure 1**). These short- and long-distance networks also offer plants a high level of plasticity, increasing their ability to cope with environmental variations and selective pressures (Kragler & Hulskamp, 2012). Scientists will eventually need to investigate the whole plant, because a given gene, protein or small molecule function may not be apparent when the elements of a complex system are considered in isolation (Casal, Fankhauser, Coupland & Blazquez, 2004).

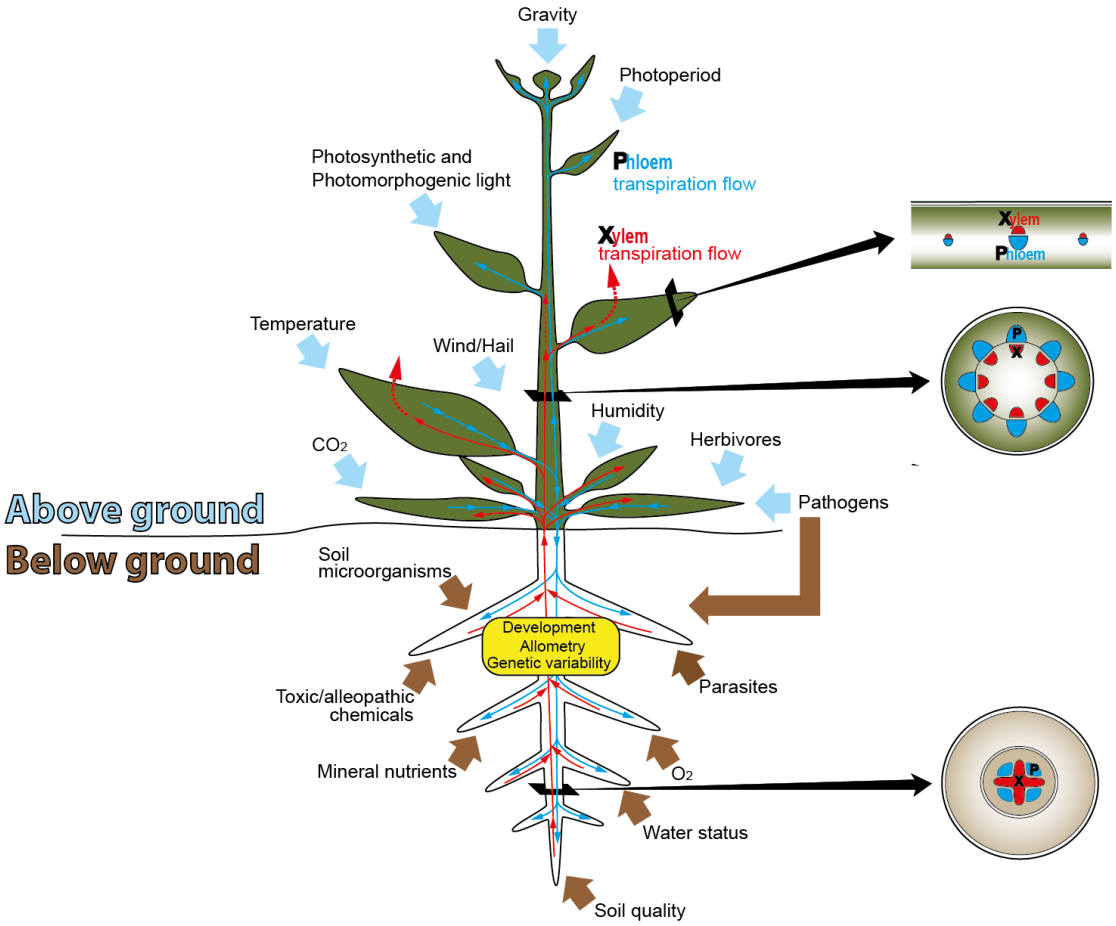

Figure 1. Schematic illustration of a plant exposed to biotic and abiotic environmental signals, above and below ground. Xylem stream (red) moves from roots to shoots, while phloem stream (blue) moves from source to sink tissues. Plant endogenous constraints (yellow box) also influence the responses to stimuli.

Similarly, the whole-organismic function of genes and molecules evolves by natural selection in an organism's natural habitat, embedded in its ecological interactions (Figure 1). Strategies employing new gene functions have emerged as solutions to the problems plants have faced over evolutionary time. When studying the whole organism, asking *how* and *in which context* often determines whether the observed outcome is comprehensible. Therefore, large portions of available sequenced genomes might only be understood when contextualized in the organism natural history (Baldwin, 2012).

The present thesis is framed within these considerations. It consists of two manuscripts that represent initial steps towards a better understanding of the ecological traits of plants. I view plants as whole organisms and wanted to study them under realistic conditions. To create the ideal toolbox, I used the wild coyote tobacco species, *Nicotiana attenuata*, and a collection of available transgenic lines.

First, in **manuscript I**, I described a new technique for this species, which allows for the independent manipulation of gene function of the above- and below-ground. Next, in **manuscript II**, I combined this development with our growing knowledge of *N. attenuata*'s natural history, and revealed novel mechanisms through which roots affect plant traits and above-ground herbivore preferences.

1.1.1. Nicotiana attenuata, a plant model

N. attenuata (Solanaceae) is a wild annual species of tobacco native to the xeric habitat of southwest North America, the Great Basin Desert (**Figure 2**). It germinates from long-lived seed banks in response to smoke cues in post-fire environments (Baldwin & Morse, 1994; Preston & Baldwin, 1999). The synchronized germination of seeds in transiently nitrogen-rich environments leads to high intraspecific competition between *N. attenuata* plants; these plants occur naturally in patches of isolated monocultures. In addition, the post-fire environment also exposes plants of *N. attenuata*, a pioneer species, to a highly unpredictable and varied community of herbivores of different levels of specialization and various guilds (**Figure 2**). In order to survive these extreme abiotic and biotic selective pressures, *N. attenuata* has evolved a short generation time with a rapid vegetative growth and prolonged lifetime seed set, as well as highly plastic adaptive responses (Baldwin, 1998, 2001; Kessler, Diezel & Baldwin, 2010; Diezel, Allmann & Baldwin, 2011). In addition, *N. attenuata* is self-compatible but an opportunistic out-crosser. This species is easily cultivated *in vitro*, and there is a well-established protocol for the generation of stably transformed lines (Krugel, Lim, Gase, Halitschke & Baldwin, 2002). These properties make *N. attenuata* a perfect model plant for the study of plant defenses, at the molecular and ecological levels.

1.2. Plant-herbivore interactions

Plants have evolved sophisticated mechanisms to cope with a myriad of inhospitable environmental conditions. Plant stresses can be abiotic (e.g. insufficient light, water, or nutrients, etc.) or be caused by other organisms, such as herbivores. Plant-herbivore interactions have been shaped through evolution in an antagonistic way: plants are eaten by herbivores but are able to defend themselves and in the process to decrease herbivore performance, ultimately decreasing its reproductive fitness. With regard to the amount of plant material consumed, insects are the most voracious herbivores and represent one of the most diverse groups of animals on the planet (van der Meijden, 1996). Interestingly, this antagonism between plants and insects and counter-adaptations can be tracked at the

molecular level. Talyzina and Ingvarsson (2006) showed that a plant gene family encoding for inhibitors of insect digestive proteins (i.e. protease inhibitors or PIs) is as diverse as the insect proteases they affect.

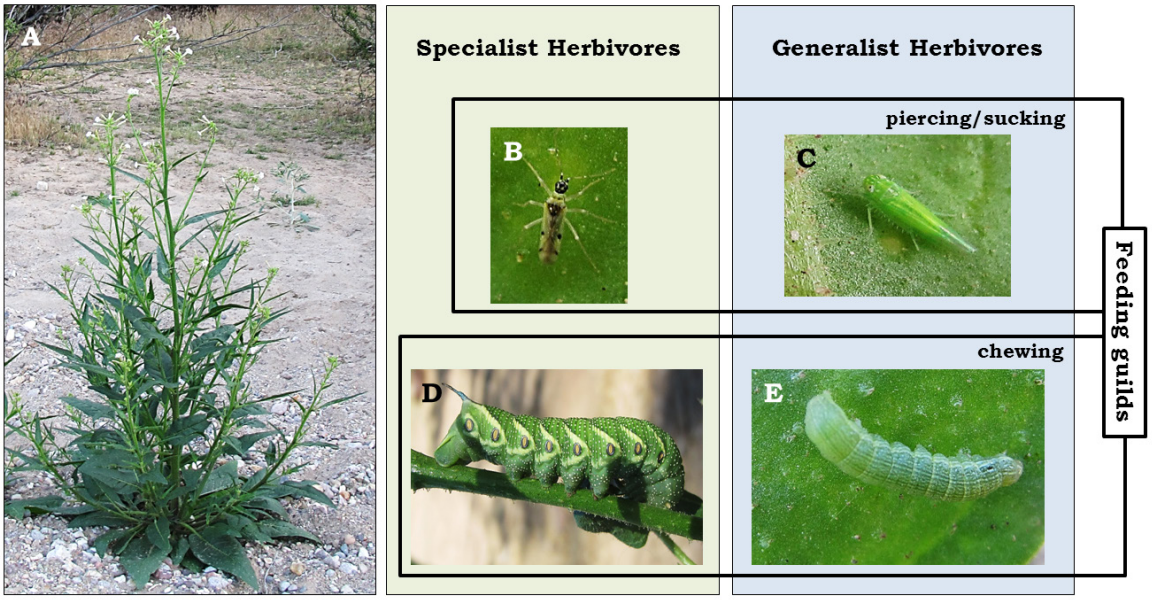

Figure 2. A. *Nicotiana attenuata* in its native habitat, the Great Basin Desert, faces the attack by an unpredictable and varied community of herbivores of different levels of specialization and feeding guilds. **B.** *Tupiocoris notatus*. **C.** *Empoasca* spp. **D.** *Manduca sexta*. **E.** *Spodoptera* spp.

From the perspective of the insect, the ability to become more tolerant to plant defense metabolites by detoxifying mechanisms can benefit their individual performance, which in turn is reflected in higher reproductive output. This defense mechanism provides a new niche, at the same time that this specialization narrows the range of possible host plants, often to a single genus. These insects become specialist herbivores (Ali & Agrawal, 2012). Indeed, more than 90% of all herbivorous insects are estimated to feed on less than three different plant families (Bernays & Graham, 1988). Some insects can even use the same plant defense traits for their own defense against predators or as mating signals, in a phenomenon known as sequestration (Conner et al., 2000; Kuhn et al., 2007; Kumar, Pandit, Steppuhn & Baldwin, 2013). Although there is a continuum in herbivore degree of dietary specialization, on the other extreme of highly specialized herbivores lie the generalist herbivores, which can feed on more than one plant family (Ali & Agrawal, 2012). Another important distinction between herbivores concerns their consumption behavior, or feeding guilds. Whereas chewing insects

use their strong mandibles to feed on plant tissue, causing extensive plant cell damage and loss of tissue (Karban & Baldwin, 1997), piercing/sucking insects use their mouthparts to penetrate and consume contents of plant cells or vasculature, inflicting minimal wounding to the plant (Walling, 2000).

From the perspective of the plant, the strategies to deter consumption by an herbivore are very diverse. Conventionally, they are categorized according to their method of action -- constitutive or inducible, and direct or indirect. Plant constitutive defenses are physical or chemical traits present in the plant regardless of the presence of herbivores; conversely, plant inducible defenses are mounted upon herbivore attack (Howe & Jander, 2008). Plant direct defenses have negative effects on herbivore growth, reproduction or fecundity, while plant indirect defenses employ another trophic level, and aid or reward the predators of herbivores (Wu & Baldwin, 2010).

The costs and benefits of these different actions and how they affect plants' Darwinian fitness also define how plants respond to herbivore attack. The optimal defense hypothesis (ODH) predicts that the within-plant allocation of defenses depends on whether, and how often, a plant organ is attacked, and how valuable this organ is to plant fitness. In other words, this theory predicts that tissues that contribute the most for plant fitness (i.e. young leaves, flowers and seeds) will be best defended (Meldau, Erb & Baldwin, 2012). In addition, defense traits are undoubtedly beneficial when herbivores are present. However, in the absence of herbivores, they are expensive (from a metabolic standpoint) and can reduce plant fitness (Baldwin, 1998). Therefore, expressing defenses traits only when necessary (i.e. upon attack) can save costs and is less expensive than are constitutive defenses. Also, inducible defenses are more flexible than constitutive defenses and allow plants to respond differently depending on the type and amount of damage and other abiotic or biotic coexisting constraints.

The substantial disadvantage of inducible defenses is the time lag between the attack and the onset of the defense (Karban, 2011). However, many studies have shown that plants can reduce this lag or strengthen defense responses through priming and/or vaccination strategies (Kessler & Baldwin, 2004; Voelckel & Baldwin, 2004; Frost, Mescher, Carlson & De Moraes, 2008). In light of all these aspects, it has been proposed that induced defenses systems might have evolved first, and only later under more constant constraints, constitutive systems were selected. Consistent with this idea of the order of development is the notion that

slower growing plants are predicted to invest more heavily in constitutive defenses than are rapidly growing plants (Karban & Baldwin, 1997).

The net fitness value of plant strategies is best assessed when we know a lot about the players involved and then experimentally manipulate them. The study of PIs offers a great example. Since their discovery more than 40 years ago, PIs are probably the most iconic example of plant direct and induced defenses (Green & Ryan, 1972). Under glasshouse conditions, and in the absence of herbivores, PI-deficient plants grow faster and produce more seed capsules than do PI-producing plants (Zavala, Patankar, Gase & Baldwin, 2004). A subsequent study, though, showed that in plants under attack, PIs mediated decreases in herbivore performance. Because this effect was translated into a fitness benefit for the plant, it outweighed the costs of producing PIs (Zavala & Baldwin, 2004). Another study showed that when a plant is less nutritious, due to the presence of PIs, herbivores can simply consume more. However, the presence of the antifeedant toxin, nicotine prevents larvae from this compensatory feeding (Steppuhn & Baldwin, 2007). More recent work has shown that PIs can also have defensive synergistic effects in combination with plants' indirect defenses, changing herbivore behavior as well as predators' feeding preferences (Schuman, Barthel & Baldwin, 2012). In summary, a single plant trait can act on many different levels, and the more we contextualize the study of the traits in the conditions under which they evolved, the more the closer we are to understand its function.

1.3. Oxylipins and jasmonic acid

Oxylipins form a broad class of chemicals derived from the peroxidation of membrane-derived lipids. Although involved in many different processes, the importance of oxylipins in plants is comparable to that of eicosanoids in animal central nervous system and inflammatory and immune reactions (Bouarab et al., 2004). A vast portion of the research on plant oxylipins has focused on the phytohormone jasmonic acid (JA) and its derivatives, collectively referred to as jasmonates (JAs). The biosynthesis of JA is initiated with the production of linolenic acid. This molecule serves as a substrate for two distinct branches of the lipoxigenase (LOX) pathway: 9-LOX and 13-LOX, yielding 9- and 13-hydroperoxy linolenic acid (HPOT), respectively, depending on the oxygen position of the C_{18} chain (Howe & Schilmiller, 2002). 13-HPOT is converted into the intermediate 12-oxo-phytodienoic acid (OPDA) by the action of an allene oxidase synthase followed by an allene oxidase cyclase (AOC; Stenzel et al., 2003; Kallenbach, Bonaventure,

Gilardoni, Wissgott & Baldwin, 2012). From the chloroplast, OPDA is then transported into the peroxisome where it is reduced and submitted to cycles of β-oxidation; this process leads to the production of JA. Although JA can be metabolized to several derivatives, such as methyl-JA (MeJA; Seo et al., 2001; Stitz, Baldwin & Gaquerel, 2011), JA is active at the molecular level only when conjugated to isoleucine (JA-Ile; Staswick & Tiryaki, 2004). JA-Ile interacts with coronatine-insensitive 1 (COI1), release its targets from jasmonate ZIM domain (JAZ) protein repression, and triggers JA-mediated responses (Xie, Feys, James, Nieto-Rostro & Turner, 1998; Chini et al., 2007; Paschold, Halitschke & Baldwin, 2007; Oh, Baldwin & Galis, 2012).

JAs play a notorious and well-documented role in plant responses to wounding (Farmer & Ryan, 1992; Glauser et al., 2008), herbivore attack (Kessler & Baldwin, 2002; Zavala & Baldwin, 2006; Browse & Howe, 2008) and pathogen infection (Glazebrook, 2005). Interestingly, other processes, such as senescence (He, Fukushige, Hildebrand & Gan, 2002; Shan et al., 2011), pollen maturation and flower development (McConn & Browse, 1996; Sanders et al., 2000; Ishiguro, Kawai-Oda, Ueda, Nishida & Okada, 2001), and fruit ripening (Fan, Mattheis & Fellman, 1998), are also under the regulation of JAs. Therefore, JAs' signaling plays a key role in plant defense strategies, as jasmonates also orchestrate plant development and reproduction (Kessler, Diezel & Baldwin, 2010).

1.4. Long-distance signaling

Long-distance signaling in plants was discovered long ago, and it is well established that it plays important roles in regulating growth, development and responses to abiotic stimuli, stress and herbivore attack (reviewed in Lough & Lucas, 2006; Kragler & Hulskamp, 2012). These mobile signals coordinate responses at the tissue and organ level, and can occur extracellularly (apoplastically) or through the cell cytoplasm (symplastically). In the latter, channels that perforate cell walls (plasmodesmatas) form a cytoplasmic continuum of molecular exchange within a field of cells in what is known as symplasmic domains (Complainville et al., 2003; Lucas & Lee, 2004). Vascular plants also make use of specialized tissues that constitute highways for the upward (xylem) and sinkward traffic of signals (phloem; Taiz & Zeiger, 2006). Because the phloem interconnects all the symplasmic domains, plants operate above the cell level, supracellularly.

Almost all kinds of biomolecules have been shown to be transported throughout plants organism. Low molecular weight mobile signals include iron (Curie & Briat, 2003), nitrate (Forde, 2002), sugars (Stitt, 1996), nitric oxide (Wendehenne, Durner & Klessig, 2004), and phytohormones like auxin (Leyser, 2002) and abscisic acid (ABA; Wilkinson & Davies, 2002). The macromolecules mobilizing signals can be protein-protein or protein-RNA complexes (Haywood, Kragler & Lucas, 2002; Wigge et al., 2005), transcription factors (Sessions, Yanofsky & Weigel, 2000), messenger RNA (Haywood, Yu, Huang & Lucas, 2005), and, currently of special interest to researchers, small RNAs (Yoo et al., 2004).

Small RNAs (sRNAs) are non-coding sequence-specific negative regulators of gene expression. Silencing by sRNAs can occur through direct cleavage of target mRNA, through the inhibition of translation, or by DNA methylation. Small RNAs are divided in two major classes: short or small interfering (si) and micro (mi) RNAs. They depend on the activity of Dicers or Dicer-like (DCL) ribonucleases, the multi-component RNA-induced silencing complex (RISC) and argonaute (AGO) proteins (Vazquez, Legrand & Windels, 2010; Kragler & Hulskamp, 2012). Small interfering RNAs are 21 to 25 nucleotide-long, and derive from double-stranded RNA (dsRNA). Small interfering RNAs play a crucial role in a process called post-transcriptional gene silencing (PTGS), an innate, widespread plant defense mechanism against foreign DNA, transgenes, transposable elements, pathogens and viruses (Waterhouse, Wang & Lough, 2001; Ellendorff, Fradin, de Jonge & Thomma, 2009; Vazquez, Legrand & Windels, 2010). In contrast, miRNA are usually composed of 21 nucleotides and derived from a long single-stranded precursor in a hairpin structure, with imperfect base pairing. As most known plant miRNAs target endogenous genes that encode for transcription factors, it is safe to say that miRNAs are mainly involved in plant development (Achard, Herr, Baulcombe & Harberd, 2004; Jones-Rhoades, Bartel & Bartel, 2006; Carlsbecker et al., 2010).

Both si- and miRNA are found in the phloem sap (Yoo et al., 2004). However, it has been suggested that although miRNAs silencing action is spatially restricted to the producing or nearby cells, siRNA can move over many different layers of cells and reach distant organs (Schwab et al., 2009). Interestingly, just as it moves from source to sink tissues, siRNA can also find their way into pollen grains, ovules and embryo sacs, and can make epigenetic alleles heritable (reviewed in Martienssen, 2010). This mechanism establishes a molecular mechanism in which environmental signals modifying the growth and development of the soma can be transmitted and inherited by the progeny (Martienssen, 2010).

1.4.1. Grafting and plant systemic wound responses

Arguably, the two greatest goals in plant systemic signaling research have been the identification of the flower induction and of the wound alert signals. The length of the day, or photoperiod, is perceived by leaves, and the signal that is transported to shoot apex switches the meristem identity from vegetative to floral (Chailakhyan, 1936). Similarly, the seminal work that led to the discovery of PIs also showed for the first time that the damage in one leaf can cause a systemic wound response in undamaged portions of the plant (Green & Ryan, 1972). Since then, many studies with ingenious manipulations have led to progress on both fronts (Wigge et al., 2005; Mousavi, Chauvin, Pascaud, Kellenberger & Farmer, 2013).

Arguably, among all techniques applied to the study of these signals, the one that contributed the most is grafting. Grafts provided the first molecular evidences of *florigen*, in the induction of flowering (Chailakhyan, 1936); *systemin* precursor gene, in the systemic induction of PIs upon wounding (Mcgurl, Orozcocardenas, Pearce & Ryan, 1994); and, more recently, of siRNAs in systemic PTGS (Palauqui, Elmayan, Pollien & Vaucheret, 1997). The use of grafts dates from ancient-Greek times, and grafts are currently widespread in the cultivation of species such as tomato, grape, eucalyptus, apple, etc. The grafting procedure consists of combining two or more different genotypes into a single chimeric organism. Genotypes can be fused in many different ways, but generally the plant part on top, the *scion*, is grafted onto a receiver plant, the *rootstock* (Pina & Errea, 2005). However, unless automated, this manual and time-consuming technique requires a certain level of skill.

Regarding the mechanisms of the wound systemic signal, grafting has also provided evidence on the additional players involved (reviewed in Schilmiller & Howe, 2005). In a series of important reports, researchers have studied grafts done with mutants of tomato deficient in the production of systemin (*spr1*; Lee & Howe, 2003), or the perception (*jai1*; Li, Li, Lee & Howe, 2002) or the synthesis of JA (*acx1*; Li et al., 2005). Interestingly, a common feature of these studies is the use of PI, a leaf-derived protein, as a marker of the systemic wound-response. In addition, for all grafts analyzed, the graft junction was above ground, and rootstocks also contained leaves, which were wounded. In other words, these studies were carried out above ground, in the framework of leaf-to-leaf wound systemic signaling. Rootstocks of *jai1*, *acx1* or *spr1* failed to induce PIs either locally or systemically in wild-type (WT) scions. This failure suggests that *jai1*, *acx1* or *spr1* are required

locally to produce the systemic signal. In contrast, when the leaves of WT rootstocks were wounded, PI induction was observed locally but not in scions of *jai1*, although scions of *spr1* and *acx1* were still able to systemically induce PIs. In summary, this evidence suggests that the local wound response requires JA perception and synthesis, as well as, to a lesser extent, systemin. However, systemic PI induction is not dependent on systemic *de novo* biosynthesis JA or systemin, and requires simply JA perception.

Moreover, the idea of a wound signal that is transported in the phloem is also supported by evidence that sieve elements, companion and parechyma cells are able to produce JA and prosystemin (Hause, Hause, Kutter, Miersch & Wasternack, 2003; Narvaez-Vasquez & Ryan, 2004). Recently, Mousavi and colleagues (2013) recorded cell surface potential changes in response to wounding in Arabidopsis using non-invasive electrodes (Zimmermann, Maischak, Mithofer, Boland & Felle, 2009). Within seconds after wounding, an apoplastic electrical wave spread from damaged to undamaged leaves, triggering systemic JA-Ile accumulation and the expression of JA-responsive genes. These authors suggest that, similar to animal synaptic activity, wound-activated surface potential changes (WASPs) are capable of transmitting information from sensor organs to effector organs in plants. Next, the authors tested for a direct link between electrical activity and systemic JA. In the absence of wounding, they mimicked the electrical signal by injecting a current of similar endogenous input into the petiole. The current injection, however, was not able to induce all the wound-related genes. This is consistent with the idea that symplastic and apoplastic mechanisms together orchestrate wound responses in plants. Nevertheless, whether these signals also travel to the below-ground parts of the plant remains largely unexplored.

For almost 80 years, grafting and increasingly refined genetic manipulations have been contributing substantially to the study of long-distance signaling. However, most of these studies focus on above-ground signaling. In **manuscript I**, I established a micrografting protocol for seedlings of *N. attenuata*, a model plant for ecological studies. Initially, I compare the rate of grafting success amongst other species of *Nicotiana* and observe that *N. attenuata* is very suitable for this procedure. Almost 80% of all grafted plants result in completely healed and connected grafts after only five to six days after grafting. Further, I tested for the absence of grafting effects on plant growth and development by comparing non-grafted WT versus WT/WT grafts (referred in a shoot/root manner). Finally, I analyzed the profiles of gene expression of grafts that combined WT to stably

transformed lines of *N. attenuata*. Unlike Arabidopsis and other species of the Solanaceae family, *N. attenuata* has no available mutant library. However, it comprises a growing list of transgenic lines, in which target genes are either overexpressed (*ov*) or silenced (antisense –*as* -- or inverted repeat – *ir* -- constructs). Because the silencing technique makes use of RNA interference (RNAi), I was also able to test for the spread of PTGS in chimeric plants. Consistent with the source-to-sink movement of the sRNA silencing signal, *ir* scions affected the expression of their target gene in WT rootstocks. Conversely, in WT/*ir* grafts, the RNAi of the scion did not affect the endogenous expression of the scion's transcripts in rootstocks. This work represents the first step towards a better understanding of root gene function in traits hitherto evaluated mostly in above ground.

1.4.2. Nicotine and systemic JA signaling

After almost 20 years of research, we have learned about many of the defensive molecular mechanisms employed by *N. attenuata* to withstand herbivore attack. Many components of the JA pathway have been identified, and, with the use of stably transformed lines, these have been proven to aid *N. attenuata*'s performance under herbivore pressure (Kessler & Baldwin, 2004; Paschold, Halitschke & Baldwin, 2007; Kallenbach, Bonaventure, Gilardoni, Wissgott & Baldwin, 2012). Among the best-studied defense compounds employed by *N. attenuata* are PIs (Zavala & Baldwin, 2004); volatiles, such as trans-α-bergamotene, produced by terpene synthases (TPS; Schuman, Heinzel, Gaquerel, Svatos & Baldwin, 2009; Schuman & Baldwin, 2012) or green leaf volatiles (GLVs; Allmann & Baldwin, 2010); and nicotine (Steppuhn, Gase, Krock, Halitschke & Baldwin, 2004).

Nicotine is an antifeedant pyridine alkaloid that is highly toxic to most herbivores (Glendinning, 2002). Its synthesis occurs exclusively in the roots, in particular the growing toot tips (Dawson, 1942; Solt, 1957; Saedler & Baldwin, 2004). Synthesized nicotine is then loaded into the xylem, where it accumulates in the vacuoles of mesophyll cells of the leaves, but not exclusively (Saunders, 1979; Baldwin & Karb, 1995). Above ground, nicotine allocation follows the predictions of the ODH: seed capsules and young leaves receive proportionally greater allocations of nicotine than do older leaves (Baldwin, Sims & Kean, 1990). Therefore, as a defense strategy, nicotine relies substantially on transporters mediating its efflux and intake through cell and vacuolar membranes. The vacuolar sequestration of nicotine is made by multidrug and toxic compound extrusion (MATE) transporters (Morita et al., 2009; Shoji et al., 2009); a cell membrane transporter, called nicotine

uptake permease 1 (NUP1), moves nicotine from the apoplast into the cytoplasm of root tip cells (Hildreth et al., 2011).

Nicotine is the most abundant alkaloid found in leaves, with basal levels of 0.1 to 1% of dry mass (Baldwin, 1999). Treatments such as leaf wounding or the application of MeJA dramatically increase the above-ground accumulation of nicotine up to 10-fold (Baldwin & Ohnmeiss, 1993). This induction is effected by *de novo* synthesized nicotine, and it is tightly associated with increased transcripts levels of *NaPMT* in roots (Winz & Baldwin, 2001). Not surprisingly, nicotine induction "costs" a plant a lot of nitrogen: 6-8 % of a plant's total N in nicotine alone, without considering its biosynthesis, transport and storage (Baldwin, Karb & Ohnmeiss, 1994). Also, it is estimated that the synthesis of 1 g of nicotine requires 3.62 g of glucose (Gershenzon, 1994). Interestingly, once effected, this investment does not seem to be recovered and reinvested in growth, even when plants are grown in nitrogen-limited conditions, meaning that nicotine-induced levels are a one-way investment (Baldwin & Ohnmeiss, 1994). Therefore, the mechanisms underlying nicotine synthesis, transport and accumulation after induction are likely to be tightly regulated.

Stem girdling experiments provided the first evidence of a phloem-borne wound signal mediating nicotine production in the roots of tobacco (Baldwin, 1989). The phloem vascular connection was killed in sites on the stem immediately above or below the damaged leaves, and nicotine content was measured in an undamaged leaf of the same position across treatments 4 days after wounding. Damaged leaves above the girdle (no phloem connection to roots) failed to induce nicotine; therefore, the signaling is assumed to occur via phloem. Although the involvement of an electrical signal was not ruled out, the kinetics of increases in endogenous JA pools in damaged leaves (30 min after wounding) and in roots (2 h after wounding) were consistent with the kinetics of a slow-moving chemical cue moving on an *intra-plant* route (Baldwin, Schmelz & Ohnmeiss, 1994). Also, that the exogenous application of MeJA to the roots mimics the wound-induced nicotine response in the absence of wounding finally establishes JAs as potential central players in below-ground systemic signaling (Baldwin, 1996).

Labeled compounds were used to determine whether plants produce root JA autonomously in response to leaf elicitation. Although ^{14}C-labeled JA applied to the wounded leaves was recovered in roots, the ^{13}C-labeled Ile precursor was found as a ^{13}C-labeled JA-Ile only in local leaves, not in roots (Zhang & Baldwin, 1997; Wang,

Allmann, Wu & Baldwin, 2008). However, labeled compounds are "foreigners" to the plant - molecules that fundamentally differ from their plant-derived respective - and it is possible that physiologically they are treated as such. That labeled compounds might not be recognized as endogenous compounds weakens the interpretation of these results.

Regardless of the identity of the mobile signal, our understanding of whether root JA-dependent defenses, if any, affect plant fitness remains elusive. The role of root JA is timely particularly now that Grebner and colleagues (2013) have shown that wounded roots can synthesize JA independently of the shoots, relying on a single member of the LOX family (LOX6) of the JA pathway for that. These authors focused on the local JA responses of the roots when directly wounded. In contrast, Acosta and colleagues (2013) reported that the application of the wounding treatment to cotyledons of Arabidopsis seedlings induced the expression of GUS gene reporter fused to the JAZ10 promoter, a JA-responsive gene (*JAZ10-GUSPlus*). Although very exciting, this recent evidence does not explicitly validates root JA function in plant defenses.

In **Manuscript II**, I investigated the function of root JAs in the resistance of *N. attenuata* under glasshouse and natural conditions. I performed a series of grafts using WT, COI1- and AOC-silenced plants, and restrained JA impairment to the roots. I then asked whether root JA synthesis or perception contributes to defense traits employed in the above-ground plant, and whether these changes are translated into differences in herbivore performance or preference. I initially use nicotine levels in leaves to report the systemic function of root JA. Nicotine induction after leaf wounding is almost fully dependent on the COI1 activity of the roots. In addition, the root *de novo* synthesis of JA by the action of AOC is necessary but not sufficient to effect maximum nicotine levels. I showed that COI1 and AOC root activity regulate nicotine production as well as nicotine uptake by leaves. Knocking down roots' ability to synthesize or perceive JA in roots also caused an over-accumulation of JAs in damaged leaves after wounding. This suggests a novel shoot-root-shoot loop regulating the JA response. In addition, I observed that above-ground herbivore performance and preference are influenced by a plant's ability to synthesize and perceive JA in the roots. These evidences suggest that root JAs signaling tailors leaf responses to above-ground attack.

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2. Overview

2.1. Manuscript I

A simple and efficient micrografting method for stably transformed *Nicotiana attenuata* plants to examine shoot-root signaling

Variluska Fragoso, Hannah Goddard, Ian T. Baldwin and Sang-Gyu Kim

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In this manuscript, I established an efficient protocol of micrografting for *Nicotiana attenuata*, allowing for the independent gene manipulation of the below- and above-ground parts of the plant. I showed that grafted wild-type plants (WT/WT, grafts are indicated here in a shoot/root manner) do not display any growth or developmental compromises when compared to intact WT *N. attenuata* plants. Additionally, I analyzed the transcript accumulation profiles of grafts combining WT and stably transformed *ov* (overexpressing) or *ir* (inverted repeats, i.e. silenced) genotypes. I observed that the ectopic expression of ov or ir constructs was restrained to the transgenic plant part in WT/*ov*, *ov*/WT and WT/*ir* grafts. However, the *ir* scion reduced the expression levels of the target gene in WT roots of *ir*/WT grafts. This protocol, combined with a collection of available transformed lines, represents the first step of the study of gene function employed in long-distance signaling controlling plant processes in an ecological model species.

Variluska Fragoso performed all the grafts, designed and carried out all the experiments, analyzed the data and wrote the manuscript. Hannah Goddard contributed with the initial grafts. Ian T. Baldwin designed the experiments and wrote the manuscript. Sang-Gyu Kim designed the experiments, coordinated the work, and wrote the manuscript.

2.2. Manuscript II

Root jasmonic acid synthesis and perception regulate folivore-induced shoot metabolites and increase *Nicotiana attenuata* resistance

Variluska Fragoso, Eva Rothe, Ian T. Baldwin, and Sang-Gyu Kim

New Phytologist - in press (accepted 15 January 2014)

While many studies focus on the root production of jasmonates (JAs) after local damage, or the first downstream targets of root systemic (jasmonic acid) JA signaling after wounding the cotyledons of seedlings; in this manuscript, we have provided the first explicit evidence that below-ground JA signaling contributes to resistance traits observed in the shoots of *Nicotiana attenuata*. I dissected the root systemic function of JA by micrografting wild-type *N. attenuata* shoots onto roots of transgenic plants impaired in their JA perception and *de novo* synthesis. I showed that, upon leaf wounding, systemic root JAs regulate nicotine production in roots as well as nicotine transport to leaves. Also, I described a novel shoot-root-shoot loop by which systemic root JAs regulate the accumulation of JAs in leaves. Finally, in mimicked herbivore attack, root systemic JAs control the shoot accumulation of other shoot metabolites which account for enhanced plant resistance to generalist and specialist herbivores under glasshouse and nature conditions.

Variluska Fragoso performed all grafts, designed and carried out all the experiments, and analyzed the data. Eva Rothe helped in experiments involving simulated herbivory and with field plants. Ian T. Baldwin discussed the results and helped design petiole-feeding experiment. Sang-Gyu discussed the results and helped collect field data. The manuscript was written by Variluska Fragoso and was significantly improved after Ian T. Baldwin and Sang-Gyu's revision.

3. Manuscript I

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PLANT METHODS

METHODOLOGY Open Access

A simple and efficient micrografting method for stably transformed *Nicotiana attenuata* plants to examine shoot-root signaling

Variluska Fragoso[1], Hannah Goddard[2], Ian T Baldwin[1] and Sang-Gyu Kim[1*]

Abstract

To adjust their development to the environment, plants rely on specific signals that travel from shoot to root and *vice versa*. Here we describe an efficient micrografting protocol for *Nicotiana attenuata*, a useful tool for identifying these signals and understanding their functions. Additionally we analyzed transcript accumulation profiles of scions and rootstocks of grafts performed with wild-type and stably transformed *N. attenuata*. Our results are consistent with the source-to-sink movement of an sRNA silencing signal.

Keywords: Grafting, *Nicotiana attenuata*, root and shoot signaling, systemic signals

Background

Many studies have shown that plants use long-distance or systemic signals to coordinate and adjust their growth. These signals convey messages throughout the whole plant, from sensor to effector tissues or organs, and they seem to operate with great specificity which can depend not only on the message itself but also on the spatial and temporal scales over which they act [1]. One of the interesting examples of long-distance signaling in plants is activated by herbivore attack and results in the production of a complex bouquet of plant defenses. Plants are capable of priming defenses systemically in tissues that are distal to the sites of attack, suggesting that an herbivory alert signal is transmitted from attacked to unattacked leaves and roots [2].

Grafting has provided important insights into the study of these systemic wound signals in plants. Grafting a JA biosynthesis mutant with a JA response mutant clearly showed that the production of jasmonic acid (JA) in damaged leaves and the perception of JA by distal leaves are necessary for inducing systemic responses [3]. Moreover, the *de novo* biosynthesis of JA in systemic leaves was further determined not to be required for the systemic transmission of the wound signals [4]. This research focused on the long-distance communication within shoots, and there remains much to be learned about the role of roots in the production and propagation of these important signals throughout the plant that mediate ecological interactions [5].

A wild tobacco, *Nicotiana attenuata* has been studied in plant-herbivore interaction in its natural habitat, the Great Basin Desert of Utah. Several local and systemic defense responses are induced in *N. attenuata* during herbivore attack. Defense traits, which include trypsin proteinase inhibitors (TPI) and specific volatiles, such as trans-α-bergamotene produced by terpene synthases (TPS), are increased in *N. attenuata* when this plant is attacked by herbivores or elicited by herbivore-specific elicitors [6-8]. A notable example is of nicotine, which requires the activity of putrescine *N*-methyltransferase (PMT) in the roots for its synthesis and accumulates the alkaloid in leaves and other above-ground parts of tobacco plants [9]. This alkaloid restrains the consumption of *N. attenuata*'s leaves by its attackers, even by *Manduca sexta*, a specialist and nicotine-tolerant herbivore [10]. The identity of the signal which travels from attacked leaves and activates nicotine production in the roots, and whether this signal is the same that activates other systemic defenses in undamaged leaves are questions yet to be addressed [1].

Research in plant ecology has been greatly enhanced by the manipulation of gene expression in stably

* Correspondence: skim@ice.mpg.de
[1]Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Straße 8, D-07745 Jena, Germany
Full list of author information is available at the end of the article

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transformed plants. Isogenic lines expressing either low or high levels of transcripts for a particular gene provide a powerful tool to dissect its organism-level function and fitness consequences of the gene under real-world conditions [11]. However, one drawback of such genetic changes is when the transgene is driven by nonspecific promoters which therefore ectopically express transgenes or silencing constructs, preventing tissue- or organ-specific manipulations.

Here, we describe a simple and highly efficient micrografting protocol for *N. attenuata*, and characterize the influence of the grafting procedure on plant growth. Given that the graft junction is located between the shoots and roots of grafted plants, this technique allows for the independent manipulation of the below- and above-ground parts and the study of their interplay in *N. attenuata* for defense. Our results represent the first step towards the study of shoot-to-root interplay by micrografting in *N. attenuata*.

Results

Simple micrografting method for *N. attenuata* has a high success rate

Preliminary cleft grafting experiments with adult plants of *N. attenuata* resulted in low success rate (approximately 10 ~ 15%). Therefore, we tested a method using plants in an earlier phase of development. One-week-old seedlings (approximately 2 mm in height) of different *Nicotiana* genotypes grown on agar plates were excised below the apical meristem (Figure 1A, B) to prevent adventitious rooting [12]. With a stereomicroscope and the seedlings lying horizontally on the media, the scion of one seedling was placed as close as possible to the rootstock of another. To stabilize the contact between scions and rootstocks, small blocks of agar were placed over the junction of the grafted seedlings. Five days after grafting, a visually apparent connection between the combined parts was observed, surrounded by feeble callus growth (Figure 1B).

Micrografting efficiency varied dramatically among different species of *Nicotiana* (Figure 1C). *N. attenuata* proved to be the most suitable among the species tested for this procedure, with an 80% success rate, as scored one week after grafting. The lowest grafting success rate was observed with *N. benthamiana* (ca. 1%) in which scions tended to produce roots under these *in vitro* grafting and growth conditions.

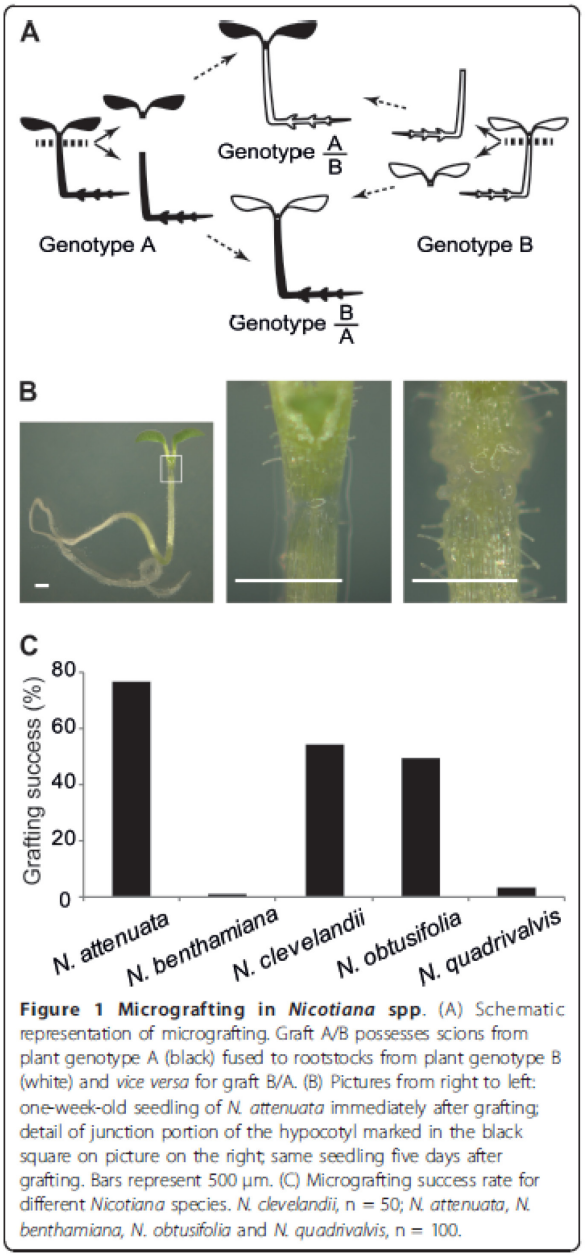

Figure 1 Micrografting in *Nicotiana* spp. (A) Schematic representation of micrografting. Graft A/B possesses scions from plant genotype A (black) fused to rootstocks from plant genotype B (white) and *vice versa* for graft B/A. (B) Pictures from right to left: one-week-old seedling of *N. attenuata* immediately after grafting; detail of junction portion of the hypocotyl marked in the black square on picture on the right; same seedling five days after grafting. Bars represent 500 μm. (C) Micrografting success rate for different *Nicotiana* species. *N. clevelandii*, n = 50; *N. attenuata*, *N. benthamiana*, *N. obtusifolia* and *N. quadrivalvis*, n = 100.

Micrografting does not affect *N. attenuata* growth and development

Growth parameters of scions and rootstocks of grafted WT/WT *N. attenuata* plants (chimeras are named in a shoot/root manner throughout this paper) were compared weekly to intact WT individuals and no significant differences were observed (Figure 2). Rosette diameter and height (Figure 2A, B) as well as the length of the longest leaf (data not shown) of grafted WT/WT plants were of similar sizes compared to intact WT plants. The same was also true for biomass comparisons: shoot (Welch's *t*-test; $p = 0.34$) and root (Welch's *t*-test; $p = 0.14$) dry mass were similar in intact versus grafted plants (Figure 2A). Comparisons of the number of flowers (Welch's *t*-test; $p = 0.94$) (Figure 2B), capsules (Welch's *t*-test; $p = 0.17$) and seeds per capsule (Welch's

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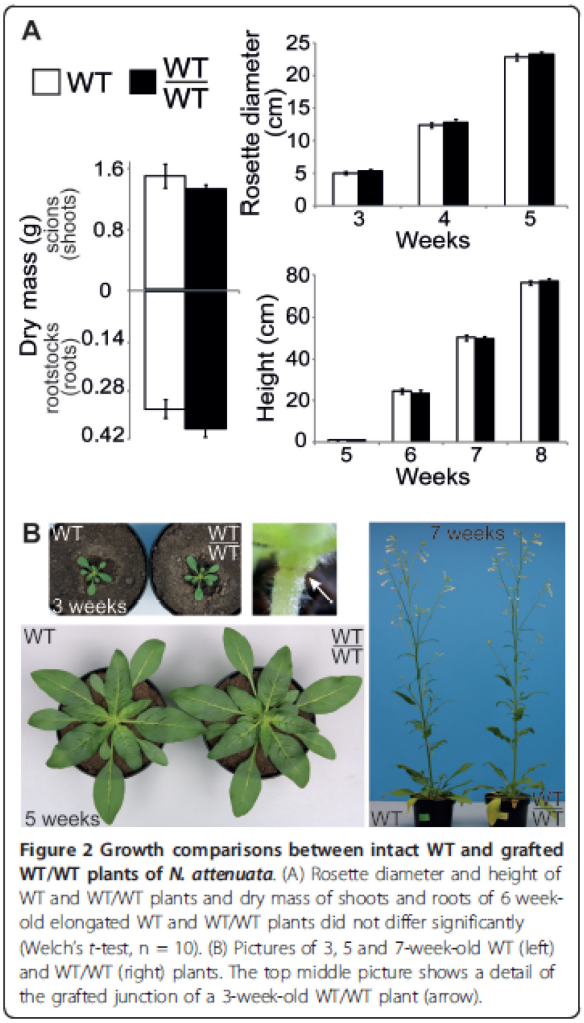

Figure 2 Growth comparisons between intact WT and grafted WT/WT plants of *N. attenuata*. (A) Rosette diameter and height of WT and WT/WT plants and dry mass of shoots and roots of 6 week-old elongated WT and WT/WT plants did not differ significantly (Welch's *t*-test, n = 10). (B) Pictures of 3, 5 and 7-week-old WT (left) and WT/WT (right) plants. The top middle picture shows a detail of the grafted junction of a 3-week-old WT/WT plant (arrow).

t-test; $p = 0.31$) (data not shown) were also not significantly different.

The graft junction of three-week-old grafted plants was located just below the rosette (Figure 2B, top middle picture). This means that for this work, scions refer to whole shoot or above-ground portions of grafted plants, while rootstocks refer to the entire root system.

Inverted repeat construct of scions affects the transcript accumulation in WT rootstocks

The main reason for establishing a grafting method for *N. attenuata* is to manipulate defense-related shoot-root signaling in lines already developed by our group (Additional file 1). Such stably transformed plants harboring constructs designed either to silence (antisense - *as* or inverted repeat - *ir*) or overexpress (*ov*) a particular gene are the main approaches by which gene function can be studied in *N. attenuata* system. However, the value of micrografting is mainly limited by the transmission of signals through the graft site [13]. Therefore, to validate the utility of the grafting method using these lines, we verified whether the transcript accumulation of WT scions and rootstocks was altered by their transgenic grafted counterpart. We made use of *TPI* and *PMT* genes as innate reporters of the up- and downward spread of silencing since the endogenous expression of these target genes in *N. attenuata* are particularly useful for this purpose: *TPI* is mainly expressed in shoots and *PMT* in roots [14]. We further investigated if the ectopically overexpressed (*ov*) mRNA of a foreign gene could also be transmitted to WT scions or rootstocks. Importantly, these lines are not morphologically distinguishable from WT *N. attenuata* plants [7,10,15], hence the effects of grafting on plant growth of these transgenic lines were comparable to those observed in grafts performed with WT plants (Figure 2A, B).

As expected [16], accumulation levels of *TPI* transcripts in leaves of *irTPI* plants (Figure 3A) were found to be dramatically reduced to below 1% of WT *TPI* transcript levels (Fisher's PLSD test; $p < 0.0001$). Likewise, *PMT* transcript levels in roots of *irPMT* plants (Figure 3B) were also found to be reduced to less than 5% of those found in the roots of WT plants (Fisher's PLSD test; $p < 0.01$), as previously characterized by Steppuhn et al. [10]. These significant differences found in transcript levels of *TPI* and *PMT* of intact plants were maintained when comparing grafted *irTPI*/*irTPI* and *irPMT*/*irPMT* plants to grafted WT/WT plants, respectively. *irTPI*/*irTPI* and *irPMT*/*irPMT* plants yield only 0.8% and 3% of *TPI* and *PMT* transcript levels of WT/WT plants, respectively (Figure 3A, B).

Leaves of *irTPI*/WT grafts (Figure 3A) failed to accumulate transcripts of *TPI* and didn't differ significantly from leaves of intact *irTPI* and grafted *irTPI*/*irTPI* plants (Fisher's PLSD test; $p > 0.98$). In addition, *TPI* transcript levels of scions of WT/*irTPI* plants (Figure 3A) didn't significantly differ from those found in WT or grafted WT/WT leaves (Fisher's PLSD test; $p = 0.25$ and $p = 0.37$, respectively). Roots of WT/*irPMT* grafts (Figure 3B) expressed similarly low levels of *PMT* transcripts when compared to the roots of *irPMT* and *irPMT*/*irPMT* grafted plants (Fisher's PLSD test; $p = 0.92$ and $p = 0.85$, respectively). However, the same low levels of *PMT* transcript (Figure 3B) were found in WT rootstocks of *irPMT*/WT plants, which didn't differ significantly from *irPMT* and *irPMT*/*irPMT* (Fisher's PLSD test; $p = 0.94$ and $p = 0.86$, respectively).

Transcript levels of *TPS* in shoot or roots of non-grafted *ovTPS* plants (Figure 3C) didn't differ significantly from those found in scions or rootstocks of grafted *ovTPS*/*ovTPS* (Fisher's PLSD test; $p = 0.29$ and $p = 0.09$, respectively). In addition, levels of *TPS*

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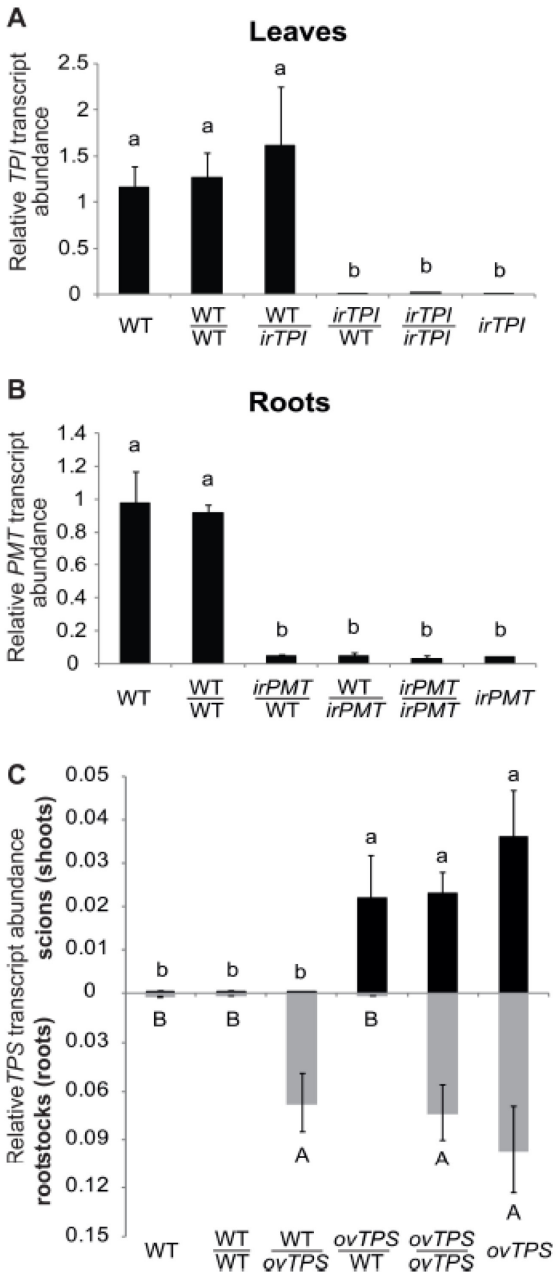

Figure 3 Molecular analysis of leaves and roots of grafted plants transformed with different RNAi and overexpression constructs. (A) *TPI* (normally expressed only in scions) transcript levels of leaves (scions) of intact or grafted WT and *irTPI*-silenced plants. (B) *PMT* (normally expressed only in roots) transcript levels of roots (rootstocks) of intact or grafted WT and *irPMT*-silenced plants. (C) *TPS* transcript levels of leaves (scions) and roots (rootstocks) of intact or grafted WT and *ovTPS*-overexpressing plants, in which a *TPS* from *Z. mays* was ectopically expressed behind a 35S promoter. Bars sharing same letters do not significantly differ (Kruskal-Wallis test followed by Fisher's PLSD, n = 5).

transcripts in scions of WT/*ovTPS* and rootstocks of *ovTPS*/WT (Figure 3C) resembled those of WT or WT/WT plants (Fisher's PLSD test; $p > 0.96$).

Discussion

As previously described for other species [12,17,18], including *N. benthamiana* [19,20], here we describe a simple and highly efficient micrografting protocol for *N. attenuata* with transgenic lines. Our data support the employment of *N. attenuata* grafts that combine WT genotype either to *ov*- or *ir*-transgenic lines, this latter only when used as rootstocks, given the altered transcript accumulation of WT roots promoted by silenced shoots. The motivation to adopt this well-known grafting method for *N. attenuata* is grounded in the several layers of inter-related plant defenses that have described in *N. attenuata*. Systemic signals produced upon herbivore attack enhance the indigestibility of *N. attenuata* leaves by augmented TPI activity [8] and increase its toxicity by producing nicotine [10] and diterpene glucosides [21]. Systemic defense signals are also involved in recruiting predators to attacking herbivores by producing volatiles that betray the location of the herbivores on attacked plants [6,22], as well as in reallocation of energy to roots that are later remobilized for reproduction and thereby enhances a plant's tolerance of herbivore attack [23]. In addition, it is known that *N. attenuata* can change its flower opening time in order to recruit new pollinators that do not have larval stages that are herbivores of the plant and thereby reduces future herbivore loads [24]. Taken together, the establishment of micrografting method for *N. attenuata* will allow us to examine the systemic signals and gene function in above- versus below-ground parts of a plant in an ecological context.

Meristematic activity in the graft junction is an important determinant of attaining high grafting success rates [25]. The reason why previous attempts of cleft grafting with adult *N. attenuata* plants resulted in low success rate may be due to poor callus formation in stem tissues. The hypocotyls of germinated seeds have proved to be the most reliable explant to induce meristematic activity for tissue culture and regeneration of *N. attenuata* [26] and this tissue's particularly high meristematic activity appears also to be the reason behind the success of the of the grafting procedure described here (Figure 1C).

Other than its simplicity and high efficiency, a major advantage of this method for *N. attenuata* is that its impact on a plant's adult life is expected to be minor because the grafting takes place in an early phase of plant development. Only five to six days after grafting, completely healed and healthy grafted seedlings are obtained (Figure 1B), which do not show morphological

or fitness compromises when compared to intact WT *N. attenuata* plants at later stages in development (Figure 2). Moreover, the wounding inflicted by the grafting procedure itself could potentially lead to activation of defense related traits such as augmented *TPI* expression or nicotine accumulation [27,28]. However *TPI* and *PMT* transcript accumulation levels of five-week-old WT/WT resembled those of intact WT *N. attenuata* plants (Figure 3A, B). In addition, given that the graft junction is established at the shoot-root interface, this protocol allows for the manipulation of a larger long-distance signaling system in plants, rather than only within shoots [29].

Recent molecular studies suggest the graft hybridization can occur by the exchange of genetic material among neighboring cells and across the graft junction [30-32]. However, as reported for *N. benthamiana*, micrografted roots harboring 35S-derived RNAi constructs were unable to promote silencing of its target in nonsilenced shoots [19]. To validate the micrografting method for *N. attenuata* with transgenic lines, transmission of the silencing effect in rootstocks to scions or *vice versa* should be examined. Our data are consistent with the lack, or very weak upward transmission of the silencing signal (Figure 3A). On the other hand, when *irPMT* shoots were grafted onto WT roots of one-week-old seedlings, the roots' ability to accumulate *PMT* transcripts in later developmental stages was reduced (Figure 3B), consistent with the concept of source-to-sink facilitated movement of sRNA silencing signals [32]. Regardless of the molecular mechanism underlying the spread of the silencing from shoot to root observed in *irPMT*/WT grafts, these data suggest a limitation to the use of grafts consisting of *ir* construct-derived transgenic lines scions and WT rootstocks for addressing ecological questions.

Roots are thought to play a role in the control of developmental processes of the aboveground parts of plants, such as shoot branching and flowering [12,33]. As for plant defenses, it has been shown that roots of *N. attenuata* account for both plant resistance (e.g. nicotine production, [10]) and tolerance (e.g. changes in within-plant carbon allocation, [23]) to herbivore attack. However, micrografting can further extend our understanding of a plant's below-ground interactions and molecular mechanisms and their final contribution to the whole-plant performance. For instance, the signaling underlying the JA-induced nicotine synthesis in the roots of *N. attenuata* after leaf damage can be investigated by analyzing grafted plants that have WT shoots and roots deficient in JA perception (*irCOI1*, [34]), synthesis (*irLOX3*, [35]) or activation (i.e. conversion of JA to its active JA-Ile form, [36]) (Additional file 1). In addition, ethylene is known to attenuate MeJA-induced accumulation of *PMT* transcripts as well as the production of nicotine [9]. Given the readily available ethylene-related transgenic lines (*ovETR1* and *irACO*, [37]) of *N. attenuata* (Additional file 1), it would be interesting to determine whether ethylene biosynthesis or perception in the roots is the limiting step in the regulation of nicotine synthesis upon herbivore attack. The movement of small RNA is also important in long-distance signaling. Silenced lines of RNA-directed RNA polymerase genes (*irRdR1, irRdR2* and *irRdR3*, [38-40]) enable us to find small RNAs that move from WT shoot to small RNA-deficient root after herbivore attack. In addition, silenced lines of Dicer-like (DCL) proteins, which will be available soon, are also useful in manipulating the role of small RNA in shoot-root signaling. Finally, scrutiny of field-grown micrografted *N. attenuata* plants displaying markedly differences in performance could lead to the identification of novel root-derived traits that account for plants' Darwinian fitness as well as serve as a complementary approach to the molecular characterization of genes [12]. Therefore we predict that the protocol reported here will be valuable for unraveling potential root-based traits that profoundly affect plant development and fitness.

Conclusions

Micrografting combined with a collection of available stably transformed lines, especially for a non-model plant such as *N. attenuata* which has no available mutant libraries, represents a key tool to evaluate gene-function in the many developmental and physiological processes that are governed by long-distance signals. The ectopic expression of *ov* or *ir* constructs was restrained in WT/*ov*, *ov*/WT and WT/*ir* grafts. Micrografting thus represents an important advance towards organ-specific characterization of gene function and detection of currently unrecognized long-distance signals, particularly focusing on root physiology which determines the relationships between their below- and above-ground parts and the contribution of this root-shoot communication to whole-plant performance. Given the potential of this method in unraveling root and shoot interplay and the hitherto overlooked importance of roots, we envisage that this procedure will be commonly applied to the study of gene function in *N. attenuata*.

Materials and methods

Plant materials and growth

N. attenuata seeds are derived from an inbred collection from the DI Ranch, Utah [41]. Seeds of additional *Nicotiana* sp. were kindly supplied by Dr. Verne A. Sisson (Oxford Tobacco Research Station, Oxford, NC) and originated from collections made by Dr. T. H.

Goodspeed [42]. For *TPI* (Accession number: AY426751) and *PMT* (Accession number: AF280402) silencing in *N. attenuata*, inverted repeat constructs (IR) containing either a fragment of the *TPI* gene (*irTPI*) or a consensus fragment for the two *N. attenuata*'s *PMT* genes (*irPMT*) in inverted orientation were used and characterized by Zavala et al. [15] and Steppuhn et al. [10], respectively. As previously described by these authors, these transgenic lines are not morphologically distinguishable from WT plants and show reduced resistance to herbivores. Transcript levels of *TPI* and *PMT* are found reduced to below 1% in *irTPI* and 18% in *irPMT* plants, respectively, of levels found in WT plants. Consistent with the transcript analysis, no TPI activity was detected in leaf tissues of *irTPI* plants while levels of nicotine were reduced to 15% in leaves of *irPMT* plants [16]. *TPS10*-overexpressing *N. attenuata* plants (*ovTPS*) harbor a sense sequence based on the *TPS10* gene of maize (Accession number: AY928078), driven by CaMV 35S promoter [7]. The maize *TPS10* gene encodes a terpene synthase involved in the herbivory-induced production of (*E*)-α-bergamotene and (*E*)-β-farnesene which are the major components of the volatile blend emitted by attacked plants and function as attractants of herbivore's natural enemies [43]. As for the *ir* lines included in this study, no morphological or developmental differences between WT and *ovTPS* are observed [7]. All seeds were sterilized and treated with 0.1 mM gibberellic acid in 1:50 smoke-distilled water solution for 1 h [26]. Petri dishes containing 40 mL of Gamborg's B5 media with minimal organics containing 0.8% (w/v) plant agar (Duchefa) were used for germination, manipulation and growth of grafted seedlings. The plates were maintained in growth chambers (Percival, Perry Iowa, USA) at 26 ± 2°C, under 16/8 h of light/dark regime and plants were further transferred to the glasshouse under same conditions when necessary. Grafts were performed in sterile bench under an Olympus SZ51 stereomicroscope. Seedling pictures were obtained using a stereomicroscope equipped with a digital CCD camera (SteREO Discovery.V8, 14 Carl Zeiss Microimaging) and processed with AxioVision LE software (Carl Zeiss 15 Microimaging). Growth parameters were measured weekly (n = 10). Flowers were counted weekly from the 6^{th} until the 8^{th} week of development and the total number of produced flowers was compared. For seed production, mature capsules were collected from plants (n = 5) over 5 days starting from the first mature capsule harvested.

RNA isolation and real time quantitative PCR (qPCR)

RNA was extracted from shoots of twenty-day-old or from leaves of five-week-old plants using Tri Reagent [44]. For root RNA extraction, the Tri protocol was modified: 300 mg of ground frozen root material was used and one extra round of centrifugation (12000 g, 15 min) was adopted before addition of chloroform. Extracted RNA was checked on agarose gel and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Synthesis of cDNA from 0.5 μg of RNA per sample and qPCR analyses were conducted as in Wu et al. [45] using a Mx3005P qPCR system (Stratagene, Santa Clara, CA, USA, http://www.stratagene.com) and qPCR Core Kit for SYBR® Green I (Eurogentec, Seraing, Belgium, http://www.eurogentec.com). Transcript levels were quantified relative to *N. attenuata* elongation factor 1A (NaeEF1A) and primers were designed according to Steppuhn et al. [10] and Zavala et al. [15]. All reactions were performed with at least 5 biological replicates.

Statistical analysis

After verifying data for *t*- or normal distribution, Welch's *t*-test or Kruskall-Wallis test, followed by Fisher's PLSD as a *post-hoc* test, were performed using R-2.11.1 http://www.R-project.org or StatView5 softwares (SAS Institute, Cary, NC, USA).

Additional material

Additional file 1: Table 1: List of stably transformed lines of *Nicotiana attenuata*. Lines harbor sense (*ov*), antisense (*as*) or inverted repeats (*ir*) constructs and were created in two different accessions of natural population (Arizona, Az or Utah, Ut) [46-74].

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Author details
[1]Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Straße 8, D-07745 Jena, Germany. [2]MitoSciences, Inc., Eugene, Oregon 97403, USA.

Authors' contributions
VF carried out the lab work, HG helped with the *irTPI* and *irPMT* grafts, ITB and SK conceived the project and oversaw the research. All authors wrote, read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Table 1: List of stably transformed lines of *Nicotiana attenuata*.
Lines harbor sense (*ov*), antisense (*as*) or inverted repeats (*ir*) constructs and were created in two different accessions of natural population (Arizona, Ar or Utah, Ut)

Line	Accession	Vector/Cross	Selectable marker	Gene(s)	Publication
Empty Vector (EV)	Ar and Ut	pRESC2NC	hygromycin	empty vector	[15]
Empty Vector (EV)	Ut	pNATNC	nourseothricin	empty vector	[15]
Empty Vector (EV)	Ut	pCAMBIA1300	hygromycin	empty vector	[46]
Empty Vector (EV)	Ut	pSOL3NC	hygromycin	empty vector	[47]
asLOX3	Ut	pNATLOX	nourseothricin	lipoxygenase 3	[48]
ovHPTII	Ut	pCAMBIA1300	hygromycin	hygromicin phosphotransferase II	[49]
ovGUS	Ut	pCAMBIA1301	GUS	β-glucuronidase A	[49]
asAOS	Ut	pNATAOS2	nourseothricin	allene oxide synthase	[50]
asHPL	Ut	pNATHPL1	nourseothricin	hydroperoxide lyase	[50]
asTPI	Ut	pNATPI1	nourseothricin	trypsin proteinase inhibitor	[15]
ovTPI	Ar	pRESC2PIA2	hygromycin	trypsin proteinase inhibitor	[15]
asPMT	Ut	pCAMPMT1	hygromycin	putrescine *N*-methyl transferase	[10]
irPMT	Ut	pRESC5PMT	hygromycin	putrescine *N*-methyl transferase	[10]
asGAL83	Ut	pNATGAL83	nourseothricin	β-subunit of SNF1-related kinase	[23]
asGLP	Ut	pRESC2GER	hygromycin	germin-like protein	[51]
asTD	Ut	pNATTD1	nourseothricin	threonine deaminase	[52]
TDpromoter::GUS	Ut	pCAMBIA1301	hygromycin	threonine deaminase promoter::GUS	[46]
irRAFL	Ut	pRESC5RALF	hygromycin	rapid alkalinization factor	[53]
irCOI1	Ut	pSOL3COI1	hygromycin	coronatine insensitive 1	[34]
irACO	Ut	pRESC5ACO1	hygromycin	ACC oxidase	[37]
sETR1	Ut	pRESC2ETR1	hygromycin	ethylene receptor 1	[37]
irJAR4	Ut	pRESC5JAR4	hygromycin	jasmonate resistant 4	[54]
irJAR6	Ut	pRESC5JAR6	hygromycin	jasmonate resistant 6	[54]
irPMT	Ut	pRESC5PMT	hygromycin	putrescine *N*-methyl transferase	[16]
irTPI	Ut	pSOL3PI	hygromycin	trypsin proteinase inhibitor	[16]
irPMT/irTPI	Ut	pRESC5PMT::pSOL3PI	hygromycin	putrescine *N*-methyl transferase::trypsin proteinase inhibitor	[16]
irPMT/irTPI	Ut	pRESC5PMT::pSOL4PI	nourseothricin	putrescine *N*-methyl transferase::trypsin proteinase inhibitor	[16]
irNPR1	Ut	pRESC5NPR1	hygromycin	non-expressor of PR-1	[55]
irRdR1	Ut	pRESC5RdR1	hygromycin	RNA-directed RNA polymerase 1	[38]
irSYS	Ut	pRESC5SYS2	hygromycin	Systemin (preproTobHypSys)	[56]
ovSYS	Ut	pRESC2SYS2	hygromycin	Systemin (preproTobHypSys)	[56]
irJAR4/irJAR6	Ut	*irJAR4* x *irJAR6*	hygromycin	jasmonate resistant 4 and jasmonate resistant 6	[36]
irPMT	Az	pRESC5PMT	hygromycin	putrescine *N*-methyl transferase	[57]
ovTPI	Az	pRESC2PIA2	hygromycin	trypsin proteinase inhibitor	[57]
ovWRKY3	Ut	pRESC2WRKY3	hygromycin	WRKY transcription factor	[58]
ovWRKY6	Ut	pRESC2WRKY6	hygromycin	WRKY transcription factor	[58]
irWRKY3	Ut	pSOL3WRKY3	hygromycin	WRKY transcription factor	[58]
irWRKY6	Ut	pSOL3WRKY6	hygromycin	WRKY transcription factor	[58]
irWRKY3/irWRKY6	Ut	*irWRKY3* x *irWRKY6*	hygromycin	WRKY transcription factor	[58]
irPR-1	Ut	pRESC5PR1	hygromycin	pathogenesis-related protein 1	[59]
irThionin	Ut	pRESC5Thionin	hygromycin	thionin (pathogenesis-related protein 13)	[59]
irDefensin	Ut	pRESC5Defensin	hygromycin	defensin (pathogenesis-related protein 12)	[60]
irRdR3	Ut	pRESC5RdR3	hygromycin	RNA-directed RNA polymerase 3 (RdR6 homolog)	[40]
irRdR2	Ut	pRESC5RdR2	hygromycin	RNA-directed RNA polymerase 2	[39]
asRUB	Ut	pRESC2RUB	hygromycin	ribulose-1,5-bisphosphate carboxylase/oxygenase	[61]
irRCA	Ut	pRESC5RCA	hygromycin	RuBPCase activase (RCA)	[61]
irCHAL	Ut	pRESC5CHAL	hygromycin	chalcone synthase	[47]
irCHAL	Ut	pRESC1CHAL	nourseothricin	chalcone synthase	[47]
irPMT/irCHAL	Ut	*irPMT* x *irCHAL*	nourseothricin	putrescine *N*-methyl transferase and chalcone synthase	[47]
irSIPK	Ut	pRESC5SIPK	hygromycin	salicylic acid-induced protein kinase	[62]
irWIKP	Ut	pRESC5WIPK	hygromycin	wound-induced protein kinase	[62]
asLOX3/asHPL	Ut	*asLOX3* x *asHPL*	nourseothricin	lipoxygenase 3 and hydroperoxide lyase	[62]
irPME	Ut	pRESC5PME1	hygromycin	pectin methylesterases	[63]
irSYS	Az	pRESC5sys2	hygromycin	Systemin (preproTobHypSys)	[64]
iraDOXS	Ut	pRESC5αDOX1	hygromycin	α-dioxygenase 1	[65]
iraDOXM	Ut	pRESC5αDOX1	hygromycin	α-dioxygenase 1	[65]
irLOX2	Ut	pSOL3LOX2	hygromycin	lipoxygenase 2	[66]
irLOX3	Ut	pRESC5LOX3	hygromycin	lipoxygenase 3	[66]
irLOX2/irLOX3	Ut	*irLOX2* x *irLOX3*	hygromycin	lipoxygenase2 and lipoxygenase3	[35]
irSIPK/irWIPK	Ut	*irSIPK* x *irWIPK*	hygromycin	salicylic acid-induced protein kinase and wound-induced protein kinase	[22]
sETR1/asLOX3	Ut	*sETR1* x *asLOX3*	hygromycin	lipoxygenase 3 and ethylene receptor 1	[67]
irMYB8	Ut	pSOL8MYB8	hygromycin	MYB transcription factor	[68]
irGGPPS	Ut	pRESC5GGPPS	hygromycin	geranylgeranyl diphosphate	[21]
irNOA1	Ut	pSOL8NOA1	hygromycin	nitric oxide-associated protein 1	[69]
irMYB3	Ut	pSOL8MYB3	hygromycin	R2R3-MYB transcription factor	[70]
irGLA1	Ut	pSOL8GLA1	hygromycin	glycerolipase A1	[71]
irHPL	Ut	pSOL3HPL	hygromycin	hydroperoxide lyase	[72]
ovJMT	Ut	pRESC2JMT	hygromycin	JA O-methyltransferase	[73]
ovJMT/irMJE	Ut	pRESC2JMT::pSOL8MJE	hygromycin	JA O-methyltransferase::methyl jasmonate esterase	[73]
irLecRK	Ut	pSOL8LECRK	hygromycin	lectin receptor-like kinase	[74]
ovNahG	Ut	pSOL1NAHG1	hygromycin	salycilate hydroxylase	[74]
irLecRK/ovNahG	Ut	*irLECRK x ovNahG*	hygromycin	lectin receptor-like kinase and salycilate hydroxylase	[74]

4. Manuscript II

Root jasmonic acid synthesis and perception regulate folivore-induced shoot metabolites and increase *Nicotiana attenuata* resistance

Variluska Fragoso, Eva Rothe, Ian T. Baldwin, Sang-Gyu Kim

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07745 Jena, Germany

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Summary

- While jasmonic acid (JA) signaling is widely accepted as mediating plant resistance to herbivores, and the importance of the roots in plant defenses is recently being recognized, the role of root JA in the defense of aboveground parts remains unstudied.

- To restrict JA impairment to the roots, we micrografted wild-type *Nicotiana attenuata* shoots to the roots of transgenic plants impaired in JA signaling and evaluated ecologically relevant traits in glasshouse and nature.
- Root JA synthesis and perception are involved in regulating nicotine production in roots. Strikingly, systemic root JA regulated local leaf JA and abscisic acid (ABA) levels, which in turn, explain differences in nicotine transport from roots to leaves via the transpiration stream. Root JA signaling also regulated the accumulation of other shoot metabolites; together these account for the differences in resistance against a generalist, *Spodoptera littoralis*, and a specialist herbivore, *Manduca sexta*. In *N. attenuata*'s native habitat, silencing root JA synthesis increased the shoot damage inflicted by *Empoasca* leafhoppers, which are able to select natural jasmonate mutants. Silencing JA perception in roots also increased damage by *Tupiocoris notatus*.
- We conclude that attack from aboveground herbivores recruits root JA signaling to launch the full complement of defense responses.

Introduction

Plants have evolved refined signaling mechanisms capable of inducing responses specifically according to different stimuli. These signals can act locally, but can also travel to distal systemic parts within the plant, fine-tuning the whole-plant metabolism and maximizing its performance and Darwinian fitness. An interesting case of these systemic events is the inducible responses of plants when attacked by leaf-feeders: within minutes, defense mechanisms are triggered locally in attacked leaves and also in unattacked remote parts of the plant (Wu & Baldwin, 2010). In maize, transcriptional changes in roots in response the attack of leaves by *Spodoptera littoralis* larvae were shown to exceed the responses observed in the infested leaves (Erb *et al.*, 2009). Also, in the wild tobacco species *Nicotinana attenuata*, transcriptional and metabolic reorganization after mimicked herbivore attack were more pronounced in roots than in local leaves (Gulati *et al.*, 2013). Interestingly, in both cases, minor or no overlap in induced genes was found between the local and the systemic tissues, suggesting that the responses elicited by herbivore attack are triggered in a tissue-specific manner. However, the signaling mechanisms that integrate these belowground events with the defense responses observed in the shoots remain unknown.

Considerably more is known about the defense responses observed in aboveground plant parts, in which jasmonic acid (JA) and its derivatives, collectively known as jasmonates (JAs), play a well-described regulatory role for the defense responses in aboveground (Howe & Jander, 2008). In attacked leaves, the initial step of JA biosynthesis is the production of linolenic acid, a precursor that through the 13-lipoxigenase (13-LOX) pathway culminates in the formation of 12-oxo-phytodienoic acid (OPDA) by the action of an allene oxidase cyclase (AOC; Stenzel *et al.*, 2003). OPDA is then reduced and subjected to cycles of β-oxidation, which finally leads to the production of JA. JA can be subsequently conjugated to isoleucine (JA-Ile) by jasmonate-resistant 1 (JAR1; Staswick & Tiryaki, 2004). JA-Ile, the bioactive conjugated form of JA, interacts with coronatine-insensitive 1 (COI1) and triggers the degradation of jasmonate ZIM domain (JAZ) proteins, which releases downstream positive regulators of the JA-mediated responses (Xie *et al.*, 1998; Chini *et al.*, 2007). In *N. attenuata*, plants silenced for *NaAOC* (*irAOC*; Kallenbach *et al.*, 2012) or for *NaCOI1* (*irCOI1*; Paschold *et al.*, 2007) are more vulnerable to herbivore attack. In addition, wound-treated leaves of *irAOC* completely lack the capacity to induce JAs levels, while those of *irCOI1* present a delayed and more pronounced accumulation of JA-Ile compared to WT plants, as a

consequence of a lower JA-Ile catabolism in leaf tissues of *irCOI1* plants (Paschold *et al.*, 2008).

Following the JA burst observed in local attacked leaves, JA signaling is also required in the systemic responses of distal leaves. Grafting experiments have dissected the local *versus* systemic requirements of the JA signaling components in the shoots of tomato (Li *et al.*, 2002; Li *et al.*, 2005). However, for the systemic events of the belowground, JA has been suggested to mediate root responses to shoot elicitation. One of the best studied examples of this JA-dependent shoot-root interplay is given by nicotine induction in tobacco plants. Although nicotine is synthesized in roots, it is the most abundant alkaloid found in the leaves. It is highly toxic to most herbivores (Glendinning, 2002), and it is effective in thwarting compensatory leaf consumption of generalists, such as *Spodoptera exigua*, as well as specialists, like *Manduca sexta* (Steppuhn *et al.*, 2004). Upon leaf damage, levels of nicotine in shoots dramatically increase (up to 10-fold) as a consequence of induced root-specific expression of the putrescine *N*-methyltransferase (*NaPMT*) gene (Winz & Baldwin, 2001). These events are tightly associated with increased JA pools in roots that follow the rapid increase in locally damaged leaves (Zhang & Baldwin, 1997). When ^{14}C-labeled JA was applied to wounded leaves, this labeled compound was recovered in roots, at similar rates of endogenous root JA, suggesting that leaf-derived JA transported to roots could alone account for the systemic increase in root JA after leaf wounding. In a later study, Wang *et al.* (2008) showed that, after a treatment that mimicked herbivore attack combined to the application of ^{13}C-labeled Ile, newly synthesized^{13}C-labeled JA-Ile was only detected in elicited leaves, but not in roots. However, these results should be interpreted with caution, because it is possible that labeled compounds are not metabolized or transported in the same way as plant-derived compounds. And although a plant's nicotine investment is elicited in the roots through enhanced JA levels in roots (Baldwin, 1996b), it remains unknown whether shoot-derived JA alone can account for nicotine induction.

Recently, Grebner *et al.* (2013) showed that Arabidopsis roots when wounded can synthesize JA independently of the shoots by the action of a single member of the LOX family (LOX6) that is putatively activated by a rapidly propagated electrical signal. Acosta *et al.* (2013) reported that after wound treatment applied to cotyledons of Arabidopsis seedlings the activity of JAR1 and NINJA, a member of the JA transcriptional repression complex, are indispensable to induce the roots systemic expression of *JAZ10-GUSPlus*, a JA-responsive reporter. However, whether

root JA *de novo* synthesis and perception is involved in ecological relevant traits, which in turn affect plant performance under herbivore pressure, remains an open question.

Here we dissected the function of systemic JA synthesis and perception in roots of *N. attenuata* in response to shoot elicitation. To test whether the disruption of root JA signaling has ecological consequences, we generated chimeric plants consisting of shoots of transgenic plants harboring an empty vector (EV) grafted onto roots of transgenic lines impaired in JA synthesis (*irAOC*) or perception (*irCOI*). We then evaluated resistance traits of these grafts in both glasshouse and field experiments. The results reveal that JAs synthesis and JA-Ile perception of both shoot and roots are required to induce nicotine production in the roots as well as its transport to the shoots. Strikingly, root JA signaling systematically regulates leaf levels of JA and ABA after leaf wounding. Finally, we show that root JA synthesis and perception contribute to the metabolic profile of leaves, which in turn, influence aboveground herbivore preference in both the glasshouse and nature.

Materials and Methods

Plant material and treatments

All lines were derived from seeds originally collected in a natural population of *N. attenuata* at the DI Ranch, near Santa Clara, USA. Seed germination and plant growth are described in Kruegel *et al.* (2002). WT or transgenic plants harboring an empty vector (EV) construct were used as controls; all transformed and WT plants were from the same inbred generation of the same original accession. Silenced (*ir*) stably transformed plants were used to knock-down JA-Ile perception (*irCOI1*; Paschold *et al.*, 2007), JA synthesis (*irAOC*; Kallenbach *et al.*, 2012), or nicotine production (*irPMT*; Steppuhn *et al.*, 2004). Seven-day-old seedlings were grafted as described in Fragoso *et al.* (2011), with average rate of grafting success of 77%, that did not differ significantly amongst all graft combinations (p =0.1377; approximately 300 to 400 seedlings were grafted per each graft combination).

Glasshouse plants were grown in sand or in soil, and kept at 26-28°C under 16 h of light and 8 h of dark. In order to induce nicotine levels in a standardized way, five-week-old plants had 3 of their rosette leaves punctured with a pattern wheel, run 4 times on each side of leaf, parallel to the midrib (wounding treatment). After designated time points, systemic tissues (roots and pooled stalk leaves) and

local leaves of control and wounded plants were sampled and stored at -80°C until analysis. In order to normalize leaf damage among plants for global metabolic analysis, herbivore attack was mimicked by wounding, as previously described, and the applying 20 µL per leaf of a 1:5 dilution of oral secretions in water directly to the freshly created puncture wounds. Oral secretions were collected from 3rd to 4th instar larvae of *M. sexta* or *S. littoralis* reared on WT plants. To detected differences in metabolite profiles in response to mimicked herbivory, three days after treatments, undamaged systemic leaves were sampled and stored at -80°C until analysis.

For field experiments, seeds were imported under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) notification number 11-350-101r, and planted in a randomized manner to an experimental plot at the Lytle Ranch Preserve, Utah, USA, in 2012.

Nicotine extraction and quantification by HPLC-PDA detector

To test whether disrupting root JA signaling influences nicotine induction, approximately 150 mg of leaf or 300 mg of root tissue of control or wound-treated grafts was extracted for nicotine quantification as described by Onkokesung *et al.* (2012). Plant tissue was ground with two 4 mm steel balls by Genogrinder 2000 (SPEX CertiPrep, New Jersey, USA). Samples were extracted with 1 mL of methanol:water (40:60, v/v) acidified by 0.1% (v/v) acetic acid and homogenized by vortex for 10 min. Supernatants were subjected to two rounds of centrifugation at 16,100 g at 4°C for 20 min. Sample aliquots of 1 µL were analyzed by an Agilent HPLC 1100 Series device (http://www.chem.agilent.com) in a Chromolith FastGradient RP-18e column (endcapped 50 x 2 mm; Merck) attached to a precolumn (Gemini NX RP-18e, 3 um, 2 x 4.6 mm; Phenomenex) as described in Oh *et al.* (2012). For quantification, an external standard curve of an authentic and purified nicotine standard was used, and peaks were identified based on their retention time, spectra, and samples were spiked with purified compounds to estimate matrix effects. All extractions were performed with at least 6 biological replicates, and the resulting nicotine amount was expressed per gram fresh mass plant material.

RNA extraction and real time RT-PCR

To check whether *NaAOC* and *NaCOI1* expression in EV shoots was silenced when grafted onto rootstocks of *irAOC* and *irCOI1*, respectively, and to check expression levels of *NaPMT* in roots, which encodes the first enzyme committed to nicotine biosynthesis, total RNA was extracted from leaf or root tissues with the Trizol reagent. After normalizing total RNA concentration of all samples to 500 ng, cDNA was synthesized as described in Fragoso *et al.* (2011). All primers were previously described (Paschold *et al.*, 2007; Kallenbach *et al.*, 2012), and for *NaPMT* a new pair of primers was designed and tested for their ability to amplify a 93-bp-long consensus cDNA fragment of both *NaPMT1* and *NaPMT2* genes (NaPMT12-for 5'- TCATTGGACCAAGATCGAG-3' and rev 5'-TGGAAATTATGATAATTACTGCAGA-3'; Winz & Baldwin, 2001). The efficiency of the primers and the estimated initial amount of template were calculated as described in Fragoso *et al.* (2011) and relativized to *N. attenuata*'s elongation factor1A (*NaEF1A*). All reactions used qPCR Core Kit for SYBR Green I (Eurogentec, Seraing, Belgium, http://www.eurogentec.com), and performed with at least 5 biological replicates.

Petiole-feeding experiment

To test whether disruption in root JA signaling influences nicotine uptake by leaves, a petiole-feeding assay was performed (Fig. 3a, later). One day after leaf wounding, when systemic leaves of non-grafted WT plants start to differentially increment nicotine in response to wounding treatment, younger unwounded systemic leaves from control or wound-treated plants were carefully excised at the base of their petioles, weighed and transferred to vials with water or a 1 mM nicotine-containing solution. After one day of feeding, leaf laminas were dissected and stored at -80°C until HPLC analysis for their nicotine content, as described above. All tubes containing solutions were weighed prior to and after the petiole-feeding, so that the volume of solution transpired by the leaf could be estimated. In the nicotine treatment, the remaining solution was also analyzed for its nicotine content, so in comparing initial and final nicotine solution concentrations, nicotine uptake was calculated. Treatments were performed with at least 8 biological replicates.

Phytohormone analysis

Phytohormones were quantified in order to evalutate whether root-silencing of *NaAOC* (EV/*irAOC*) or *NaCOI1* (EV/*irCOI1*) resulted in impaired root jasmonates and ABA accumulation in response to leaf wounding, or whether, alternatively, these molecules would be transported from EV shoots to the silenced roots. Damaged leaves of EV/EV, EV/*irAOC*, and EV/*irCOI1* grafts, along with their respective undamaged roots, were sampled prior to and at 1, 2, and 3 h after wounding treatment. Approximately 100 mg leaf or 200 mg root frozen tissue material was ground as described above. Phytohormones were extracted with 1 mL of ethyl acetate spiked with internal standards (200 ng of [$^{2}H_{2}$]JA and 40 ng of each JA-[$^{13}C_{6}$]-Ile, [$^{2}H_{4}$]SA, and [$^{2}H_{6}$]ABA). After extraction by vortexing for 10 min, 500 µL of the organic phase was obtained by centrifugation at 16,100 g at 4°C for 15 min. Samples were evaporated almost to dryness in a vacuum concentrator (Eppendorf) under reduced pressure at 30°C. Leaf samples were then diluted in 200 µL of methanol:water (70:30, v/v), while 100 µL was used for root samples, once roots accumulate smaller amounts of phytohormones. Analysis was performed with a Varian 1200 HPLC-MS/MS system as described in Vadassery *et al.* (2012), with a modified shortened chromatographic gradient. Sample-derived phytohormones were calculated by the ratios of their ion intensity and of their respective internal standards, for cis-OPDA, [$_{2}H_{2}$]JA was used as an internal standard applying an experimentally determined response factor of 0.5. All quantifications were corrected according to the sample dilution, and extractions were performed with 6 biological replicates. The resulting amount of different phytohormones was then expressed per gram fresh mass plant material.

Extraction and unbiased analysis of metabolites

In order to evaluate whether root JA disruption influences leaf accumulation of metabolites other than nicotine, an unbiased metabolomics screen was performed (Gaquerel *et al.*, 2010). Plants were elicited by mimicked and standardized herbivore attack and, 3 days after treatment, systemic leaves were sampled and ground as described above. Metabolites were extracted from frozen ground tissue with 1 mL of 50 mM acetate buffer (40 mM acetic acid plus 44 mM ammonium acetate; 4.8 pH) in methanol (60:40, v/v). Samples were homogenized, and supernatant was recovered after two rounds of centrifugation. After separation by an Agilent HPLC 1100 Series device (http://www.chem.agilent.com), the eluted

compounds were positively charged by Electrospray Ionization (ESI) and had their masses detected by mass spectrometry (MS), carried out with a MicroToF (Time-of-Flight, Bruker Daltonik, Bremen, Germany). Extractions used 4 to 5 biological replicates for analysis.

Raw data files were converted to netCDF format and processed by XCMS (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Peak_Alignment/xcms/) and CAMERA (http://bioconductor.org/packages/devel/bioc/html/CAMERA.html) R packages according to Kim *et al.* (2011). All peaks from 40 to 450 s of ions in the mass range *m/z* 90 to 1400 were selected and normalized by the exact amount of plant material used. Only peaks that were found in at least 75% of the replicates with absolute intensities higher than 5 mega counts per s of the total ion count within same graft kind were analyzed by PCA using MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp), following a normalization by the median value and Pareto scaling.

Herbivore assays

To explore whether disruption of JA signaling in roots influence herbivore mass gain or preference, herbivore assays were performed with EV/EV, EV/*irAOC*, and EV/*irCOI1* plants using adapted herbivores, under glasshouse or open field conditions. Eggs of *M. sexta* were obtained from Carolina Biological Supply and were derived from an in-house colony; and *S. littoralis* eggs were obtained from Syngenta Crop Protection AG (Stein, Switzerland). All eggs were kept in growth chambers (Snijders Scientific) at 22-26°C under 16 h light and 8 h dark conditions until hatching. For glasshouse experiments, 4 freshly hatched neonates were placed on the rosette leaves of each grafted plant (n = 10); due to a high mortality rate in the first days of life, after 3 days, only 2 neonates per plant were used for the performance assays. Larvae performance was estimated by their mass gain, measured on the 12th day of *M. sexta* and on the 10th day of *S. littoralis* feeding. Under field conditions, insect damage was measured on June 4th, 2012, on plants between the 5th and 6th week after transplanting to the plot. Insect-specific damage signatures were identified according to Gaquerel *et al.* (2013), and quantified in standardized units of leaf area consumed relative to insect size (i.e. 5 units of mirids damage ≈ 5 units of leafhoppers damage ≈ 1 cm^2 of heavily attacked leaf area).

Statistical analysis

Data were verified for assumptions of normal distribution and homogeneity of variances, and log-transformed when adequate. Parametric or non-parametric (Kruskal-Wallis test) ANOVA, followed by Fisher LSD or Dunnett's as *post-hoc* tests, were performed using StatView5 (SAS Institute, Cary, NC, USA) or SigmaPlot 12.0 (Systat Software Inc. 2008).

Results

Complete nicotine induction in systemic leaves in response to leaf-wounding requires root JA synthesis and perception.

To examine the contribution of the belowground parts of *N. attenuata* plants to JA-dependent nicotine induction in systemic leaves, we grafted EV scions onto rootstocks of transgenic lines of *N. attenuata*. We used grafts of the scions and rootstocks of the same genotypes as controls. As previously described (Fragoso *et al.*, 2011), the silencing of target genes of *ir* lines is only restrained to the transgenic counterpart of the grafts when those are used as rootstocks (Fig. S1). We first observed that grafted empty vector plants (EV/EV) responded to leaf wounding with amounts of nicotine 10-fold higher than of unwounded control EV/EV levels (Fig. 1; p <0.001). EV shoots grafted onto silenced *irPMT* roots (EV/*irPMT*) completely lacked nicotine, and did not differ from fully silenced *irPMT*/*irPMT* grafted plants (Fig. 1; p >0.94 for both control and induced nicotine levels).

A 55% decrease in wound-elicited nicotine induction in systemic leaves of plants with impaired JA synthesis in roots (EV/*irAOC*) was observed compared with the nicotine induction in EV/EV plants (Fig. 1; p <0.001). An even more pronounced impairment of nicotine induction in systemic leaves was observed in plants lacking JA perception in roots: wounded EV/*irCOI1* plants accumulated less than 20% of the induced EV/EV nicotine levels (Fig. 1; p <0.001). Both graft combinations accumulated more nicotine in systemic leaves than their respective entirely silenced graft combinations, *irAOC*/*irAOC* and *irCOI1*/*irCOI1* (Fig. 1; p <0.001 and p =0.023, respectively). Moreover, only EV/EV, EV/*irAOC*, and EV/*irCOI1* responded to wounding with increased levels of nicotine in systemic leaves compared to control nicotine levels (p <0.001, p <0.001, and p =0.009, respectively). When analyzing nicotine induction in roots, all graft combinations

accumulated similar or lower levels of nicotine than those found in EV/EV roots (Fig 1).

Root JA synthesis and perception control the induction of *NaPMT* expression in roots.

To test whether JA synthesis or perception in roots regulates the expression of nicotine biosynthetic genes, we analyzed the accumulation of *NaPMT1* and *NaPMT2* transcripts, here collectively referred as *NaPMT*. One day after the leaf wounding treatment, *NaPMT* transcript levels in roots of both EV/*irPMT* and *irPMT*/*irPMT* were less than 5% of the *NaPMT* transcript levels of EV/EV grafts (Fig. 2; p <0.001 in both comparisons). Roots of induced EV/*irAOC* plants accumulated 63% less *NaPMT* transcripts than did the roots of induced EV/EV plants (Fig. 2; p <0.001). An even more pronounced impairment in *NaPMT* transcript abundance was found in the roots of induced EV/*irCOI1* plants compared to those of EV/EV grafts (76% less, Fig. 2; p <0.001). *NaPMT* levels in EV/EV, EV/*irAOC*, and EV/*irCOI1* were significantly increased in response to wounding relative to their respective untreated levels (p <0.001, p <0.001, and p =0.005, respectively).

Root JA synthesis and perception control the transport of *de novo* synthesized nicotine to leaves.

To further investigate whether JA synthesis or perception in roots regulates the transport of wound-induced nicotine from roots to leaves, a petiole-feeding experiment was performed (Fig. 3a). Systemic leaves of control and wounded EV/EV, EV/*irAOC*, and EV/*irCOI1* were excised and had their petioles submerged for one day in a nicotine-containing solution at a physiologically relevant concentration of 1 mM. We observed that nicotine levels in excised systemic leaves of wounded EV/EV plants were 66% higher than those of control EV/EV plants (Fig. 3b; p <0.001), which matches the level of the nicotine induction observed in intact systemic leaves of non-grafted WT plants between the 24 h and 48 h time interval after leaf wounding (Fig. S2). Conversely, nicotine levels of excised systemic leaves of both EV/*irAOC* and EV/*irCOI1* after leaf wounding were significantly lower than those of EV/EV, although the genotype of all leaves was the same (Fig. 3b; p <0.001 in both comparisons). In comparison to nicotine-fed undamaged controls, wounding treatment increased nicotine levels in excised systemic leaves of

EV/*irAOC* only by 35% (Fig. 3b; p =0.016). Most strikingly, for nicotine-fed EV/*irCOI1* systemic leaves, wounding treatment failed to induce nicotine levels, these remained as low as their respective nicotine-fed control levels (Fig. 3b; p =0.963).

When leaves were fed a nicotine-free solution, the wounding treatment failed to induce nicotine in leaves of all grafts, including EV/EV, indicating that induced nicotine levels in nicotine-fed systemic leaves are entirely derived from the nicotine-containing solution (Fig. S3). Interestingly, the presence of nicotine in the solution did not increase nicotine levels of control EV/EV leaves, these remained similar to those found in water-fed control EV/EV leaves (Fig. 3b,S3; p =0.654). However, systemic leaves of EV/*irAOC* and EV/*irCOI1* plants fed with a nicotine-containing solution accumulated more nicotine than did their respective leaves fed only with water (Fig. 3b,S3; nicotine- *versus* water-feeding for EV/*irAOC* control, p =0.029 and wounding, p =0.025; for EV/*irCOI1* control, p =0.013 and wounding, p =0.052). Although the interaction of factors was statistically significant (Feeding*Treatment: p =0.047), treatment factor had a stronger effect than feeding factor in explaining how EV/EV leaves incorporated nicotine from the feeding solution (Treatment: p <0.001; Feeding: p =0.099). In contrast, taking EV/*irAOC* and EV/*COI1* leaves together, the feeding factor contributed more to explaining how samples varied in leaf-nicotine, and made the interaction between factors no longer significant (Feeding solution: p <0.001; Treatment: p <0.001; Feeding*Treatment: p =0.337; for both grafts analyzed individually). In other words, for EV/*irAOC* and EV/*irCOI1* plants, systemic leaves of either control or wound-treated plants accumulated more nicotine whenever this molecule was offered in the solution, regardless the treatment.

The size of the leaves did not differ significantly across different grafts (p =0.104), treatments (p =0.536), and feedings (p =0.515; data not shown). As expected, nicotine uptake from the solutions was highly correlated to the volume of solution transpired by the leaf (correlation coefficient =0.99, p =7.5e-09), and wounding significantly increased the volume of solution transpired (Fig. S4; wounding *versus* control nicotine-fed EV/EV, p <0.001).

Impaired JA synthesis and perception in roots systemically up-regulate jasmonates and ABA accumulation in wound-induced leaves.

To evaluate whether the impairment in JA synthesis or perception in roots changes phytohormone accumulation in wounded leaves, we measured the levels of phytohormones in damaged leaves of EV/EV, EV/*irAOC*, and EV/*irCOI1* plants and in their respective undamaged roots after leaf wounding. A burst in JA and JA-Ile accumulation was detected in local leaves of EV/EV 1 h after wounding, and this pattern was also observed in EV/EV roots 2 h after leaf wounding at much lower concentrations (Fig. 4a). However, local tissues of EV/*irAOC* and EV/*irCOI1* attained strikingly higher levels of phytohormones than those in the local tissues of EV/EV (Fig. 4a; p <0.001, for all comparisons), while EV/*irAOC* and EV/*irCOI1* roots completely lacked the JA and JA-Ile burst found in EV/EV roots (Fig. 4a; p <0.001, for all comparisons). JA levels in local leaves of EV/*irAOC* and EV/*irCOI1* 1 h after wounding were 1.7-fold higher than those of EV/EV leaves, and JA-Ile levels were 5-fold higher than those of EV/EV leaves (Fig. 4a). Levels of leaf OPDA were also higher in EV/*irAOC* and EV/*irCOI1* than in EV/EV (Fig. 4a; p =0.01, for both grafts compared individually to EV/EV). In addition, ABA levels of control and treated leaves were also higher in EV/*irAOC* and EV/*irCOI1* when compared to those of EV/EV (Fig. 4b; p <0.001). Initial basal levels of JA, JA-Ile, and OPDA in local tissues were similar among all grafts (p >0.8 for all comparisons against EV/EV, within control time-point at 0 h), while EV/*irAOC* roots had reduced basal levels of jasmonates compared to EV/EV (JA, p =0.002; JA-Ile, p =0.011; OPDA, p <0.001).

JA synthesis and perception in roots contribute to plant resistance in glasshouse and nature.

To investigate the contribution of root JA synthesis or perception to plant resistance to aboveground herbivore attack, we performed a series of experiments exploring plant-insect interactions under glasshouse and field conditions using EV/EV, EV/*irAOC*, and EV/*irCOI1* plants. Initially, we compared the global metabolic profile of systemic leaves of these grafts 3 days after simulated herbivore attack, which standardizes the induction treatment across plants. Oral secretions of two herbivore species were tested: *M. sexta*, a specialist, and *S. littoralis*, a generalist. Principal component analysis (PCA) revealed that metabolic profiles of EV/EV, EV/*irAOC*, and EV/*irCOI1* grafts were more clearly discriminated when

treated with regurgitant of *S. littoralis* than when treated with regurgitant of *M. sexta* (Fig. 5a,c). When comparing the principal components (PC) individually across the two herbivores examined, PC1 and PC2 explained similar levels of the total variance (PC1: *M. sexta*, 50.5% and *S. littoralis*, 49.6%; PC2: *M. sexta*, 16.3% and *S. littoralis*, 12.8%).

We further tested whether root JA signaling influences caterpillar performance. Larvae of *M. sexta* and *S. littoralis* were reared on EV/EV, EV/*irAOC*, and EV/*irCOI1* grafts and had their mass measured. While larvae of both *M. sexta* and *S. littoralis* reared on EV/EV plants showed the smallest mass gain (Fig. 5b,d), larvae reared on *irAOC*/*irAOC* and *irCOI1*/*irCOI1* plants attained the largest masses, followed by those reared on EV/*irAOC*. Larvae of *M. sexta* fed on EV/*irCOI1* gained the same mass as those fed on EV/EV (Fig. 5b; p =0.150). However, *S. littoralis* larvae fed on EV/*irCOI1* plants gained significantly more mass than those on EV/EV plants (Fig. 5d; p =0.002).

We also measured herbivore preference between EV/EV, EV/*irAOC*, and EV/*irCO1* plants in *N. attenuata*'s natural habitat, the Great Basin Desert of Utah, USA. Under field conditions, EV/*irAOC* plants were significantly more damaged by leafhoppers (*Empoasca* spp., p =0.044), and less attacked by mirids (*Tupiocoris notatus*, p <0.001) compared to EV/EV plants (Fig. 6a). EV/*irCOI1* plants were more heavily damaged by mirids (p =0.038), but were consumed as much by leafhoppers as EV/EV plants (Fig. 6b; p =0.096). The silencing of target genes in field-grown grafts was confirmed to be restricted to the transgenic rootstocks (Fig. S1).

Discussion

In this study, we investigated the role of root JA synthesis and perception for the resistance of aboveground plant parts. For this, we used genetically transformed plants disrupted in different components of the JA pathway and dissected the JA-dependent function of roots using micrografted plants. JA synthesis and perception in roots tightly controlled nicotine accumulation (Fig. 1), *NaPMT* transcript levels (Fig. 2), and nicotine transport from roots to the shoots (Fig. 3b). Like nicotine, JA signaling in roots also regulated the concentration of other shoot-accumulated metabolites (Fig. 5a,c), and significantly promoted plant resistance against leaf-feeders in both glasshouse and natural conditions (Fig. 5b,d,6).

Nicotine and micrografting in *N. attenuata*: the toolbox for the study of root-dependent JA signaling in systemic responses.

Recently, Mousavi *et al.* (2013) showed that changes in electrical potentials of the leaf surface were triggered by larval feeding. This electrical wave spread throughout unattacked portions of the shoots, inducing jasmonate biosynthesis and defense-responsive gene expression in systemic leaves. However, it remains unknown whether this signal is also inducing systemic responses in the belowground. Regardless of the identity of the systemic signal conveying the information of leaf wounding to roots, we focused on its downstream events, and investigated whether JA synthesis or perception in roots regulate systemic root responses after leaf wounding. We used nicotine induction as a case study to explore the function of JA in roots, because nicotine is synthesized in roots and induced by leaf wounding. EV/EV plants accumulated 10-fold more nicotine in response to leaf-wounding than undamaged control grafted EV/EV plants (Fig. 1), and nicotine induction was completely absent in systemic leaves of EV/*irPMT* and *irPMT*/*irPMT*. These observations are in agreement to previous reports of wounding in *Nicotiana* species (Ohnmeiss *et al.*, 1997) and confirm that nicotine biosynthetic genes are required only in roots (Winz & Baldwin, 2001). These data also highlight the value of micrografting to test root responses to leaf induction, and demonstrate that the mobile wound signal is graft-transmissible.

JA *de novo* synthesis and perception in roots tightly regulate nicotine production in roots and transport to leaf lamina.

Nicotine induction in response to leaf wounding was almost fully dependent on JA-Ile perception (COI1) in roots. JA *de novo* synthesis (AOC) in roots was also required for nicotine induction in shoots (Fig. 1). However, nicotine levels of EV/*irAOC* and EV/*irCOI1* leaves were induced in response to leaf wounding, suggesting a JA-independent root signaling pathway involved in nicotine induction. Alternatively, the residual nicotine induction found in leaves of these grafts might be due to minor expression levels remaining in silenced roots (Fig. S1) or a consequence of the rapid turnover of shoot-derived JAs transported to roots.

The expression levels of *NaPMT* transcripts in roots of all grafts were strongly correlated to the nicotine amounts found in leaves (Fig. 2), and roots did not over-accumulate nicotine (Fig. 1), suggesting that JA signaling in roots controls nicotine

induction at the transcriptional level. *NaPMT* expression and nicotine production are known to be attenuated by ethylene emission (Kahl *et al.*, 2000; Winz & Baldwin, 2001). However, wounding alone did not induce leaf ethylene emission in EV/EV grafts and intact WT plants (Diezel *et al.*, 2011); also, EV/*irAOC* and EV/*irCOI1* showed similar or even reduced levels of ethylene emission compared to EV/EV (data not shown). It is also noteworthy that nicotine induction in *Nicotiana* species has allometrically-determined setpoints that control the amount of nicotine accumulated in the shoots in response to wounding. The allometric nicotine induction is proportional to the biomass of the plant, and it seems to be mainly dictated by the rate of *de novo* synthesized nicotine in roots, as well as by other factors regulating nicotine storage in the shoots (Baldwin, 1996a; Baldwin, 1999). Hence, a plants' ability to store nicotine aboveground is presumably controlled by the same mechanisms as those that control nicotine synthesis in the belowground.

In a petiole-feeding experiment, we mimicked the endogenous increase in the transport of nicotine via the apoplast from roots to shoot that occurs between the first and second day after leaf wounding (Fig. S2; Baldwin, 1989). As a control, water-fed leaves failed to induce nicotine in systemic leaves after wounding (Fig. S3), indicating that the wound-induced nicotine increment of nicotine-fed leaves was due to the nicotine loaded into the leaf by transpiring the nicotine-containing solution. Even when nicotine was equally offered, systemic leaves of EV/*irAOC* and EV/*irCOI1* failed to allocate nicotine to the leaf lamina, suggesting that JA signaling in roots regulates physiological changes in shoots required to transport the root-derived nicotine to the leaf lamina (Fig. 3b), as had been previously suggested with *N. sylvestris* (Baldwin & Callahan, 1993; Baldwin, 1996a). It was also observed that leaves of EV/*irAOC* and EV/*irCOI1* have significantly fewer trichomes than EV/EV leaves on their abaxial surfaces (around 20~30% less, p =0.007), and trichomes represent one site of nicotine accumulation (Roda *et al.*, 2003). These data add another regulatory step by which root JA signaling fine-tunes the wound-induced nicotine response of shoots (Baldwin & Schmelz, 1994).

Interestingly, as shown in the petiole-feeding experiment, wounding significantly induced solution uptake in excised systemic leaves of EV/EV plants. These data are consistent with a mechanism by which wound-induced nicotine transport via xylem is facilitated through higher rates of transpiration (Baldwin, 1989; Baldwin & Schmelz, 1994). Conversely, nicotine-fed leaves of EV/*irAOC* and EV/*irCOI* tended to absorb more solution than did their water-fed leaves, whether wounded or not (data not shown), and basal levels of nicotine in these grafts were

already reduced compared to EV/EV. Therefore, it is possible that nicotine *per se* serves as a signal to promote transpiration in shoots of these nicotine-deprived *N. attenuata* grafts. However these hypotheses require further tests, and changes in transpiration rates are likely one of the mechanisms regulating nicotine accumulation in tobacco leaves. Taken together, these results suggest that root JA signaling regulates directly and/or indirectly leaf nicotine uptake. To explore these differences further, the levels of phytohormones were analyzed.

Root JA synthesis and perception systemically tune local leaf JA and ABA accumulation in response to leaf-wounding.

As expected from previous study with WT intact plants of *N. attenuata* (Von Dahl & Baldwin, 2004), leaf wounding induced neither JA nor MeJA in systemic leaves of EV/EV (data not shown). Since AOC enzyme activity leads to OPDA production that serves as a substrate for JA and JA-Ile formation, basal levels of all these JAs were reduced in EV/*irAOC* roots (Fig. 4a). Although basal levels of root jasmonates in EV/*irCOI1* plants were similar to those of EV/EV, roots of both EV/*irAOC* and EV/*irCOI1* did not show the systemic wound-induced burst of OPDA, JA, and JA-Ile observed in EV/EV roots. Surprisingly, the reduced JAs levels of EV/*irAOC*, and EV/*irCOI1* roots were associated with a hyper-responsive JA accumulation in induced leaves, suggesting the existence of a novel shoot-root-shoot loop in regulating the JA response. The impaired accumulation of systemic root JAs possibly boosts JAs responses in the local leaf, as a compensatory effect. We ruled out the possibility that the result was an artifact (i.e. silencing of JA components in shoots) by checking gene expression in both shoot and roots of these grafted plants (Fig. S1).

Moreover, levels of ABA were found to be surprisingly higher in leaves of EV/*irAOC* and EV/*irCOI1* when compared to those of EV/EV (Fig. 4b), suggesting a crosstalk between JA and ABA regulating shoot-root-shoot interplay in plant defenses. This agrees with our petiole-feeding data, and also with the notion of facilitated nicotine transport through higher transpiration rates: higher ABA levels in EV/*irAOC* and EV/*irCOI1* leaves would inhibit transpiration, ultimately leading to reduced nicotine contents. Furthermore, ABA-regulated water stress, rather than ABA-induced defenses, has been already suggested to be involved in leaf resistance induced by root-herbivory in maize plants (Erb *et al.*, 2010). However, the involvement of ABA in JA-dependent responses to wounding and to herbivore attack

is shown to be beyond the control of guard cells and transpiration rates, and JA-ABA signaling cross-talk likely regulates a more complex range of processes. For instance, reduced levels of ABA in leaves, and its consequent augmented transpiration rates were recently associated with reduced emission of defensive organic volatile compounds (VOCs) in *N. attenuata* plants silenced for a novel protein that suppress ABA catabolism after herbivore attack (Dinh *et al.*, 2013).

JA synthesis and perception in roots enhance plant resistance against aboveground herbivores.

The treatment using regurgitate of a generalist herbivore caused EV/*irAOC* and EV/*irCOI1* to be metabolic more distinct from EV/EV when compared to the weak grouping found for these graft combinations when elicited with *M. sexta* regurgitant (Fig. 5a,c). These data suggest that other defensive metabolites in addition to nicotine are dependent on JA signaling in roots. This result is consistent with the notion of an herbivore-induced carbon sequestration to roots as a resistance mechanism regulating defensive metabolites, rather than solely a tolerance mechanism (Schwachtje *et al.*, 2006; Machado *et al.*, 2013).

The more distinct pattern of grouping found in PCA of plants induced by *S. littoralis* (generalist) oral secretion was also reflected in more pronounced effect on *S. littoralis* larval performance between EV/EV and EV/*irCOI1* grafts compared to those of *M. sexta*, a specialist (Fig. 5). Under field conditions, COI1 activity in roots accounted for enhanced plant resistance against mirids, while it likely had no influence on the feeding choice of *Empoasca* leafhoppers (Fig. 6). On the other hand, the lack of AOC activity only in roots enhanced the vulnerability of plants to these leafhopper species. *Empoasca* spp. is able to identify in native populations natural *N. attenuata* JA-mutants with impaired capacity to mediate JA signaling (Kallenbach *et al.*, 2012). Despite its hyper-response in JA accumulation after wounding (Fig. 4a), EV/*irAOC* plants were preferably attacked by *Empoasca*, suggesting that the function of AOC in roots profoundly influences JA-mediated responses of the shoots. In addition, our data support the notion that JA dependent responses are employed in an herbivore-specific way (Hettenhausen *et al.*, 2013), and suggest a COI1-independent JA signaling in the roots.

In addition, we observed that damage caused by mirids was negatively correlated to damage inflicted by *Empoasca* leafhoppers in *irAOC* grafts. It would be

interesting to test whether the density of these herbivores is directly tailored simply by their presence/absence, or indirectly, through plants' responses mediated by JA signaling (Kessler & Baldwin, 2004; Kallenbach *et al.*, 2012). Mirids are specialist herbivores, and might be more adapted to *N. attenuata* defense metabolites than to the presence of other generalist herbivores, and this becomes apparent only in *irAOC* plants. In other words, mirids might prefer plants more defended against generalists. Moreover, the negative correlation between damage inflicted by mirids and leafhoppers found in *irAOC* grafts was not found in grafts using *irCOI1*. This suggests that the interaction between these herbivores is very likely plant mediated, and CO1-dependent.

The revival of the root-brain theory originally proposed by Charles and Francis Darwin (Baluska *et al.*, 2009) has renewed attention to the function of roots as a regulatory organ of plants. How changes in roots affect shoot responses, and vice-versa, is the subject of current intense study. Here, we focused on the aboveground changes induced by leaf-attack that engage roots in a more comprehensive shoot-root-shoot loop. Based on the dramatic changes observed in how leaves respond to attack when roots are depleted of JA signaling, we conclude that roots play a central role in orchestrating aboveground processes.

Acknowledgements

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List of supporting information

Fig. S1. Silencing of target genes is restricted to transgenic scions or rootstocks of grafted plants under glasshouse and field conditions.

Fig. S2. Kinetic of endogenous nicotine (in systemic leaves) and *NaPMT* expression (in roots) of non-grafted WT plants in response to leaf wounding.

Fig. S3. Water-fed systemic leaves failed to induce leaf-nicotine levels in response to leaf wounding.

Fig. S4. Systemic leaves excised from wound-treated EV/EV plants and fed with a nicotine-containing solution transpired more than those detached from untreated plants.

Figures

Figure 1

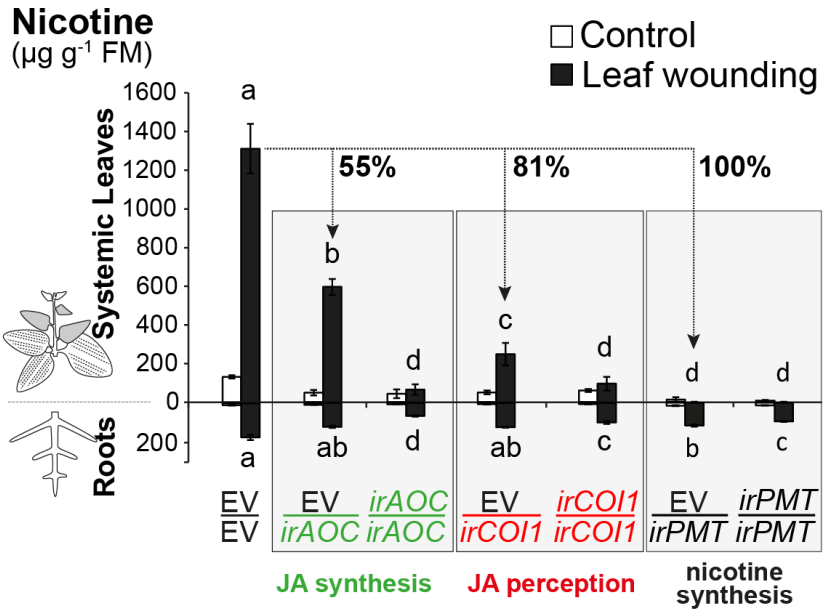

Fig. 1. *Jasmonic acid (JA) de novo synthesis and perception in roots contribute to nicotine accumulation induced in systemic leaves in response to leaf wounding in Nicotiana attenuata.* Mean ± SE levels of nicotine accumulated in undamaged roots and systemic leaves (shaded) of control and leaf wounding-treated (dashed lines) grafted plants displaying roots impaired in JA synthesis (EV/*irAOC*), JA perception (EV/*irCOI1*), and nicotine synthesis (EV/*irPMT*), 3 days after leaf wounding. Grafts of the scions and rootstocks of the same genotypes (EV/EV, *irAOC*/*irAOC*, *irCOI1*/*irCOI1*, and *irPMT*/*irPMT*) were used as controls. Bars sharing same letters do not differ significantly (Two-way ANOVA followed by Fisher LSD test, n = 6). Dashed arrows indicate % of reduction in nicotine induction compared to wounded EV/EV grafts. EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line; *irPMT*, putrescine *N*-methyltransferase silenced line.

Figure 2

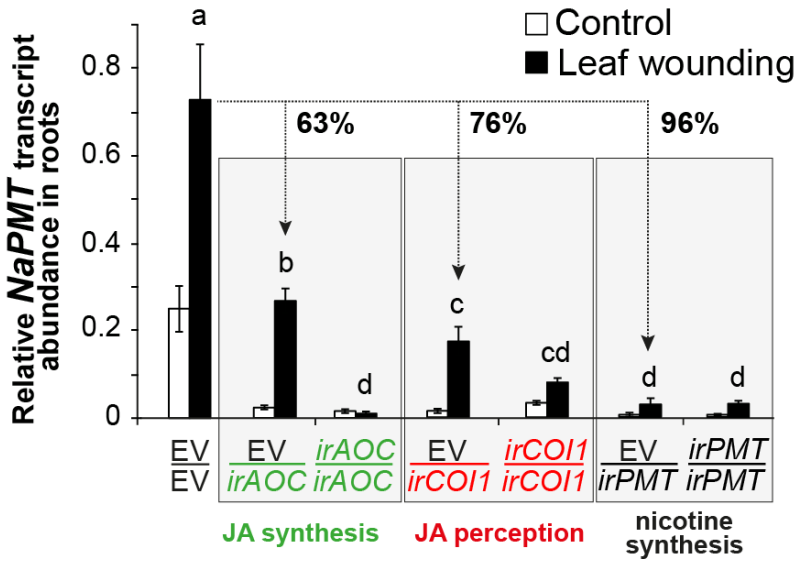

Fig. 2. *Jasmonic acid (JA) de novo synthesis and perception in roots contribute to induced NaPMT expression in roots in response to leaf wounding.* Mean ± SE levels of relative *NaPMT* transcript accumulation in undamaged roots of control and leaf wounding-treated grafted plants displaying roots impaired in JA synthesis (EV/*irAOC*), JA perception (EV/*irCOI1*), and nicotine synthesis (EV/*irPMT*), 1 day after leaf wounding. Grafts of the scions and rootstocks of the same genotypes (EV/EV, *irAOC*/*irAOC*, *irCOI1*/*irCOI1*, and *irPMT*/*irPMT*) were used as controls. Bars sharing same letters do not differ significantly (Two-way ANOVA followed by Fisher LSD test, n = 6). Dashed arrows indicate % of reduction in nicotine induction compared to wounded EV/EV grafts. EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line; *irPMT*, putrescine *N*-methyltransferase silenced line.

Figure 3

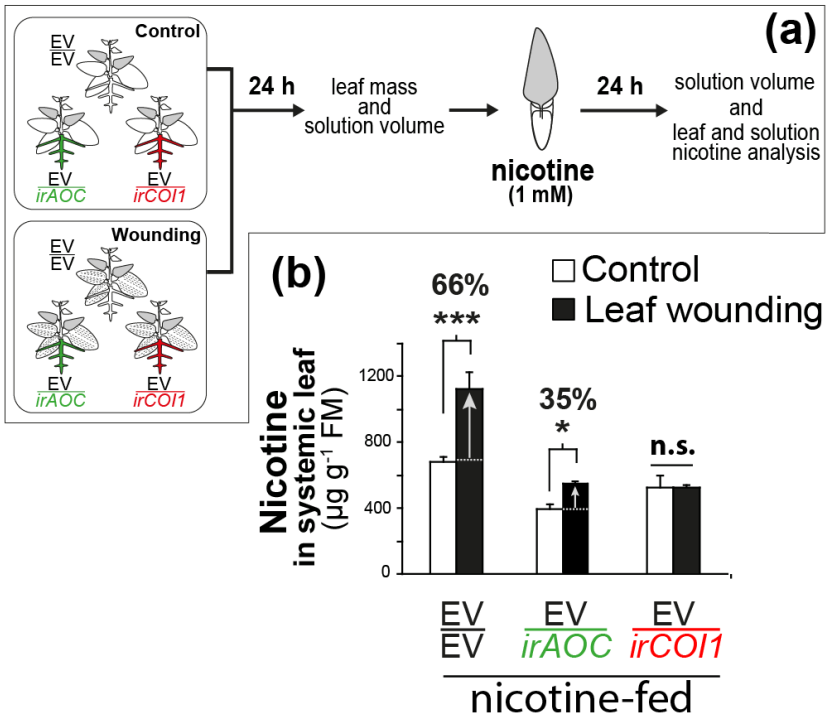

Fig. 3. *Jasmonic acid (JA) synthesis and perception in roots tightly control induced nicotine transport to systemic leaves after leaf wounding.* (a) Experimental set-up of petiole-feeding assay: control or leaf wounded (dashed lines) grafts had systemic leaves (shaded) detached 24 h after treatment, and fed for the following 24 h with a 1 mM nicotine solution. (b) Mean ± SE levels of nicotine accumulated in leaf lamina systemic leaves (shaded) of control and induced grafted plants. Asterisks refer to comparisons between control and leaf wounding treatment within same graft kind (***, p <0.001; *, p <0.05; n.s., not significant; Two-way ANOVA followed by Fisher LSD test, n = 8). EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line.

Figure 4

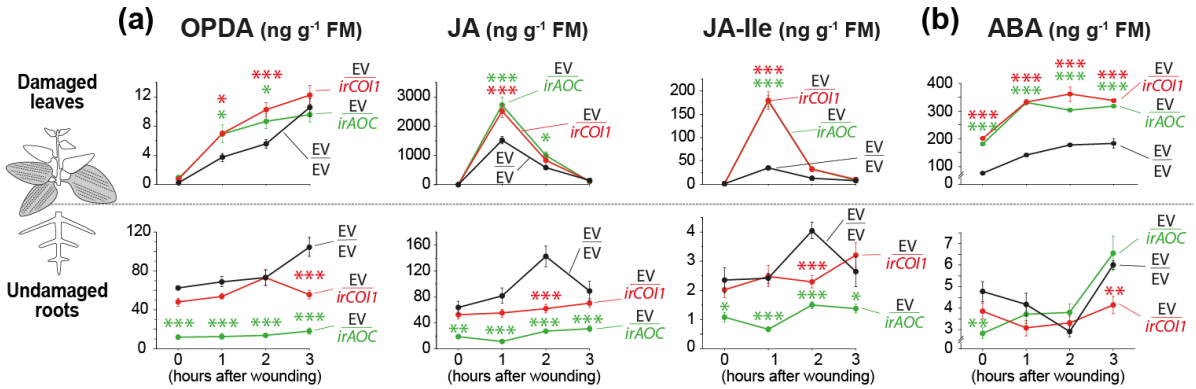

Fig. 4. *Impaired Jasmonic acid (JA) synthesis and perception in undamaged roots up-regulate jasmonates and abscisic acid (ABA) accumulation in damaged leaves in response to wounding.* Mean ± SE levels of phytohormone accumulation in response to wounding of undamaged roots and damaged leaves (shaded with dashed lines) of grafted plants with roots impaired in JA synthesis and perception (***, p <0.001; **, p <0.01; *, p <0.05; Two-way ANOVA followed by Dunnett's test, n = 6). EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line. OPDA, 12-oxo-phytodienoic acid; JA-Ile, jasmonic acid isoleucine.

Figure 5

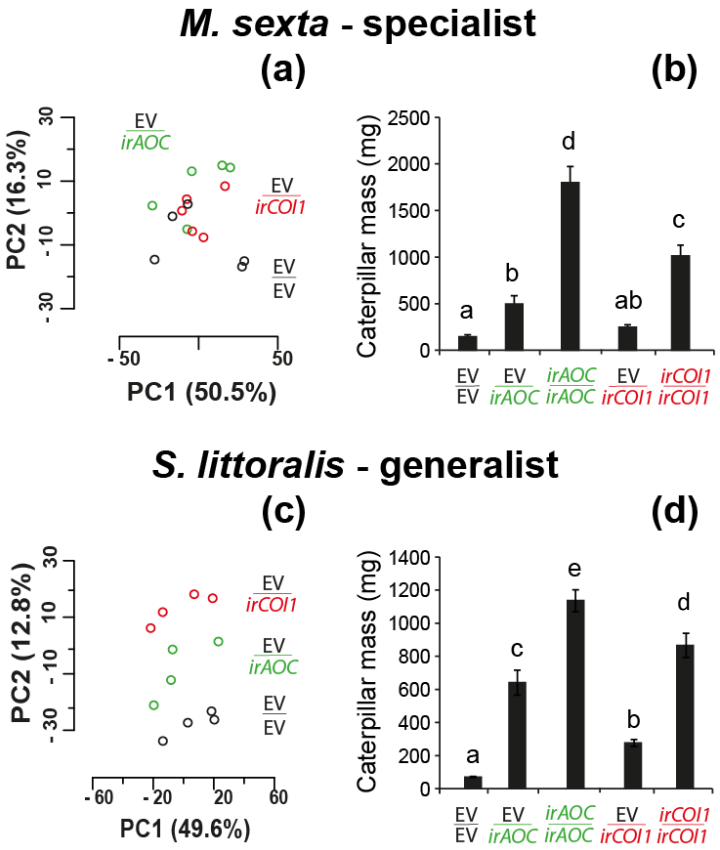

Fig. 5. *Jasmonic acid (JA) synthesis and perception in roots contribute to plant resistance against leaf-attackers, and are differentially employed depending on the level of adaptation of the herbivore species.* Untargeted principal component analysis (PCA) of metabolic profile of systemic leaves of EV/EV, EV/*irAOC*, and EV/*irCOI1* plants 3 days after simulated *M. sexta* (a) or *S. littoralis* attack (c) under glasshouse conditions. Mean ± SE levels of *M. sexta* (b) and *S. littoralis* (d) mass after 12 and 10 days, respectively, of feeding on EV/EV, EV/*irAOC*, and EV/*irCOI1* plants under glasshouse conditions. Grafts of the scions and rootstocks of the same genotypes were used as controls. Bars sharing same letters do not significantly differ (One-way ANOVA followed by Fisher LSD test, n ≈ 20). EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line.

Figure 6

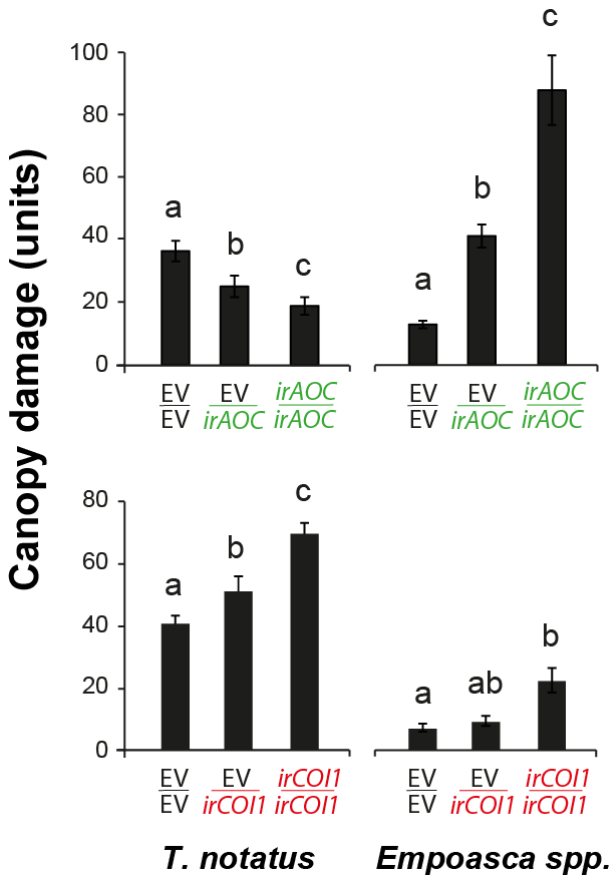

Fig. 6. *Under field conditions, plants impaired in (a) Jasmonic acid (JA) synthesis and (b) perception in roots are differentially preferred by Empoasca spp. and Tupiocoris notatus herbivores.* Mean ± SE levels of cumulative plant damage over 6 weeks after transplantation to the plot. Grafts of the scions and rootstocks of the same genotypes (EV/EV, *irAOC*/*irAOC*, and *irCOI1*/*irCOI1*) were used as controls. Bars sharing same letters do not significantly differ (One-way ANOVA followed by Fisher LSD test, n = 25). EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line.

Supporting information

Figure S1

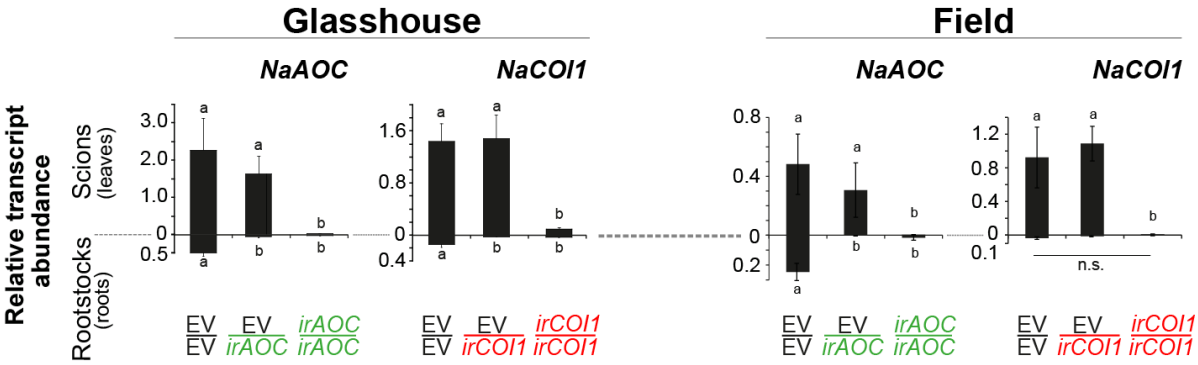

Fig. S1. Silencing of target genes is restrained to transgenic rootstocks of EV/*irAOC* and EV/*irCOI1* grafted plants under glasshouse and field conditions. Grafts of the scions and rootstocks of the same genotypes (EV/EV, *irAOC*/*irAOC* and *irCOI1*/*irCOI1*) were used as controls. Average ± SE transcript abundance of *NaAOC* and *NaCOI1* in systemic leaves of control plants or roots of induced plants, 1 day after leaf wounding (glasshouse) or 7 weeks after planted in field plot. Bars sharing same letters do not differ significantly (Two-way ANOVA followed by Fisher LSD test, n = 6, glasshouse; n = 7, filed). *NaAOC*, *N. attenuata*'s allene oxidase cyclase gene; *NaCOI1*, *N. attenuata*'s coronatine insensitive1 gene.

Figure S2

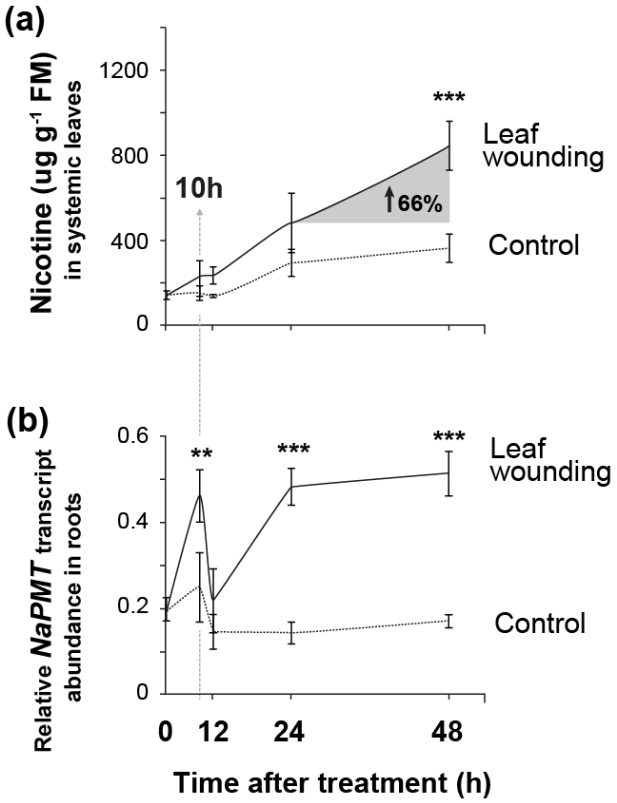

Fig. S2. The largest increase (66%) in endogenous nicotine in systemic leaves of non-grafted WT plants takes place between the first and second day after leaf wounding (top panel), which is associated with induced *NaPMT* transcript abundance in roots (bottom panel). Average ± SE values of control or induced plants. Asterisks refer to comparisons between control and wounding treatment within same time point (***, $p < 0.001$; **, $p < 0.01$; Two-way ANOVA followed by Dunnett's test, n = 5). *NaPMT*, *N. attenuata*'s putrescine *N*-methyltransferase gene.

Figure S3

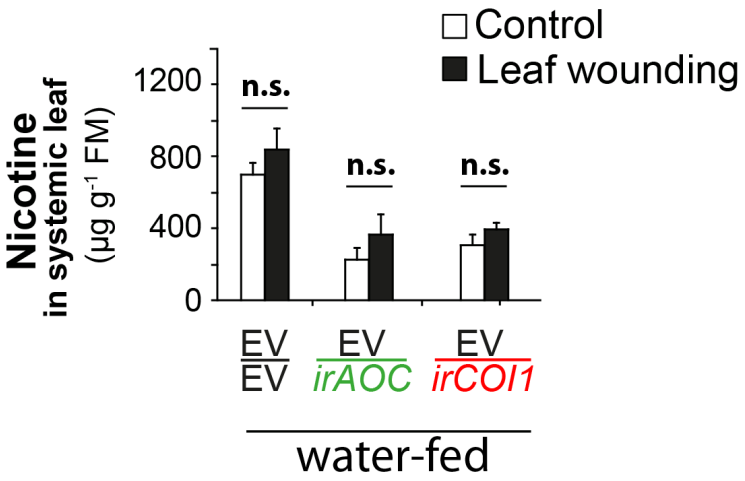

Fig. S3. In water-fed leaves, wounding failed to induce leaf-nicotine levels in EV/EV, EV/*irAOC*, and EV/*irCOI1* grafts. (a) Average ± SE nicotine accumulated in leaf lamina of detached systemic leaves of control or induced grafted plants incubated in a nicotine-free water solution. Control and wounding treatment within same graft kind did not differ significantly (n.s., not significant; Two-way ANOVA followed by Fisher LSD test, n = 8). EV, empty vector; *irAOC*, allene oxidase cyclase silenced line; *irCOI1*, coronatine insensitive1 silenced line.

Figure S4

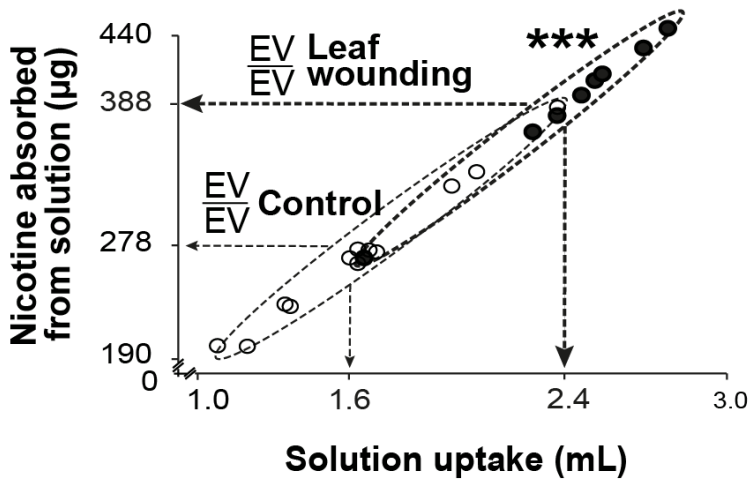

Fig. S4. Systemic leaves detached from wound-treated EV/EV plants and fed with a nicotine-containing solution transpired significantly more than those detached from untreated plants. Correlation plot of solution uptake (mL) versus nicotine absorbed from solution (µg). Asterisks refer to comparison between control and wounding treatment (***, $p < 0.001$; t-test, $n = 8$).

5. General Discussion

Roots are increasingly being recognized as plants' headquarters, where "decisions" are made that coordinate traits observed at the organismic level. Early evidence has been reported showing that roots can determine whole-plant growth. For example, water deficits in roots have been shown to reduce shoot growth, which was recovered by watering or by excising root portions with inadequate water supply (Gowing, Davies & Jones, 1990). Even with leaves in full turgor, stomatal conductance was reduced in response to soil desiccation by supplying positive pneumatic potentials to the soil water in sealed soil chambers (Gollan, Passioura & Munns, 1986). Increased mechanical impedance of the soil was also shown to impair the growth of wheat seedlings (Masle & Passioura, 1987). Root tips can synthesize ABA and cytokinins, and it is suggested that changes in the environment of roots can modify root hormone production; these changes are then spread via the xylem to the shoots, eliciting growth changes at the level of the organism (Torrey, 1976). Electrical signals might also be involved in the control that roots have over shoot growth responses. Electric stimulations to roots of willow plant cuttings were propagated through the plant and elicited changes in transpiration and photosynthesis in the leaves (Fromm & Eschrich, 1993).

Increasingly, evidence from electrophysiology and molecular biology indicates very close parallels between animals and plants in terms of their mechanisms of rapid signal transmission (Baluska, Mancuso, Volkmann & Barlow, 2009). According to Dustin and Colman (2002), animal synapses are characterized as actin-based asymmetric adhesion domains specialized for the kind of rapid cell-to-cell communication that is accomplished by vesicle trafficking. Detailed analyses of plant cell connections (also known as cross-walls), especially in the transition zone of the root apex, reveal that cross-walls technically fall into the same definition, meaning that plant cell-to-cell contact can occur as actin-based synapses (reviewed in Baluska, Mancuso, Volkmann & Barlow, 2004). Instead of long projections of a typical neuron (i.e. axions), plants present rigid tubular cells that come into contact with each other at their end-poles, forming lengthy cell files which compose the basic unit of plant diffuse brain. Baluska and colleagues (2004) further reviews aspects typically found in animal neurotransmitter molecules which are also present in auxins' mechanism: they both elicit a range of electrical responses; modulate ion channels and free calcium (Zimmermann, Thomine, Guern &

Barbierbrygoo, 1994). Darwin's purely speculative notion of root apices working like brains of lower animals has been gradually demonstrated (Darwin, 1880).

Recently, Mousavi and colleagues (2013) reported that the wound systemic response also involves an electric signal that spreads from damaged tissues to distal leaves, triggering systemic defense gene expression. Whether this alarm electrical wave also spreads to the roots is unknown. However, the inclusion of roots in the study of plant defense responses is timely. Roots represent a strategic site for managing plant responses to above-ground feeders. First, these organs are protected from attack by the depth of the soil. Second, roots are active biosynthetic sites of plant defense compounds (Erb, Lenk, Degenhardt & Turlings, 2009). Third, roots can centralize information coming from the shoots in order to buffer the right dose of the defense according to the degree of damage and/or tissue lost (Baldwin (Baldwin & Schmelz, 1994). Last, roots have ready access to nitrogen-containing compounds from the soil as well as photosynthetic assimilates from the shoots; in combination, these resources can be invested in plant resistance and/or plant tolerance (i.e. regrowth; Schwachtje et al., 2006).

We are only starting to uncover root-based plant defense strategies. However, in doing so, it is crucial that we describe at the physiological and molecular level the mechanisms underlying processes studied under ecologically relevant conditions (Baldwin, 2012; Soler, Erb & Kaplan, 2013). Meeting all these criteria, stably transformed lines of *N. attenuata* represent a powerful tool to test gene function in ecological traits. The use of transgenic lines of *N. attenuata* allows not only for the description of correlated traits in different environments but also for the manipulation of gene expression and the falsification of hypotheses of ecological gene function (Gase, Weinhold, Bozorov, Schuck & Baldwin, 2011). However, gene manipulation in *N. attenuata*, as it does in many other plant models, relies on nonspecific promoters that ectopically express the transgene throughout the whole plant over its entire development. This approach is clearly inadequate to study traits employed only at specific spatial levels or in temporal scales.

5.1. Micrografting in *N. attenuata* and systemic PTGS

To overcome the inflexibility of available gene manipulation techniques in *N. attenuata*, recently, an inducible expression system was applied in this species (Schafer et al., 2013). The dexamethasone (DEX)-inducible pOp6/LhGR method involves two constructs: one that constitutively expresses a chimeric transcription factor (LhGR) containing a repressor domain, a transcription activator region, and

the ligand-binding domain of a glucocorticoid receptor. The second construct contains the glucocorticoid receptor (pOp6), which is the target for the LhGR construct, fused to a given gene of interest (GoI). LhGR is consitutively expressed, but only in the presence of a inducer compound, DEX, does it become active and trigger the expression of pOp6:GoI. These authors tested whether cytokinins (CK) play a role in plant-insect interactions within this system, using a gene involved in CK biosynthesis (*ipt* - isopentenyl transferase). By applying a paste of lanolin containing DEX in only some branches of the plant, these authors showed that a local and restrained CK increase led to a local increased attack by mirid bugs, *Tupiocoris notatus*. This method allows for temporally and spatially specific gene manipulation. However, this system cannot be used in the study of traits involving tissues inaccessible to treatment with DEX, such as the roots of non-hydroponically grown plants.

In **manuscript I**, I described a simple alternative method for root gene manipulation in *N. attenuata*, micrografting. This technique allows for the independent manipulation of gene expression of plants below- and above-ground, by combining two different plant genotypes, one for the shoots (scion) and another for the roots (rootstock). Seedlings obtained seven days after germination were grafted under the microscope partially fitted in sterile benches.

In the beginning, each graft used to take around 5 minutes to be done. However, with practice (this thesis involved around 1,800 grafted seedlings), this time was reduced, and at this time on average around 20 seedlings are grafted within 30 to 35 minutes. Also, although micrografting was performed under sterile conditions for glasshouse experiments, this protocol was also effective under non sterile conditions in the field. Open bench surfaces were simply cleaned with 70% ethanol and a germicide UV-C lamp prior to starting work. The grafting success rate was maintained, at both conditions, at around 80%. However, under sterile conditions the main reason for graft failure consisted of rooted scions, while in Utah, bacterial contamination prevented explants from surviving in the media.

Because micrografting takes place in the early stages of plant development, grafted seedlings are prompt to recover (Pina & Errea, 2005). Moreover, micrografting involves wounding the hypocotyl. While wounding is beneficial for grafting, once it induces callus growth that favors the adhesion of the graft partners (Miller & Barnett, 1993), it can induce defense-related traits such trypsin protease inhibitor (*TPI*) or nicotine, (Ohnmeiss, McCloud, Lynds & Baldwin, 1997) that can

confound results of experiments involving wounding. However, micrografting is performed with seedlings, normally 3 weeks ahead of further experimental manipulation, and *TPI* and putrescine *N*-methyltransferase (*PMT*) expression of WT/WT plants resembled those of non-grafted WT plants.

Nevertheless, it is of particular importance that grafted plants be similar in growth and development to intact plants as the graft junction might impose a barrier for long-distance mobile signals controlling growth and development in plants (Searle et al., 2003; Yoo et al., 2004; Lough & Lucas, 2006). In other words, the value of micrografting is mainly dictated by what the graft junction transmits between scion and rootstock.

Given that the main purpose of establishing this technique is to provide independent above- and below-ground gene manipulation, I investigated whether RNAi of scions or rootstocks alters the endogenous expression of the target gene in the WT counterparts. To that end, I used *NaTPI* and *NaPMT* endogenous expression patterns. *NaTPI* is mainly expressed in leaves, and it was used to report the spread of root-to-shoot silencing signals in WT/*irTPI* grafts; *NaPMT* is mainly expressed in roots, so it was used to report the spread of shoot-to-root silencing signals in *irPMT*/WT grafts. Although *NaTPI* expression in WT scions was not reduced in WT/*irTPI* plants, *NaPMT* expression in WT rootstocks was dramatically reduced in *irPMT*/WT grafts. These results suggest that the mobile silencing signal travels downward, in a shoot-to-root fashion.

It has been proposed that the silencing mobile signal moves both cell-to-cell and via phloem. Guard cells overexpressing green fluorescent protein (35S:GFP) that had lost plasmodesmatal connections to other cells failed to be silenced in response to induced systemic silencing (Voinnet, Vain, Angell & Baulcombe, 1998). That silencing signals move through the phloem is supported by the detection of silencing along major and minor veins prior to the spread into mesophyll cells. Also, the spread of the silencing that is initiated in one leaf is restricted to shoots that have emerged from the same side of the stem (Palauqui, Elmayan, Pollien & Vaucheret, 1997; Voinnet, Vain, Angell & Baulcombe, 1998). The phloem connection is the last step in the establishment of the graft, and it is considered the basic requirement for a successful graft. Non-functional phloem connections or the formation of wide areas of undifferentiated callus cells on the graft junction are typical in incompatible grafts (Pina & Errea, 2005). Neither of these symptoms was observed in successfully micrografted *N. attenuata* plants.

The work of Palauqui and colleagues (1997), Voinnet and colleagues (1998) and Sonoda and Nishigushi (2000) suggests that the silencing signal movement occurs more efficiently upward than downward. However, all these studies used top- or clef-grafting; that is to say, the rootstocks had at least 2 basal leaves. Moreover, these studies mainly focused on systemic post-transcriptional gene silencing PTGS targeting transgenes as opposed to endogenously expressed targets. In an attempt to establish the requirements for the TPGS observed, Palauqui and colleagues (1997) in an example of a different approach, used WT scions and nitrate reductase as endogenous target (*Nia*), and tested whether *Nia*-silenced rootstocks promote upward silencing. However, in this case, none of the WT became silenced, suggesting that the highly abundant expression of the transgene was required for the patterns of upward silencing observed. Moreover, Voinnet and colleagues (2000) also recognized the limitations of transgenes targets in obtaining a more accurate picture of natural PTGS in plants (i.e. caused by virus infection). Therefore, these authors used WT plants and endogenously expressed ribulose biphosphate carboxylase small subunit (rbcs) or phytoene desaturase (PDS) as targets. Unlike the extensive and persistent silencing of transgenes, rbcs and PDS silencing were transient and restrained to regions near the veins of new emerging leaves.

In an approach more similar to that of **manuscript I**, Kasai and colleagues (2011) micrografted WT scions onto rootstocks of *N. benthamiana* silenced for an endogenous gene (glutamate-1-semialdehyde) by RNAi. In agreement with the results of manuscript I, none of the 72 micrografted plants showed endogenous PTGS in WT scions caused by silenced rootstocks. In addition, Molnar and colleagues (2010) also showed that for micrografted Arabidopsis, a silencing signal was more effective from shoots to roots than from roots to shoots. In summary, in the study of systemic PTGS signal it is important to establish first a common ground for what are sink or recipient tissues (i.e. leaves or roots), and, most important, to determine the nature of the silencing signal target (i.e. transgenes or endogenous genes; reviewed in Mlotshwa et al., 2002).

The micrografting protocol described for *N. attenuata* in **manuscript I** has been applied to the study of growth promotion effects on seedling colonized by the fungus *Piriformospora indica* (Schuck et al., 2012). This approach can also be applied to further investigate the generation and/or amplification of systemic PTGS signal, since silenced lines of DCL (*irDCL1*, *irDCL2*, *irDCL3* and *irDCL4*, as well as crosses) and RNA-direct RNA polymerase (*irRdR1*, *irRdR2* and *irRdR3*) are available for this species (Pandey & Baldwin, 2007, 2008; Bozorov et al., 2012). In addition,

micrografting *N. attenuata* can further extend our understanding of the below-ground function of phytohormones such as ethylene, abscisic acid or jasmonic acid (von Dahl et al., 2007; Dinh, Baldwin & Galis, 2013).

5.2. Root function of jasmonates

To the best of my knowledge, lipoxigenase 5 (LOX5) is the single case in the literature in which the function of root oxylipins was investigated in an ecological context (Nalam, Keeretaweep, Sarowar & Shah, 2012; reviewed in Nalam, Shah & Nachappa, 2013). *LOX5* is a 9-LOX-encoding gene (Bannenberg, Martinez, Hamberg & Castresana, 2009). In other words, it encodes for an enzyme of the LOX pathway different from the 13-LOX involved in the synthesis of jasmonates (JAs); these two enzymes peroxidase lipids at different positions (Howe & Schilmiller, 2002). Nalam and colleagues (2012) showed that this enzyme produces 9-LOX-derived oxylipins (referred collectively as 9-HPs), which promote aphid infestation and fecundity in Arabidopsis. Moreover, aphid infestation was reduced in grafted plants displaying the WT shoots and roots of a loss-of-function LOX5 mutant (*lox5*). The *lox5*/WT and WT/WT grafts presented similar high levels of infestation. To summarize, this work revealed that in contrast to the well-reported role of oxylipins in plant defenses, these insects adapted to cues derived from the roots in specific 9-HPs that facilitate infestation, which are derived from the roots.

Of the six lipoxigenase genes present in Arabidopsis, four encode 13-LOX types: LOX2, LOX3, LOX4 and LOX6. All six genes are thought to be located in plastids. LOX6 shows the lowest levels of expression (Bannenberg, Martinez, Hamberg & Castresana, 2009). Although LOX3 and LOX4 are expressed in roots and are induced by wounding the roots, these LOXs could not compensate for the loss of LOX6 function in the *dde2* mutant (Grebner, Stingl, Oenel, Mueller & Berger, 2013). However, these authors investigated the role of LOX6 in roots when mechanical damage was inflicted locally. Conversely, Acosta and colleagues (2013) studied systemic root function in response to the wounding of the cotyledons of seedlings. These authors made use of a construct containing the promoter of a gene responsive to jasmonic acid (JA) signaling, *JAZ10*, in order to drive the expression of GUS (*JAZ10-GUSPlus*) to report JA signaling. After 2 h of mechanical damage in the cotyledon, evidence of a strong *JAZ10-GUSPlus* activation was detected in the systemic cotyledon, hypocotyl and roots. Using *JAZ10-GUSPlus* in other backgrounds mutated for JA synthesis or signaling, the authors observed that a loss-of-function mutant in the *novel interactor of JAZ* (*ninja*) displayed a

constitutively expressed *JAZ10-GUSPlus*, even in unwounded seedlings. In addition, *ninja* mutants displayed JA-mediated root-growth inhibition. These observations suggest that NINJA is a repressor of JA signaling and that in the absence of NINJA, JA-like responses are constitutively recapitulated in roots, even in the absence of JA. However, although very exciting, these studies do not explicitly demonstrate the root function of JA in plant defenses.

Moreover, an important aspect to bear in mind when considering findings from Arabidopsis concerns the differences in the systemic wound mechanisms between species. For instance, in tomato, JA *de novo* synthesis in systemic leaves is not necessary for the systemic response of protease inhibitors (PIs) (Li et al., 2005). Similarly, in *N. attenuata*, also a solanaceous, leaf wounding did not induce JA or MeJA in systemic leaves (von Dahl et al., 2007). In contrast, Koo (2009) found that in Arabidopsis, the *de novo* biosynthesis of JA and JA-Ile in systemic leaves is required to activate systemic responses, and this process is independent of JA and JA-Ile production in local leaves. In addition, in Arabidopsis, defense-related phenotypes associated with loss of function of individual JAZs have been rarely reported. The lack of phenotype is mainly attributes to the functional redundancy of JAZs in Arabidopsis. However, in *N. attenuata*, the single and specific silencing of JAZd or JAZh resulted in very distinct phenotypes, and these were related to either reproduction or defenses, respectively (Oh, Baldwin & Galis, 2012, 2013).

In **manuscript II**, I investigated the contribution of systemic root JA to *N. attenuata*'s resistance to above-ground herbivore attack. Because this species has available transgenic lines harboring empty vector (EV) or *ir* constructs for the targeted silencing of components of the JA pathway, namely synthesis (*irAOC*; Kallenbach, Bonaventure, Gilardoni, Wissgott & Baldwin, 2012) and perception (*irCOI1*; Paschold, Halitschke & Baldwin, 2007), and it is at the core of a well-characterized ecological model system, *N. attenuata* constitutes the perfect system to study the function of JA in plant-herbivore interactions. In order to restrain JA impairment to below-ground tissues, I made use of micrografting.

First, it is important to point out that, depending on the function of the target gene, the expression levels above-ground might compensate for the silencing of below-ground tissues. Systemic silencing can also occur transiently or limited to veins and nearby regions (Voinnet, Lederer & Baulcombe, 2000). Therefore, silencing efficiency analyses are mandatory, and could resolve unexpected patterns of gene expression and phenotypes. This is especially true under natural

conditions, where many plant viruses which have evolved proteins that suppress RNA silencing might be present (Li & Ding, 2001).

After confirming gene expression patterns and validating the use of grafts, I first showed that, like intact WT plants, EV/EV grafts responded to leaf wounding with augmented nicotine accumulation in systemic leaves (Ohnmeiss, McCloud, Lynds & Baldwin, 1997), suggesting that the shoot-to-root wound mobile signal is graft-transmissible. Next, I showed that nicotine induction in response to leaf wounding is almost fully blocked in plants lacking COI1 activity in roots (EV/*irCOI1*). The impairment in nicotine induction in EV/*irAOC* was partial, suggesting that AOC activity in shoots and roots is necessary to induce WT-nicotine levels. In addition, both EV/*irAOC* and EV/*irCOI1* grafts were still able to marginally increase nicotine levels in response to wounding, suggesting JA-independent root signaling in the control of nicotine induction. Alternatively, the residual nicotine induction may be a product of the remaining low expression levels of *NaCOI1* and *NaAOC* in silenced roots. To avoid confounds due to potential allometric differences between plants, grafts were matched in size prior to experiments (**Figure 3A**, $p = 0.06$; **1B**, Shoots $p = 0.06$, Roots $p = 0.07$; unpublished data).

In addition, I checked the transcript levels of *NaPMT*, the gene that encodes for the first committed enzymatic step in nicotine biosynthesis (Winz & Baldwin, 2001). The degree of impairment observed in nicotine levels was well correlated to the levels of *NaPMT* in roots of all grafts. This suggests that nicotine is likely regulated by root JA at the transcriptional level of genes encoding for enzymes that synthesize nicotine.

EV/EV took up an amount of nicotine similar to levels found between the first and second day after wounding in non-grafted WT plants. This suggests that root JA can systemically control leaves' transport of nicotine. One possible explanation for leaves' reduced ability to absorb nicotine could be the fewer trichomes found in EV/*irCOI1* and EV/*irAOC* leaves, given that these sites accumulate nicotine (Roda, Oldham, Svatos & Baldwin, 2003).

In addition transporters of nicotine might play a determinant role in nicotine induction. To date, only three nicotine transporters have been identified (Morita et al., 2009; Shoji et al., 2009; Hildreth et al., 2011). Nicotine uptake permease (NUP1) is localized in the plasma membrane; and the other two consist of multigrug and toxic compound extrusion (MATE)-type of transporters localized in the tonoplast of

vacuoles. However, it has been suggested that a single gene product could manage two different transport events, given the tightly regulated movement of metabolites in plants and the limited number of transporters genes in the genome. For example, although localized in the tonoplast of vacuoles, *Nicotiana tabacum* jasmonate-inducible alkaloid transporter (Nt-JAT1) can also work as an efflux transporter if localized in the plasma membrane. Interestingly, Nt-JAT1 is coregulated with nicotine biosynthetic genes (PMT) in response to MeJA in culture cells. In summary, it seems likely that root JAs regulate nicotine induction through enhanced expression levels of shoot nicotine transporters.

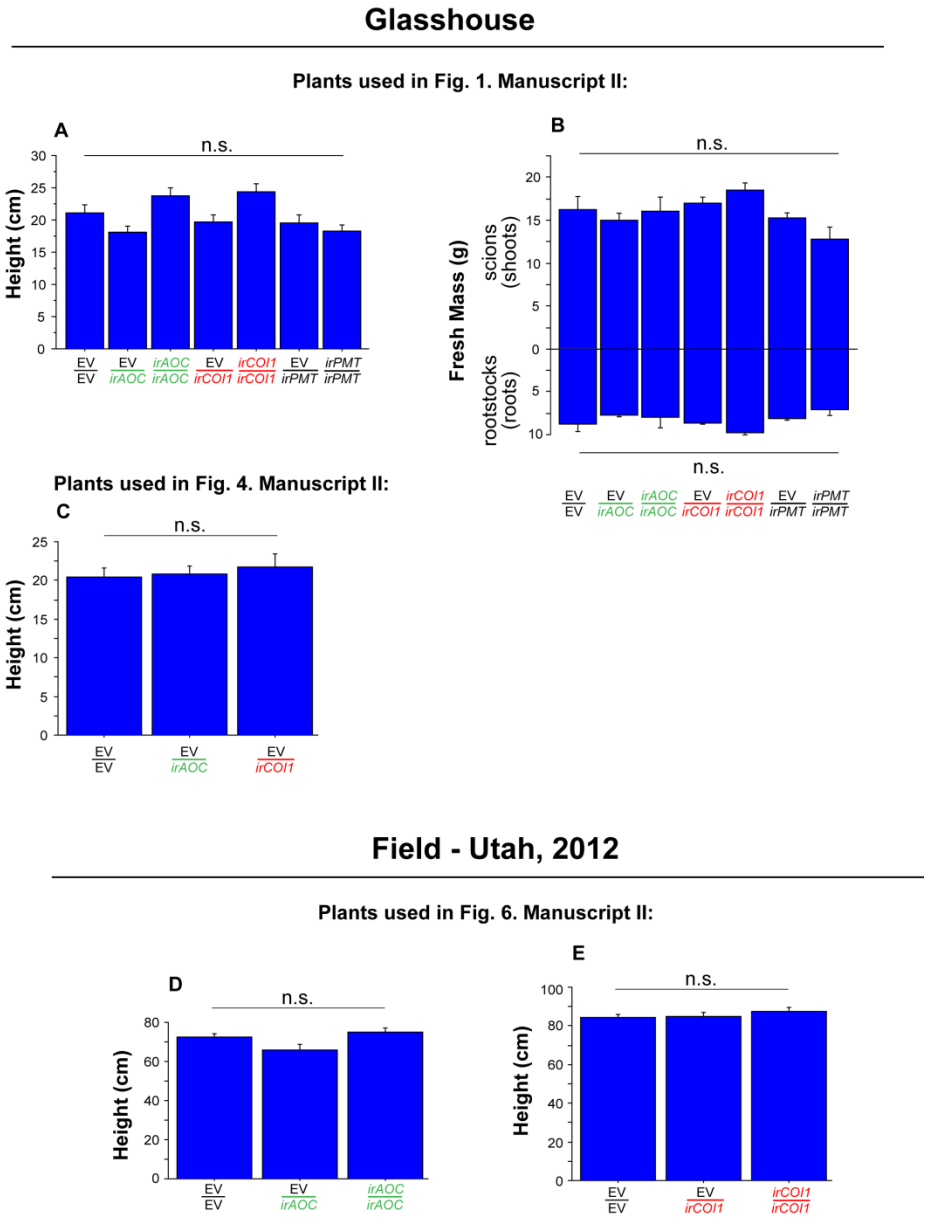

Figure 3. Growth parameters of plants used in manuscript II for experiments as indicated above graphs.

Next, I tested for the hypothesis that root JA systemically modulates the leaf JA burst in response to wounding. To falsify this hypothesis, I elicited leaves of EV/EV, EV/*irAOC* and V/*irCOI1* plants and measured JAs accumulation in roots and damaged leaves prior to and after 1, 2 and 3h of wounding. Whereas JAs levels of roots are accordingly lower in EV/*irAOC* and EV/*irCOI1* when compared to those of EV/EV roots, damaged leaves of EV/*irAOC* and EV/*irCOI1* attained strikingly higher levels of JAs and ABA than did damaged leaves of EV/EV. These patterns of JAs over-accumulation in leaves of plants deprived of root JAs suggest a novel shoot-to-root loop in regulating the JA response described in **manuscript II**. JA-deficient roots probably boost leaf responses in terms of JA accumulation in a compensatory mechanism. As previously, plants used in this experiment were matched in size (**Figure 3C**, $p = 0.89$; unpublished data).

Levels of ABA were also higher in damaged leaves of EV/*irAOC* and EV/*irCOI1* compared to those of EV/EV. Given that EV/*irAOC* and EV/*irCOI1* accumulated less nicotine, it is possible that ABA negatively effects nicotine uptake through inhibition of transpiration in these leaves. Wounding did not induce the transport of dye in the xylem of *N. sylvestris*, and nicotine induction is mainly due to increased concentrations of nicotine found in the xylem after leaf elicitation (Baldwin, 1989). However, adaptively, conditions of high evapotranspiration may facilitate and optimize nicotine delivery from roots to shoots especially for a species like *N. attenuata*, which is native to xeric habitats. However, the cross-talk of JA and ABA is shown to be more complex than the simple control of transpiration rates. For example, by silencing a new protein in *N. attenuata* involved in suppressing ABA catabolism (The Herbivore Elicitor-Regulated1 - HER1), levels of this phytohormone were reduced. However, despite increasing transpiration rates, lower levels of ABA were associated with reduced emission of volatile organic compounds in *N. attenuata*, suggesting that the ABA involvement in JA-dependent responses is likely to go beyond the control of guard cells (Dinh, Baldwin & Galis, 2013).

I also observed herbivore-specific patterns regarding global metabolic profiles accumulated in EV/EV, EV/*irAOC* and EV/*irCOI1* plants elicited by wounding combined with the application of oral secretions (OS) of two herbivores: *Manduca sexta*, a specialist, and *Spodoptera littoralis*, a generalist. The elicitation by *S. littoralis* OS caused EV/*irAOC* and EV/*irCOI1* to be metabolic more distinct from EV/EV when compared to the weak grouping observed after *M. sexta* OS elicitation. This suggests that *S. littoralis* induces a more profound reconfiguration of *N.*

attenuata's metabolism than that elicited by *M. sexta*. In addition, *S. littoralis* also showed more pronounced differences in larval mass than did *M. sexta*. For instance, while *S. littoralis* performed better on EV/*irCOI1* plants compared to EV/EV plants, *M. sexta* larvae did not benefit from the lack of COI1 activity in roots when fed on EV/*irCOI1* plants; these larvae performed similarly to larvae fed on EV/EV. Together, these results suggest that root JA is employed in above-ground resistance traits other than nicotine; root JA is employed in plant defenses in an herbivore-specific manner; and that *M. sexta* elicits COI1-independent JA signaling in roots of *N. attenuata*. However, demonstrating the existence of a second JA-Ile receptor in *N. attenuata* involves a comprehensive set of further experiments and genetic manipulations.

Many recent studies have focused on how herbivory on either the root or the shoot affects defense in another tissue (Hol, Macel, van Veen & van der Meijden, 2004; Soler et al., 2009; reviewed in Soler et al., 2012). These studies that link damage patterns below and above ground are connected to the identification of plant traits driving the diversity of plant-herbivore communities (Kessler & Halitschke, 2007; Poelman, van Loon & Dicke, 2008). In general, different herbivore species attacking the same plant can compete or facilitate one another. The way these feeders interact is mainly dictated by the responses they induce in the plant. For example, intraguild feeders (i.e. chewer x chewer) are more likely to negatively affect one another by up-regulating the same phytohormonal pathways. In contrast, interguild species (i.e. chewer x piercing/sucking-feeder) are more likely to facilitate one another, probably by inducing antagonistic phytohormonal pathways that overall attenuate plant defenses (Soler, Erb & Kaplan, 2013).

However, in **manuscript II**, I observed that damaged caused by mirids was negatively correlated to damage caused by *Empoasca*. These herbivores are both piercing/sucking; however differ in the level of specialization: comparatively, *Empoasca* spp. feeds on a wider range of plants than mirids (Kallenbach, Bonaventure, Gilardoni, Wissgott & Baldwin, 2012). Interestingly, this negative interaction was evident only in *irAOC* plants but not *irCOI1* plants. This suggests that the above-ground insect-insect interaction can be mediated by a specific step of the root JA signaling. Alternatively, mirids are specialists; therefore these herbivores might be more adapted to *N. attenuata*'s defense metabolites than to the presence of other herbivores. Plants used in the experiments were similar in size (**Figure 3D**, $p = 0.09$; **3E**, $p = 0.23$; unpublished data).

5.3. Conclusions

Plant sensory systems have long been compared to neurobiological processes of animals. However, only in recent years, has the controversial root-brain theory proposed by Charles and Francis Darwin in 1880 regained attention (Baluska, Mancuso, Volkmann & Barlow, 2009). Along with this revival, roots have been increasingly recognized as active players in plant defenses (Erb, Lenk, Degenhardt & Turlings, 2009; Meldau, Erb & Baldwin, 2012; Soler et al., 2012), and it is already known that some root-derived compounds are employed against predation by specialist herbivores (Eisner & Eisner, 1991; Hare & Eisner, 1993; Kumar, Pandit, Steppuhn & Baldwin, 2013).

Soler and colleagues (2013), in discussing factors that hinder our understanding of above- and below-ground plant-insect interactions, argue that studies to date tend to fall into one of two groups: the mechanistic or the ecological. While the former often disregards ecologically relevant aspects (i.e. co-occurring herbivores), the latter lacks physiological or molecular insights that explain observed community-level patterns.

Here I described a simple, yet elegant, micrografting protocol for *N. attenuata* that facilitates the manipulation of below-ground gene function with an ecological perspective. By applying this approach to a collection of stably transformed plants, I was able to investigate mechanisms of shoot-root interplay mediating systemic PTGS, systemic JA signals, nicotine biosynthesis and transport, and *N. attenuata*-insect and insect-insect interactions. I showed that the whole is greater than the sum of its parts, and that root JA can extensively tailor ecological processes in *N. attenuata*.

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7. Summary

Plants deploy sophisticated strategies to withstand the selective pressures of life above and below ground. In order to implement adaptive responses to environmental stimuli, plants have evolved mechanisms capable of perceiving signals from these stimuli; the mechanisms trigger local and distal reactions. However, although plants operate as a single functional system, roots have been largely ignored in the study of many plant processes which have been focused mainly on shoots, especially processes related to above-ground plant-insect interactions.

It is becoming evident that roots can profoundly influence whole-plant performance. Roots not only provide anchorage soil-based resources for plants, but they also serve as strategic sites for managing plant defenses. Roots (a) are physically inaccessible below ground; (b) constitute active sites for the synthesis of defense metabolites; (c) can centralize information on the level of damage and amount of leaf-tissue loss; and (d) have ready access to photoassimilates from plant shoots and nitrogen-containing compounds from the soil, resources that can be incorporated in either defense and/or tolerance mechanisms.

To investigate the interplay of roots and shoots in plant responses, I used *Nicotiana attenuata*, a species suitable for both ecological and genetic manipulations. Equipped with a collection of available transgenic lines that either silence (*ir*) or overexpress (*ov*) genes involved defense-related traits, I established a new protocol of micrografting for *N. attenuata*. This approach fuses two different genotypes, one for the shoots and another for the roots, and allows for the independent gene manipulation of the above- and below-ground plant parts.

Initially, I compared micrografting success rates across seven different species of *Nicotiana* spp. and observed that *N. attenuata* is highly suitable for this protocol: 80% of grafts resulted in healed healthy plants. Next, I compared the growth and development between intact WT and grafted WT/WT plants (grafts are named in a shoot/root manner), and did not observe any compromises due to the procedure. Next I investigated the traffic of graft-transmissible signals, especially those conveying systemic post-transcriptional gene silencing (PTGS). To that end, I quantified transcripts that had accumulated in shoots and roots of self- and reciprocal-grafts using WT, *ir* and *ov* genotypes. Since *NaTPI* is mainly expressed in the shoots of *N. attenuata* and *NaPMT* in the roots, the endogenous expression of

these genes was used to report the up- and down-ward spread of PTGS, respectively. I observed that the silencing of *NaTPI* in roots did not affect its expression in the WT shoots of WT/*irTPI* grafts. However, the silencing of *NaPMT* in shoots reduced the endogenous expression of *NaPMT* in the WT roots of *irPMT*/WT grafts. These results are consistent with the reported lack or very weak upward transmission of PTGS. In addition, this technique, which combines the vast collection of available transgenic lines of *N. attenuata,* will improve our understanding of root gene-function and clarify the shoot-root interplay that controls the whole-plant performance.

I further investigated the root function of (jasmonates) JAs in response to above-ground attack in *N. attenuata*. I restrained the impairment of jasmonic acid (JA) synthesis (*irAOC*) or perception (*irCOI1*) to the roots by micrografting (WT/*irAOC* and WT/*irCOI1*, respectively), and next compared the ecologically relevant traits of these plants *versus* the same traits in WT/WT control grafts. I initially used nicotine to report systemic root JA function in leaf wound responses. This toxic antifeedant alkaloid is produced in roots and accumulates at high levels in leaves upon mechanical damage. Despite the extensive literature associating nicotine induction with increased levels of JAs, so far there has been no explicit evidence for the role of root JA in this process. Nicotine induction in roots is almost fully dependent on *NaCOI1* and is partially dependent on *NaAOC* activity. Root JAs control nicotine production at the transcriptional level and also tightly regulate the systemic nicotine uptake of leaves. Strikingly, these patterns were associated with hyper-responsive JAs and abscisic acid (ABA) accumulation in the local damaged leaves. These results demonstrate a novel shoot-root-shoot loop regulating the JA burst. I also observed that root JA synthesis and perception influence the performance and preference of above-ground herbivores in the glasshouse and in nature. Interestingly, root-JAs can also mediate above-ground herbivore interactions.

Since roots were discovered to be the exclusive sites of nicotine synthesis in tobacco plants more than 70 years ago, other root-dependent compounds and strategies to defend plants against herbivore attack have been identified and investigated. However, we still have much to learn from plants' hidden lives. Clearly our knowledge of plant processes will deepen if below-ground tissues are included. Here I describe how the regulation of leaf defenses by a novel root-JA mechanism tailors above-ground plant-interactions. I conclude that the whole is greater than the sum of its parts.

8. Zusammenfassung

Pflanzen benutzen anspruchsvolle Strategien, um dem Selektionsdruck sowohl unter als auch über der Erde zu widerstehen. Um angemessen auf Umweltreize reagieren zu können, entwickelten Pflanzen verschiedene Mechanismen um in der Lage zu sein diese Signale wahrzunehmen, die lokale als auch distale Reaktionen auslösen können. Obwohl Pflanzen als ein einziges funktionales System funktionieren, wurden Wurzeln weitgehend ignoriert, wenn es um das Studium vieler Pflanzenprozesse ging, insbesondere oberirdischen Interaktionen mit pflanzenfressenden Insekten.

Es wird immer deutlicher, dass Wurzeln die gesamte Leistung von Pflanzen beeinflussen, insbesondere auch in Bezug auf Verteidigung gegen oberirdische Fraßfeinde. Wurzeln dienen nicht nur zur Verankerung im Boden und zur Lieferung von Ressourcen für die oberen Teile der Pflanze, sondern dienen auch als strategisch wichtige Standorte für die Steuerung der Pflanzenabwehr. Wurzeln sind (a) physikalisch unerreichbar, da in der Erde verborgen sind, dienen (b) als Zentren für die konstante Synthese von Verteidigungsmetaboliten, können (c) Informationen über die Höhe des Schadens und des Blattverlusts aufnehmen und haben (d) jederzeit Zugang zu Photosyntheseprodukten der Blätter und stickstoffhaltige Verbindungen aus dem Boden, die entweder für die Verteidigung und / oder für Toleranzmechanismen verwendet werden.

Um das Zusammenspiel der Wurzel-Spross Interaktionen zu untersuchen, habe ich *Nicotiana attenuata* als Modellpflanze gewählt, die sich sowohl für ökologische Untersuchungen als auch genetische Manipulationen eignet. Mit einer Vielzahl an verfügbaren transgenen Pflanzenlinien, die verteidigungsrelevante Gene entweder „*gesilenced*“ (*ir*) oder überexpremiert (*ov*) haben, habe ich ein neues Protokoll für die Mikrotransplantation von *N. attenuata* Keimlingen etabliert. Dieser Ansatz verbindet zwei verschiedenen Genotypen durch das aufpfropfen des oberen Triebes auf die Wurzel einer anderen Pflanze und ermöglicht die unabhängige Gen-Manipulation der unter- und oberirdischen Pflanzenteile.

Zunächst verglich ich die Erfolgsrate der Mikrotransplantationen in sieben verschiedene *Nicotiana* Arten und habe festgestellt, dass *N. attenuata* sehr gut für diese Methode geeignet ist: 80% der Mikrotransplantationen führte zu vollständig verbundenen und gesunde Pflanzen. Weiterhin verglich ich Wachstum und die Entwicklung von intakten wildtyp (WT) und gepfropft WT/WT -Pflanzen (diese

werden in Spross/Wurzel Schreibweise dargestellt) und konnte keine Nachteile für die Pflanzenentwicklung durch dieses Verfahren nachweisen. Ich untersuchte weiterhin die Übertragung von Signalen durch die Transplantationsstelle, insbesondere solche welche für systemisches Gen-*silencing* (PTGS) verantwortlich sind. Zu diesem Zweck verglich ich die Transkripte zwischen Spross und Wurzel von WT, *ir* und *ov* Genotypen, je gleich oder wechselseitig transplantiert. Da NaTPI hauptsächlich im Spross und NaPMT hauptsächlich in der Wurzel von *N. attenuata* exprimiert wird, verwendete ich diese Gene als Marker für den Auf- oder Abwärtstransport von Signalen, welche zu PTGS führen. Ich beobachtete, dass das *Silencing* von NaTPI in den Wurzeln nicht die Genexpression im WT Spross von WT/irTPI Pflanzen beeinflusst. Jedoch führte das *Silencing* von NaPMT im Spross dazu dass die endogene Expression in der WT Wurzel der irPMT/WT Pflanze beeinflusst wurde. Diese Ergebnisse bestätigen die Beobachtung dass PTGS kaum von der Wurzel in den Spross übertragen werden kann. Darüber hinaus wird diese Technik, in Kombination mit einer großen Vielzahl an verfügbaren transgenen Linien von *N. attenuata*, unser Verständnis für die Rolle bestimmter Wurzel Gene, als auch vom Zusammenspiel von Spross und Wurzel für die Kontrolle der gesamten Pflanzen-Verteidigung, beeinflussen.

Des Weiteren untersuchte ich die Rolle der Wurzel in der Jasmonsäure (JA) Antwort auf oberirdischen Insektenfraß in *N. attenuata*. Ich benutze Linien die in der JA -Synthese (irAOC) oder Wahrnehmung (irCOI1) beeinflusst waren für den Wurzelteil mittels Mikrotransplantation (WT/irAOC und WT/irCOI1) und erfasste ökologisch relevanten Eigenschaften dieser Pflanzen gegenüber WT/WT Kontrollpflanzen. Ursprünglich habe ich Nikotin als Reporter benutzt um die systemische JA Signalübertragung der Wurzel bei Blattverwundung zu beobachten. Dieses hochgiftige Alkaloid wird ausschließlich in den Wurzeln produziert und sammelt sich in hohen Konzentrationen in den Blättern an, wenn diese mechanisch verletzt werden. Trotz der umfangreichen Literatur die einen Zusammenhang der Nikotin Induktion auf erhöhte Werte von JA zurückführen lässt, gab es bisher keine expliziten Hinweise für die Rolle von JA in den Wurzeln. Nikotin Induktion war fast vollständig abhängig von NaCOI1 und teilweise abhängig von NaAOC Aktivität in den Wurzeln. JA in den Wurzeln steuert Nikotin Produktion auf der Transkriptionsebene und reguliert ebenfalls die systemische Nikotinaufnahme durch die Blätter. Interessanterweise war dies mit einer reaktionsschnellen JA und ABA Akkumulation in den lokalen Blättern verbunden. Diese Ergebnisse sind ein

Hinweis auf eine neuartige Spross-Wurzel-Spross Regulationschleife bei der JA induzierten Pflanzenabwehr. Ich konnte ebenfalls beobachten, dass die JA Synthese und Wahrnehmung in der Wurzel die Leistung und die Vorlieben von oberirdischen Pflanzenfressern beeinflussen kann, sowohl im Gewächshaus als auch in der freien Natur. Interessanterweise scheint JA in den Wurzeln einen Einfluss auf die Interaktionen mit oberirdischen Pflanzenfressern zu haben.

Seit der Entdeckung vor mehr als 70 Jahren, dass die Nikotinsynthese in Tabakpflanzen exklusiv in den Wurzeln stattfindet, konnten zwar viele Wurzel-abhängige Verbindungen und Strategien gegen Pflanzenfresser identifiziert und untersucht werden, aber es gibt dennoch viel über die verborgene Hälfte der Pflanzen zu lernen. Sicherlich werden wir unser Wissen über Pflanzenprozesse weiter vertiefen können, wenn wir mehr auch die unterirdischen Gewebe mit einbeziehen. Hier beschreibe ich eine neue Wurzel abhängige JA Regulation der Pflanzenverteidigung, welches oberirdische Pflanze-Insekt als auch Insekt-Insekt Wechselwirkungen mit einschließt, wodurch sich schließen lässt, dass die Pflanze als gesamtes weit mehr ist als nur die Summe einzelner Gewebeteile.

9. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Variluska Fragoso

Jena, 24-08-2014

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufenhatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

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Variluska Fragoso

Jena, 24-08-2014

10. Curriculum Vitae

Variluska Fragoso
Address: Hans-Knöll Str. 8, 07745. Jena, Germany.
E-mail: variluska@ice.mpg.de
Birth date: August 6th, 1981
Citizenship: Brazilian

Education:

2010 – present	Ph.D. in Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena, Germany.
2009	TOEFL proficiency –Final score of 108 out of 120.
2005 – 2007	M.Sc. in Plant Science, Graduate Program in Cell and Molecular Biology, Centre for Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Final grade: A (excellent)
2000 – 2004	B.Sc. in Biology, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.
2000 – 2004	Licentiate in Biology (leading to the capacity of Teacher of Biology), Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Complementary Education:

2012 Research funding – Is there life after getting your Ph.D.? – 5 hours. Max Planck Institute and International Max Planck Research School, Jena, Germany.
2012 Understanding Statistics: R – 18 hours. Jena Graduate Academy. Max Planck Institute and International Max Planck Research School, Jena, Germany.
2012 Arabidopsis protoplast transfection – 20 hours. Max Planck Institute and International Max Planck Research School, Jena, Germany.
2011 Introduction to real time PCR – 20 hours. Jena School for Microbial Communication, Jena, Germany.
2011 Analysis of protein-nucleic acid interactions by electrophoretic mobility shift assays – 15 hours. International Leibniz Research School, Jena, Germany.
2010 Statistics for beginners – 30 hours. Max Planck Institute and International Max Planck Research School, Jena, Germany.
2010 Plant secondary metabolism – 20 hours. Max Planck Institute and International Max Planck Research School, Jena, Germany.
2010 Course of Identification of small molecules/phytohormones by HPLC-MS, HPLCToF and PAS analysis – 20 hours. Max Planck Institute and International Max Planck Research School, Jena, Germany.
2004 Topics in Molecular Biology – 15 hours. Pontifical Catholic University of Rio Grande do Sul, PUCRS, Porto Alegre, Brazil.
2004 Biotechnology of Medicinal Plants – 15 hours. Pontifical Catholic University of Rio Grande do Sul, PUCRS, Porto Alegre, Brazil.
2004 Pharmacogenomics: basis and applications – 8 hours. Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil.
2004 Neurological Basis of Memory – 8 hours. Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil.
2003 Secondary Metabolism – 6 hours. Brazilian Society of Plant Physiology.
2002 Defence mechanisms in Plants – 30 hours. Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil.

2001 Medicinal Plants – 20 hours. Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil.
2000 Histological Techniques – 40 hours. Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil.

Scholarships and Honors:

2010	Ph.D. Fellowship from International Max Planck Research School (IMPRS) - Germany.
2005 – 2007	M.Sc. Fellowship from the National Committee for Improvement of University Level Personnel – Ministry of Education – CAPES - Brazil.
2002 – 2004	Undergraduate Research Fellowship from the National Council for Scientific and Technological Development – CNPq - Brazil.
2002	Recipient of the first prize for oral and poster presentation in XIII Annual Meeting for Undergraduate Students' Scientific Works – Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil.
2000 – 2002	Undergraduate Research Fellowship from the State Foundation for Research Support - FAPERGS-RS, Brazil.

Professional Activities:

2007	Part-time technician post at Plant Physiology laboratory, Centre for Biotechnology, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. Employer: Arthur Germano Fett-Neto with grants of the Regulation of the Production of Secondary Metabolites with Economic Interest Project (National Committee for Improvement of University Level Personnel – Ministry of Education – CAPES – Brazil).
2007	Part-time technician post at Plant Physiology laboratory, Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil. Employer: Janette Palma Fett with grants of the Harvest Plus Project.
2006	Teaching Assistant for the Department of Botany, UFRGS, helping in the laboratory classes of Introduction to Plant Physiology. 18 hours.
2004	Technician services in orchids micropropagation, FLORATECH, Production and Commerce of Ornamental Plants Inc. Porto Alegre, RS, Brazil. 500 hours.
2003	Teaching Assistant for the Department of Botany, UFRGS, helping in the laboratory classes of Introduction to Plant Physiology. 100 hours.
2003	Collaborator teaching (Secondary Education), Environment Municipal Office of Porto Alegre – SEMAM, RS, Brazil. 120 hours.
2000	Technician assistance for the Department of Botany in the Federal University of Rio Grande do Sul Herbaria. Porto Alegre, RS, Brazil. 600 hours.

B. PUBLICATIONS (papers and abstracts)

1. Published:

1. Fragoso, V.; Rothe, E.; Baldwin, I.T.; Kim, S.G. Root jasmonic acid synthesis and perception regulate herbivore-induced shoot metabolites and increase *Nicotiana attenuata* resistance. *New Phytologist – in press.*

2. Fragoso,V.; Goddard, H.; Baldwin, I.T.; Kim, S.G. A simple and efficient micrografting method for stably transformed *Nicotiana attenuata* plants to examine shoot-root signaling. *BMC Plant Methods* 7-34.
3. Paranhos, J. T.; Fragoso, V.; Silveira, V. C.; Henriques, A. T.; Fett-Neto, A. G. Organ-specific and environmental control of accumulation of psychollatine, a major indole alkaloid glucoside from *Psychotria umbellata*. *Biochemical Systematics and Ecology* 37(6): 707-715, 2009.
4. Fragoso, V.; Nascimento, N. C.; Moura, D. J.; Silva, A. C. R.; Richter, M. F.; Saffi, J.; Fett-Neto, A. G. Antioxidant and antimutagenic properties of the monoterpene indole alkaloid psychollatine and the crude foliar extract of *Psychotria umbellata* Vell. *Toxicology In Vitro* 22(3): 559-566, 2008. The first two authors contributed equally to this work.
5. Nascimento, N. C.; Fragoso, V.; Moura, D. J.; Silva, A. C. R.; Fett-Neto, A.G.; Saffi, J. Antioxidant and antimutagenic effects of the crude foliar extract and the alkaloid brachycerine of *Psychotria brachyceras*. *Environmental and Molecular Mutagenesis* 48(9): 728-734, 2007. The first two authors contributed equally to this work.
6. Paranhos, J. T.; Fragoso, V.; Henriques, A. T; Ferreira, A. G.; Fett-Neto, A. G. Regeneration of *Psychotria umbellata* Vell. and production of the analgesic indole alkaloid umbellatine. *Tree Physiology* 25(2): 251-255, 2005.

2. Talks:

1. Fragoso V. Do roots defend shoots? JA-dependent root defenses against folivores. *11th IMPRS Symposium, MPI for Chemical Ecology*, Dornburg, DE, Feb 2012

3. Poster Presentations:

1. Fragoso, V. Systemic root jasmonic acid synthesis and perception regulate nicotine production and transport and account for plant performance against folivores. *12th IMPRS Symposium, Max Planck Institute for Chemical Ecology*, Jena, DE, Apr 2013.
2. Fragoso, V. Grafting in *Nicotiana attenuata*: an ancient approach to modern ecological questions. *10th IMPRS Symposium, MPI for Chemical Ecology*, Dornburg, DE, Feb 2011

4. Published abstracts:

a. Expanded abstracts

1. Paranhos, J. T.; Fragoso, V.; Henriques, A. T.; Fett, J. P.; Fett-Neto, A. G.; Changes in contents of the indole alkaloid Umbellatine and chlorophylls in plants of *Psychotria umbellata* Vell. (Rubiaceae) exposed to UV radiation. *I International Plant Physiology Congress*, 2002, Montevideo, Uruguay
2. Paranhos, J. T.; Gregianini, T. S.; Schwambach, J.; Porto, D. D.; Fragoso, V.; Camargo, F.; Fett, J. P.; Fett-Neto, A. G.; Zuanazzi, J. A.; Henriques, A. T. Adventitious rooting of southern Brazilian *Psychotria* species (Rubiaceae) producers of bioactive alkaloids. *VIII Brazilian National Plant Physiology Congress*, 2001, Ilhéus, Brazil.

b. Abstracts

1. Fragoso, V.; Nascimento, N. C.; Moura, D. J.; Silva, A. C. R.; Saffi, J.; Fett-Neto, A. G. The Crude foliar extract from *Psychotria umbellata* (Rubiaceae) and its main alkaloid psychollatine display antioxidant and antimutagenic activities. *IX Brazilian National Plant Phisiology Congress*, 2007, Gramado, Brazil.
2. Alarbace, F. S.; Fragoso, V.; Fett-Neto, A. G. Ontogenetic regulation and tecid-especific distribuction of N, β-D-Glucopyranosylvincosamide alkaloid of

Psychotria leiocarpa. *57º Brazilian National Botany Congress*, 2006, Gramado, Brazil.

3. Nascimento, N. C.; Fragoso, V.; Moura, D. J.; Saffi, J.; Fett-Neto, A. G. Antioxidant and antimutagenic activities of foliar extracts and Braquicerine alkaloid from *Psychotria brachyceras* (Rubiaceae) in *Saccharomyces cerevisiae*. *57º Brazilian National Botany Congress*, 2006, Gramado, Brazil.
4. Fragoso, V.; Paranhos, J. T.; Fett-Neto, A. G. Light induction of N, β-D-Glucopyranosylvincosamide alkaloid in seedlings of *Psychotria leiocarpa* Cham. & Schlecht. *X Brazilian National Plant Physiology Congress*, 2005, Recife, Brazil
5. Fragoso, V.; Paranhos, J. T.; Fett-Neto, A. G. Accumulation and distribuction of monoterpene indole alkaloids of *Psychotria umbellata* Vell. and *P. leiocarpa* Cham. & Schlecht. (Rubiaceae). *55º Brazilian National Botany Congress*, 2004, Viçosa, Brazil.
6. Fragoso, V.; Paranhos, J. T.; Fett-Neto, A. G. Stress and ontogenic roles in the production of N, β-D-Glucopyranosylvincosamide alkaloid from *Psychotria leiocarpa* Cham. & Schlecht (Rubiaceae). *50º Brazilian National Genetics Congress*, 2004, Florianópolis, Brazil.
7. Fragoso, V.; Paranhos, J. T.; Fett-Neto, A. G. Photoregulation of N, β-D-Glucopyranosylvincosamide alkaloid in *Psychotria leiocarpa* Cham. & Schlecht. (Rubiaceae). *XVI Annual Meeting for Undergraduate Students' Scientific Works, Federal University of Rio Grande do Sul* - UFRGS, 2004, Porto Alegre, Brazil.
8. Fragoso, V.; Paranhos, J. T.; Fett, J. P.; Fett-Neto, A. G. Regulation of N, β-D-Glucopyranosylvincosamide alkaloid contents in *Psychotria leiocarpa* Cham. & Schlecht. *XV Annual Meeting for Undergraduate Students' Scientific Works, Federal University of Rio Grande do Sul* - UFRGS, 2003, Porto Alegre, Brazil.
9. Paranhos, J. T.; Fragoso, V.; Henriques, A. T.; Fett-Neto, A. G. Organ distribution and factors affecting the accumulation of the analgesic alkaloid umbellatine in *Psychotria umbellata* Vell. (Rubiaceae). *IX Brazilian National Plant Physiology Congress*, 2003, Atibaia, Brazil.
10. Paranhos, J. T.; Fragoso, V.; Ferreira, A. G.; Fett, J. P.; Fett-Neto, A. G. *In vitro* regeneration of *Psychotria umbellata* Vell. aiming the production of Umbellatine, an analgesic indole alkaloid. *53º Brazilian National Botany Congress*, 2002, Recife, Brazil.
11. Gregianini, T. S.; Paranhos, J. T.; Porto, D. D.; Fragoso, V.; Fett, J. P.; Fett-Neto, A. G. Herbivory simulation on leaves of *Psychotria brachyceras* and *P. umbellata* affecting the production of bioactive alkaloids. *53º Brazilian National Botany Congress*, 2002, Recife, Brazil.
12. Fragoso, V.; Paranhos, J. T.; Ferreira, A. G.; Fett, J. P.; Fett-Neto, A. G. *In vitro* propagation of *Psychotria umbellata* and Umbelatine production. *XIV Annual Meeting for Undergraduate Students' Scientific Works, Federal University of Rio Grande do Sul* - UFRGS, 2002, Porto Alegre, Brazil.
13. Fragoso, V.; Paranhos, J. T.; Fett, J. P.; Fett-Neto, A. G. Production of Umbellatine – a monoterpene indole alkaloid – in *Psychotria umbellata* Vell. (Rubiaceae). *XIII Annual Meeting for Undergraduate Students' Scientific Works, Federal University of Rio Grande do Sul* - UFRGS, 2002, Porto Alegre, Brazil.

Jena, 24 August 2014 Variluska Fragoso

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