Impact of wounding and biotic stress on primary metabolism and growth processes of different plant species

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To my parents

Zusammenfassung

Im Laufe ihrer gesamten Entwicklung sind Pflanzen abiotischem und biotischem Stress ausgesetzt. In der vegetativen Entwicklungsphase regulieren sie Primär- und Sekundärmetabolismus, was zu veränderten Wachstumsprozessen von Blättern und Wurzeln infolge von Angriff durch Pathogene oder Herbivore führen kann. Sekundärmetabolite, als wichtige Substanzen für Abwehrprozesse, sind gut erforscht, während Kenntnisse über den Einfluss von biotischem Stress auf Primärmetabolimus und Wachstumsdynamiken von Pflanzen spärlich sind.

Das Ziel dieser Doktorarbeit lag in der Charakterisierung der Effekte von Herbivoren auf Primärstoffwechsel und Wachstumsprozesse verschiedener Pflanzenarten mit Hilfe von analytischen Methoden sowie Methoden, die auf Bildverarbeitung beruhen.

Substanzen in oralen Absonderungen von Herbivoren beeinflussen die Bildung von Ionenkanälen in der Plasmamembran und sind daher wichtig bei der Weiterleitung von elektrischen Signalen. Die Auswirkungen von frühen Stadien des Herbivorenbefalls auf Photosynthese, Stickstoff-Haushalt und Blattwachstum wurden an Baumwollpflanzen untersucht, die Herbivoren mit unterschiedlichen Fraßmechanismen ausgesetzt waren: Spinnmilben als saugend-stechende Herbivore, Raupen als kauend-beißende Herbivore. Blattwachstum und Wassergehalt waren bei Blättern, die von Raupen befallen waren, signifikant reduziert. Blätter, die von Spinnmilben geschädigt wurden, zeigten dagegen kein verringertes Wachstum, aber erhöhte Konzentrationen an Stickstoff und Saccharose. Dies zeigt, dass die Beeinflussung der Wachstumsaktivität bei Befall mit den unterschiedlichen Herbivoren mit einer unterschiedlich starken Veränderung des Primärmetabolismus einhergeht. Photosynthese und Transpiration wurden durch keinen der beiden Herbivoren beeinflusst; in systemischen Blättern wurden keine nennenswerten Auswirkungen auf deren Physiologie festgestellt.

Für die gut untersuchte Interaktion von *Nicotiana attenuata* und dem Spezialisten *Manduca sexta* wurde gezeigt, dass das Wurzelwachstum infolge von Jasmonsäure (JS)-vermittelten Prozessen stärker reduziert ist als das Blattwachstum. Eine einfache Verwundung, auch in Kombination mit Applikation

von oralen Sekreten von *M. sexta*, führte zu ähnlichen Verringerungen des Wurzelwachstums wie eine mehrfache Verwundung. Eine Verlangsamung des Wurzelwachstums durch JS-vermittelte Signalwege wurde in verschiedenen Entwicklungsstadien von Tabakpflanzen beobachtet, was darauf hinweist, dass dies eine allgemeine Reaktion von *N. attenuata* auf Verletzung und Herbivorenfraß ist.

Eine ähnliche Verringerung des Wurzelwachstums infolge von Verwundung oder simuliertem Raupenfraß wurde bei der Modellpflanze *Arabidopsis thaliana* dagegen nicht gefunden. Daraus kann man schließen, dass das Abwehrverhalten von Pflanzen abhängig ist von der ökologischen Nische, an welche die Pflanzen angepasst sind. Bei *A. thaliana* führte mechanische Verwundung zu einem Anstieg der JS-Konzentrationen im Blatt, während das Wurzelwachstum nur kurzzeitig über einen JS-unabhängigen Signalweg reduziert wurde. Infektion mit den Bakterien *Pseudomonas syringae* pv. tomato DC3000 reduzierte das Wachstum der Primärwurzel von *A. thaliana* stark. Dies war unabhängig von JS-Signalwegen und konnte auf das Bakterientoxin Coronatin und von ihm ausgelöste Ethylensignalwege zurückgeführt werden.

Diese Doktorarbeit zeigt, dass in frühen Entwicklungsstadien von verschiedenen Kultur- und Modellpflanzen ein weites Spektrum von physiologischen Reaktionen vorhanden ist. Anpassungen von Blatt- und Wurzelwachstum werden in der verschiedenen Pflanzenarten über verschiedene Signalwege vermittelt and sind nicht zuletzt abhängig vom Fraßfeind. Dies zeigt die Komplexität des molekularen Netzwerks, welches die Reaktion auf mechanische Verwundung und biotischen Stress reguliert.

Abstract

Plants suffer from abiotic and biotic stress throughout their ontogeny. In the vegetative phase, they adjust the primary and secondary metabolism which may result in altered growth processes of leaves and roots upon attack by pathogens or herbivores. Secondary metabolites can be necessary for defence responses and are well investigated while knowledge about the impacts of biotic stresses on primary metabolism and growth dynamics of plants is still scarce.
The aim of this PhD thesis was to characterize the effects of herbivory on primary metabolism and growth processes of several plant species using analytical and image-processing-based methods. Elicitors in the oral secretions of herbivores influence the formation of ion channels at the plasma membrane level and are thus important in electrical signalling. Effects of two herbivores with different feeding mechanisms on photosynthesis, nitrogen metabolism and leaf growth were investigated by elucidating the reaction of cotton towards the early stages of attack by spider mites and Lepidopteran larvae. Leaf growth and water content was reduced significantly by the chewing-biting caterpillars while plants were able to compensate for injuries to leaf tissue by the piercing-sucking spider mites by increasing the concentrations of nitrogen and sucrose at the site of damage and thereby maintaining leaf growth. Both herbivores did not affect photosynthesis and transpiration and they had little effects on the physiology of systemic cotton leaves. For the well-investigated interaction between the specialist herbivore *Manduca sexta* and *Nicotiana attenuata* it has been shown that root growth decreases more severely than leaf growth and that this reaction involves the jasmonate (JA) signalling pathway. Single leaf wounding with application of oral secretions of *Manduca sexta* leads to similar growth responses as multiple wounding treatments. JA-induced root growth decrease was observed in various developmental stages of the plant, suggesting that this is a general response pattern of *Nicotiana attenuata*. Yet, such a response pattern was not observed in the model species *Arabidopsis thaliana,* demonstrating that the ecological niche to which the plant is adapted, is of major importance for its defence mechanisms. In *Arabidopsis thaliana*, mechanical wounding of seedlings increased the foliar concentration of jasmonates, but only temporarily reduced root growth in a JA-independent way.

The bacteria *Pseudomonas syringae* pv. tomato DC3000 severely inhibited growth of the primary root of *Arabidopsis* seedlings independently of JA signalling but via the toxin coronatine and subsequent ethylene signalling.
In summary, this PhD thesis shows that even in early developmental stages of several model and crop species, a wide range of physiological response patterns can be observed. Leaf and root growth responses are mediated via different signalling pathways in different species and depend on the herbivore, demonstrating the enormous complexity of the molecular network regulating the response towards wounding and biotic threats.

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Abbreviations

1-MCP	1-methyl cyclopropene
ABA	abscisic acid
ACC	1-cyclo-propanocarboxylic acid
AP	action potential
aos	*Arabidopsis* mutant with defects in the gene coding for allene oxide synthase
Asat	CO_2 assimilation in saturating light
Asn	asparagine
Asp	asparagic acid
cat	caterpillar treatment (*Spodoptora littoralis*) in cotton experiment
CCD	charge-coupled device
coi	coronatine-insensitive mutant of *Arabidopsis*
Col-0 and Col-6	wild types of *Arabidopsis*
con	control plants
DISP	digital image sequence processing
E	transpiration
EFN	extrafloral nectar
FW	fresh weight
GDBH	growth-differentiation balance hypothesis
Gln	glutamine
Glu	glutamic acid
Gly	glycin
gs	stomatal conductance
JA	jasmonic acid
JA-Ile	jasmonate conjugated to isoleucin
Lys	lysine
MeJA	methyl jasmonate
MeSA	methyl salicylate
Met	methionine
N	nitrogen
ODH	optimal defence hypothesis

OS	oral secretions
Phe	phenylalanine
Pro	proline
Pst	*Pseudomonas syringae* pv. tomato
Rd	dark respiration
RGR	relative growth rate
ROS	reactive oxygen species
SA	salicylic acid
SE	standard error of mean
Ser	serine
SP	system potential
Sys	systemic leaves
Trp	tryptophane
UV-B	ultraviolet B
VOC	volatile organic compounds
VP	variation potential
Vtip	velocity of the root tip
Vtip(norm)	velocity of the root tip normalized to pre-treatment Vtip

Citations of publications in this dissertation

The publications belonging to this cumulative dissertation are cited in the following way. See section 3.1 for complete references.

Number	Citation	Journal	Status
1	Lühring *et al.* (2007)	FEBS Letters	published
2	Schmidt *et al.* (2009)	Plant, Cell and Environment	published
3	Schmidt *et al.* (submitted)	Plant, Cell and Environment	submitted

1 Introduction

Like most living organisms, plants are exposed to many abiotic threats in their natural environment, such as changes in temperature, light or availability of nutrients. As sessile organisms, they are also forced to deal with a lot of biotic stresses, such as herbivores or pathogens. These biotic attackers can severely reduce crop yield and thus cause serious economic damage. While, for this reason, the effects of biotic stresses during the reproductive phase of plant development are relatively well investigated, the vegetative phase of development is studied less intensely. During the vegetative phase, plant biomass increases rapidly and primary metabolites are invested in root and leaf growth, whereas they are directed towards fruit and seed development during the generative phase. Biotic stress can lead to an accumulation of defence compounds at the expense of primary metabolites which reduces leaf and root growth. Moreover, wound signalling pathways are activated that can regulate a decrease of vegetative growth which in turn can lead to diminished yield.

Cotton is a well-studied crop for fibre production. During its vegetative development, the effect of biotic stressors such as herbivores has mostly been studied in the context of ecological investigations. Topics include the synthesis of feeding deterrents (Alborn *et al.* 1996), the emission of volatiles (Loughrin *et al.* 1994; Röse *et al.* 1996; Röse *et al.* 1998; Röse & Tumlinson 2004; Röse & Tumlinson 2005) or the production of extrafloral nectar (Wäckers *et al.* 2001; Röse *et al.* 2006).

Tobacco presents another important crop plant but, in contrast to cotton, it is bred for maximal leaf production and hence for an optimized vegetative development. The wild tobacco *Nicotiana attenuata* is an annual plant which suffers little from generalist herbivory but is susceptible to attack by the specialist herbivore *Manduca sexta*. Thus, this model system of plant-insect interaction is extensively studied in terms of ecological aspects and defensive mechanisms (for instance Pluskota *et al.* 2007; Steppuhn *et al.* 2008) but little attention has been paid to growth processes, especially at the seedling stage (but see Hummel *et al.* 2007 & 2009). The same holds true for another annual

plant, the weed *Arabidopsis thaliana*, which is used as model plant for genetic and molecular studies as its genome is fully sequenced.
The fact that growth processes at high spatial and temporal resolution have been ignored is due to the lack of adequate technical possibilities. The establishment of methods such as digital image sequence processing (DISP) growth analysis allow the characterization of growth dynamics of leaves and roots in more detail. These techniques lead to interesting insights into the reactions of plants in diverse environmental conditions. For example, a recent study showed that carbohydrate availability affects the diel growth patterns of *Arabidopsis* leaves (Wiese *et al.* 2007). Nagel *et al.* (2007) reported that an elevated export of sucrose from the leaves to the root is responsible for rapid acclimation of root growth when the shoot is exposed to increasing light intensities. However, there are little data on the effects of herbivory on leaf growth and primary metabolism at the initial stages of insect feeding. A study investigated the short-term effects of wounding or simulated herbivory by the specialist *Manduca sexta* on *Nicotiana attenuata* seedlings, finding that leaf growth is less susceptible to herbivory than root growth (Hummel *et al.* 2007). As only little is known about the effects of herbivory on root growth dynamics at high temporal resolution, it is important to test, whether the findings by Hummel *et al.* (2007 & 2009) can be applied to other plant species.

The aim of this PhD thesis was to elucidate the effects of wounding and biotic stress on primary metabolism and growth processes of leaves and roots. The focus of interest was on the initial phase of biotic attack with respect to different plant organs of different model plants interacting with typical herbivores. It is recognized in literature that no ideal model arthropod or plant system exists (Schmelz *et al.* 2009), thus the plant-insect model systems were chosen according to their appropriateness for the respective question of research. This may allow general insights about possible mechanisms that plants use to adjust primary metabolism as well as leaf and root growth processes following attack by herbivores or pathogens.

1.1 Classification of herbivores and pathogens

Herbivores are classified according to their feeding mechanisms into piercing-sucking and biting-chewing herbivores. Piercing-sucking herbivores can either feed on the phloem or on the cell content, and thus cause relatively little damage to the plant (Mewis *et al.* 2005; Leitner *et al.* 2005). Nevertheless, cell-macerating herbivores (like mites) are predicted to damage the plant more severely than phloem-feeding herbivores (for instance aphids, planthoppers, whiteflies; see Leitner *et al.* 2005).

Biting-chewing herbivores, in contrast, remove large parts of the leaf tissue. Thus, the water loss through the holes is assumed to be bigger than upon feeding of piercing-sucking insects as verified within this PhD thesis (Schmidt *et al.* 2009). First-instar larvae usually avoid leaf veins and produce small holes while older caterpillar larvae make bigger holes and consume veins (Tang *et al.* 2006). Thus, the damage to the plant imposed by leaf consuming herbivores is strongly dependent on the age of the caterpillar.

Pathogens of plants can be fungi, oomycetes, bacteria and viruses (Berger *et al.* 2007; Pieterse *et al.* 2009). According to the lifestyle of the pathogen, it can be divided into biotrophic, necrotrophic and hemi-biotrophic pathogens. Biotrophic pathogens need healthy plant tissue for their growth and reproduction while necrotrophic pathogens kill the host and then feed on the dead tissue. Hemi-biotrophic pathogens (for instance *Pseudomonas syringae*) are biotrophic at the beginning of the plant infection but at a later stage switch to the necrotrophic lifestyle.

Plant-pathogen interactions can also be classified into compatible and incompatible interactions (Baron & Zambryski 1995). Pathogens that carry a functional avirulence gene will be recognized by the plant´s resistance gene. The plant will mount defence responses which reduce pathogen invasion and thus disease symptoms (incompatible interaction). In contrast, virulent pathogens will cause diseases as they are not recognized by the host plant (compatible interaction).

Another way of classifying pathogens is based on the plant organ or tissue where they occur. Above-ground pathogens (like *Pseudomonas syringae*) are found on the green tissues while the main target of below-ground pathogens is the root.

1.2 Plant strategies to cope with biotic stress

For interactions of plants and herbivores or pathogens, two main strategies are employed: tolerance or resistance (cited in Oliver *et al.* 2009).
Resistance is found mainly in predator-prey interactions and aims at minimizing the damages and thus reducing the loss of fitness of the attacked plant. Resistance traits are mechanical (such as trichomes, thorns) and chemical (toxic compounds) features that reduce the performance or preference of the herbivores (cited in Leimu & Koricheva 2006).
Tolerance traits are typical for mutualistic interactions (meaning interactions of two species with benefits for both partners). By employing the tolerance strategy, the loss of the plant´s fitness is minimized but the fitness of the attacker is not reduced. Possibilities of tolerating herbivory are: compensatory tissue re-growth, increased photosynthetic activity or utilization of stored resources (Leimu & Koricheva 2006; Oliver *et al.* 2009).
Neither tolerance nor resistance exclude the existence of the other strategy in the same plant species. Several crops and wild plants do not show a trade-off between the two defence strategies (Leimu & Koricheva 2006). It is known that weak antagonistic interactions result in both types of defence (Oliver *et al.* 2009). One example is the interaction of *Nicotiana attenuata* with its specialist herbivore *Manduca sexta*. Upon *M. sexta* feeding on the leaves, *Nicotiana attenuata* allocates sugars to the roots as they will not be consumed by the herbivore. The stored reserves are later used for re-growth and reproduction when the biotic threat has passed (Schwachtje *et al.* 2006). *A*t the same time, root growth is reduced following herbivory which was shown in this PhD thesis (Fig. 4, 5, 6), supporting the results by Hummel *et al.* (2007 & 2009), and alkaloids such as nicotine are synthesized in the roots, incorporating up to 8% of the plant´s total nitrogen pool (Baldwin & Preston 1999; Baldwin 2001 and references therein). The alkaloids are highly toxic and reduce the feeding

activity and the fitness of the specialized herbivore, but may also impair photosynthetic activity of the attacked tobacco plant itself (Nabity *et al.* 2009).

1.3 Defence mechanisms

1.3.1 Direct defences

Plant responses to herbivore damage include direct and indirect defence reactions. Direct defences can be mechanical barriers for herbivores such as trichomes and thorns, as well as toxic secondary metabolites (phytoalexins) or proteinase inhibitors produced by the plant in order to decrease nutrient availability and retard the growth of the herbivores (Baldwin & Preston 1999). Cotton plants, which are important crops, store terpenoid aldehydes like gossypol, hemigossypolone and heliocides in pigment glands so that these substances can rapidly be released upon herbivore attack in order to serve as immediate feeding deterrents. Rapid systemic *de novo* synthesis of the terpenoid aldehydes after caterpillar feeding (Alborn *et al.* 1996; Bezemer *et al.* 2004) lowers the food quality for the herbivores and reduces further consumption. Early stages of herbivore attack (meaning a small number of herbivores and a relatively short time of feeding, in this case 8-9 spider mites feeding for 96 hours or two caterpillars feeding for 48 hours on one single leaf) do not affect gossypol concentrations of cotton leaves either locally nor systemically (L. Schmidt, unpublished). The direct defence traits are negatively correlated in cotton species, meaning that a high density of trichomes corresponded to a reduced investment into gossypol glands and *vice versa* (Rudgers *et al.* 2004).

The weed *Arabidopsis thaliana* produces glucosinolates, phenolics and terpenoids (Mewis *et al.* 2005 and references therein). Upon tissue damage, glucosinolates are hydrolyzed and the resulting substrates are repellent to generalist herbivores (Mewis *et al.* 2005 and references therein). Tobacco, in contrast, increases the production of toxic alkaloids, such as nicotine (Baldwin & Preston 1999), which repel potential herbivorous insects. In tomato plants, an increase of the feeding-deterring non-protein amino acid γ-aminobutyrate follows wounding, herbivory or simply crawling of caterpillar larvae on leaves (Bown *et al.* 2006).

Invasion of pathogens can be prevented both by cell wall fortification, and the accumulation of antimicrobial secondary metabolites (Pieterse *et al.* 2009). As most pathogens invade the plants via stomata or wounds, the first phase of defence consists of stomatal closure which is mediated by the plant hormone abscisic acid (Ton *et al.* 2009). Some pathogens use their effector molecules (such as coronatine from *Pseudomonas syringae* pv. tomato DC3000) to re-open the stomata and thus facilitate invasion (Melotto *et al.* 2008). In the second phase of plant defence, the cell walls are reinforced by deposition of callose, and reactive oxygen species (ROS) are generated (Ton *et al.* 2009). The ROS burst induces programmed hypersensitive cell death at the infection site, thus preventing further spreading of the pathogen (after Pieterse *et al.* 2009). Also, ROS production can occur locally and systemically after wounding, MeJA application or herbivore attack in several plant species, probably in order to minimize pathogen invasion via wounded leaves (Orozco-Cardenas & Ryan 1999).

1.3.2 Indirect defences

Indirect defence aims at attracting enemies of the herbivores via emission of volatile organic compounds (VOCs) or secretion of extrafloral nectar (EFN). This allows the plant to adjust the investments in defence according to the existing herbivore pressure as it is proposed by the optimal defence hypothesis (ODH; Heil 2008 and references therein). According to the ODH, EFN secretion and VOC emission are higher in the youngest leaves of a plant as recently proved for *Phaseolus lunatus* and *Ricinus communis* (Radhika *et al*. 2008).

EFN mainly consists of mono- and disaccharides and amino acids and represents a food source for ants, but also for predatory mites and parasitic wasps (Heil 2008). Odour components may be contained in the EFN to facilitate attraction of parasitic wasps (Röse *et al.* 2006). It is suggested that a baseline production of EFN in cotton and castor bean plants is maintained to minimize the lag time between herbivore attack and increased nectar secretion (Wäckers *et al.* 2001). Early stages of spider mite or caterpillar attack on cotton plants seem to have little effect on the composition of sugars in the leaves as reported in Schmidt *et al*. (2009) which might reflect the composition of carbohydrates of the EFN as well.

Foliar VOCs are also emitted by plants upon insect feeding in order to attract parasites or predators of the herbivores (Röse *et al.* 1998; Choudhary *et al.* 2008). The most commonly released VOCs include indole, MeSA, C6 volatiles, terpenoids, oximes and nitriles (Choudhary *et al.* 2008). Röse *et al.* (1996) have found that some volatiles, such as indole, isomeric hexenyl butyrates and 2-methyl butyrates, are released locally while others, like β-ocimene and linalool, are released systemically upon caterpillar feeding on cotton plants. Changes in the pool of some free amino acids which are precursors of VOCs may be due to the increased VOC production of cotton following caterpillar feeding as suggested in this PhD thesis (Schmidt *et al.* 2009).

In two closely related *Arabidopsis* species, a blend of sesquiterpenes (such as nerolidol), homoterpenes and MeSA are emitted from the leaves upon caterpillar feeding (Abel *et al.* 2009). The emission of VOCs in *Arabidopsis thaliana* following herbivore attack is mediated by jasmonic acid (JA) signalling but probably also via other signalling substances (Bruinsma *et al.* 2009).

The blend of VOCs emitted upon herbivore attack is variable and depends – besides the abiotic conditions - on both plant and herbivore species (Reymond & Farmer 1998; Gatehouse 2002; Kessler & Baldwin 2002; van Poecke & Dicke 2004; Choudhary *et al.* 2008; Heil 2008; Olson *et al.* 2008; Bruinsma *et al.* 2009). Paré & Tumlinson (1999) and Bruinsma *et al.* (2009) reported that tobacco, maize, cotton and *Arabidopsis* emit herbivore-specific compositions of VOCs which are used by herbivore-specific predators as cues for location of their prey. Interestingly, the emission of so-called infochemicals, such as MeSA, MeJA or cis-jasmonates upon herbivore attack can prime neighbouring plants by triggering their JA-induced defence responses (Engelberth *et al.* 2004; Choudhary *et al.* 2008). In Lima bean (*Phaseolus lunatus*), VOC emission in response to herbivory can induce EFN secretion in neighbouring healthy plants, suggesting an interaction of both indirect defence mechanisms (Kost & Heil 2006).

1.4 Signalling pathways

1.4.1 Hydraulic signalling

Hydraulic signals are rapid self-propagating changes in water pressure and may play a role in systemic signalling of wound responses. In unwounded plants, changes in pressure can be buffered by the hydraulic capacity of the entire plant (Malone 1993). Wound-induced hydraulic signalling is postulated to consist of two components: First, there is a rapid pressure wave which is followed by a slower flow of cell sap (containing elicitor from damaged cells) from the site of wounding (cited in Boari & Malone 1993). The hydraulic wave leads to changes in water uptake and growth and can modify the ion fluxes of the neighbouring cells (depolarization) which can be transmitted to the xylem and then induce electrical responses in distant tissue (Stahlberg & Cosgrove 1995; Fromm & Lautner 2007).

In cucumber and pea seedlings, a sustained growth inhibition after excision of the hypocotyl was reported (Stahlberg & Cosgrove 1995). This was ascribed to hydraulic effects and subsequent variation potentials rather than to release of wound substances from the damaged cells. Feeding of caterpillars causes cumulative loss of water at the feeding site and induces mass flow which can drive the dispersal of elicitors from damaged cells within the whole plant (Alarcon & Malone 1994). Hydraulic signals also seem to be the reason for rapid reductions of the velocity of the root tip of *Arabidopsis thaliana* and *Nicotiana attenuata* seedlings following mechanical wounding as suggested by Hummel *et al.* (2007 & 2009) and supported by findings within this PhD project (Schmidt *et al.*, submitted).

1.4.2 Electrical signalling

Electrical signals are fast and thus precede the slower-travelling signalling substances such as phytohormones (Zimmermann *et al.* 2009). Besides hydraulic waves, they may be of importance for rapidly reduced root growth following mechanical wounding treatments to leaves of *Arabidopsis thaliana* or

Nicotiana attenuata seedlings (Hummel *et al.* 2007 & 2009; Schmidt *et al*., submitted).

Three types of long-distance electrical signals in plants are known:

Action potentials (AP) appear with constant propagation from the place of stimulation when plants are exposed to non-damaging stimuli (like electrical and mechanical stimuli, illumination). Excitation of plant cells requires an increase in intercellular concentrations of Ca^{2+}. Thus the activation of Ca^{2+}-permeable channels is regarded as the initial step in the generation of APs (Fromm & Lautner 2007). APs are transmitted to other symplasmic cells via plasmodesmata (Fromm & Lautner 2007).

Stimuli that damage the plant, such as wounding, organ excision or flaming, induce *variation potentials* (VPs) (Fromm & Lautner 2007). VPs are slow-travelling signals induced by changes in the ion fluxes of the cell due to a hydraulic pressure wave or wounding substances. The VPs are transported to the sieve elements of the xylem via plasmodesmata and are then distributed to distant parts of the plant (Fromm & Lautner 2007). The intensity of the VPs decreases with distance from the site of damage (Dziubinska 2003).

System potentials (SP) appear upon wounding of higher plants and are neither caused by a hydraulic pressure wave nor by activation of ion channels (Zimmermann *et al.* 2009). The intensity of the SP varies with the intensity of the stimulus unlike AP and VP.

Upon feeding of Lepidopteran larvae or application of caterpillar oral secretions to plants, ion fluxes are induced which depolarize the cell membranes in the vicinity of the bite zone. This leads to voltage-dependent opening of Ca^{2+} channels and thus an increase in cytosolic Ca^{2+} (Maffei *et al.* 2004; Maischak *et al.* 2007). The ion channel-forming compounds were found to be amphipathic molecules which seem to be oligopeptides whose components of less than 3 kDa form small pores and larger peptides forming larger pores (Lühring *et al.* 2007). In distances up to about 6 mm from the bite zone, the plant membrane is constantly depolarized (Maffei *et al.* 2004).

1.4.3 Hormonal signalling

1.4.3.1 Jasmonates

Jasmonic acid (JA) and its volatile derivative methyl jasmonate (MeJA) are synthesized from α-linolenic acid via the octadecanoid pathway. Jasmonates are important for growth and developmental processes of plants, such as flower development, fruit ripening or pollen production. Jasmonates inhibit root growth when they are applied to the growth medium (Ellis & Turner 2002; Devoto & Turner 2003) which is confirmed for *Arabidopsis thaliana* seedlings in cultivation systems with shoots growing outside the agar within this dissertation (Schmidt *et al*., submitted). In that manuscript (Schmidt *et al*., submitted), it was shown that wounding-induced plant-internal jasmonates do not lead to root growth reductions in *Arabidopsis*, unlike *Nicotiana attenuata* (Hummel *et al*. 2007 & 2009).

In general, jasmonates are regarded as important signalling substances upon wounding or biotic attack (Creelman & Mullet 1997). The JA signalling pathway is important for defence against necrotrophic pathogens and against insect herbivores (Pieterse *et al*. 2009).

Wounding-induced JA can be transported to systemic tissues via the phloem (in *Nicotiana sylvestris*; Zhang & Baldwin 1997) while MeJA can be transported in both phloem and xylem due to its volatility (in *Nicotiana tabacum;* Thorpe *et al*. 2007).

In several plant species, JA is known to mediate an increase in VOC emission and / or EFN secretion in response to herbivore attack (Heil 2008 and references therein). JA also influences the production of feeding repellents, for instance glucosinolates in *Arabidopsis* or nicotine in tobacco, upon herbivore feeding (Mewis *et al*. 2005 and references therein; Shoji *et al*. 2008). In cotton, the enzyme which catalyzes the key step of gossypol biosynthesis pathway is induced by MeJA (Wang *et al*. 2009).

Thus, JA and MeJA application is often used to mimick herbivory-induced JA signalling (for example see van Dam & Oomen 2008) as in this dissertation (Schmidt *et al*., submitted, unpublished data), although this practice is increasingly criticized (Mewis *et al*. 2005; Bruinsma *et al*. 2009).

Several mutants of the JA signalling pathways have been isolated, and they are preferable used for verifying the role of JA in plants. One important mutant is *coi* (coronatine insensitive) whose root growth is not affected by JA nor by the bacterial toxin coronatine (Feys *et al*. 1994). Thus, *coi,* which lacks the F-box protein COI1 and is male sterile, represents an ideal mutant to study the involvement of jasmonates in root growth processes following wounding or biotic stress, such as in this PhD thesis (Schmidt *et al*., submitted).

1.4.3.2 Ethylene

Ethylene is a plant hormone which triggers senescence processes in plants synergistically with jasmonates. Thus, ethylene is also involved in defence responses towards necrotrophic pathogens and herbivores (Pieterse *et al*. 2009). Moreover, an increased concentration of ethylene is reported for plants after wounding and after exposure to increased concentrations of ozone or high doses of UV-B (Wang *et al*. 2002). The emission quantity of ethylene differs between mechanical wounding treatment and herbivore feeding: the increase in ethylene is more pronounced following herbivore attack (von Dahl & Baldwin 2007). The amount of ethylene which is emitted upon herbivore feeding varies strongly between plant species and depends on herbivore species, too (von Dahl & Baldwin 2007).

Several defence responses of plants, like nicotine accumulation in Solanaceae, accumulation of phenolics, production of terpenoid resins, emission of sesquiterpenes and accumulation of glucosinolates in *Brassica* species, are dependent on ethylene (von Dahl & Baldwin 2007). The precursor of ethylene is the amino acid methionine which is converted via various enzymes to 1-cyclo-propanocarboxylic acid (ACC). ACC is transported to the leaves via the xylem and is then converted to ethylene (Starck 2006).

Ethylene by itself is a volatile substance which is transported via intercellular spaces thus can spread within the whole plant in a short time (Dziubinska 2003). It is involved in growth and developmental processes of plants. Several studies showed that ethylene, in interaction with auxin and gibberellins, is an important inhibitor of root growth as it reduces cell elongation and cell division (Ruzicka *et al*. 2007; Swarup *et al.* 2007; Dugardeyn & van der Straeten 2008).

The role of ethylene in suppressing root elongation was confirmed with *Arabidopsis* seedlings by applying the ethylene reception blocker 1-methyl cyclopropene (1-MCP) in one experiment of this PhD thesis (Schmidt *et al*., submitted).

1.4.3.3 Salicylates

Salicylic acid (SA) and its derivative methyl salicylate (MeSA) are synthesized mainly following pathogen attack, and are thus important in establishing the systemic acquired resistance of plants towards biotrophic pathogens. The hemi-biotrophic bacteria *Pst* may overcome the SA-mediated defence by their toxin coronatine, which mimicks JA signalling.
Mechanical wounding does not increase the plant-internal concentrations of SA (confirmed for *Arabidopsis thaliana*, unpublished data) suggesting that SA signalling is not involved in wound responses.
The biosynthesis of salicylic acid starts with chorismic acid which derives from the shikimate pathway. Chorismic acid can be metabolized to salicylic acid via isochorismate, but other pathways via phenylalanine and successively cinnamic acid are known (Métraux 2002). Until now it is not clear which one of the three known pathways is induced for synthesis of basal SA (meaning the SA level in uninjured plants) or SA biosynthesis following attack by biotrophic pathogens or piercing-sucking herbivores (Métraux 2002, Heil 2008; Pieterse *et al*. 2009).
SA is transported from the site of infection to the systemic plant organs via its volatile derivative MeSA rather than in the form of SA itself (Heil & Ton 2008).

1.4.3.4 Cross-talk of jasmonates, salicylates and ethylene

The cross-talk between the signalling pathways can be variable. It is known that JA induces ethylene biosynthesis and *vice versa* (Maleck & Dietrich 1999; Wang *et al*. 2002). The synergism of JA and ethylene may decrease upon mechanical treatments: When plants are wounded, ethylene may suppress the

JA-dependent signalling pathway in the damaged tissue while the systemic JA-dependent reactions are not affected by ethylene (Wang *et al*. 2002).

SA, in contrast, has an inhibiting effect on the enzymes of the octadecanoid pathway (Maleck & Dietrich 1999; Devoto & Turner 2003; Beckers & Spoel 2006), but JA and SA can act synergistically as well (reviewed in Reymond & Farmer 1998; Pieterse *et al*. 2009).

SA and ethylene are known to act synergistically in pathogen defence (Pieterse *et al.* 2009), but negative interactions are reported as well (Wang *et al*. 2002). JA and ethylene can inhibit the accumulation of SA and thus the expression of SA-induced genes following attack by pathogens (Wang *et al.* 2002).

This holds true for the bacterial strain *Pseudomonas syringae pv. tomato* (Pst) whose toxin coronatine is suggested to modify the JA and ethylene signalling pathways (Laudert & Weiler 1998; He *et al*. 2004; Glazebrook 2005). It is hypothesized in the literature that coronatine induces the JA signalling pathway (Laudert & Weiler 1998; Thilmony *et al*. 2006) thereby impeding the establishment of the antagonistic SA pathway. This suppression of the SA-mediated plant defence can counteract growth processes of the host plant (as shown for root growth of *Arabidopsis thaliana* in this PhD thesis; Schmidt *et al*., submitted) and thus enables the bacteria to establish within the plant tissue before the systemic immunity is mounted (Kloek *et al*. 2001; Spoel *et al*. 2003; Block *et al.* 2005; Mittal & Davis 1995; Truman *et al*. 2007). The SA-mediated defence against *Pst* can also be impeded by abscisic acid and by auxin signalling, as well as by inhibition of SA accumulation in the chloroplast via the type III effector avrRpt2, or the virulence factor HopI of the bacteria (Pieterse *et al.* 2009).

Thus, the interaction of the plant hormones is variable and depends on both plant and herbivore species.

1.4.3.5 Involvement of other signalling substances

Abscisic acid (ABA) is known as a plant hormone that accumulates upon water shortage. In seeds, germination and root growth are inhibited by ABA (Ellis & Turner 2002). Upon loss of water after wounding of leaves or herbivore feeding, plant water reduces, resulting in increased ABA concentrations (as observed for

Arabidopsis thaliana, Schmidt *et al*., unpublished data). ABA signalling induces stomatal closure which prevents severe water loss and desiccation (for example see results on cotton infested with spider mites as reported in Schmidt *et al.* 2009). ABA accumulation upon water shortage also enables continuous root growth by promoting the elimination of reactive oxygen species and thus reducing ethylene biosynthesis (Starck 2006). For *Arabidopsis* seedlings it was shown that ABA inhibits root growth via the ethylene response pathway and not via increased ethylene biosynthesis (Beaudoin *et al*. 2000; Ghassemian *et al*. 2000). In biotic interactions, ABA signalling is known to have a positive effect on JA-responsive gene expression, and thus on JA-dependent defence against necrotrophic pathogens (Pieterse *et al*. 2009). Thus, ABA counteracts SA signalling and enables establishment of virulent bacteria, such as *Pseudomonas syringae*, within the plant tissue (Ton *et al*. 2009).

Auxin signalling negatively interacts with SA signalling, thus preventing the establishment of SA-mediated defence against biotrophic and hemi-biotrophic pathogens. Auxins, as important plant growth regulators, are known to promote root growth (Devoto & Turner 2003), but in *Arabidopsis* seedlings, accumulation of auxins via cytokinin and ethylene signalling pathways strongly reduces root growth (Beaudoin *et al.* 2000).

Similar to auxins, gibberellins are hormones that control plant growth and development. They counteract the JA and the ethylene signalling pathways but have a positive effect on SA signalling (Pieterse *et al*. 2009).

Cytokinins are important in plant development, they work in concert with auxins, but little is known about their interactions with the JA-, SA- and ethylene signalling pathways. Also, no connection has been reported between these three main signalling substances and the brassinosteroids in terms of defence (Pieterse *et al*. 2009). Nevertheless, brassinosteroids are important promoters of root growth processes in *Arabidopsis*, increasing the transport of auxins from the base of the root to the root tip (Bao *et al*. 2004).

1.5 Impact of herbivory on primary metabolism

1.5.1 Photosynthesis

Photosynthetic activity can be strongly influenced by herbivore feeding, depending on the duration of feeding, the herbivore species and the plant species as well as the capacity of the attacked plant to cope with the herbivore. Direct effects of herbivore feeding are due to removal of leaf area or disruption of photosynthetically active tissue. Besides this, herbivory can have indirect impacts on the photosynthetic activity of the remaining leaf tissue via (Nabity *et al.* 2009)

- severed vasculature (disruption of the leaf veins and subsequent water loss by transpiration leads to increased stomatal closure and reduced photosynthetic CO_2 assimilation)
- altered sink demand of the plant, resulting in increased photosynthesis of the remaining leaves
- autotoxicity, as many defensive secondary compounds (e. g. nicotine, furanocumarins) are toxic to the plant itself
- down-regulation of photosynthesis-related genes, for example due to the signalling substance JA.

Piercing-sucking herbivores can either feed via stylets on the phloem (like whiteflies and aphids) or on the cell content, thereby causing relatively little damage to the photosynthetic active tissue. Aphid feeding on cotton for nine days has no effect on photosynthetic rates and dark respiration but, after 18 days, led to decreased CO_2 assimilation and increased transpiration (Shannag *et al*. 1998; Gomez *et al*. 2006). Infestation with silverleaf whitefly for 60 days significantly reduced the photosynthetic rate of cotton plants but there were no effects on stomatal conductances, intercellular CO_2 concentration or leaf chlorophyll content (Lin *et al*. 1999).
In a similar way, the cell content-feeding thrips (Lei & Wilson 2004) or spider mites (Bondada *et al*. 1995; Reddall *et al*. 2004; Reddall *et al*. 2007) reduce net photosynthesis in the oldest leaves of cotton after a long infestation time. Besides this, long-term infestation with spider mites strongly decreases

chlorophyll concentration, stomatal conductances, and consequently transpiration, of cotton plants even in the un-damaged leaf portions (Bondada *et al*. 1995; Reddall *et al*. 2004). This is due to grana disintegration of the chloroplast which is probably caused by toxins in the mites´ saliva (Bondada *et al*. 1995). In contrast, this PhD project showed that short-term infestation of cotton plants with a small number of spider mites on one leaf, simulating the initial stage of infestation, has no effect on photosynthetic CO_2 assimilation or transpiration but results in locally increased dark respiration and reduced stomatal conductances (Reddall *et al.* 2004; Schmidt *et al.* 2009).
Caterpillars, as chewing herbivores, are supposed to severely affect photosynthetic activity of the host plant, as they remove large amounts of plant tissue. Studies of CO_2 assimilation upon caterpillar feeding show variable results: For *Spodoptera littoralis* feeding on cotton, it was shown that stomatal conductances were reduced locally while transpiration was not affected (Schmidt *et al.* 2009). Tang *et al*. (2006) suggested that water loss upon *Trichoplusia ni* larvae feeding led to decreased stomatal conductances and thus decreased photosynthetic activity of *Arabidopsis thaliana*, especially when fourth-instar larvae were feeding. Stomatal aperture can be reduced by degradation products of cell walls, such as oligalacturonic acid, by pathways which increase cytosolic Ca^{2+} concentrations, and H_2O_2 production to reduce potential invasion of pathogens via the stomata, as suggested by Lee *et al*. (1999). It is also hypothesized in literature that damage to the cell membranes release water which can reach the xylem and travel as hydraulic signal to systemic tissues where it can close stomata hydropassively (Malone 1993). This probably can result in impaired photosynthesis in the un-damaged parts of the leaves as it was shown for *Trichoplusia ni* feeding on wild parsnip (Zangerl *et al.* 2002). Izaguirre *et al.* (2003) reported that several photosynthesis-related genes were down-regulated upon application of *Manduca sexta* regurgitant on mechanically wounded *Nicotiana longiflora* leaves. In contrast, *Cucumis sativus* compensated for the loss of leaf area, due to snail feeding, with higher photosynthetic efficiency (Thomson *et al.* 2003), which was reported for severe defoliation of *Nicotiana sylvestris* as well (Baldwin & Ohnmeiss 1994). For *Gastrophysa viridula* larvae feeding on *Rumex obtusifolius,* it is reported that there was no effect on photosynthetic activity although the leaf expansion was

reduced systemically (Moore *et al.* 2003). When *Ricinus communis* and *Phaseolus lunatus* were treated with JA, which is commonly used to simulate herbivory-mediated wound signalling, they did not show significant alterations in the photosynthetic rate (Radhika *et al.* 2008). In *Zea mays*, whole-plant photosynthesis was not affected by grasshopper grazing (Holland *et al.* 1996) and cotton plants did not show alterations in light-saturated photosynthetic activity after 48 hours of *Spodoptera littoralis* feeding, as also reported in one manuscript of this dissertation (Schmidt *et al.* 2009). Nevertheless, dark respiration increased locally after caterpillar feeding, as observed for mild spider mite attack as well, and thus Schmidt *et al.* (2009) suggest it to be a general effect of localized herbivory on cotton.

Herbivory damages the leaf tissue, and may facilitate the invasion of pathogens via the wounds. Pathogen attack can rapidly decrease photosynthetic activity of the plant, while respiration and photorespiration increase (Berger *et al*. 2007). For instance, after 48 hours of infection with *Pst* DC3000, the maximum and effective quantum yield of photosystem II decreased in parallel with the non-photochemical quenching at the infection site of *Arabidopsis thaliana* leaves. Differences in the dynamics were found between virulent and avirulent *Pst* strains: upon infection with virulent bacteria, photosynthesis of *Arabidopsis* decreases more slowly compared to avirulent *Pst* (Bonfig *et al.* 2006), corresponding to a slower increase in plant-internal JA (Spoel *et al*. 2003; Grun *et al.* 2007).

1.5.2 Carbohydrates

Wounding, herbivory and pathogen attack can affect the carbon status of plants. Severe defoliation of *Casearia nitida* saplings resulted in increased starch breakdown, suggesting increased allocation of reserves towards sink organs such as new leaves (Boege 2005). By spraying JA onto Lima bean leaves in order to simulate herbivory-induced JA signalling, Radhika *et al.* (2008) demonstrated that photosynthates were transported from mature to young leaves according to the ODH. Henkes *et al.* (2008) also showed that ^{11}C labelled photosynthates were diverted from JA-treated root parts of barley to the untreated part of the root. Upon grazing of grasshoppers on *Zea mays*, carbon allocation to the roots - and concomitantly root exudation and rhizosphere respiration - increased at the expense of carbon allocation to the shoot (Holland *et al.* 1996). An increased diversion of sugar reserves to the roots in a JA-independent manner after simulated herbivory by the specialist *Manduca sexta* was reported for the annual plant *Nicotiana attenuata* (Schwachtje *et al.* 2006) and for JA application to Populus tremuloides (Babst *et al.* 2005) while silverleaf whitefly stress resulted in reduced carbon export from source leaves of cotton (Lin *et al.* 2000). Unlike these findings, no differences in glucose, fructose, sucrose and starch concentrations were detectable in cotton leaves after 48 hours of caterpillar feeding as reported in a manuscript of this PhD thesis (Schmidt *et al.* 2009). Nor did feeding by phloem-piercing aphids for 9 days have any impact on foliar non-structural carbohydrates of cotton plants (Gomez *et al.* 2006). This is surprising as it is known that cotton plants produce increased amounts of EFN, both locally and systemically, in response to 48 hours of caterpillar feeding (Wäckers *et al.* 2001), and thus changes in the carbohydrates pool of the whole leaf were expected to occur in the experiments of Schmidt *et al.* (2009).

In contrast, spider mite-infested cotton plants showed locally increased sucrose concentrations (Schmidt *et al.* 2009). Increases in sucrose content of the leaf were also observed in *Brassica oleracea* shoots treated with JA (van Dam & Oomen 2008) and in cotton subjected to silverleaf whitefly stress (Lin *et al.* 2000). Sucrose is important, not only for transport, but also a signalling molecule that can play a role in the protection against reactive oxygen species (Couée *et al.* 2006). Thus it can be of importance for defence responses towards pathogen invasion.

Upon pathogen attack, invertase expression increases, resulting in increased amounts of hexoses. According to Berger *et al.* (2007), these hexoses can then (i) down-regulate photosynthesis-related gene expression (feedback-inhibition) or (ii) be used either as nutrients for the pathogen or to activate defence reactions in the plant.

1.5.3 Nitrogen

Photosynthesis is strongly coupled not only to the carbohydrate status of a plant, but also to its nitrogen (N) content. A large fraction of leaf N is bound in soluble proteins (such as Rubisco or Calvin cycle enzymes) and in the thylakoid membranes of the chloroplast (Evans 1989), and so the leaf N correlates with the concentration of chlorophyll and the N content of Rubisco, as well as with CO_2 assimilation and electron transport of many plant species (Evans 1989). Major amino acids are present in high concentrations, and are closely linked to N assimilation and primary carbon assimilation, while minor amino acids are less abundant, and their content in leaves is independent of photosynthetic rates of wheat and potato (Noctor *et al.* 2002).

Carbon skeletons and energy which derive from photosynthetic processes are used for assimilation of N, and thus production of the primary amino acids glutamate and glutamine (Noctor *et al.* 2002). These amino acids are converted to other amino acids.

The composition of free amino acids changes in stressed or senescent tissue, which may then become unfavourable to herbivores (Cockfield 1988). Free amino acids affect ion transport across membranes, as well as enzyme activities, and can act as osmolytes or scavengers of reactive oxygen species (such as proline, Rai 2002). They may be precursors for defence-related secondary metabolites such as VOCs or alkaloids, as well.

Free amino acids are found in solutions and are thus more accessible than proteins for vascular-feeding insects, such as aphids or planthoppers (Cockfield 1988). Planthopper feeding on *Spartina alterniflora* reduces the concentrations of several proteinogenic free amino acids, as well as the concentrations of γ–amino butyrate, which results in a lower quality of the host plant (Olmstead *et al.* 1997). When cotton plants are exposed to a small number of spider mites, there is no effect on the composition of foliar free amino acids, but the total leaf N increases (Schmidt *et al.* 2009). Interestingly, no increased leaf N concentrations were observed upon localized caterpillar feeding on cotton (Schmidt *et al.* 2009) which is in accordance to reports on another cotton variety attacked by another leaf herbivore as well as root nematodes (Olson *et al.* 2008), and also for *Nicotiana sylvestris* plants upon defoliation (Baldwin &

Ohnmeiss 1994). This fits well to reports about a two-fold increase in activity of proteinase inhibitors in spider mite-infested tomato leaves (Kant *et al.* 2004), thus representing a possible explanation for the higher N levels in cotton at the early stages of spider mite infestation (Schmidt *et al.* 2009).

Upon long-term infestation with spider mites, N is translocated from the infested and thus senescing leaves to the reproductive tissues, resulting in a decrease in the leaf N content (Sadras & Wilson 1997). This reduction of leaf N may aim to make the infested plant less attractive for other herbivorous arthropods, thus decreasing the risk of further feeding damage, and at the same time saving N reserves for reproduction (Sadras & Wilson 1997). An overall reduction in concentrations of most free amino acids was also observed following treatment of *Brassica oleracea* or cotton with jasmonates (van Dam & Oomen 2008, Fig. 1).

The amino acids glutamine (Gln), glutamate (Glu), asparagine (Asn) and aspartate (Asp) are responsible for transport of N within the plant. Upon feeding of the caterpillar *Spodoptera littoralis* on one single cotton leaf, the Gln concentrations were decreased while concentrations of the other N-transporting amino acids were not altered. The early stages of spider mite infestation did not have any effect on the N-transporting amino acids (Schmidt *et al.* 2009). In contrast, treatment of *B. oleracea* shoots with JA or treatment of cotton with MeJA reduced the concentrations of Asn, Asp, Glu and Gln (Fig. 1a; see too van Dam & Oomen 2008).

Tryptophan (Trp) can be a precursor of indole or indole-3-acetic acid (auxin), the latter being important for the regulation of growth. In cotton, caterpillar feeding for 48 hours results in a local increase in Trp concentrations (Schmidt *et al.* 2009) as well as in locally increased indole emission (Röse *et al.* 1996; Olson *et al.* 2008). Nevertheless, no conclusions can be drawn from these patterns as it has been shown that there was no correlation between glucosinolates and their respective precursor amino acids in JA-treated *B. oleracea* shoots (van Dam & Oomen 2008). Upon application of MeJA to cotton plants, no significant changes in Trp concentrations were observed (Fig. 1a).

Spodoptera littoralis feeding caused significant reductions in local phenylalanine (Phe) concentrations in cotton, which was also reported for *B. oleracea* shoots treated with JA (van Dam & Oomen 2008), and which was observed in cotton after 18 hours of MeJA treatment (Fig. 1b). Phe is a precursor for cinnamic acid which, in turn, can be a precursor for synthesis of salicylic acid. Thus, the decrease of the Phe concentration can be attributed to an increased requirement for methyl salicylate synthesis, as it was shown that 48 hours of caterpillar feeding on *Medicago truncatula* does not affect SA concentrations, but causes increased emission of its volatile derivative MeSA (Leitner *et al.* 2005). No increases in methionine (a precursor of ethylene) concentrations were detectable after caterpillar or spider mite feeding, or MeJA application, in cotton (Fig. 1c). This contrasts with reports about ethylene emission in wild tobacco and maize in response to caterpillar attack (Kahl *et al.* 2000; Schmelz *et al.* 2003).

Changes in the concentrations of free amino acids upon herbivory may additionally result from infection with pathogens at the site of wounding. Pathogen attack can result in accumulation of polyamines such as putrescine, spermine and spermidine (Yoda *et al.* 2009). The cleavage of the polyamines by polyamine oxidase releases hydrogen peroxide, which may induce cell death and thereby limit spreading of pathogens within a plant, as shown for several plant-pathogen interactions (Yoda *et al.* 2009). In general, polyamines play an important role in plant growth and developmental processes, as they delay senescence by inhibiting ethylene biosynthesis (Slocum *et al.* 1984).

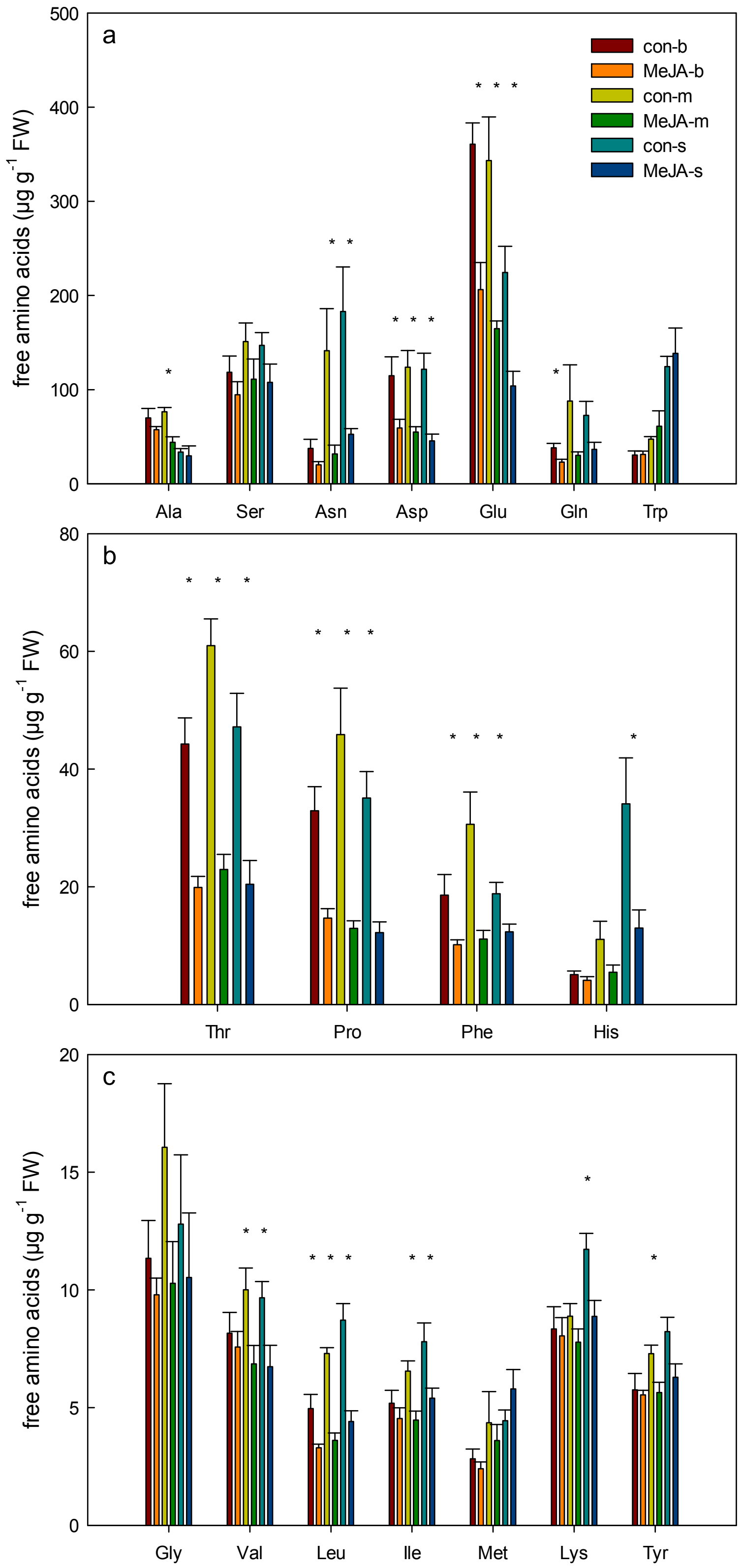

Fig.1 Concentrations of free amino acids in big (b), medium (m) and small (s) leaves of cotton after 18 hours of continuous exposure to MeJA. Statistically significant differences ($P \leq 0.05$, t-test) between controls and MeJA-treated plants are indicated by asterisks. Mean ± SE. N = 4-5.

1.6 Plant growth dynamics

1.6.1 Growth patterns of leaves and roots

Leaves have to cope with a fluctuating environment. Nevertheless, the dynamics of leaf growth are not strongly correlated to short-term fluctuations of light intensity or concentrations of CO_2, presumably it would waste resources to acclimate growth processes to every single change in the environment. Instead, the plant is capable to buffer the fluctuations by maintaining strong endogenous rhythms of leaf growth. Diel cycles of leaf growth can show different patterns. As classified by Walter *et al*. (2009), type I plants, such as *Nicotiana attenuata*, *Nicotiana sylvestris* and *Arabidopsis thaliana*, have maximal rates of leaf growth at the beginning of the day and show a base-tip gradient of leaf growth. In contrast, type II plants (for example *Glycine max., Populus deltoides*) show maximal leaf growth at the end of the day and may lack a base-tip gradient (Ainsworth *et al*. 2005; Matsubara *et al*. 2006).

In contrast to leaves, roots are exposed to relatively constant conditions, as the buffer capacity of the soil is very high (Walter *et al.* 2009 and references therein). Thus, root growth is constant when external conditions are stable but can be very sensitive to variations in temperature or soil water potential.

Direct exposure to light can modify the diel patterns of root growth. When roots of *Arabidopsis* seedlings are exposed to relatively high light intensities (50-60 µmol m^{-2} s^{-1}), they show a decrease of the velocity of the root tip (v_{tip}) in the course of the day and an increase during the night, resulting in maximal v_{tip} values around dawn as reported by Schmidt *et al*. in a recent manuscript (submitted). This unusual fluctuation of root growth dynamics disappeared in seedlings that were grown with darkened roots (Fig. 2). The inhibition of root growth velocity upon exposure of the roots to light may be due to ethylene accumulation inside the Petri dish (Eliasson & Bollmark 1998) which was proved by applying the ethylene receptor blocker 1-MCP (Schmidt *et al*., submitted).

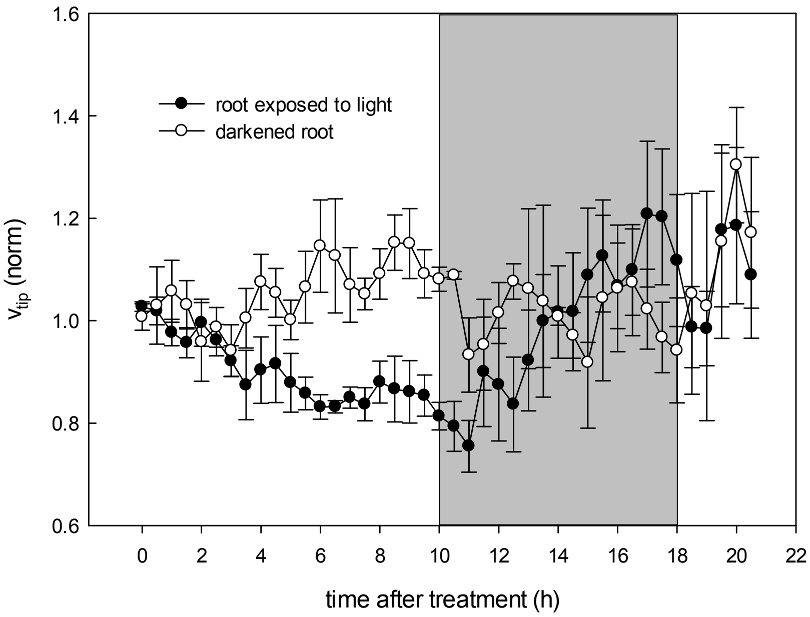

Fig. 2: Normalized values of velocity of the root tip (v_{tip}) of *Arabidopsis thaliana* seedlings whose roots were exposed to light and seedlings whose roots were darkened by wrapping aluminium foil around the Petri dish throughout the growth period. Vtip was normalized to the average value of the population before treatment. Shaded areas indicate the night period. Mean ± SE. N=4.

1.6.2 Regulation of leaf and root growth

The growth processes of leaves and roots are controlled biomechanically, metabolically and via hormones (reviewed in Walter *et al*. 2009). Biomechanical control includes turgor and cell wall extensibility. Root growth is sensitive to changes in turgor, caused by alterations in environmental conditions such as rapid increases in the light intensity (Nagel *et al*. 2006), or by feeding of herbivores (Hummel *et al.* 2007; Schmidt *et al*., submitted).

Concerning metabolites, carbohydrates are recognized to be essential for root growth (Freixes *et al*. 2002; Nagel *et al*. 2006) but also for leaf growth processes (Wiese *et al.* 2007). Besides sucrose, nitrogen is of importance in maintaining leaf growth following herbivore attack as shown in the experiments performed on cotton plants by Schmidt *et al*. (2009).

1.7 Impact of foliar wounding, herbivory and pathogen attack on growth processes

1.7.1 Leaf growth processes

Plant growth is affected by abiotic and biotic stressors mainly due to decrease in nutrient uptake and reduced photosynthetic rate (Starck 2006). Wounding and biotic stress is often correlated with metabolic costs for synthesis of defence compounds which might counteract growth processes, as it is assumed by the ODH.

Young leaves are known to grow faster than old leaves, as it was shown for several plant species, such as *Arabidopsis thaliana* (Barto & Cipollini 2005) or cotton (Schmidt *et al*. 2009). Thus, they are expected to suffer more from defoliation by herbivores as they act as sinks and thus have less storage reserves than older leaves.

A reduction of the leaf area following repeated wounding was reported for *Nicotiana benthamiana* (Zhang & Turner 2008) while excision of cucumber seedlings caused a sustained inhibition of hypocotyl growth (Stahlberg & Cosgrove 1995). Repeated wounding of *Arabidopsis* leaves reduces the leaf area in a similar way to the effect of MeJA application to the leaves, suggesting that wound-induced jasmonates suppress growth by reducing cell divison (Zhang & Turner 2008).

Phloem-sap-feeding herbivores such as planthoppers are known to increase leaf mortality and decrease the production of new leaves of *Spartina alterniflora* (Olmstead *et al.* 1997). Herbivores that feed on the cell content via stylets, such as spider mites or thrips, can have different impacts on the leaf growth of cotton. Mild spider mite infestation on one cotton leaf does not affect local or systemic leaf growth of cotton plants after 4 days, while the total leaf area of cotton plants can be reduced by 30% by thrips after cessation of infestation (Lei & Wilson 2004). The recovery of leaf area after exposure of cotton plants to thrips was neither attributed to improved carbon assimilation, nor to increased allocation of biomass for leaf construction, but to changes in the pattern of development, e.g. earlier expansion of leaves. The differences between the effects on leaf growth observed after thrips feeding (Lei & Wilson 2004), and spider mite attack (Schmidt *et al.* 2009), could have been due to smaller damage to the cotton leaves by the mites, so that growth processes of the attacked leaf were less affected. It is hypothesized that the accumulation of sucrose and N was responsible for maintaining leaf growth of cotton leaves upon mild spider mite attack. This suggestion is underpinned by the study of Cazetta *et al.* (1999) which showed that both nitrogen and sucrose are essential for maximum growth of maize kernels.

Herbivory by chewing-biting *Spodoptera littoralis* larvae on tomato increased water loss at the sites of feeding but did not have an effect on leaf thickness (Alarcon & Malone 1994). In cotton, caterpillar feeding for 48 hours caused a decrease of the relative growth rate of the affected leaf, probably due to reduced water content, as demonstrated by Schmidt *et al.* (2009). This is in accordance with *Nicotiana attenuata* treated with *Manduca sexta* regurgitant following mechanical wounding, which resulted in a decrease of the relative growth rate of the attacked leaf within 8 hours, while systemic leaf growth was less affected (Hummel *et al*. 2007). In contrast, Moore *et al*. (2003) found that removal of less than 5% of a fully expanded leaf of *Rumex obtusifolius* by two *Gastrophysa viridula* larvae reduced the expansion rate of newly developing leaves, which was probably caused by an up-regulation of the activity of cell wall peroxidase, leading to cell wall rigidification, and thus to a limitation of leaf growth (Moore *et al.* 2003). Seedlings of *Sinapis arvensis* had reduced plant height and shoot mass, as well as delayed onset of flowering, when being attacked by *Pieris rapae* larvae, but this was compensated later on so that reproduction was not affected (Poveda *et al.* 2003).

1.7.2 Root growth processes

Root growth can be reduced in many plant species by application of jasmonates to the growth medium (Staswick *et al.* 1992; Feys *et al.* 1994; Vellosillo *et al.* 2007; Yan *et al.* 2007; Henkes *et al.* 2008). For *Nicotiana sylvestris*, wounding-induced JA was shown to be transported from the treated leaf to younger leaves and roots, but not to older leaves (Zhang & Baldwin 1997). As JA is important for growth processes of plants, changes in root growth are expected to occur

following wounding, herbivore feeding, pathogen attack or application of signalling substances due to increased transport of jasmonates to the root.
When MeJA was repeatedly applied to leaves of *Arabidopsis thaliana*, the leaf size was reduced significantly in the study of Zhang & Turner (2008) due to reduced cell division, but no effects on root growth were detected by Schmidt *et al.* (submitted). As JA or MeJA application to plants is often used to mimic herbivory-induced JA signalling, it is expected that herbivore feeding can also reduce root elongation. In seedlings of the ecological model plant *Nicotiana attenuata*, root growth was reduced more than leaf growth upon simulated herbivory with *Manduca sexta,* or application of MeJA to the leaves (Hummel *et al*. 2007 & 2009), which was ascribed to JA signalling. While Hummel *et al.* (2007 & 2009) used single wounding treatments, it was proposed that multiple treatments are more realistic as they more closely mimic the feeding behaviour of caterpillars. This hypothesis was not confirmed, as multiple wounding reduced root growth of *Nicotiana attenuata* to the same extent as single wounding treatments (Fig. 3).

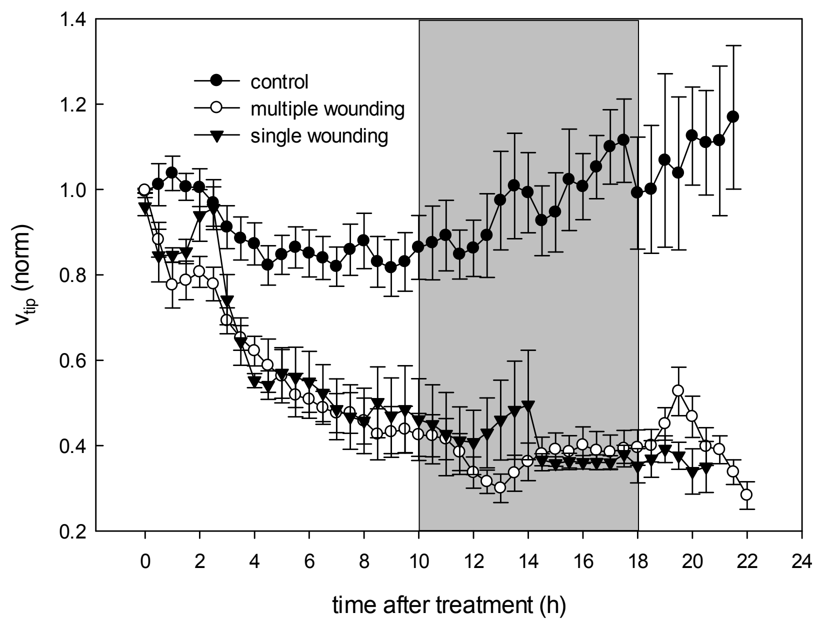

Fig. 3 Root growth dynamics of *Nicotiana attenuata* seedlings after single and multiple wounding treatments. Vtip was normalized to the average value of the population before treatment. Wounding treatments were applied at time points 0 h (single treatments) or at the time points 0 h, 2 h and 4 h (multiple treatments). Controls were not treated. Shaded areas represent the night period. Mean ± SE. N = 5-8.

Multiple wounding and/or OS treatments showed comparable results as those reported for single treatments by Hummel *et al.* (2007) for up to 3 days. (Fig. 4). Thus, a single wounding procedure, combined with application of bacteria suspension or herbivore oral secretions, is shown to be sufficient to elicit plant responses.

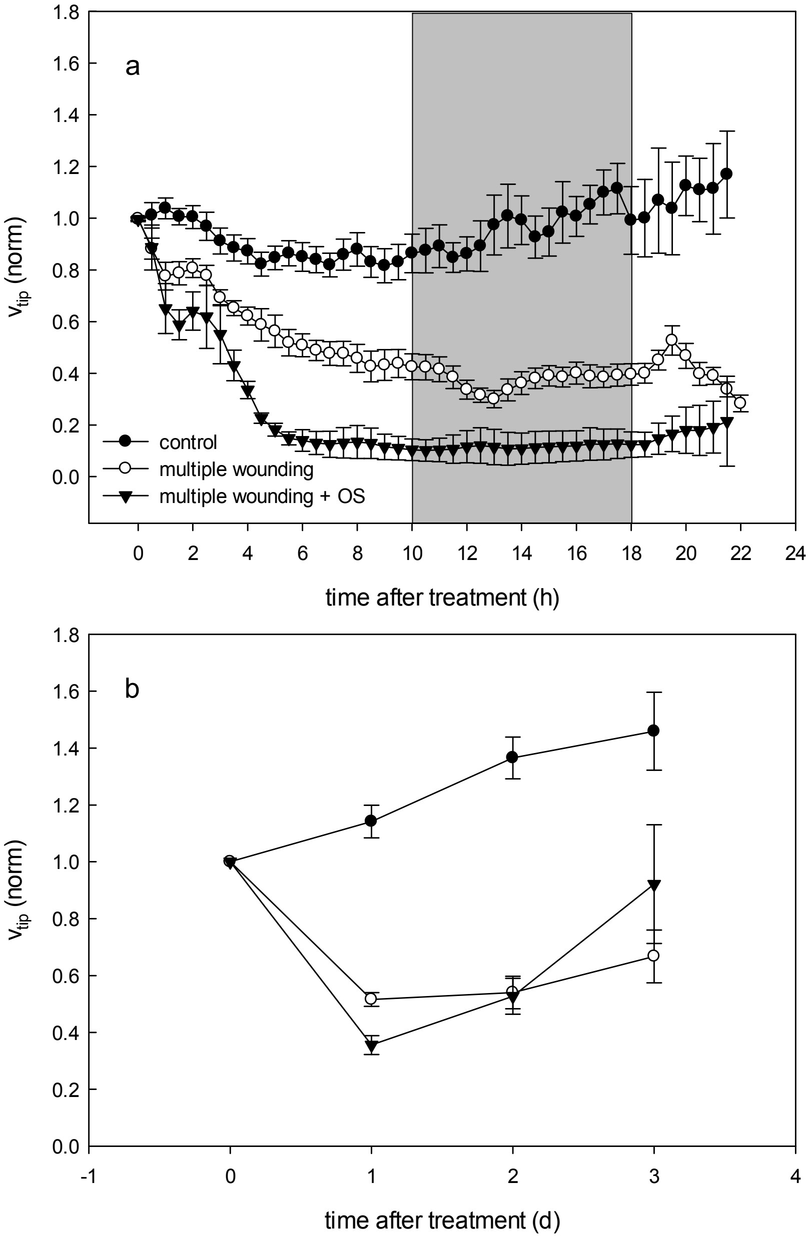

Fig. 4 Root growth dynamics of *Nicotiana attenuata* seedlings after multiple wounding and multiple wounding with subsequent application of oral secretions of *Manduca sexta* (OS) at high temporal resolution over 24 hours (a) and at a lower resolution over 3 days (b). Vtip was normalized to the average value of the population before treatment. Wounding treatments were applied at time points 0 h, 2 h and 4 h on day 0. Controls were not treated. In (a), shaded areas represent the night period. Mean ± SE. N = 5-8.

To extend the results on root growth dynamics following specialist herbivory to real herbivores, freshly hatched *Manduca sexta* larvae were allowed to feed on leaves of *Nicotiana attenuata* seedlings. Mean feeding times were 3-7 minutes, which already caused reductions of root growth for 3 hours in comparison to untreated control plants (Fig. 5). The differences of root growth depression following larvae feeding, and a single `wounding + OS` treatment, can be ascribed to less severe damage by the caterpillar in comparison to the commonly used practice to simulate herbivore feeding (mechanical wounding and subsequent application of caterpillar oral secretions).

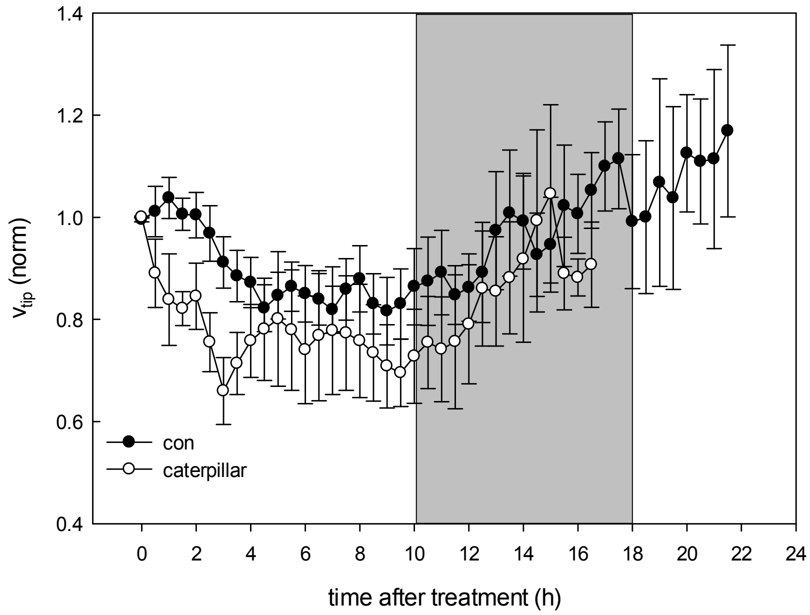

Fig. 5 Root growth dynamics of untreated *Nicotiana attenuata* seedlings (con) and of seedlings attacked by freshly hatched *Manduca sexta* larvae (caterpillar). Vtip was normalized to the average value of the population before treatment. Larvae were feeding for 3-7 minutes. Shaded areas represent the night period. Mean ± SE. N = 5-8.

Older seedlings (4 weeks-old) of *Nicotiana attenuata* showed similar patterns of root growth reductions upon wounding and/or simulated herbivory, compared to the younger seedlings investigated by Hummel *et al.* (2007 & 2009) (Fig. 6).

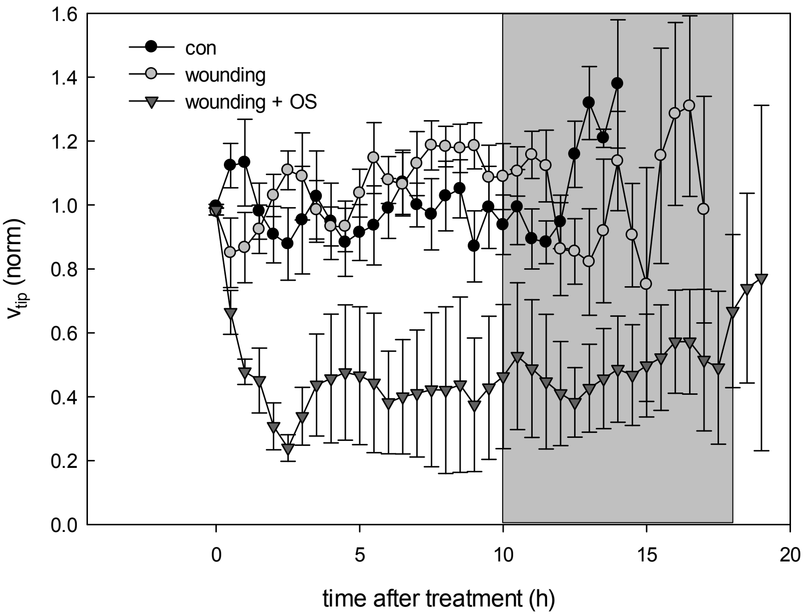

Fig. 6 Root growth dynamics of 4-to-5-weeks-old *Nicotiana attenuata* seedlings after single mechanical wounding, and after single mechanical wounding with subsequent application of oral secretions of *Manduca sexta* (OS). Vtip was normalized to the average value of the population before treatment. Shaded areas indicate the night period. Mean ± SE. N = 4-7.

In contrast, application of *Spodoptora littoralis* regurgitant to wounded leaves of *Arabidopsis* seedlings did not have any effect on root growth compared to a mere wounding treatment, as reported by Schmidt *et al.* (submitted). Upon infection with the avirulent bacteria *Pseudomonas syringae* pv. tomato DC3000 avrRpt2, root growth of *Arabidopsis* was reduced, which Schmidt *et al.* (submitted) ascribe to the bacterial toxin coronatine rather than to JA.

1.8 Ecological significance of defence strategies

When faced by biotic stress, plants are in the dilemma to invest their resources in either growth or defence. The *growth-differentiation balance hypothesis* (GDBH) predicts that, as defence is costly, plant parts with slow growth contain more resources that are available for defence while faster growing plant parts are less defended (see Barto & Cipollini 2005). Thus, young leaves that grow fast have lower levels of defence compared to older, slow-growing leaves (Barto & Cipollini 2005). This is in accordance with the results on cotton obtained by Schmidt *et al.* (2009) showing that leaves with lower growth rates accumulate more nitrogen and sucrose following spider mite attack, which suggests increased investment in growth processes. In the same leaves, more changes in the concentrations of defence-related free amino acids were found after caterpillar feeding, compared to younger leaves with a higher growth rate, which implies that older leaves are better defended.

In contrast, the *optimal defence hypothesis* (ODH) predicts that the most valuable parts of a plant contain more defensive compounds or structures than less valuable parts. This hypothesis is based on the assumption that defence is preferably located in plant parts with both a high fitness value for the plant and also a high risk of herbivore attack (Stamp 2003; Barto & Cipollini 2005). During the vegetative growth phase, the leaves, which are still developing, represent the most valuable parts, as their photosynthetic potential is higher than that of older leaves (Barto & Cipollini 2005; Schmidt *et al.* 2009). The young leaves act as sink but as they are important for the future fitness of the plant, they receive defensive compounds from the mature leaves (see e.g. Radhika *et al.* 2008). Nevertheless, in the study of Schmidt *et al.* (2009), there were only very little differences between young, rapidly growing leaves (referred to as \`systemic\`, see Schmidt *et al.* 2009) and leaves which almost reached their final size (referred to as \`local\` leaves) in terms of gossypol concentrations after attack of herbivores to the \`local\` leaves (unpublished data). In consequence, for cotton, faced with the early stages of spider mite infestation or caterpillar attack, the ODH has to be rejected while the GDBH can be confirmed.

The balance between shoot and root growth has not yet been addressed in literature adequately. Hence, it is an open question how insect attack affects this balance and how this relates to defence strategies. In this PhD thesis, important results were achieved to this end by investigation of seedling systems, where root and shoot growth could be studied at the same time. Root growth was chosen as the major subject of investigation as other studies have already dealt with seedling leaf growth under the influence of herbivory (for instance see Hummel *et al.* 2007). Seedlings are increasingly coming into the focus of research, as they are more vulnerable to herbivory-induced mortality than adult plants (Fenner 1987). Several studies showed that defence responses of young plants are different from those of adult plants. For instance, juvenile *Raphanus sativus* plants were less tolerant, and had higher glucosinolate concentrations upon defoliation than plants in the reproductive stage of their ontogeny (Boege *et al.* 2007). Saplings of the tropical tree *Casearia nitida* compensated a 75%-loss of foliage by mobilizing stored resources in order to replace the lost tissue, while reproductive trees were not able to do this (Boege 2005). In seedlings of *Nicotiana attenuata*, proteinase inhibitors are not inducible by *Manduca sexta* feeding, which differs from adult plants (van Dam *et al.* 2001).

It is important to note that seedlings are in the more severe dilemma than adult plants, on whether to allocate their limited resources to growth or to the production of defensive substances (Barton 2008). For instance, *Plantago lanceolata* seedlings compensated severe defoliation by specialist caterpillars by increasing growth and decreasing chemical defence, while older plants showed chemical induction at the expense of root biomass (Barton 2008). The same may be true for *Arabidopsis thaliana* seedlings, whose root growth velocity is not affected by wounding or herbivory by a specialist caterpillar (Schmidt *et al.*, submitted). These data pointed out that seedlings use different strategies compared to adult plants when they are attacked by biotic stressors. It seems that seedlings invest more of their resources into growth processes of roots. Growth of the belowground organs can thus either be continued while the attacker is feeding (see *Arabidopsis*, Schmidt *et al.*, submitted), or the reserves are stored in the roots, thus enabling rapid continuation of growth when the

threat has passed (see *Nicotiana attenuata*, Schwachtje *et al*. 2006; and *Populus tremuloides*, Babst *et al.* 2005).
As plants live in different habitats, they are faced with different strengths and modes of herbivory and pathogen attack. Schmidt *et al*. (submitted) demonstrated that wound-induced JA does not affect root growth in *Arabidopsis*, while this is not the case for *Nicotiana attenuata* (Hummel *et al*. 2007 & 2009). Hence, the same wound signal triggers different root growth reactions in the two species. This difference in growth response might be explained by the ecologies of the habitats in which the plants evolved. *Arabidopsis* is found in temperate climates and has to cope with shorter growth periods than *Nicotiana,* which is a subtropical plant. *Arabidopsis* therefore has to finish its life cycle more quickly than *Nicotiana attenuata,* and does not suffer from high herbivore pressure. For instance, no specialist herbivore is known to attack *Arabidopsis*. Thus, *Arabidopsis* is focused on a rapid turnover and adjusts its growth response only marginally upon wounding, by suppressing the rate of leaf cell division. The reduced cell numbers require fewer nutrients, which saves resources for the completion of the life cycle of the plant (Zhang & Turner 2008). In contrast, *Nicotiana attenuata* has longer growth periods and mostly grows in monocultures. Hence, herbivore pressure and intraspecific competition is far higher compared to *Arabidopsis* (Baldwin 2001). Often when *N. attenuata* is attacked by its specialist herbivore *Manduca sexta*, the shoot is entirely defoliated. In this case, tolerance is suggested to be the best strategy for a plant to cope with its species-specific herbivores (Schwachtje *et al.* 2006).

Overall, this study demonstrates that the relationship between plant defence patterns, resource capture and vegetative growth is regulated in a complex manner with different species following different strategies that optimize their fitness within their specific ecological habitat. Moreover, the results reveal limits for the use of model organisms. It seems likely that their responses towards wounding or biotic stress are strongly dependent on the ecological context in which a species has evolved.

2 Synopsis

The objective of this PhD thesis was the investigation of plant growth processes upon wounding or biotic stress by focussing on several time scales, plant species and plant organs.

It was shown that peptides of oral secretions from Lepidopteran larvae induce pore formation in plant membranes, and thus might be important for herbivore-specific signalling processes in the attacked plant. Initial stages of attack by two generalist herbivores, with either of two different feeding mechanisms, did not severely affect the leaf growth of cotton plants. In contrast, specialist herbivores reduced root growth of the annual *Nicotiana attenuata,* while the root growth dynamics of the annual weed *Arabidopsis thaliana* were less sensitive to wound-induced plant-internal jasmonates than to ethylene.

Altogether, this work sheds a light on the regulation of leaf and root growth processes in several plant-herbivore or plant-pathogen interactions, which will be part of the knowledge-base for breeders to improve plant breeding for increased resistance to biotic stress.

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5 Publications of the dissertation

5.1 First publication: Caterpillar regurgitant induces pore formation in plant membranes

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Own contribution:

- Rearing of *Spodoptera littoralis*
- Collection of caterpillar regurgitant
- Discussion

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Caterpillar regurgitant induces pore formation in plant membranes

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Abstract Formation of channel-like pores in a plant membrane was induced within seconds after application of an aqueous solution containing regurgitant of the insect larvae *Spodoptera littoralis*. Gated pore currents recorded on the tonoplast of the Charophyte *Chara corallina* displayed conductances up to several hundred pS. A voltage-dependent gating reaction supports the assumption that pore-forming molecules have amphipathic properties. Regurgitant samples separated into masses smaller or larger than 3 kDa were evaluated by patch–clamp and mass spectroscopy. Fractions containing peptides larger than 3 kDa constituted pores of large conductances, peptides smaller than 3 kDa constituted pores of small conductances. Peptide-free eluates did not constitute conducting pores, indicating that pore-forming components in regurgitant are membrane-spanning oligopeptides.

Keywords: Alamethicin; Caterpillar; Herbivory; Ion channel; Regurgitant; *Chara*

1. Introduction

Plant species like corn, cotton, lima beans and cultivated tobacco that are under attack by herbivorous insects are known to emit a variety of volatile compounds [1–4] that are partially de novo synthesised by the plant in response to herbivory [5]. Several of these plant-emitted volatiles function as signals in tritrophic interactions and may attract parasitoids or predators of the herbivores and therefore function as an indirect defence signal of the plant [2,6–8]. While caterpillar-damaged leaves of corn seedlings emit a complex blend of volatile compounds, mechanical wounding alone led to a far less complex blend. However, the application of caterpillar regurgitant to mechanically injured leaves resulted in a local and systemic emission of volatiles that was comparable to that emitted in response to herbivory [9–11] whereas regurgitant administered to intact leaf surfaces had no effect. Even when the mechanical damage to cotton plants was continued over several days, the systemically emitted amounts of several compounds were quantitatively lower than in those plants that were additionally treated with regurgitant [11]. It appears that a specific elicitor in the regurgitant of the caterpillar enhances the amount of several systemically released volatiles.

A compound isolated from the regurgitant of *Spodoptera exigua* larvae feeding on corn was identified as *N*-[17-hydroxylinolenoyl]-L-glutamine and was shown to induce the emission of herbivore-inducible volatiles when applied to mechanically wounded leaves. An application of this synthesised key component, named volicitin, elicited the production of volatiles [12]. Additional compounds in the oral secretion of *Manduca sexta* have been identified as *N*-linolenoyl-L-glutamine [13], and fatty acid–amino acid conjugates in herbivore oral secretions were shown to be necessary and sufficient for herbivore-specific plant responses and activation of the jasmonate signaling cascade [14]. Besides elicitors from herbivore regurgitant, fungal derived elicitors like alamethicin, an ion channel forming peptaibol from *Trichoderma viride*, may induce the emission of some of the volatiles emitted in response to herbivory. Alamethicin is well-known to produce pores in artificial membranes as well as in animal cell membranes, but has to date not been shown to form ion-channels in plant membranes. In *Phaseolus lunatus*, application of alamethicin induced the production of two homoterpenes and methyl salicylate whereas the complex blend of volatiles could not be observed [15].

From those results, a succession of primary events can be postulated: only intact cells in the neighbourhood of destroyed cells can produce metabolites; regurgitant must contain components (elicitors) triggering a cascade that leads to metabolite production; these components have to cross the plasma membrane to reach their adequate receptor molecules; to cross the membrane, a facilitator must mediate this transit; it appears reasonable that this facilitator molecule is also present in the caterpillar regurgitant. Assuming that the elicitor molecule is notably larger than an ion, the translocator of the elicitor should be a specific carrier-like molecule or simply an unspecific channel that may also transport ions. On artificial planar bilayer membranes, the existence of ion channel-like facilitators has recently been demonstrated [16]. Also elicitors with different functions like cellulolytic enzymes employed in cell wall digestion that had previously been presumed to act on plant cells via receptor binding sites proved to directly interact with membranes by formation of channel-like membrane pores [17].

To demonstrate the existence of an ion channel-like facilitator in caterpillar regurgitant and the pore formation of alamethicin in a plant membrane, we have chosen cytoplasmic droplets derived from internodal cells of the giant green alga *Chara corallina* that are delineated by the original tonoplast [18,19]. These droplets served as a model membrane system to evaluate permeation characteristics. The *Chara* tonoplast contains a highly specific maxi-K channel [19–21], a Ca^{2+}-activated Cl^- channel [22], and, as H^+ pumps, a H^+-ATPase as

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well as a H^+-PPase [23–26]. The H^+ pumps fuelled by ATP and pyrophosphate hydrolysation, respectively, are silent in the absence of their substrates and maxi-K and Cl^- channel can be blocked by Cs^+ ions or by withdrawal of Ca^{2+} ions, respectively. Thus, the *Chara* tonoplast provides a simple but adequate membrane to explore a possible new formation of membrane pores by exogenously applied caterpillar regurgitant and alamethicin.

2. Materials and methods

2.1. Collection of regurgitant of *Spodoptera littoralis*

Third instar larvae of *S. littoralis* (Lepidoptera: Noctuidae), reared on bean diet (shredded beans with vitamins) were placed in a container and fed on cotton leaves for 2–3 days before regurgitant was collected. Regurgitation of oral secretion was induced by holding the caterpillar in the head region with a pair of light-weight forceps. Oral secretion was collected with two 100-μl pipettes inserted in a vial through a septum. One pipette was connected to a low vacuum while the other pipette tip was held in front of the mouth part of the caterpillar. About

Table 1
Conductance levels (g) in picoSiemens (pS) measured from alamethicin-induced pores in different types of membranes

Lipid bilayer	Frog muscle	Rat muscle	*Chara corallina* tonoplast
g/pS			
20	20	27	
110	100	125	202
250	260	340	345
430	460		449
620	660	590	592
	860	840	749
		1090	1033

Results from *C. corallina* tonoplast recordings are compared to data collected from [29] and tabulated for comparison. Matching data are aligned, blank positions are meaningless.

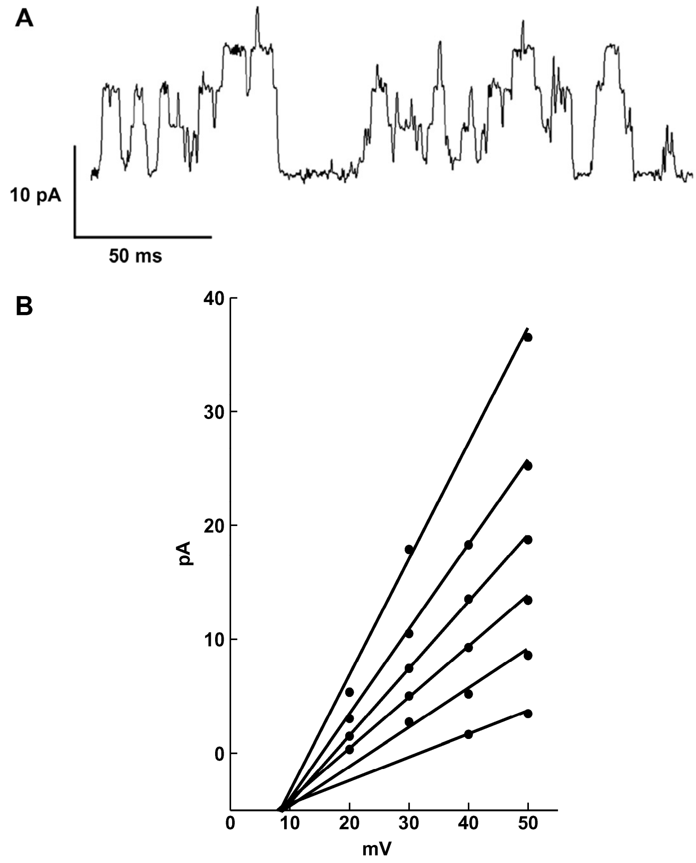


Fig. 1. Alamethicin-induced conductances in the tonoplast of *Chara corallina*. In a cell-attached configuration, formation of a high-resistance seal and the absence of any current fluctuation at positive pipette voltages could be reconfirmed until alamethicin was inserting into the membrane. At negative voltages, current through the native maxi-K channel could be observed from the beginning. (A) Current fluctuations at +40 mV clamp voltage. (B) Voltage-dependency of alamethicin-mediated current. Currents of subsequent amplitude levels emerging in activity bursts were plotted versus clamp voltage. Lines connect the observed current levels that presumably belong to one conductance state. Conductances are summarised in Table 1. Experimental conditions – pipette and bath contained 120 mM CsCl, 5 mM Tris/HCl, pH 7.5; pipette additionally 11 μM alamethicin, 1 kHz Bessel low-pass, 50 μs/sample, R_{pip} 17 MΩ.

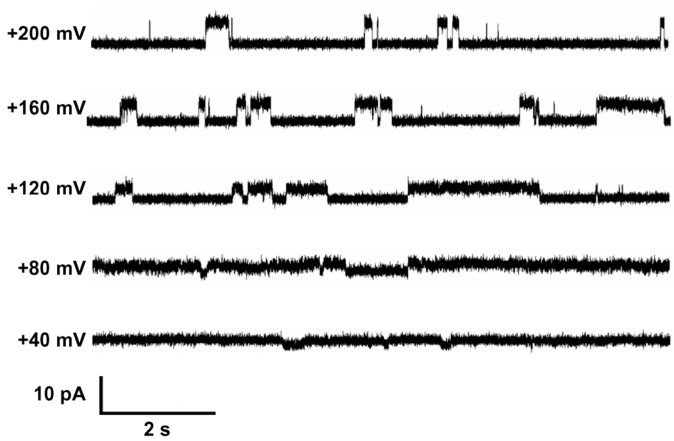


Fig. 2. Current fluctuations occurring after spontaneous insertion of *Spodoptera* regurgitant components in the presence of ethanol contained in the aqueous pipette solution at five different clamp voltages. Clamp voltages are given left-hand side of the traces; closed levels are indicated right-hand side by a short line. Experimental conditions – bathing solution contained 120 mM KCl, 5 mM Tris, pH 7.5; pipette solution: 120 mM CsCl, 5 mM Tris, pH 7.5, 12.6 mg/ml regurgitant, 0.1‰ EtOH; R_{pip} 5.8 MΩ, 1 kHz Bessel low-pass, inside-out configuration.

5–10 µl of oral secretion could be collected per larva. The crude oral secretion was stored in the freezer at −80 °C until a sufficient amount was collected.

2.2. Electrophysiology

C. corallina was cultivated in large plastic buckets at 23 °C under a 12:12 h light:dark regime. Internodal cells of about 10 cm length and 0.8 mm diameter were isolated from adjacent cells and wilted to turgor loss. To prepare cytoplasmic droplets, one cell end submersed in K^+ solution was cut and the cytosol gently squeezed out. Cytoplasmic droplets that formed readily in the bathing solution were picked up with a 20 µl pipette and transferred to the experimental chamber. Those droplets surrounded by the tonoplast were accessible by patch pipettes without further treatment. The solution for droplet preparation and the bathing solution in the experimental chamber contained 120 mM KCl and 5 mM Tris adjusted to pH 7.5. The patch pipettes were filled with solutions containing 120 mM CsCl, 5 mM Tris adjusted to pH 7.5, and additionally the substance under examination.

In cell-attached an inside-out configurations, Cs^+ in the patch pipette did not admit inwardly directed current fluctuations across

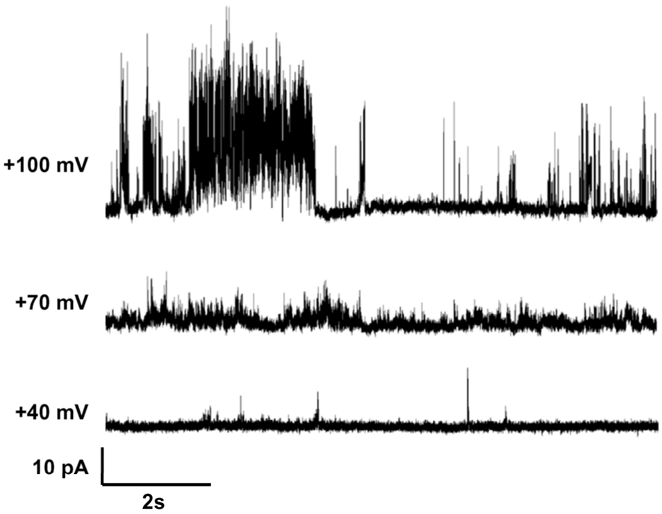


Fig. 3. Current fluctuations occurring after spontaneous insertion of *Spodoptera* regurgitant components contained in the aqueous pipette solution in the absence of ethanol at three different clamp voltages. Clamp voltages are given on the left-hand side of the traces; closed levels are indicated on the right-hand side by a short line. Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris, pH 7.5; pipette solution: 120 mM CsCl, 5 mM Tris, pH 7.5, 5 µl/ml regurgitant; R_{pip}: 15 MΩ, 1 kHz Bessel low-pass, cell-attached.

intrinsic maxi-K channels at positive pipette voltages. Availability of K^+ ions in the bathing solution allowed outward fluctuations at negative pipette voltages, thus providing information on the actual state of the membrane patch. Patch–clamp data were recorded and digitised with a MultiClamp 700A amplifier and Digidata 1322A acquisition hardware (both by Axon Instruments, Union City, CA).

Alamethicin (Fluka, Buchs CH) was presolved in ethanol and diluted to a final concentration of 11 μM in the aqueous pipette solution with less than 1‰ v/v ethanol.

Caterpillar regurgitant was added to the pipette solution containing less than 1‰ ethanol to give final concentrations of 8–13 mg ml^{-1} (depending on the collected sample size).

For a crude separation of molecular masses above 3 kDa and below 3 kDa, regurgitant was diluted into 120 mM CsCl to a final concentration of 11.7 mg ml^{-1} and filtered through a 0.2 μm membrane filter (DynaGard, Spectrum Laboratories, Rancho Dominguez, CA) to remove undissolved particles for patch–clamp experiments. Subsequently, samples were separated according to their molecular weight above and below 3 kDa by filtering through a Microcon cell (YM-3, Amicon, Millipore, Billerica, MA) by centrifugation at $14000 \times g$ for 30 min. Both fractions, $\geqslant$3 kDa and $\leqslant$3 kDa, were analysed electrophysiologically and by mass spectroscopy. To determine whether the pore forming compound is a peptide, regurgitant was further concentrated by ZipTip pipette (Millipore, Billerica, MA). The peptide-free eluate from the ZipTip was tested for pore forming activity by patch–clamp and analysed by mass spectrometry. For subsequent mass spectrometry of peptides, peptides were eluted from the ZipTip with 0.05% trifluoro acetic acid and 50% acetonitrile.

2.3. Mass spectroscopy

Mass spectra were acquired with a MALDI-TOF spectrometer (Bruker Daltonik Biflex III, Bremen D), equipped with a multiprobe inlet and N_2 laser (337 nm, 3 ns pulse width). For the analysis of proteins, the linear-mode technique with an acceleration voltage of 19 kV and the reflectron voltage of 15.1 kV was applied. An ethanolic (<1‰) solution containing 117 mM CsCl, 4.65 mM Tris/HCl and 2.55 μM alamethicin was used for analysis. This solution was 1:100 diluted with a 0.1% aqueous solution of trifluoroacetic acid, and 5 μl thereof mixed with 5 μl of the matrix solution that was prepared as a 2:1 mixture of 0.1% trifluoroacetic acid and acetonitrile. CHCA (α-cyano-4-hydroxycinnamic acid) was added to a concentration of 20% w/v, and 1 μl of this final solution deposited on the Bruker Scout 26 target (dried droplet method). Mass spectra were acquired with delayed extraction of 200 ns. Angiotensin II (*m/z* 1047.2 $[M+H]^+$ avg.), ACTH (adrenocorticotropic hormone fraction 18–39, *m/z* 2466,73 $[M+H]^+$ avg.), and cytochrome *c* (*m/z* 12361.09 $[M+H]^+$ avg.) were used as standards for calibration of the spectra, supported by software XTOF version 5.1.0 (Bruker Daltonics), as described elsewhere [27].

Of *Spodoptera* regurgitant (47.66 mg stocked in 1 ml of 120 mM NaCl), 200 μl were desalted and concentrated to 5 μl using a C_{18} Millipore ZipTip pipette (Millipore) before analysis by mass spectrometry. Those pipettes are applicable for peptides and proteins of low molecular weight up to 50 kDa. Of the concentrated sample, mixed 1:1 with matrix solution, 1 μl was deposited on the target. For calibration of the spectra, the same standards were used as mentioned above. Size fractionated regurgitant samples and peptide-free and peptide-enriched samples were prepared as described above and analysed with the same method.

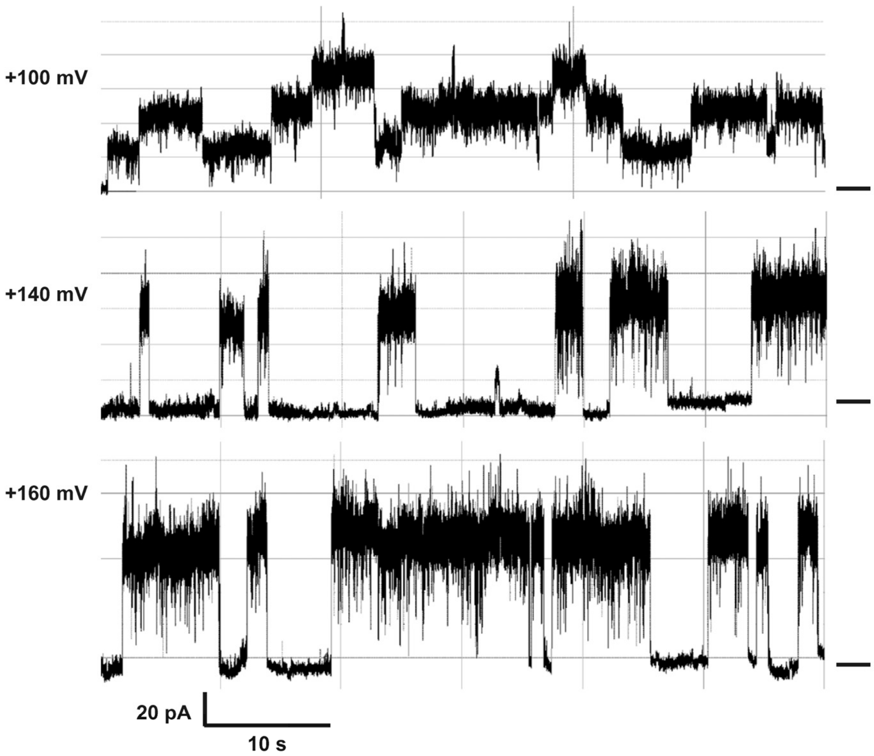


Fig. 4. *Spodoptera* regurgitant components forming high-conductance pores after spontaneous insertion into *Chara* tonoplast. Clamp voltages are given on the left-hand side of the traces; closed levels are indicated on the right-hand side. Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris/HCl, pH 7.5, pipette solution: 120 mM CsCl, 5 mM Tris/HCl, pH 7.5, 12.6 mg/ml regurgitant, 0.1‰ ethanol; R_{pip}: 5.2 MΩ, 20 kHz Bessel low-pass, inside-out.

3. Results and discussion

3.1. Alamethicin-induced pores in a plant membrane

To investigate the ability of alamethicin to induce pore formation in a plant membrane of *Chara*, alamethicin was applied to the *Chara* tonoplast at a concentration of 11 μM as a component of the patch pipette filling solution. Within about 30 s of administration of alamethicin in a cell-attached configuration, the first Cs^+-conducting pores could be recorded (Fig. 1A). The different conductances of an alamethicin channel were obtained by measuring current levels at varying clamp voltages and plotting the current–voltage relationship for the detected conductant states (Fig. 1B). Alamethicin channels formed in the *Chara* tonoplast displayed conductance levels similar to those reported from artificial planar lipid bilayers, and frog or rat muscle membranes [28] showing that a spontaneous incorporation of alamethicin into the plant membrane may facilitate the intrusion of otherwise impermeant substances (Table 1). Conducting pores formed after administration of alamethicin at both membrane faces, the vacuolar as well as the cytosolic face, depending on the polarity of the applied voltage. Only a positive potential at the *cis*-side (locus of alamethicin application) compared to a more negative potential at the *trans*-side of the membrane, led to the formation of conducting pores. This is in accordance with observations on tobacco suspension cells where the tonoplast remains impermeable for low-molecular-mass molecules in response to alamethicin treatment [29]. The transitions between conductance levels are obviously faster than those reported for alamethicin pores in lipid bilayers. One reason may be that the commercially obtained alamethicin is a mixture of alamethicin analogues, wherein the Rf-30 component, the so-called major component, is contained by at least 50%. In lipid bilayers, functional pores formed by different alamethicin classes, like the Rf-50 type or mixtures of them, exhibit marked variances in dwell times for their conductance levels. The mean life time of the Rf-50 type of alamethicin, e.g., is by a factor of 5–10 lower than that of the Rf-30 type [30–32].

The oligopeptide alamethicin, well-known to produce pores in artificial and in animal cell membranes, and to elicit the production of volatile compounds in plant cells [15] is shown here to form conductive pores depending on the polarity of the applied voltage in plasma membranes of Charophytes, the class of green algae which is considered to be the immediate predecessor of land plants [33]. *Chara* qualifies as an excellent membrane model system, particularly seen in combination with its adequacy to serve as a heterologous expression system for, e.g., ion channels of mammal tissues provenience like acetylcholine receptor [34], $5\text{-}HT_3$ serotonin receptor and CX32 gap junction protein (Lühring, unpublished data).

3.2. Caterpillar regurgitant contains a pore-forming compound

To explore the ability of caterpillar regurgitant to induce the formation of pores in plant cell membranes, patch pipettes were filled with an aqueous solution containing CsCl, Tris buffer and *Spodoptera* regurgitant. Setting the clamp voltage of the pipette in a cell-attached configuration to positive values of about +50 mV, the incorporation of pore-forming molecules could be detected by the induction of current fluctuations. With a positive pipette voltage, Cs^+ prevents a current through the tonoplast-resident maxi-K channel and the observed current therefore had to be ascribed to an alien conductance induced by one or more compounds in the regurgitant. At negative pipette voltage, current fluctuations through the native maxi-K channel proved the integrity of the membrane. After patch excision into the inside-out configuration, current bursts were recorded at various clamp voltages (Fig. 2). Regurgitant-induced pores appear to be unspecific at least to monovalent cations, because changes in current amplitudes and also in reversal voltages could not be observed (results not shown). It is noteworthy that the presence of ethanol in the pipette solution (<1‰ v/v), increased the life-time of current bursts of regurgitant-induced channels significantly (Fig. 2), whereas current fluctuations through the membrane-integrated regurgitant components in the absence of ethanol were rather short-lived (Fig. 3). Components in *Spodoptera* regurgitant may constitute pores of unusual long open times and very high conductance (Fig. 4). The long-lived open state of those high-conductance pores is extremely noisy and more than 10-fold that of the open state observed for low-conductance pores (Fig. 2), whereas the closed states of low- and high-conductance pores display a similar noise. Despite unusual great noise, current fluctuations could be recognised as single-channel events. Current fluctuations, recorded at a high sampling rate (100 kHz) were conditioned with an anti-aliasing filter set to 20 kHz corner frequency in order to prevent loss of information. These current fluctuations suggest that the regurgitant-induced membrane pores are not uniform, but rather that their formation should be very complex. This could be due to a changing number of components forming the transmembranal structure or perhaps to a single unit that may vary its configuration over a wide range. Application of volicitin, a compound isolated from caterpillar regurgitant that induces volatile emissions in plants [12], formed pores that exhibited a similar noisy current after reconstitution in artificial bilayers

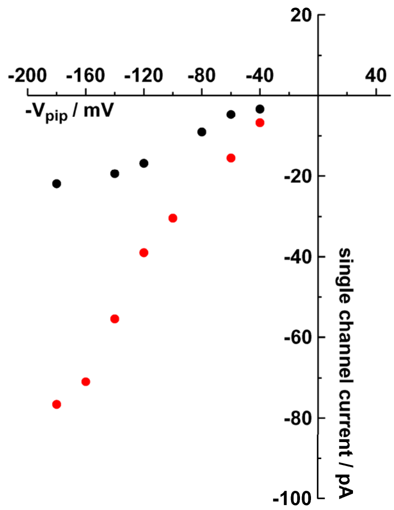


Fig. 5. Current–voltage relationships of *Spodoptera* regurgitant-induced membrane conductances. Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris/HCl, pH 7.5, pipette solution: 120 mM CsCl, 5 mM Tris/HCl, pH 7.5, 12.6 mg/ml regurgitant, 0.1‰ ethanol; 2 kHz Bessel low-pass, inside-out.

[16] as we observed for raw caterpillar regurgitant in plant membranes. However, in artificial bilayers, raw caterpillar regurgitant induced almost noise-free signals [16].

To detect and ascertain various conductance levels by means of amplitude histograms, whole data files were partitioned into time-limited fractions, rather than using the entire 1-min recording. Peak values of fractionated amplitude histograms, which were generated from recordings with apparently one conductant level, allowed constructing current–voltage relationships (Fig. 5). Here, conductances were determined to be

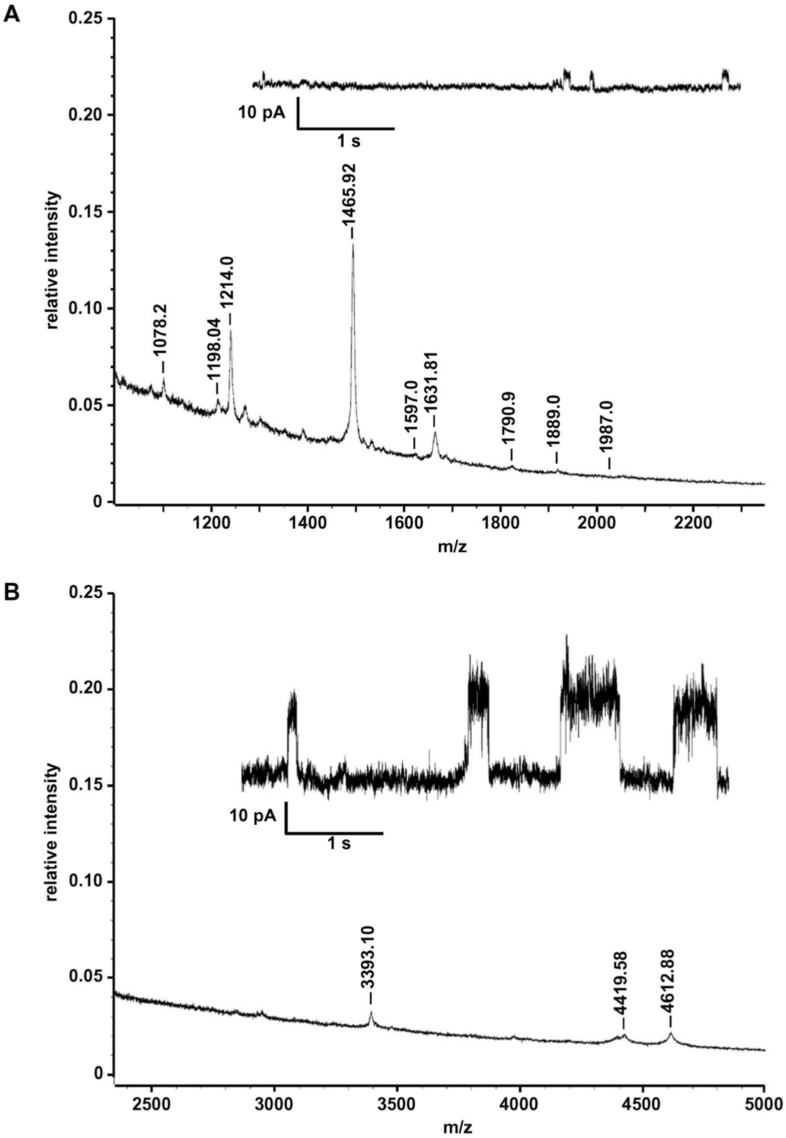


Fig. 6. Distributions of molecular masses of peptides A: smaller than 3 kDa (A) and larger than 3 kDa (B), from *Spodoptera* regurgitant dissolved in 120 mM NaCl and analysed by linear-mode MALDI-TOF MS. For comparison with the sample, calibration standards were deposited on the same target. Mass spectra of 234 µg each of *Spodoptera* regurgitant were generated in the presence of less than 30 nmol NaCl. The insets display electrical conductances typically found after spontaneous insertion of regurgitant compounds into *Chara* tonoplast. Conductances correspond to their fraction of molecular masses. Current traces recorded at +100 mV, cell-attached configuration, R_{pip} 5 MΩ (A) and +140 mV, inside-out configuration, R_{pip} 15 MΩ (B), low-pass filtered at 1 kHz.

about 167 pS and 517 pS, respectively. Obviously, there are even higher conductances which, however, could not be assigned to single pores. Size fractionation of regurgitant revealed that compounds in the regurgitant of molecular size below 3 kDa induced only formation of small pores with approximately 50 pS (Fig. 6A) in patch–clamp experiments, whereas compounds with a molecular size of more than 3 kDa induced the formation of large pores (ca. 600 pS, Fig. 6B). However, small amounts of low molecular mass compounds could not be excluded from the ⩾ 3 kDa fraction. We

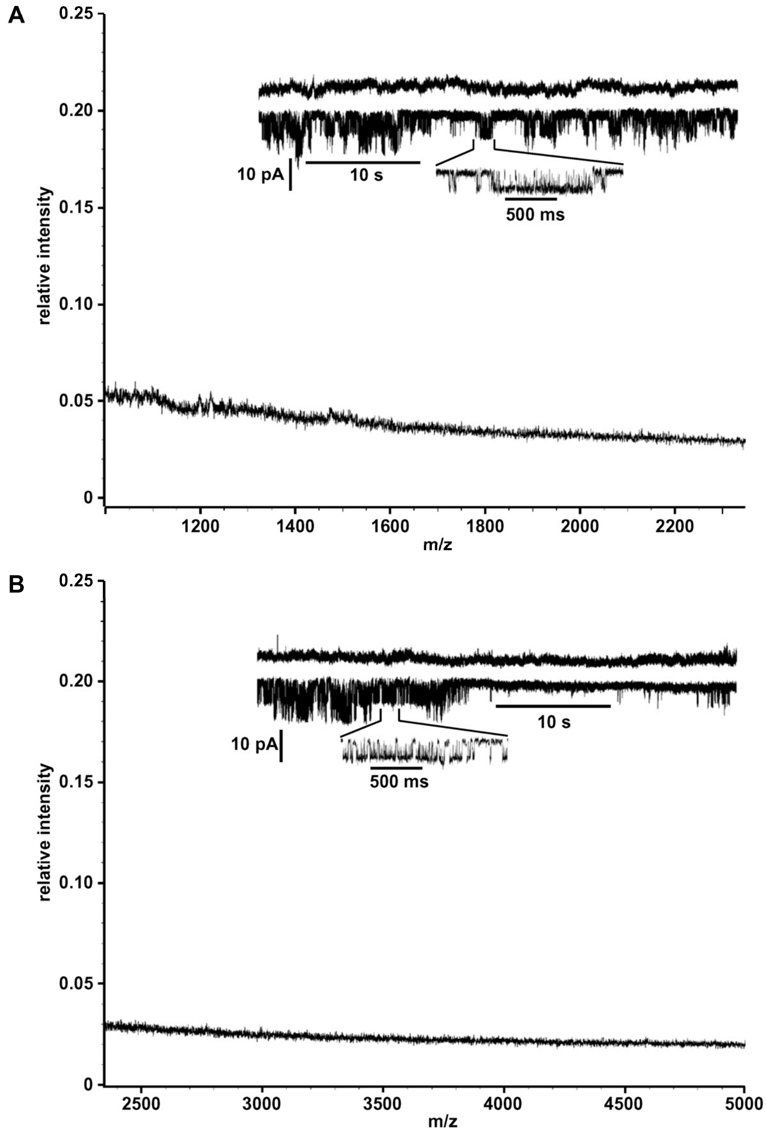


Fig. 7. Mass spectra after mass separation of *Spodoptera* regurgitant solutions from which peptides had been specifically removed by ZipTip treatment: (A) ZipTip-treated fraction smaller than 3 kDa. (B) ZipTip-treated fraction larger than 3 kDa. *Spodoptera* regurgitant had been dissolved in 120 mM NaCl, spectra were generated by linear-mode MALDI-TOF MS. For comparison with the sample, calibration standards were deposited on the same target. Less than 30 nmol NaCl were present in samples which contained 234 µg of *Spodoptera* regurgitant each. The insets display patch–clamp recordings on *Chara* tonoplast at the respective condition. Upper traces: no current events at peptide-free conditions, lower traces: membrane integrity shown by maxi-K channel activity at negative clamp voltage (low-pass filtered, 1 kHz).

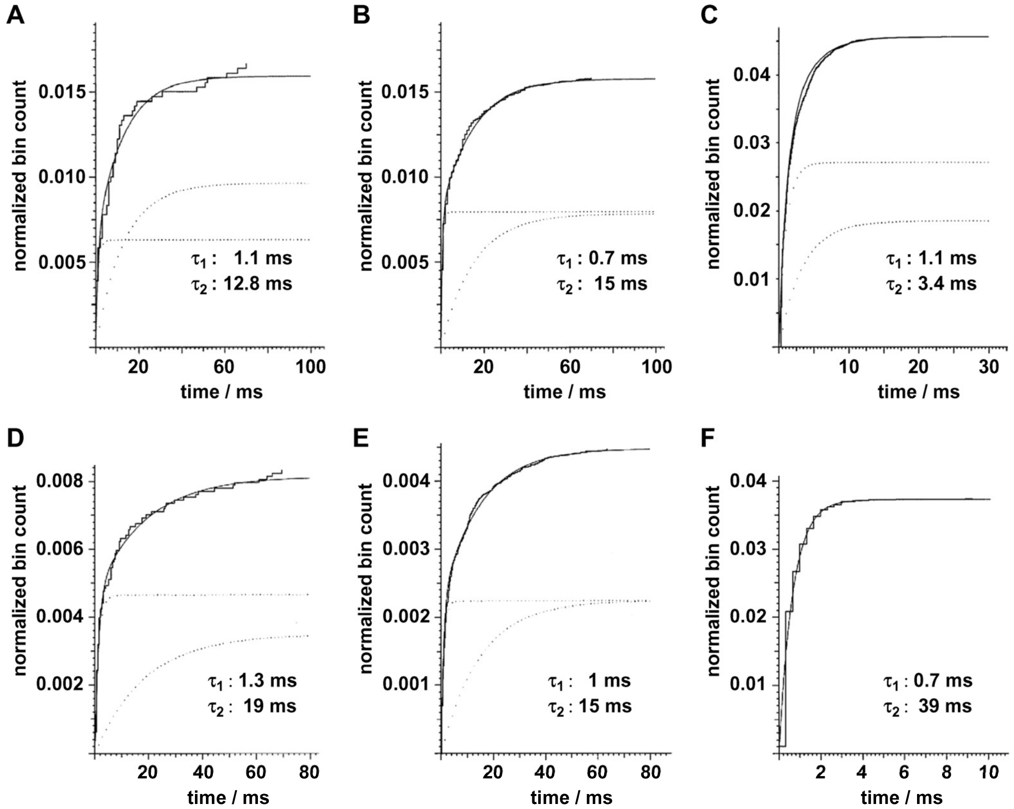


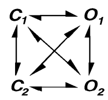
Fig. 8. Distributions of open and closed dwell-times of the channel induced by *Spodoptera* regurgitant at different clamp voltages and at high temporal resolution, sampled with 100 kHz and conditioned by a Bessel low-pass filter set to 20 kHz corner frequency. Open dwell-time distributions at +200 mV (A), +160 mV (B), and +120 mV (C). Closed dwell-time distributions at; +200 mV (D), +160 mV (E), and +120 mV (F). Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris/HCl, pH 7.5; pipette solution: 120 mM CsCl, 5 mM Tris/HCl, pH 7.5, 12.6 mg regurgitant, 0.1‰ ethanol.

can therefore conclude that compounds with masses above 3 kDa are necessary for the formation of large pores, either in combination with small amounts of lower molecular size compounds or by itself. Regurgitant filtered to specifically remove any peptides did not induce any formation of conducting pores in the plant membrane (Fig. 7A and B), although membrane patches were intact as shown by the activity of maxi-K channels at negative pipette voltage. These results strongly suggest that the compounds responsible for the formation of pores are peptides.

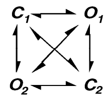
Frequently, open states could not be assigned to the appearance of a second (or third) pore, or reliably be diagnosed to be a subconductant state of the previously observed pore (Fig. 4, +100 mV trace). Nevertheless, kinetics of channel gating could be analysed in some recordings showing single events. For three recordings on one membrane patch mean open and closed life-times could be determined (Fig. 8A–F). Both, closed state as well as open state decayed in two reaction steps. The two open states were clearly distinguishable by their mean life times of about 1 ms (O_1) and 15 ms (O_2). Similarly, the closed states decayed with 1 ms (C_1) and 15 ms (C_2), respectively. Both rapidly decaying states, O_1 and C_1, were voltage-independent, at least over the range of 200–120 mV pipette voltage, whereas the slowly decaying states, O_2 and C_2, appeared to be voltage sensitive when stepping the pipette voltage from 160 to 120 mV. While the time constant of the long-lived open state O_2 was reduced from 15 ms to about 3 ms, the time constant of the long-lived closed state C_2 increased concomitantly from about 15 to almost 40 ms at the same voltage step. The simultaneous change of both time constants suggests a direct linkage in the gating reaction between O_2 and C_2. The time constants of O_1, O_2 and C_1, belong to transitions during burst activity. Since the open state may be abandoned via two different pathways, where only one of those is voltage-sensitive, the

$$C_1 \leftrightarrow O_1 \leftrightarrow O_2 \leftrightarrow C_2$$

Scheme 1.



Scheme 2.



Scheme 3.

voltage-dependent pathway may lead out of the burst into C_2, leaving the C_1–O_1 reaction pathway unaffected.

Here we propose minimum schemes that may represent gating reactions, in which C_1–O_1–O_2 constitute the burst. Scheme 1 allows a transition into C_2 only from O_2, while in Schemes 2 and 3, as an alternative, C_2 may be attained from all other states C_1, O_1 or O_2. Transitions between O_1 and O_2, and C_1 and C_2 are permitted.

3.3. Mass spectroscopy

To analyse the chemical composition of our alamethicin and to carry out a first analysis of peptides in caterpillar regurgitant that may contribute to pore formation MALDI-TOF mass spectroscopy was employed. Spectrum analysis of alamethicin shows a peak with a *m/z* value of 2098.43 Da, which corresponds to the mean values of the alamethicin–Cs complex $[\text{alamethicin} + \text{Cs}]^+$, comprising 132.9 Da for Cs and 1965.53 Da for alamethicin. The range of peptides from *Spodoptera* regurgitant covered masses from 1 kDa up to 5 kDa. The molecular weight distribution of peptides in samples of 234 μg of *Spodoptera* regurgitant shows 12 peptides, greater than 1 kDa each (Fig. 6). Surprisingly, it contained a peptide which exhibits a mass close to that of alamethicin, namely 1987.0 $[\text{peptide} + \text{Na}]^+$, comprised 23 Da for Na and 1964.0 Da for the peptide. Analysis of regurgitant in fractionated samples of $\geqslant 3$ kDa and $\leqslant 3$ kDa confirmed that fractions contained the expected sizes with the $\geqslant 3$ kDa sample containing small amounts of the smaller fragments (Fig. 6A and B). Analysis of the eluate from the ZipTip-treated fractions revealed that none of them contained peptides (Fig. 8).

3.4. Conclusion

Regurgitant of insect larvae feeding on leaves contain components which spontaneously insert into plant cell membranes and form ion channel-like pathways. Noisy current through the conducting pores suggests that pore formation could be due to a changing number of components forming the transmembranal structure or perhaps to a single unit that may vary its configuration over a wide range. Size fractionation into compounds $\leqslant 3$ kDa showed only formation of small pores, whereas fractions $\geqslant 3$ kDa showed formation of large pores. Those pores exhibit voltage-dependent gating reactions, supporting the assumption that the pore-forming molecules have amphipathic properties. With mass spectroscopy, a number of peptides contained in caterpillar regurgitant were detected, several of them large enough to completely bridge the membrane. Removal of peptides from regurgitant completely removed the ability of the regurgitant to induce pore formation in the *Chara* tonoplast, which strongly suggests that peptides constitute these pores. Future work is directed to analyse either peptide with reference to its functionality.

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5.2 Second publication: Local and systemic effects of two herbivores with different feeding mechanisms on primary metabolism of cotton leaves

Status: published

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Own contribution:

- Experimental design
- Experiments
- Data analysis
- Preparation of manuscript

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Local and systemic effects of two herbivores with different feeding mechanisms on primary metabolism of cotton leaves

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ABSTRACT

Caterpillars and spider mites are herbivores with different feeding mechanisms. Spider mites feed on the cell content via stylets, while caterpillars, as chewing herbivores, remove larger amounts of photosynthetically active tissue. We investigated local and systemic effects of short-term caterpillar and spider mite herbivory on cotton in terms of primary metabolism and growth processes. After short-term caterpillar feeding, leaf growth and water content were decreased in damaged leaves. The glutamate/glutamine ratio increased and other free amino acids were also affected. In contrast, mild spider mite infestation did not affect leaf growth or amino acid composition, but led to an increase in total nitrogen and sucrose concentrations. Both herbivores induced locally increased dark respiration, suggesting an increased mobilization of storage compounds potentially available for synthesis of defensive substances, but did not affect assimilation and transpiration. Systemically induced leaves were not significantly affected by the treatments performed in this study. The results show that cotton plants do not compensate the loss of photosynthetic tissue with higher photosynthetic efficiency of the remaining tissue. However, early plant responses to different herbivores leave their signature in primary metabolism, affecting leaf growth. Changes in amino acid concentrations, total nitrogen and sucrose content may affect subsequent herbivore performance.

Key-words: *Gossypium hirsutum*; *Spodoptera littoralis*; *Tetranychus urticae*; amino acids; herbivory; leaf growth; nitrogen; photosynthesis; starch; sucrose.

INTRODUCTION

When plants are attacked by herbivores, they face the dilemma to either allocate their resources to growth and reproduction or to the synthesis of defence-related compounds. Plant responses to herbivory include direct and indirect defences that are closely linked to primary metabolism and depend on the duration of herbivore feeding and the prevailing environmental conditions, as well as on plant and herbivore species.

According to their feeding mechanisms, herbivores are classified into piercing–sucking and into chewing herbivores with different impacts on plant performance.

Piercing–sucking herbivores feed on the phloem (whiteflies, aphids) or on the cell content (spider mites, thrips) via stylets. Thus, they remove relatively little of the photosynthetically active tissue. For cotton plants, it is known that photosynthesis is not affected by short-term aphid feeding (Gomez *et al.* 2006), but is significantly reduced in response to infestation with different piercing–sucking herbivores for more than 7 d (Bondada *et al.* 1995; Shannag, Thorvilson & El-Shatnawi 1998; Lin, Schwartz & Saranga 1999; Lei & Wilson 2004; Reddall *et al.* 2004, 2007). Cell content-feeding spider mites may dehydrate the spongy mesophyll causing stomatal closure, which, in turn, may decrease photosynthesis and alter primary metabolism (Bondada *et al.* 1995). Salicylic acid (SA) is known to be the primary signalling substance in long-term spider mite feeding experiments; only within the first 24 h of infestation, the jasmonic acid (JA) and ethylene pathways are additionally induced (Ozawa *et al.* 2000; Kant *et al.* 2004; Leitner, Boland & Mithöfer 2005). This may lead to early senescence as recent evidence indicates that SA can cause mitochondrial dysfunction and induce senescence-associated genes (Maxwell, Nickels & McIntosh 2002). The enhanced senescence processes triggered by spider mite feeding may result in increased allocation of nitrogen from leaves to stems and reproductive tissues (Sadras & Wilson 1997).

In contrast to piercing–sucking insects, chewing herbivores, such as caterpillars, induce the JA signalling pathway (Paré & Tumlinson 1999; Ozawa *et al.* 2000; Leitner *et al.* 2005). Herbivory, or treatment of leaves with JA methyl ester, may cause the closure of stomata, and consequently, the rates of transpiration and photosynthesis may decrease (Beltrano *et al.* 1998). JA treatment of the leaves inhibits activity of PSII electron transport (Maslenkova, Zanev & Popova 1990), and application to the shoot and to the roots will additionally alter carbon transport in the plant and reduce root growth (Henkes *et al.* 2008).

Altering photosynthesis will affect primary metabolism and growth processes of the plant. In fact, *Trichoplusia ni*

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larvae feeding impaired photosynthetic activity even in undamaged leaf parts by increasing leaf water loss (Zangerl *et al.* 2002; Tang *et al.* 2006). In the same manner, application of *Manduca sexta* regurgitant to mechanically wounded leaves of different *Nicotiana* species resulted in down-regulation of photosynthesis-related genes (Izaguirre *et al.* 2003) and in short-term reduction of leaf and root growth (Hummel *et al.* 2007). In contrast, *Gastrophysa viridula* larvae feeding on *Rumex obtusifolius* did not affect photosynthetic activity but systemically reduced leaf expansion (Moore *et al.* 2003a), whereas *Cucumis sativus* compensated snail feeding with higher photosynthetic efficiency (Thomson *et al.* 2003). These studies of CO_2 assimilation and leaf growth upon attack of chewing herbivores show variable results, and are strongly linked to the primary metabolic processes of the plant. While the interaction between the carbon and nitrogen status of the plant (Morcuende *et al.* 1998; Fritz *et al.* 2006) and the feedback to photosynthesis and photorespiration (Matt *et al.* 2002; Novitskaya *et al.* 2002) has been an intense matter of recent research activities, only little is known on how this network is affected by herbivory.

For most crops, the vegetative phase of development is regarded as less important than the reproductive phase. This is also the case for cotton, which is a well-studied crop for fibre production. Therefore, most studies focus on boll yield, and thus on the reproductive phase of cotton plant development. Nevertheless, the vegetative phase, in which the developing leaves represent the most valuable parts of a plant and thus may accumulate defence compounds upon herbivory at the expense of primary metabolites, may be of importance for later boll development. In contrast, during the reproductive phase, flowers and seeds are the most valuable parts of the plant in terms of plant fitness, thus may be more defended upon herbivore attack at the expense of growth and defence of the leaves (Coley, Bryant & Chapin 1985). Little is known about the effects of spider mite or caterpillar feeding on the primary metabolism of cotton plants in the vegetative phase. During this developmental stage most studies focus on defence responses like the synthesis of feeding deterrents (Alborn, Röse & McAuslane 1996), the production of extrafloral nectar (Wäckers *et al.* 2001; Röse, Lewis & Tumlinson 2006) or the local and systemic emission of volatiles (Loughrin *et al.* 1994; Röse *et al.* 1996; Röse, Lewis & Tumlinson 1998; Röse & Tumlinson 2004; Röse & Tumlinson 2005). Volatiles emitted in response to caterpillar herbivory (Paré & Tumlinson 1997) and mite infestation (Röse *et al.*, unpublished results) are partially *de novo* synthesized and, therefore, largely depend on the primary metabolism of the plant.

Severe damage by spider mites and herbivores may lead to defoliation of the entire plant with clear impact on primary metabolism, and injured cotton plants may fail to develop and eventually drop squares and bolls. Our aim was to investigate the early local and systemic effects of lighter levels of spider mite and caterpillar feeding on leaf primary metabolism in the vegetative plant developmental stage, where cotton plants may still be able to out-compete herbivore damage.

Effects of herbivory on photosynthetic gas exchange and carbohydrate concentrations in the leaves were investigated, and the concentrations of starch as a storage compound and sugars (glucose, fructose and sucrose) were investigated. Additionally, leaf growth and nitrogen content were determined. Because changes in amino acid concentrations, total nitrogen and sucrose content may affect subsequent herbivore performance (Bernays & Chapman 1994), we analysed alterations in the profile of free amino acids that are involved in the biosynthesis of defence compounds (Phenylalanine, Tryptophan, Methionin), as well as nitrogen-transporting amino acids.

MATERIALS AND METHODS

Plant material

Cotton plants (*G. hirsutum* var. 'Stoneville 474'; Bayer CropScience, Monheim, Germany) were grown in pots with soil (ED 73, Einheitserdewerk, Uetersen, Germany) in the greenhouse. Plants were grown in cages covered with a high-transparency foil (Nowoflow, Siegsdorf, Germany) to allow transmission of photosynthetic active radiation at the top and on two sides of the cage. To exclude herbivores from the plants and to allow air circulation provided by three fans in the growth cage, the remaining two sides were covered with a fine mesh. The light intensity was according to environmental conditions with a 16/8 h (day/night) photoperiod. When the environmental light intensity dropped below a minimum of 350 μmol photons $m^{-2} s^{-1}$, it was supplemented by artificial light (SON-T Agro 400 W lamps; Philips, Cologne, Germany). The temperature in the greenhouse was maintained at 26/22 °C (day/night) and the relative humidity was adjusted to 65%.

After 4–5 weeks, cotton plants with three to four true leaves were transferred to a modified hood with 29/20 °C (day/night) and a photoperiod of 15/9 h (day/night) provided by the artificial light of 350 μmol photons $m^{-2} s^{-1}$ (Gewiss GW 84466, Gewiss Deutschland GmbH, Wenden, Germany). Experiments were performed after plants were allowed to adapt to the modified hood for two days.

Herbivores

Two-spotted spider mites (*T. urticae* Koch) were collected in the greenhouse and kept on cotton plants in a modified laboratory hood at 29/20 °C (day/night) with a 15/9 h (day/night) photoperiod.

Spodoptera littoralis Boisduval eggs (Syngenta Crop Protection, Stein, Switzerland) hatched and larvae were reared on an artificial bean diet (500 g beans with 9 g vitamin C, 9 g benzoic acid, 0.7 g vitamin E in 10 mL of corn oil (Mazola; Unilever Deutschland GmbH, Hamburg, Germany) and 1.7 mL formaldehyde in 380 mL of tap water). Larvae were kept in the laboratory in plastic boxes under low indirect continuous light and 22 ± 1 °C.

Experimental treatments

For *S. littoralis* treatments, third-instar *S. littoralis* larvae were starved for 24 h prior to the experiment to encourage immediate feeding of herbivores after being placed on the plants. At the beginning of the experiment (day 1), two larvae were confined to a cage (Röse *et al.* 1996) that covered around 50–75% of the leaf area. The cage was positioned on the distal part of the blade of the second-youngest leaf of a 4-week-old cotton plant [cat-local; initial leaf size 120.1 ± 8.4 cm^2 (average ± SE)], and caterpillars were allowed to feed for 48 h. Potential systemic effects of herbivory were assessed on the youngest leaf (cat-sys; 97.5 ± 13.5 cm^2), immediately above the 'cat-local' leaf. Because clip cages may alter leaf expansion and photosynthetic traits of leaves (Crafts-Brandner & Chu 1999; Moore *et al.* 2003b) cages were also clipped on comparable leaves of control plants ('con-local').

For the spider mite treatments, eight to nine adult female two-spotted spider mites were placed on the second-youngest leaf (mites-local; 122.7 ± 7.5 cm^2) of 4-week-old cotton plants and were allowed to feed for 4 d only, because eggs laid by adults will hatch in approximately 4 d and would increase the mite population. The petiole of the 'mites-local' leaf was surrounded with Vaseline to prevent the spider mites from escaping. The petiole of control leaflets was also surrounded by Vaseline to control for effects due to the barrier substance. Although mites were allowed to range freely on the leaf for 4 d, they were mainly found on the basal portion of the leaf blade. Like in the caterpillar treatment, the youngest leaf above the spider mite-infested leaf was defined as 'mites-sys' (81.8 ± 8.3 cm^2).

Leaf growth measurements and determination of the relative water content

To determine leaf growth, leaf length and width was measured with a ruler. The area of the leaves was calculated by multiplying the leaf rectangular area with a previously determined cotton-specific factor 0.6934. The factor represented the leaf rectangular area calculated from the length and width of 50 comparable cotton leaves divided by the true leaf area, determined by scanning the harvested leaves. Leaf area was measured on days 1 and 5 (spider mite experiments, $n = 15$) or on days 1 and 3 (caterpillar experiments, $n = 16$) at the same time of day, and relative growth rates (RGR) were calculated (Walter & Schurr 1999). For determination of relative water content, three (caterpillar experiments) and five (spider mite experiments) 'local' and 'sys' representative leaves were weighed and then dried at 70 °C for 5 d.

Photosynthetic gas exchange

Gas exchange was measured on the distal portion of the leaf blade of the 'local' and 'sys' leaves using a Li-6400 with a red/blue LED light source (6400-02B; Li-Cor, Lincoln, NE, USA) in the greenhouse (caterpillar exp., $n = 4$–5) or in the modified hood (spider mite exp., $n = 5$). The cuvette covered 6 cm^2 of the leaf blade and was clipped on the second lobe of the 'local' leaf and on the main lobe of the 'sys' leaf. Measurement of the directly damaged 'local' leaf area was avoided, aiming at yielding a full coverage of the leaf cuvette, which was not possible on the part of the leaf where caterpillars were feeding, as they consumed most of the leaf area inside the clip cage within 48 h. Additionally, care was taken not to squash the herbivores during the gas exchange measurements, as they were mainly found on the basal leaf portion (mites) or were caged onto the main lobe (caterpillars). The CO_2 concentration of the incoming air was adjusted to 400 μmol CO_2 at a flow rate of 500 μmol m^{-2} s^{-1}. Relative humidity corresponded to ambient conditions. Prior to measurements, the leaf in the measuring cuvette was adjusted stepwise to a PAR of 1200 μmol photons m^{-2} s^{-1}. When the CO_2 assimilation rate was stable for at least 2 min, a light response curve was recorded.

Measurements were carried out before herbivores were allowed to feed on the plant on day 1 and several days after herbivory (day 3 for caterpillars; day 5 for spider mites) on the same leaves and at the same time of day (±1 h). Stomatal conductances, transpiration and CO_2 assimilation were calculated from measurements at saturating light. Dark respiration was calculated from measurements in the darkness, i.e. at 0 μmol photons m^{-2} s^{-1}. The respective values of each leaf after 48 h (day 3) or 96 h (day 5) of exposure to herbivores ('day 3' or 'day 5', respectively) were divided by the values before herbivores were placed on the plant ('day 1'). The 'day 3/day 1' or 'day 5/day 1' ratios, respectively, were then averaged.

Sampling and sample preparation

On day 5 (spider mite exp.) or day 3 (caterpillar exp.), the 'local' and 'sys' leaves of each plant were cut with a razor blade at the base of the petiole. The herbivores were removed and the leaves were immediately shock-frozen in liquid nitrogen. Samples were stored at –80 °C until frozen leaf material was homogenized in a mortar with liquid nitrogen for further analyses.

Soluble sugars and starch

Soluble sugars were extracted from 30–50 mg of frozen and homogenized leaf material by adding 400 μL of 80% ethanol. Samples were incubated under agitation for 20 min. at 80 °C. After centrifugation at 15 300 *g*, the supernatant was transferred to a new reaction tube. The leaf material was subsequently extracted by adding 400 μL of 50% ethanol followed by two extractions with 200 μL of 80% ethanol. The respective supernatants were pooled and stored at –30 °C. Glucose, fructose and sucrose were analysed via a coupled enzyme assay (Jones, Outlaw & Lowry 1977) with a multiplate photometer (ht2, Anthos Mikrosysteme GmbH, Krefeld, Germany). Starch was extracted by autoclaving the remaining pellet with 500 μL of distilled water for 2 × 20 min at 120 °C and 1 bar, and starch concentration was enzymatically determined (Walter *et al.* 2005). For each experimental treatment, six leaf samples were analysed.

Total nitrogen in leaf tissue

Total nitrogen concentrations in leaf samples ($n = 7$ for caterpillar exp.; $n = 5$ for spider mite exp.) were determined with Hach Lange test kits (LCK 338, Dr. Bruno Lange GmbH, Düsseldorf, Germany). Leaf material (5–10 mg) was weighed into extraction tubes and 200 μL of Millipore water was added. The total nitrogen was extracted according to the instructions of the Hach Lange LCK338 test kit and determined with a UV/VIS spectrophotometer (DR5000, Dr. Bruno Lange GmbH). Concentrations of total leaf nitrogen were comparable with those analysed with a near-infrared method (see Sadras & Wilson 1997) justifying the use of the Hach Lange test kit in our experiment.

Analysis of free amino acids

To extract free amino acids from the leaf tissue, frozen leaf material ($n = 7$ for caterpillar exp.; $n = 8$–9 for spider mite exp.) was ground in a cooled mortar with 1000 μL of ethanol/0.1 M HCl (1:1) and 10 nmol norvalin added as an internal standard. Free amino acids were purified with the EZ-faast kit (Phenomenex, Torrance, CA, USA) and measured on a gas chromatography-mass spectrometry (GC-MS) system (GCQ; Finnigan MAT, San Jose, CA, USA) with an injector (optic2; Ai Qualitek Ltd., Cambridge, UK) coupled to an autosampler (CTC A200SE; CTC analytics AG, Zwingen, Switzerland). The injector temperature was set to 250 °C, and 1 μL of the sample was injected with a 1:10 split on a Zebron fused-silica capillary column (ZB-AAA 0.25 mm/15 m; Phenomenex). Helium was used as carrier gas at a flow rate of 19 cm s^{-1}. The initial oven temperature was set to 110 °C and increased at a rate of 15 °C min^{-1} to a final temperature of 300 °C, which was held for 5 min. Compounds were analysed by mass spectrometry in the electron impact mode. The free amino acids were identified and quantified by comparing spectra and retention time with original compounds obtained from free amino acid standard solutions (SD1 and SD2; Phenomenex) using the Excalibur software (Thermo Electron Corp., San Jose, CA, USA).

Statistical analysis

The local and systemic effects of herbivory were analysed by *t*-test using the Sigmastat 2.0 package (San Jose, CA, USA).

RESULTS

Local and systemic effects of herbivory on leaf growth

Caterpillar feeding for 48 h significantly reduced the RGRs of 'cat-local' leaves by 58% ($P < 0.001$, Fig. 1a), whereas the RGR of the 'cat-sys' leaves did not differ from those of control plants. In contrast, mite infestation for 96 h had neither local ('mites-local') nor systemic ('mites-sys') effects on leaf growth (Fig. 1b).

The control plants of the caterpillar experiment had lower RGRs compared with those of the mite experiment, probably due to the clip cages.

Photosynthetic gas exchange and relative water content

Light-saturated photosynthesis (Asat) at 1800 μmol photons m^{-2} s^{-1} yielded 18.3 ± 2.4 μmol CO_2 m^{-2} s^{-1} for 'con-local' leaves of the caterpillar exp., while Asat at 1500 μmol photons m^{-2} s^{-1} was 9.8 ± 0.6 μmol CO_2 m^{-2} s^{-1}

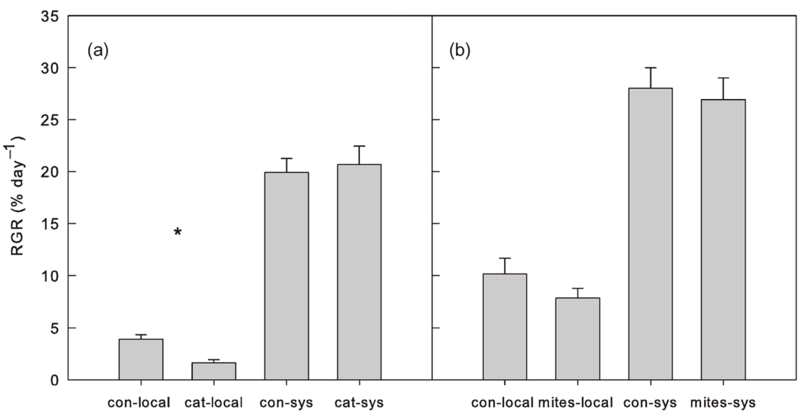

Figure 1. Local and systemic ('sys') relative growth rates (RGR) after 48 h of caterpillar feeding ('cat', a) or 96 h of spider mite infestation ('mites', b) on a single cotton leaf. Asterisks indicate statistically significant differences of treated plants compared to the controls (con) at $P \leq 0.05$. Mean ± SE. $n = 16$ (caterpillars) and $n = 15$ (spider mites).

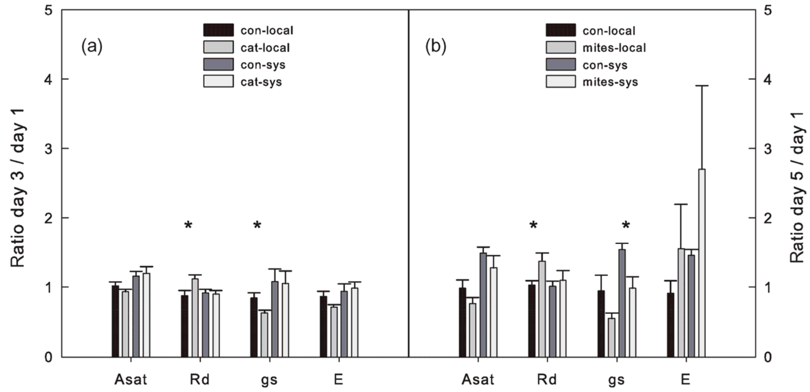

Figure 2. Local and systemic ('sys') changes in parameters of photosynthetic gas exchange after 48 h of caterpillar feeding ('cat', a) or 96 h of spider mite infestation ('mites', b) on a single cotton leaf. Asat = light-saturated photosynthesis, Rd = dark respiration, gs = stomatal conductances, E = transpiration. Asterisks indicate statistically significant differences of treated plants compared to the controls (con) at $P \leq 0.05$. Mean ± SE. $n = 4$–5 (caterpillars) and $n = 5$ (spider mites).

for 'con-local' leaves of the spider mite exp. The differences in the Asat values were due to the conditions that the plants were exposed to before the gas exchange measurements were taken, for example 'caterpillar' plants were in the greenhouse while 'mite' plants were kept in the modified hood at lower light intensities. After 48 h of caterpillar feeding, there were no significant effects on light-saturated CO_2 assimilation and transpiration rates in 'cat-local' and 'cat-sys' leaves (Fig. 2a). Dark respiration rates were increased significantly by 28% ($P = 0.045$), while stomatal conductances and the relative water content were decreased in 'cat-local' leaves by 25% and 13%, respectively (Figs 2a & 3a). Spider mite feeding for 96 h resulted in an increased dark respiration of 34% in 'mites-local' leaves, whereas stomatal conductances were reduced by 36% in 'mites-sys' leaves (Fig. 2b). Assimilation of CO_2 and transpiration, as well as the relative water content (Fig. 3b), were not altered by spider mite infestation.

Soluble sugars and starch

Concentrations of glucose, fructose, sucrose and starch did not change in response to 48 h of caterpillar feeding compared to control plants (Fig. 4a). In contrast to caterpillar feeding, sucrose concentrations in response to 96 h of spider mite infestation were increased significantly by 65% in 'mites-local' leaves compared to the corresponding 'con-local' leaves ($P = 0.009$; Fig. 4b). No effects of mites on systemic sucrose concentrations were observed compared to 'con-sys' leaves (Fig. 4b).

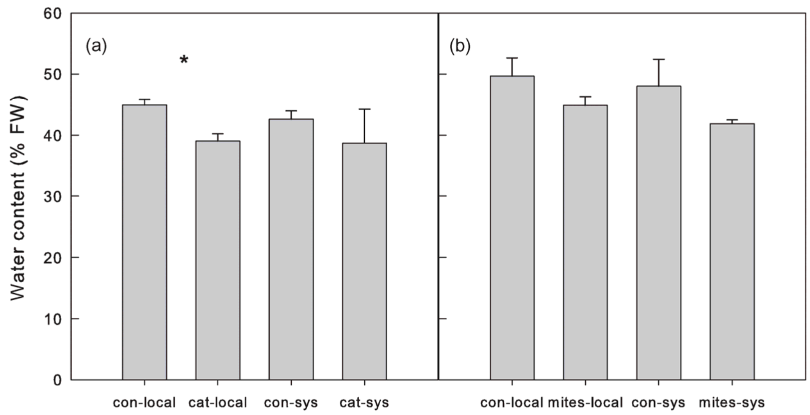

Figure 3. Local and systemic ('sys') relative water contents after 48 h of caterpillar feeding ('cat', a) or 96 h of spider mite infestation ('mites', b) on a single cotton leaf. Asterisks indicate statistically significant differences of treated plants compared to the controls (con) at $P \leq 0.05$. Mean ± SE. $n = 3$ (caterpillars) and $n = 5$ (spider mites).

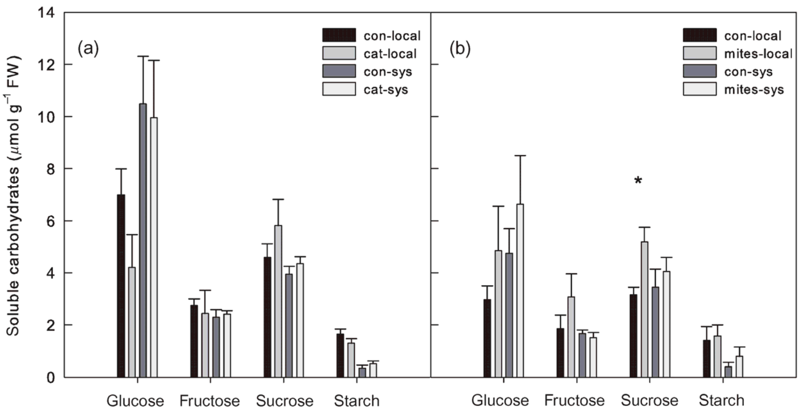

Figure 4. Local and systemic ('sys') concentrations of glucose, fructose, sucrose and starch in cotton leaves after 48 h of caterpillar feeding ('cat', a) or 96 h of spider mite infestation ('mites', b). Asterisks indicate statistically significant differences of treated plants compared to the controls (con) at $P \leq 0.05$. Mean ± SE. $n = 6$.

Total nitrogen in leaf tissue

Caterpillar feeding had no statistically significant effect on local or systemic total leaf nitrogen concentrations; however, nitrogen concentrations were slightly higher in the 'cat-local' leaves ($P = 0.083$; Fig. 5a).

Mite infestation had a significant effect on total nitrogen concentrations in 'mites-local' leaves, where concentrations of total nitrogen were significantly higher compared to 'con-local' leaves ($P = 0.015$; Fig. 5b). No effect was observed for nitrogen concentrations in 'con-sys' and 'mites-sys' leaves ($P > 0.05$).

Concentrations of free amino acids in the leaves

Caterpillar feeding for 48 h significantly changed the concentrations of several amino acids in the leaves (Fig. 6a,c): Glutamine (Gln) concentrations were lower in both 'cat-local' and 'cat-sys' leaves after 48 h of caterpillar feeding (Fig. 6a). Because Glutamate (Glu) concentrations did not change, the Glu/Gln ratio, representing photosynthetic CO_2 assimilation and availability of amino groups (Novitskaya *et al.* 2002) was significantly higher in 'cat-local' leaves compared to 'con-local' leaves. Phenylalanine (Phe) concentrations were significantly reduced in 'cat-local' leaves but not

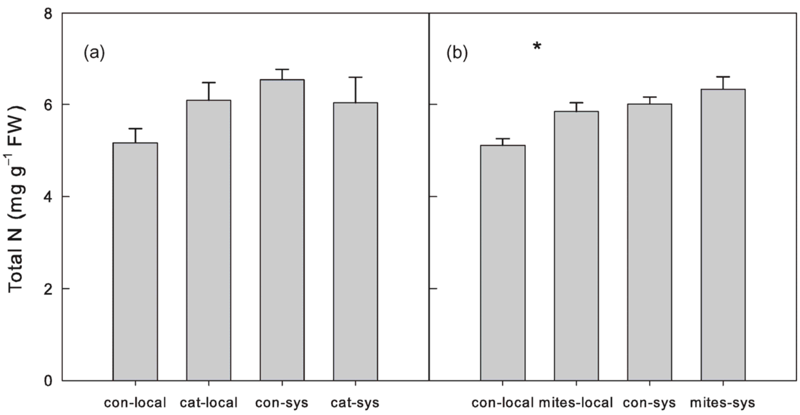

Figure 5. Local and systemic ('sys') concentrations of total nitrogen in cotton leaves after 48 h of caterpillar feeding ('cat', a) or 96 h of spider mite infestation ('mites', b). Asterisks indicate statistically significant differences of treated plants compared to the controls (con) at $P \leq 0.05$. Mean ± SE. $n = 7$ (caterpillars) and $n = 5$ (spider mites).

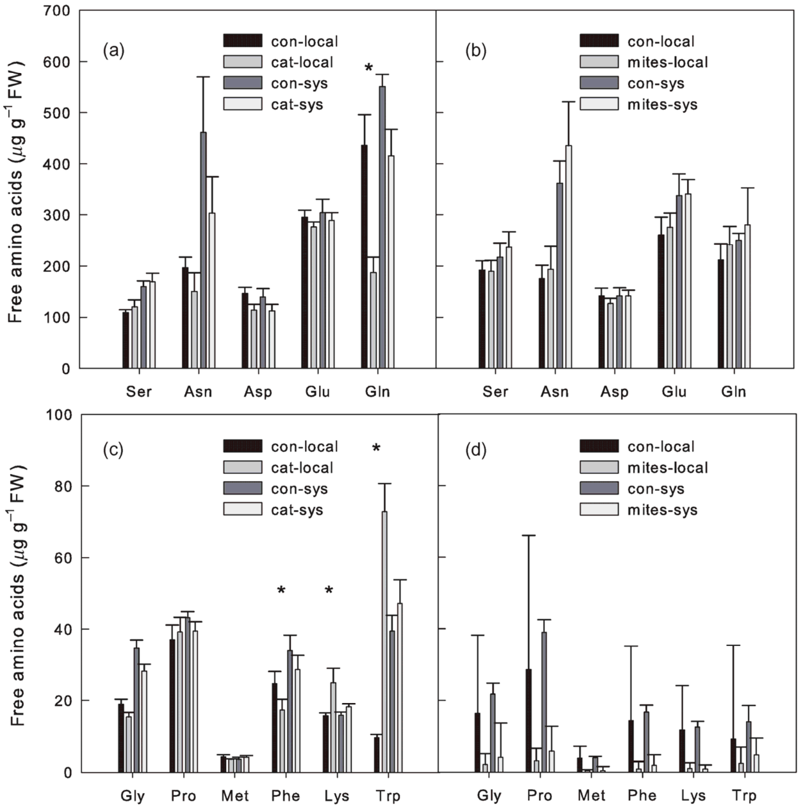

Figure 6. Local and systemic ('sys') concentrations of some free amino acids in cotton leaves after 48 h of caterpillar feeding ('cat', a,c) or 96 h of spider mite infestation ('mites', b,d). Asterisks indicate statistically significant differences of treated plants compared to the controls (con) at $P \leq 0.05$. Mean ± SE. $n = 7$ (caterpillars) and $n = 8$–9 (spider mites).

in 'cat-sys' leaves (Fig. 6c). Tryptophan (Trp) concentrations were considerably higher in 'cat-local' leaves compared to 'con-local' leaves (Fig. 6c). In response to mite infestation, no significant changes in the concentrations of the measured free amino acids in local or systemic leaves were detected (Fig. 6b,d).

DISCUSSION

Caterpillar feeding reduces leaf growth and alters the concentrations of free amino acids

We hypothesized that feeding of *S. littoralis* larvae will reduce leaf growth within 48 h, because resources are partially allocated to defence responses and because CO_2 assimilation is decreased by caterpillar feeding, reducing the amount of available carbon for growth in two ways. Our results show that by removing 3% of the leaf area, two *S. littoralis* larvae were able to decrease the water content (Fig. 3a) and the relative growth rate locally by 58%, while the growth of systemically affected leaves was not reduced (Fig. 1a). Young cotton leaves are highly protected against herbivory by accumulating large amounts of terpenoid aldehydes that will even increase systemically within 6 h in response to herbivory (Alborn *et al.* 1996). However, this resource allocation apparently does not affect short-term systemic leaf growth. In contrast, two *G. viridula* larvae feeding on a fully expanded *R. obtusifolius* leaf reduced the expansion rate of newly developing leaves systemically (Moore *et al.* 2003a). In *Nicotiana attenuata* plants, a single application of *M. sexta* oral secretion to mechanical wounding decreased the local and systemic leaf growth only temporarily (Hummel *et al.* 2007), suggesting that upon removal of the herbivore, leaf growth is re-established whereas continuous damage on our plants even for only 48 h has more pronounced effects.

Nevertheless, feeding of *S. littoralis* larvae on cotton did not affect CO_2 assimilation and transpiration (Fig. 2a). This is in accordance with studies on chewing herbivores on understory hardwood saplings (Aldea *et al.* 2006), *G. viridula* larvae feeding on *R. obtusifolius* (Moore *et al.* 2003a) and cabbage looper feeding on *Arabidopsis thaliana* (Tang *et al.* 2006) or *Pastinaca sativa* (Zangerl *et al.* 2002). Thus, we hypothesize that water loss (Fig. 3a) and degradation products of the cell wall because of tissue destruction (Lee *et al.* 1999) result in local stomatal closure, but do not limit photosynthetic CO_2 assimilation or transpiration on the whole-leaf scale (Fig. 2a). In our experiment, two indicators for water stress, soluble sugars (Fig. 4a) and proline (Pro, Fig. 6c), were not affected by caterpillar feeding. Thus, we conclude that water stress was mild and not the main reason for impaired leaf growth (Fig. 1a).

However, 48 h of caterpillar feeding affected the concentrations of several free amino acids (Fig. 6a,c). With locally decreased Gln concentrations, and the concentrations of the other nitrogen-transporting amino acids (Glu, Asp and Asn) not being altered by caterpillar feeding (Fig. 6a,c), the Glu/Gln ratio increased, suggesting an increased need for supply of amino groups. Simulation of herbivory by application of JA to *Brassica oleracea* shoots had comparable effects on the concentrations of Asp, Asn, Glu and Gln (van Dam & Oomen 2008), thus the observed alterations in amino acid concentrations might depict a general response pattern to chewing herbivory.

As consequences of caterpillar feeding, we expected changes in the profile of free amino acids that are involved in the biosynthesis of defence compounds (Phe, Trp, Met). Phenylalanine, locally reduced in concentration in response to caterpillar feeding (Fig. 6c), is an intermediate of the phenylpropanoid metabolism that leads to an array of secondary compounds with defensive functions (Bennett & Wallsgrove 1994). Phenylalanine is also a precursor for the synthesis of methyl salicylate, which is emitted in increased amounts in response to herbivory (Leitner *et al.* 2005). Tryptophane that was locally increased after 48 h of caterpillar herbivory (Fig. 6c) provides a number of important secondary metabolites for the plant including the growth regulator indole-3-acetic acid. Tryptophane is also related to an increased local emission of indole, which has been reported in response to caterpillar herbivory on cotton (Röse *et al.* 1996). The concentration of methionine, a known precursor for ethylene, was not affected by caterpillar or spider mite feeding in our experiments (Fig. 6c,d). For wild tobacco and maize, it was shown that ethylene is released in response to caterpillar attack (Kahl *et al.* 2000; Schmelz, Alborn & Tumlinson 2003), suggesting rapid methionine turnover for ethylene production.

Leaf growth is maintained upon localized spider mite infestation

Upon spider mite infestation we expected reduced leaf growth and photosynthesis due to chloroplast disintegration by toxins in the mites' saliva as suggested from long-term studies on cotton (Bondada *et al.* 1995; Reddall *et al.* 2007). However, spider mite feeding for 96 h on a single cotton leaf induced systemic stomatal closure (Fig. 2b), thereby reducing water loss (Fig. 3b), but did not have any impact on light-saturated photosynthesis (Fig. 2b) and leaf growth (Fig. 1b). On the other hand, spider mite feeding in our experiments increased sucrose concentrations in the attacked leaf (Fig. 4b). A similar local increase in sucrose concentrations as in our experiments was also observed in response to JA treatment of *B. oleracea* shoots (van Dam & Oomen 2008). Because long-term infestation of *Medicago truncatula* with spider mites has also been reported to induce the local production of JA and SA (Leitner *et al.* 2005), and because injections of sucrose and SA into corn stems have been reported to increase photosynthetic rates (Zhou *et al.* 1999), one may also have expected positive effects on photosynthesis.

In our experiments, increased concentrations of sucrose are not the result of increased photosynthesis or reduced growth, but may rather be related to increased sink strength of the locally damaged leaf or to an impaired transport of photo-assimilates. Increased sucrose concentrations due to an impaired transport of photo-assimilates were observed in response to silverleaf whitefly stress on cotton (Lin *et al.* 2000). However, inhibition of phloem export is expected to result in a decrease of starch synthesis (Lin *et al.* 2000) that we did not detect in our experiment. An increase in the sink demand of the attacked leaf has been reported in response to wounding and elicitors (Roitsch 1999). As a result, sucrose unloading from the phloem may increase and induce the gene expression of the extracellular invertase that results in the cleavage of sucrose. The resulting monosaccharides may have been used in the locally increased dark respiration for sugar signalling and regulation of defence-related genes or for other metabolic responses in the sink cell. Sucrose is known to be important in signalling pathways that regulate respiratory and nitrogen metabolism, and thus can affect amino acid concentrations (Morcuende *et al.* 1998).

In our study, spider mite feeding also increased the local concentration of total leaf nitrogen (Fig. 5b) that can not be attributed to increased concentrations of free amino acids (Fig. 6b,d). We postulate that the increased nitrogen was protein-bound suggesting an increase in proteinase inhibitor activity as reported for tomato plants upon 4 d of spider mite infestation (Kant *et al.* 2004).

As spider mites did not affect the local or systemic leaf growth after 4 d of localized infestation (Fig. 1b), we hypothesize that leaf growth was maintained because of increased allocation of nitrogen and sucrose to the site of damage (Figs 4b & 5b). This may have occurred at the expense of defence compounds as there was no effect on concentrations of defence-relevant amino acids (Fig. 6b,d) and as caterpillars did not show any preference for un-infested plants over mite-infested plants in choice experiments [data not shown, also reported by Karban (1988)].

Comparison between the effects of the different herbivores

Caterpillar and spider mite feeding on cotton differentially affect local leaf growth, leaf water content, stomatal conductances, local sucrose and total nitrogen concentrations, as well as local concentrations of the free amino acids Gln, Phe, Lys and Trp. In general, chewing caterpillars remove more leaf area than spider mites. While the damage caused by small numbers of piercing–sucking spider mites can be tolerated and leaf growth can be maintained by increasing the concentrations of growth-relevant compounds at the site of damage, caterpillar feeding affects the plant more severely. Cotton leaves that are attacked by caterpillars show reduced growth, probably because of an increased investment of resources into the synthesis of defence compounds.

Chewing caterpillars and piercing–sucking spider mites induce different signalling pathways and defence reactions, but nevertheless, upon short-term feeding, they have some similar effects on primary metabolism. Both caterpillar and spider mite feeding increased dark respiration of the affected leaf (Fig. 2), as it was shown also for *T. ni* feeding on *A. thaliana* (Tang *et al.* 2006). This suggests a response to wounding or an increased mobilization of storage compounds for the synthesis of defensive substances (Zangerl, Arntz & Berenbaum 1997). Aside from this, both herbivores did not affect transpiration and CO_2 assimilation [as upon mechanical wounding of *Pastinaca sativa* (Zangerl *et al.* 1997); Fig. 2]. Furthermore, neither caterpillar nor spider mite feeding had an impact on concentrations of glucose, fructose, starch (Fig. 4) and most of the free amino acids analysed (Fig. 6).

Moreover, practically no systemic effect was observed, which we ascribe to the short duration of feeding and the relatively mild exposure of the plants to the herbivores.

These impacts on primary metabolism, amino acid composition and leaf growth of cotton plants during the vegetative phase can be relevant for fibre yield and fibre quality in the reproductive stage. Caterpillar feeding during the transition from vegetative to reproductive phase may impair boll development, while relatively moderate spider mite infestation may not have any effect. In future studies comparing the effects of different herbivores on cotton, this phase of plant development should be investigated more closely.

In summary, we have shown that caterpillar and spider mite feeding have different effects on the concentrations of sugars, total nitrogen and amino acids, as well as on water content and growth rates of cotton leaves. Caterpillar feeding on a single leaf reduces local leaf growth in parallel to prominent alterations of the amino acid spectrum. In contrast, when attacked by a limited number of spider mites on a single leaf, cotton plants are able to compensate the damage by increasing concentrations of sucrose and nitrogen to maintain leaf growth. Nevertheless, mild exposure to both herbivore species in our study did not influence photosynthesis and had little systemic effects.

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5.3 Third publication: Jasmonic acid does not mediate root growth responses to wounding in *Arabidopsis thaliana*

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- Experimental design
- Experiments
- Data analysis
- Preparation of manuscript

Jasmonic acid does not mediate root growth responses to wounding in *Arabidopsis thaliana*

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Running title:

Root growth wound-response in *Arabidopsis thaliana*

Keywords: coronatine, ethylene, image analysis, phytohormones, *Pseudomonas syringae* pv. tomato, wounding

Abstract

Jasmonic acid (JA) is a crucial plant defence signalling substance which has recently been shown to mediate herbivory-induced root growth reduction in the ecological model species *Nicotiana attenuata*. To clarify whether JA-induced reduction of root growth might be a general response increasing plant fitness under biotic stress, a suite of experiments was performed with the model plant *Arabidopsis thaliana*. JA bursts were elicited in leaves of *A. thaliana* in different ways. Root growth reduction was neither induced by foliar application of herbivore oral secretions nor by direct application of methyl jasmonate to leaves. Root growth reduction was observed when leaves were infected with the pathogen *Pseudomonas syringae* pv. tomato, which persistently induces the JA signalling pathway. Yet, high resolution growth analyses of this effect in wild type and JA biosynthesis knock-out mutants showed that it was elicited by the bacterial toxin coronatine which suggests ethylene- but not JA-induced root growth reduction in *A. thaliana*. Overall, the results demonstrate that the reaction of root growth to herbivore-induced JA-signalling differs among species, which is discussed in the context of different ecological defence strategies among species.

Introduction

Plants have to cope with many abiotic and biotic stresses during their development in order to grow and reproduce successfully. A number of plant defence hypotheses exist, each attempting to explain how plants follow their optimal strategy within the triangle of resource availability, generation of defence measures and sustained growth (for reviews see Grime 1977; Stanton et al. 2000; Stamp 2003). Biotic stressors such as herbivores and pathogens damage plants in many ways and thereby induce different signalling pathways in the plant. The phytohormone jasmonic acid (JA) is one of the most important components of the plant defence signalling system (Rojo et al. 2003). In the model plant *Arabidopsis thaliana*, JA accumulates in the leaves after mechanical wounding (Laudert et al. 1996; Laudert & Weiler 1998; Park et al. 2002; Yan et al. 2007; Glauser et al. 2008; Zhang & Turner 2008), after pathogen attack and during feeding of herbivores (Stotz et al. 2002, Reymond et al. 2004, De Vos et al. 2005). *Arabidopsis* plants that are insensitive to JA, such as the *coi1* (coronatine insensitive) or the *jar1-1* (jasmonate resistant) mutants, are more readily consumed by herbivores (Vijayan et al. 1998, Reymond et al. 2004, Bodenhausen & Reymond 2007, Moreno et al. 2009) and are more susceptible to pathogen attack (Thomma et al. 1998; Kunkel & Brooks 2002), demonstrating the importance of an intact JA signalling pathway in *Arabidopsis thaliana* as well.

The JA signalling pathway is most readily induced by the bacterial toxin coronatine, which is structurally similar to jasmonates (Bender, Alarcon-Chaidez & Gross 1999; Glazebrook 2005). Infection of plants with *Pseudomonas syringae* pv. tomato (*Pst*) leads to coronatine-induced activation of the JA signalling pathway (Laudert & Weiler 1998; Thilmony, Underwood & He 2006). The pattern of JA accumulation upon infection with *Pst* differs between avirulent and virulent bacteria: JA accumulates more rapidly in *A. thaliana* upon infection with avirulent *Pst* compared to an infection with virulent *Pst* (Spoel et al. 2003, Grun et al. 2007).

In contrast to pathogens, herbivores can severely damage leaves not only by introduction of toxins, but also by removing photosynthetically active tissue. Thus, they impact primary metabolism and may reduce growth of the damaged leaf (Moore et al. 2003; Hummel et al. 2007; Schmidt et al. 2009). In *N.*

attenuata, an increase of carbon allocation to the root (Schwachtje et al. 2007) and a pronounced reduction of root growth (Hummel et al. 2007) in response to a single wounding or simulated herbivory event were discovered recently. While this reaction pattern seems paradoxical at first, it might be the logical consequence of a defence strategy optimized to retain important resources in the 'safe' root for re-growth after herbivoral attack, while fostering leaf growth to be stronger than root growth to maximize carbon acquisition via photosynthesis. Such a 'functional equilibrium' for the balance between above- and below-ground growth, which responds very sensitively to the availability of resources offered above- and belowground, has been described in the context of abiotic resource capture (Poorter and Nagel 2000) and this is also a conceivable strategy to cope with biotic stress situations.

Reduction in root growth of *Nicotiana attenuata* seedlings upon mechanical wounding and simulated herbivory was ascribed to JA and not to ethylene signalling, as shown by a suite of experiments involving JA signalling mutants and different experimental treatments (Hummel et al. 2007; Hummel et al. 2009). The involvement of JA signalling in rapid alterations of root growth and carbon allocation patterns was demonstrated in other species, such as barley, as well (Henkes et al. 2008). Moreover, in *A. thaliana*, jasmonates strongly reduce root length (Staswick, Su & Howell 1992; Feys et al. 1994; Vellosillo et al. 2007; Yan et al. 2007) and leaf fresh weight (Zhang & Turner 2008) when applied directly to the growth medium. It was reported recently that repeated wounding of mature *Arabidopsis* leaves mediates a rapid increase in endogenous JA concentrations, resulting in reduced cell divisions and thus reduced systemic leaf growth (Zhang & Turner 2008). However, it is not known whether wounding and biotic stress-induced JA bursts mediate a reduction in root growth in *Arabidopsis*, a species which completes its life cycle very quickly, and which will rather avoid or tolerate herbivory, while species like *N. attenuata* and 'typical crop plants' induce a substantial number of mechanisms to defend themselves against herbivore attack (Baldwin 2001; Nunez-Farfan, Fornoni & Valverde 2007).

Thus, the aim of this study was to investigate whether root growth of the model species *A. thaliana* is affected by wounding and pathogen attack via the JA signalling pathway. To test this we induced a JA burst via different treatments and then monitored root growth. First of all, methyl jasmonate (MeJA), which induces defence responses similar to caterpillar feeding (Moreno et al. 2009), was directly applied to leaves and roots. To simulate herbivory, oral secretions of larvae of the generalist herbivore *Spodoptera littoralis* were applied to mechanically wounded leaves. In further experiments, root growth of two JA signalling mutants of *A. thaliana* was investigated: The *coi1-1* mutant is insensitive to jasmonates and coronatine (Feys et al. 1994) while the transgenic knock-out mutant *aos* is lacking the allene oxide synthase and is thus defective in the rate-limiting step of JA biosynthesis (Léon, Rojo & Sanchez-Serrano 2001). In another set of experiments, *Arabidopsis* seedlings were mechanically wounded and infected with a coronatine-producing avirulent *Pst* strain, and with a virulent *Pst* strain that is unable to synthesize coronatine. Finally, an experiment with the ethylene reception blocker 1-methylcyclopropene (1-MCP) was performed to distinguish between ethylene and JA responses of *Pst* infection. Ethylene generally inhibits root elongation (e.g. Ellis & Turner 2002) and its production increases following *Pst* infection (De Vos et al. 2005).

Materials and methods

Plant material & cultivation systems

Arabidopsis thaliana seedlings were grown in sterile agar in square Petri dishes (120 x 120 x 17 mm). Five holes were melted into one side of the Petri dish with a glowing bolt under sterile conditions. Subsequently the Petri dishes were completely filled with sterile 1% plant agar (Duchefa, Haarlem, The Netherlands) in one-third Hoagland nutrient solution. When the medium had solidified, the Petri dish was closed and sealed with fabric tape (Micropore; 3M Health Care, Neuss, Germany).

The seeds of the *aos* knock-out mutant (N8149), the Col-6 wild type (N8155) and the Col-0 wild type were surface-sterilized with 70% ethanol and 20% \`glorix original\` (Unilever Belgium S.P.R.L., Brussels, Belgium) and placed into the holes of the Petri dishes. The seeds on the agar were covered with laboratory film (Parafilm, Pechiney Plastic Packaging, Menasha, WI, USA) to avoid water loss until germination and were stratified in the dark. After three days, the Petri dishes were transferred into a climate chamber with a constant air temperature of 21°C and 60% RH. Light was provided for 16 hours with a phase of dawn and dusk of 15 minutes, each, and reached 150 µmol photons m^{-2} s^{-1} at seedling height. The Petri dishes were held vertically until germination and were then set to an angle of 5° from the vertical to ensure that the roots grew along the bottom of the Petri dish. Roots of the seedlings were inside the Petri dish, while the shoots grew outside. When the seedlings were 10-14 days old, they were used for experimental treatments.

For selection of homozygous *coi1-1* mutants, the seeds were sterilized as described above and placed on Murashige and Skoog medium (Sigma Aldrich Chemie GmbH, Steinheim, Germany) supplemented with 3% sucrose and 30 µM methyl jasmonate. After three days of stratification, the plates were transferred into the climate chamber. Approximately 5-7 days later, the homozygous *coi1-1* mutants, were transferred to Petri dishes filled with one-third Hoagland agar as described above.

Throughout the cultivation and the experimental treatments, the roots of the seedlings were not covered and thus were exposed to light. To avoid possible effects of the treatments on root growth being masked by light effects, all plants were exposed to the same conditions.

The agar temperature at the root tip and the air temperature at the height of the seedlings were monitored on a representative Petri dish using a thermologger (K204, Conrad Electronic SE, Hirschau, Germany).

Pathogens

The coronatine-producing, avirulent bacteria *Pseudomonas syringae* pv. tomato DC3000 avrRpt2 (Pst DC3000 avrRpt2) were grown in an incubator set to 28°C on King´s B agar (King, Ward & Raney 1954) with kanamycin for selection. For experiments, the bacteria were transferred to liquid LB medium (Fluka Feinchemikalien GmbH, Neu-Ulm, Germany) with kanamycin and were allowed to grow overnight at 28°C. The bacteria were purified by centrifugation with washing steps in $MgSO_4$ and finally adjusted to a concentration of 1 x 10^7 cfu ml^{-1}.

The virulent strain *Pseudomonas syringae* pv. tomato NCPPB 1008 (Pst DC3000 NCPPB 1008), which is deficient in coronatine biosynthesis, was grown on LB agar plates without antibiotics. The same purification procedure as described above was used for experiments.

Herbivores

Spodoptera littoralis (Lepidoptera) eggs were reared on a diet consisting of shredded beans and vitamins. Two days before starting the collection of oral secretions, the third-instar larvae were placed in a plastic container and were allowed to feed on *Arabidopsis thaliana* Col-0 leaves. In order to induce regurgitation of oral secretions, a caterpillar was held with light-weight forceps in the head region. Collection of oral secretions used two 50-µl pipettes inserted into a vial through a septum. One of the pipettes was held onto the mouth part of the caterpillar while the other pipette was connected to a low vacuum. The oral secretions were stored at -80°C immediately after collection.

Experimental treatments

An outline of the experimental design, specifying genotypes and replicate numbers of each individual experiment, is given in table 1.

For MeJA treatment on roots, the Petri dish was opened and 1 µl of pure MeJA was applied to the agar at 5 mm distance to the root tip. In this experiment, control plants were treated with 1 µl of sterile de-ionized water (Millipore GmbH, Schwalbach, Germany) in the same manner as the MeJA-treated plants.

For the two methyl jasmonate (MeJA) treatments on leaves, 400 or 4000 ng MeJA were dissolved in 1 µl of lanolin, and applied as described by Hummel et al. (2007). Control plants were treated with 1 µl of pure lanolin.

For wounding and bacteria treatments, two leaves of the plants were wounded twice using sterile tweezers punching small holes into the leaves without damaging the major veins. For experiments with *Pseudomonas syringae* strains, 2 µl of the bacterial suspension were applied to each wounded leaf with sterile pipette tips. To facilitate the infection of the leaf, the bacteria suspension was ejected and retrieved several times with the pipette tip to ensure maximal coverage of the leaf surface with the pathogens.

To analyze long-term effects of simulated caterpillar herbivory, two leaves per seedling were wounded and immediately treated with 1 µl of *Spodoptera* *littoralis* regurgitant (diluted 1:5 with 50 mM phosphate buffer, Hummel et al. 2007). Control plants were treated with 1 µl buffered water after wounding.

For experiments with 1-methylcyclopropene (1-MCP, obtained as SmartFresh from AgroFresh Inc., Spring House, USA), a strong ethylene receptor blocker, the Petri dishes were placed in a tightly closed glass chamber as described by Hummel et al. (2009). Plants were allowed to adapt to the conditions in the glass chamber for five days. 1-MCP was applied to the chamber at a concentration of 45 µl l^{-1} by dissolving the SmartFresh powder in 100 ml of water according to Hummel et al. (2009). The glass chamber was opened only for a few minutes to mark growing root tips as described in \`basic root growth analysis\`. After each opening of the glass chamber, the 1-MCP pre-treatments were renewed. Experimental treatments were performed 24 hours after the first 1-MCP application to ensure that all ethylene receptors were blocked by 1-MCP before treatment.

Basic root growth analysis

For basic root growth analysis, the position of the root tip was marked every 24 hours with a pen on the bottom of the dishes, starting two days before the experimental treatment. The increase in length of the primary root of each seedling was monitored with a ruler. Only seedlings with initial primary root length of more than 20 mm (on treatment day, day 0) were included in experiments with caterpillar regurgitant. For experiments with 1-MCP, seedlings were selected with root lengths of more than 29 mm on the day of 1-MCP treatment. The data were normalized by dividing the velocity of the root tip (v_{Tip}) on the days following the treatment by v_{Tip} for the day before treatment (day 0). The v_{Tip} values of the individuals per Petri dish were averaged and the number of Petri dishes per treatment was taken as the replicate number.

High-resolution root growth analysis

An image of the primary root growth zone was taken every 30 s with a charge-coupled device (CCD) camera (Sony XC-55 and XC-75, Sony, Köln, Germany) at a resolution of 740 x 480 pixels which corresponds to a total area of 2.7 x 1.8 mm. During the dark phase, infrared illumination (λ = 940 nm) enabled image acquisition. The cameras were equipped with low-pass infrared filters (RG, Schott, Mainz, Germany) to block visible irradiation. The root tips were followed via a tracking algorithm which controlled a set of x-y moving stages that repositioned the Petri dish, and thus the root tip, when the tip approached the border of the image field (for more details see Hummel et al. 2007). Each replicate was measured for at least 24 hours. The velocity of the root tip (v_{Tip}) was calculated using image processing algorithms described elsewhere (Walter et al. 2002). The v_{Tip} data were normalized by dividing them by the value at the time of treatment to facilitate the comparison of the experimental treatments.

Determination of jasmonates in shoots

For analysis of the concentrations of JA in *Arabidopsis thaliana*, entire shoots of five to seven seedlings per replicate were pooled. The samples were harvested and immediately frozen in liquid nitrogen. Prior to the extraction of phytohormones, the frozen samples were homogenized with two steel balls in a Geno/Grinder 2000 (OPS Diagnostics, LLC; Bridgewater, NJ, USA) with 250 stokes min^{-1} for 30 seconds. Each sample was extracted with one millilitre of ethyl acetate spiked with 40 ng D_2-dihydro-JA and 8 ng JA-$^{13}C_6$-Ile by shaking for 10 min. After centrifugation at 16100 g for 20 min at 4°C, the extraction was repeated with 500 µl of ethyl acetate. The supernatants were combined and evaporated to dryness in a vacuum concentrator at 30°C. The dry residue was dissolved in 200 µl methanol and centrifuged at 16100 g for 10 min. An aliquot was transferred to HPLC vials and measured on a 1200 L liquid chromatography–triple quadrupole mass spectrometry system (Varian, Palo Alto, USA). 10 µl was injected onto a ProntoSIL C18-ace-EPS column (50x2 mm, 5 µm diameter, Bischoff, Germany) attached to a pre-column (C18, 4x2 mm, Phenomenex, USA). A mobile phase composed of 0.05% formic acid and methanol was used in a gradient mode for the separation. The mobile phase comprised of solvent A (0.05% formic acid) and solvent B (methanol) used in a gradient mode (time/concentration for (min/%B): 0/15; 1.5'/15; 4.5'/98; 12'/98; 13'/15; 15'/15 with a variable flow rate time/flow (mL/min): 0'/0.4; 1'/0.4; 1,5'/0.2; 10'/0.2; 10.5'/0.4; 15'/0.4). Compounds were detected as negative ions in a MRM mode. Molecular ions M-H(-) at m/z 209 and 322 generated from endogenous JA and JA-Ile and from their internal standards 213 and 328 were fragmented under 12 and 19 V CE for JA and JA-Ile, respectively. The product

Ion of JA and its internal standard is m/z 59, JA-Ile and the internal standard forms the product ions m/z 130 and m/z 136, respectively. The ratio of ion intensities of the response of the product ions was used to quantify JA and JA-Ile.

Statistical analyses

Statistical analyses were performed with SigmaStat, version 2.03 (San Jose, CA, USA). To test for significant effects of caterpillar regurgitant on root growth, the treatments “wounding” and “wounding + orals secretions” (OS) were tested with a t-test for each day following treatment. Concentrations of JA and JA-Ile were compared between controls and wounded plants for each time point following treatment with t-tests or Mann-Whitney rank sum tests.

Total increases in root length of untreated and bacteria-exposed plants following 1-MCP application were performed with one-way ANOVA followed by Fisher LSD *post hoc* test.

Results

MeJA application to roots and leaves

Direct application of MeJA to the agar next to the root growth zone immediately reduced root growth (Fig. 1a), clearly demonstrating the inhibiting growth effect of JA. Yet, neither foliar application of 400 ng MeJA nor of 4000 ng MeJA led to decreased root growth (Fig. 1b). This result shows that the perception of JA in leaves and roots can differ enormously and that root growth is not necessarily reduced when JA is sensed by leaves.

Root growth patterns of untreated plants

As the initial experiment showed that root growth decreased during the day and increased at night, 24 h (diel) growth dynamics of all investigated plant lines were analyzed carefully. The *Arabidopsis thaliana* wild types Col-0 and Col-6, as well as the JA signalling mutants *coi1-1* and *aos,* showed the same basic diel pattern of root growth (Fig. 2a). During the day, the velocity of the root tip (v_{Tip}) decreased slightly (daily average 0.25 mm h^{-1}). During the night, root growth increased, showing maximal values of up to 0.4 mm h^{-1} around the night-day-transition (Fig. 2a).

As the diel fluctuation of root growth is often strongly correlated to temperature (Hummel et al. 2007, Walter & Hummel 2008), temperature was measured within the agar during the diel cycle (Fig. 2b). Temperature varied more strongly within the agar than in the air and was 1-1.5°C higher during the day than during the night (Fig. 2b). Yet, the observed diel variation of root growth was not correlated to diel temperature variations. This temporal growth variation served as the reference frame for all subsequent experiments, with the consequence that all treatments had to be applied at the same time of the day.

Wounding

Wounding two leaves immediately reduced root growth by 26 ± 4% in all *Arabidopsis* seedlings (Fig. 3a-d). This decrease was transient; root growth recovered to pre-treatment levels at the beginning of the next day. No differences in root growth following a single wounding treatment of two leaves were found between the different *Arabidopsis* lines (Fig. 3).

The concentrations of JA and JA-Ile increased significantly within 30 minutes following the mechanical wounding treatment and then decreased slowly (Fig. 4).

Foliar wounding and application of caterpillar regurgitant

When leaves were wounded and treated with oral secretions of *Spodoptera littoralis*, the velocity of the root tip (v_{Tip}) was clearly reduced. However, no difference between mere mechanical wounding and the wounding and application of oral secretions was found concerning root growth (Fig. 5).

Wounding and application of different* Pseudomonas syringae *pv. tomato DC3000 strains

Immediately after wounding and application of *Pst* DC3000 avrRpt2, root growth decreased strongly in the *aos* mutant and the wild types (Fig. 6a). Compared to the mere mechanical wounding treatment (Fig. 3), root growth was decreased more severely here and the reduction lasted for at least two days. Throughout the two days analysed, the decrease in root growth was more pronounced in the *aos* mutant than in the wild types (Fig. 6a). In the *coi1-1* mutant, root growth decreased only slightly during the first hours following the treatment and rapidly recovered to the pre-treatment level (Fig. 6a). Immediately after joint mechanical wounding and application of the virulent coronatine-deficient *Pst* DC3000 strain NCPPB 1008, root growth decreased transiently, recovering soon to values of untreated plants in all *Arabidopsis* lines (Fig. 6b).

Treatment with 1-MCP, which blocks ethylene perception in plants, led to significantly increased root growth of *Arabidopsis* seedlings compared to plants that were not exposed to 1-MCP ($p \leq 0.002$; Figs. 7a & b). Seedlings that were wounded and treated with the coronatine-producing *Pst* DC3000 avrRpt2 grew significantly slower for three days compared to untreated control plants ($p = 0.028$; Fig. 7b). Four days after 1-MCP treatment, there was no significant difference between the root length of untreated control seedlings and seedlings that were wounded and treated with bacteria ($p > 0.05$; Fig. 7b).

Discussion

Impaired JA signalling does not affect root growth of **Arabidopsis thaliana**

As root growth is regulated by different plant hormones, it was essential to test whether root growth of wild type plants and of the two JA signalling mutants applied in this study is comparable. Indeed, there was no difference between lines in control conditions (Fig. 2a) suggesting that JA does not play a major role in controlling diurnal or nocturnal root growth dynamics in *Arabidopsis* under `normal`, unstressed circumstances. The application of the ethylene blocker 1-MCP increased root growth (Fig. 7), indicating that also in conditions without experimental treatments, ethylene accumulates inside the Petri dish and thereby prevents utilization of the full root growth potential (Eliasson & Bollmark 1998).

A repetitive variation of root growth within 24 h was observed in all *Arabidopsis* lines and under all treatments, with maximal values of v_{Tip} around dawn and minimal values at the end of the day (Fig. 2a). This result contradicts other observations from our group (Walter et al. 2002; Walter & Schurr 2005, Walter & Hummel 2008) and can not be explained by temperature variation (Fig. 2b). The unusual fluctuation might originate from progressive root growth inhibition induced by the high prevailing light intensities in the climate chamber during the day, which is abrogated each night. In contrast to the studies on *N. attenuata* where illumination of the Petri dishes from the side was approximately 3 µmol PAR m^{-2} s^{-1}, the equivalent light intensity reflected was a factor of 20 higher (50-60 µmol PAR m^{-2} s^{-1}). As all plants of this study were exposed to the same light conditions during pre-cultivation and experiments, and as diel growth variations were comparable in all populations, we can exclude the possibility that treatment effects on root growth were due to exposure of the roots to light.

***MeJA applied to leaves does not reduce root growth in* Arabidopsis**

Foliar application did not affect root growth (Fig. 1b). In contrast, direct application of MeJA to the growth medium reduced root growth (Fig. 1a). Root growth reduction was comparable to that in experiments described in literature (Feys et al. 1994; Vellosillo et al. 2007; Yan et al. 2007; Zhang & Turner 2008). Foliar application was performed at different concentrations which were reported to lead to clear effects on gene expression and concentration of phenolics of the leaves (Moreno et al. 2009). There are three potential reasons why root growth might not have been decreased here, although leaves were provided with JA: (i) the jasmonate signal was not transported from leaves to root growth zones (ii) the JA signal arrived at the root growth zone but was not sensed there (iii) the signal was sensed but did not lead to a growth reduction and was interpreted differently than in *N. attenuata*. Possibility (i) is unlikely as it is shown that, in *Arabidopsis*, the JA signal is systemically distributed along the phloem (Truman et al. 2007) and reduces cell division of leaves systemically (Zhang & Turner 2008). Possibility (ii) can be excluded due to the experiment with direct application of MeJA to roots, which is also supported by literature (Spoel et al. 2003; Badri et al. 2008). Hence, the following parts of the discussion have to elaborate on possibility (iii).

***Wounding and simulated herbivory transiently reduce root growth in* Arabidopsis thaliana**

Mechanical wounding of the leaves induces strong bursts of plant-internal jasmonates (Fig. 4) in the same timing and range as in *Nicotiana attenuata* (Hummel et al. 2009) and as reported earlier for wounded *Arabidopsis* plants

(Laudert et al. 1996; Park et al. 2002; Reymond et al. 2004; Yan et al. 2007). Moreover mechanical wounding led to a transient root growth reduction of 26 ± 4% compared to the pre-treatment value in all investigated plant lines within two hours (Fig. 3). Root growth recovered throughout the treatment day but remained some percent lower compared to control plants. The initial transient root growth reduction is clearly not mediated by JA, as JA signalling mutants showed this response in the same way as wild type plants (Fig. 3). This is different to reports on JA-mediated reductions of leaf growth following repeated mechanical wounding (Zhang & Turner 2008). A combination of decreased turgor, and decreased carbohydrate availability to the root, due to the damaged photosynthetic tissue, accounts for this pronounced short-term effect, and for a minuscule root growth reduction in the following days (Nagel, Schurr & Walter 2006; Hummel et al. 2007). Application of oral secretions of the larvae of *Spodoptera littoralis* did not significantly increase the slight effect of wounding on root growth (Fig. 5).

It is conceivable that the wounding effect was too small to evoke pronounced reactions in the root, even though it resulted in increased concentrations of jasmonates in the shoot (Fig. 4). It is known that abundances of the AOS protein (Laudert & Weiler 1998) and accumulation of polar jasmonates (Glauser et al. 2008) are lower in *Arabidopsis* roots compared to leaves after mechanical wounding of leaves. It was possible to measure concentrations of JA and its conjugated form JA-Ile in the shoot only, and thus we can not predict the real JA concentrations at the root tip. Furthermore, the repeated mechanical wounding imposed by Zhang & Turner (2008) was more severe than our single wounding treatment which may explain why they observed stunted leaf growth while there were no effects on root growth of *Arabidopsis* in our study. Yet, a more severe destruction of leaf material would have affected primary metabolism in such a serious way that a distinction between defence signalling and carbon starvation would have been difficult. The severity of the wound treatments was comparable to that in experiments with *N. attenuata*, where a single wounding event induced a clear reduction of v_{Tip} to 50% of the control value, lasting for 16 hours. Hence *Arabidopsis* root growth is clearly less susceptible to leaf wounding and herbivory compared to *Nicotiana attenuata*. If the observed reaction is not only caused by hydraulic effects and reduced plant photosynthesis but by JA signalling, then the JA signal perceived by the root leads to a very subtle growth reduction.

Pseudomonas syringae *pv. tomato infection reduces root growth via coronatine-mediated ethylene signalling*

Pseudomonas syringae pv. tomato was found to induce a very strong and persistent JA formation in leaves, since, in the avirulent strain, coronatine is continually produced, leading to a continuous induction of the JA signalling pathway (Laudert & Weiler 1998; Thilmony, Underwood & He 2006).

Upon wounding and treatment with the avirulent, coronatine-producing bacteria strain *Pst* DC3000 avrRpt2, root growth decreased immediately in all *Arabidopsis* lines, and was only able to recover during the night in the coronatine-insensitive mutant *coi1-1* (Fig. 6a). Interestingly, the *aos* mutant behaves like the wild types suggesting that not the JA signalling pathway *per se* induces the observed reduction of root growth by 50% but that a JA-independent pathway is involved as well. This points to the bacterial toxin coronatine as reason for root growth reduction, which was clearly proven by the following experiment with the virulent bacteria strain *Pst* DC3000 NCPPB 1008. In this experiment, *Pst* DC3000 NCPPB 1008, which are unable to produce coronatine, led to very similar root growth reactions in wild type and JA signalling mutant plants (Fig. 6b). It was reported earlier that coronatine reduces the root length of *Arabidopsis* wild type plants similarly to MeJA, while root growth of the *coi1-1* mutants is not affected (Feys et al. 1994). Coronatine acts via a COI1 dependent pathway (He et al. 2004) which makes the *Arabidopsis coi1-1* mutant insensitive to coronatine-producing *Pseudomonas syringae* strains (Feys et al. 1994).

Up-regulation of the JA signalling pathway by a continuous supply of coronatine has probably acted synergistically with ethylene to mediate root growth reduction upon infection with *Pst*. It is known that coronatine and another *Pst* virulence factor, the type III effector avrRpt2, mediate increased ethylene biosynthesis via auxin (Chen et al. 2007). As ethylene is known to reduce the root cell elongation by locally up-regulating auxin biosynthesis and transport (Ruzicka et al. 2007, Swarup et al. 2007), we conclude that ethylene was the major factor for root growth reduction upon infection of *Arabidopsis* with *Pst*.

This hypothesis is supported by the experiment with 1-methylcyclopropene (1-MCP) which blocks ethylene perception. Upon the application of 1-MCP, the root growth increase was similar in both un-treated control plants (as reported for *Nicotiana attenuata*, Hummel et al. 2009) and in plants that were inoculated with *Pst* (Fig. 7), confirming that ethylene is involved in mediating the reduction in root growth upon infection with the bacteria.

Ecological aspects of root growth reactions after herbivory

Plants live in different habitats and are faced with different strengths and modes of herbivory and pathogen attack. Hence, they evolved specific defence responses to minimize the negative consequences. As many biochemical signalling pathways like JA, salicylic acid or ethylene pathways are highly conserved in plants, the complex sets of signals must be integrated by the different plant organs and organized into specific reactions concerning growth, defence, storage and reproduction according to their ecological context. Thus, wounding signals must be translated into specific reactions in order to reduce the negative impact of herbivory on plant fitness. In this study we demonstrated that wound-induced JA does not affect root growth in *Arabidopsis*, although JA is well known to inhibit root growth strongly. In contrast to that result, Hummel et al. (2007, 2009) demonstrated that wound-induced JA reduces root growth in *Nicotiana attenuata* markedly. Hence, the same wound signal triggers different root growth reactions in the two species. This difference in growth response might be explained by the ecologies of the habitats in which the plants evolved. *Arabidopsis* is found in temperate climates and has to cope with shorter growth periods than *Nicotiana* which is a subtropical plant. *Arabidopsis therefore* has to finish its life cycle more quickly than *Nicotiana attenuata,* and does not suffer from high herbivore pressure. For instance no specialist herbivore is known to attack *Arabidopsis*. Thus, *Arabidopsis* is focused on a rapid turnover and adjusts its growth response only marginally upon wounding, by suppressing the rate of leaf cell division. The resulting reduction of cell numbers requires fewer nutrients, which saves resources for the completion of the life cycle of the plant (Zhang & Turner 2008). In contrast, *Nicotiana attenuata* has longer growth periods and mostly grows in monocultures. Hence, herbivore pressure and intraspecific competition is far higher compared to *Arabidopsis* (Baldwin 2001).

Often when *N. attenuata* is attacked by its specialist herbivore *Manduca sexta*, the shoot is entirely defoliated. In this case, tolerance is suggested to be the best strategy for a plant to cope with its species-specific, adapted herbivores (Schwachtje et al. 2006). Thus, when attacked by its specialist herbivore *M. sexta*, *N. attenuata* (i) increases its defence mechanisms but (ii) also allocates recently fixed carbohydrates into its roots, which can be used for re-growth whenever the aboveground threat has passed by. Such a tolerance reaction only makes sense when, simultaneously, root growth is reduced and carbon is stored in roots instead of being used for root growth. At the same time, the biosynthesis of nicotine takes place in the roots of *Nicotiana*, incorporating up to 8% of the plant´s nitrogen pool (Baldwin 2001) which will not be available for growth processes. Because in *Arabidopsis* the main defensive substances, the glucosinolates, are synthesized in the leaves and not the roots, the resources of the roots would not be used for defence against herbivores, and thus help root growth to continue following herbivory.

Conclusions

Overall, this study demonstrates that the relationship between plant defence patterns, resource capture and vegetative growth is regulated in a complex manner with different species following different strategies that optimize their fitness within their specific ecological habitat. Moreover, the results reveal limits for the use of model organisms. Although *A. thaliana* and *N. attenuata* use the JA signalling pathway and although JA leads to a reduction of root growth in both species when applied directly to roots, the two species follow different rules for the response of their belowground growth when biotic stresses occur aboveground. It seems likely that these rules are strongly dependent on the ecological context in which a species has evolved.

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Tables

Table 1 Experimental design. Overview of the *Arabidopsis thaliana* lines used in the individual experiments.

Experiment	**Genotypes**	**Replicates**
MeJA on roots	Col-0	3-4
MeJA on leaves	Col-0	3-6
control	Col-0, Col-6, *coi1-1*, *aos*	4
wounding	Col-0, Col-6, *coi1-1*, *aos*	5-8
wounding + OS	Col-0	5-6
Pst DC3000 avrRpt2	Col-0, Col-6, *coi1-1*, *aos*	4-5
Pst DC3000 NCPPB 1008	Col-0, Col-6, *coi1-1*, *aos*	3-4
Pst DC3000 avrRpt2 and 1-MCP	Col-0	3-4

Figures

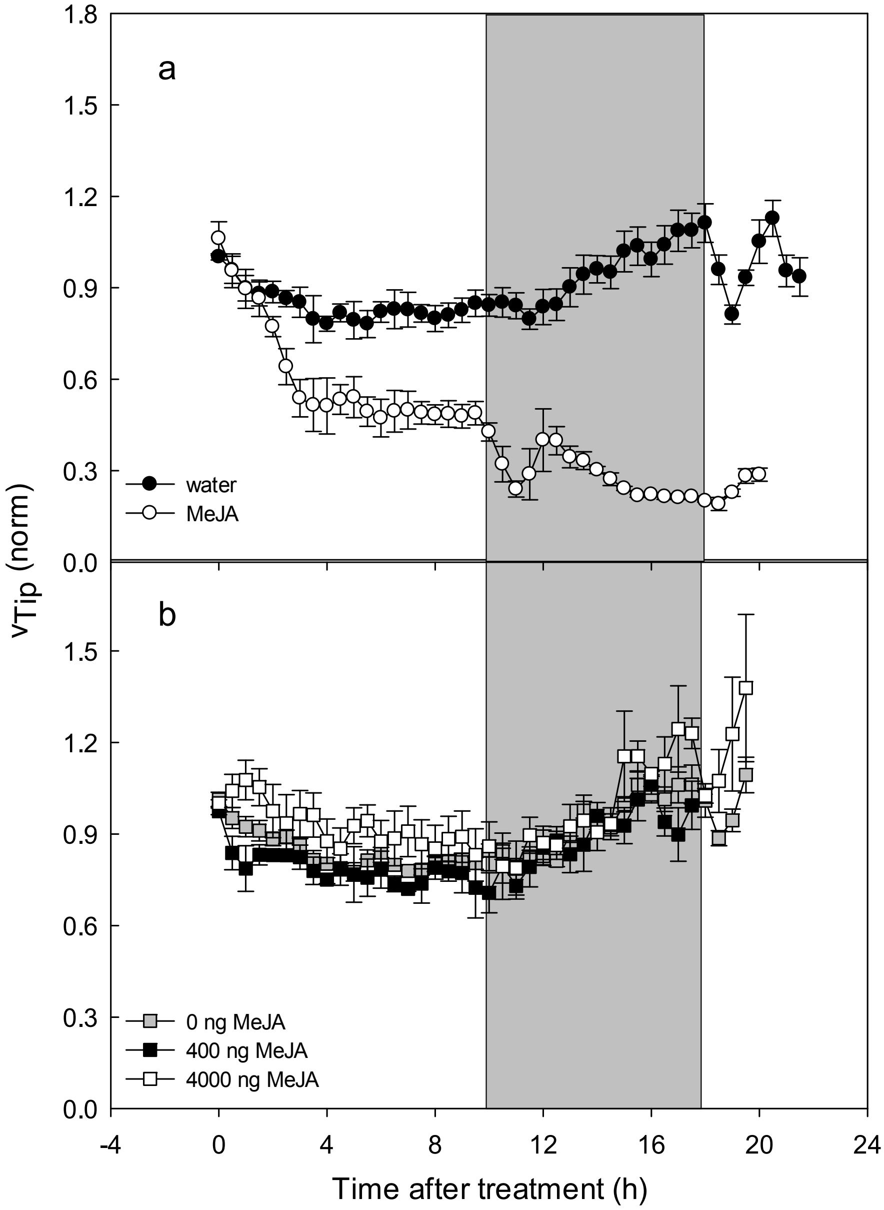

Figure 1 Normalized values of root tip velocities (v_{Tip}) of *Arabidopsis thaliana* wild types (a) after application of either 1 µl water or 1 µl methyl jasmonate to the agar near the root tip and (b) after application to a leaf of 0, 400 or 4000 ng methyl jasmonate dissolved in 1 µl lanolin. The night period is highlighted in grey. (Mean ± SE, n = 3-6).

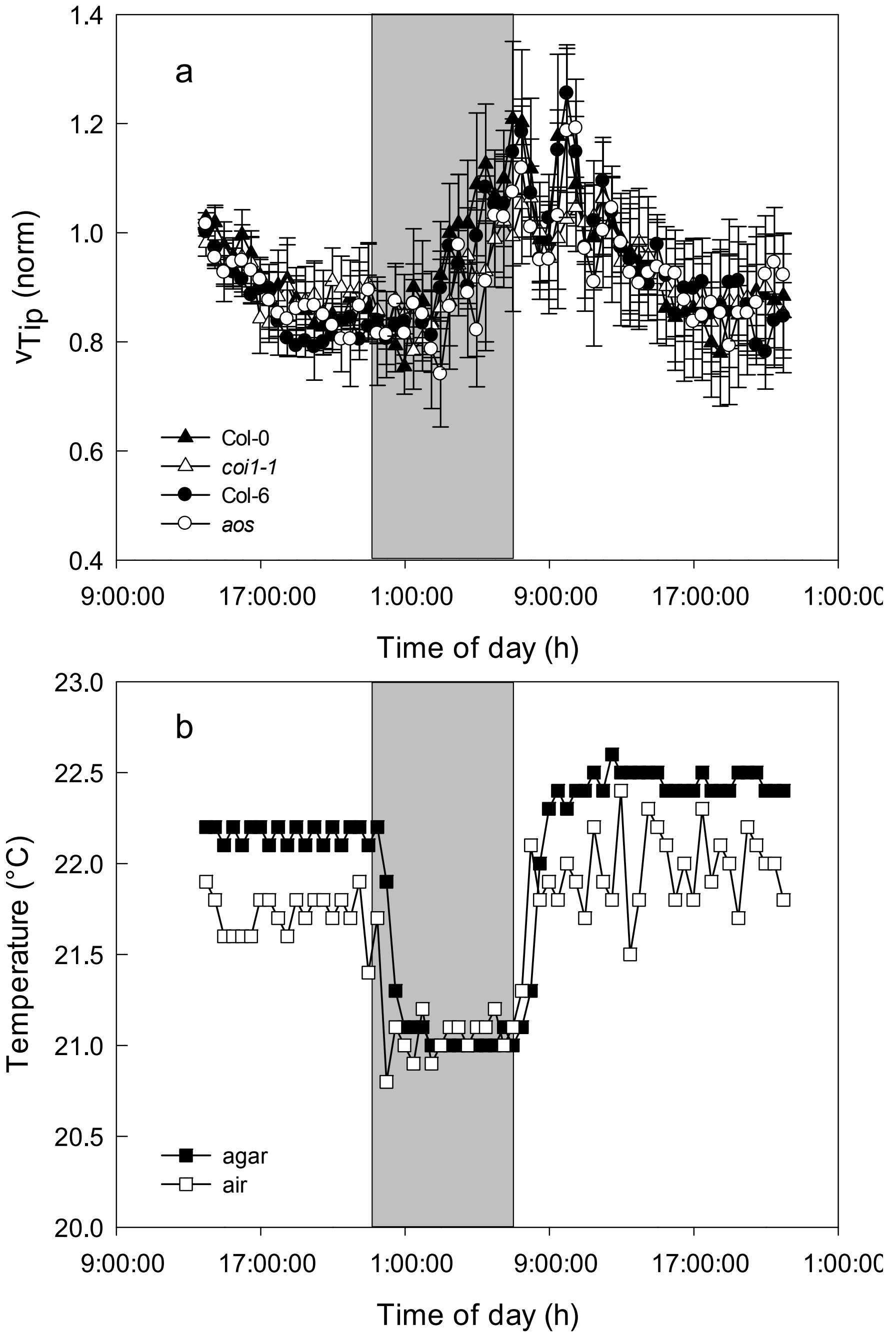

Figure 2 Time courses of (a) normalized values of root tip velocities (v_{Tip}) of two wild types (Col-0 and Col-6) and two JA signalling mutants (*coi1-1* and *aos*) of *Arabidopsis thaliana* and of (b) corresponding temperature of the air and the agar of a representative Petri dish. The night period is highlighted in grey. For (a), (Mean ± SE, n = 4).

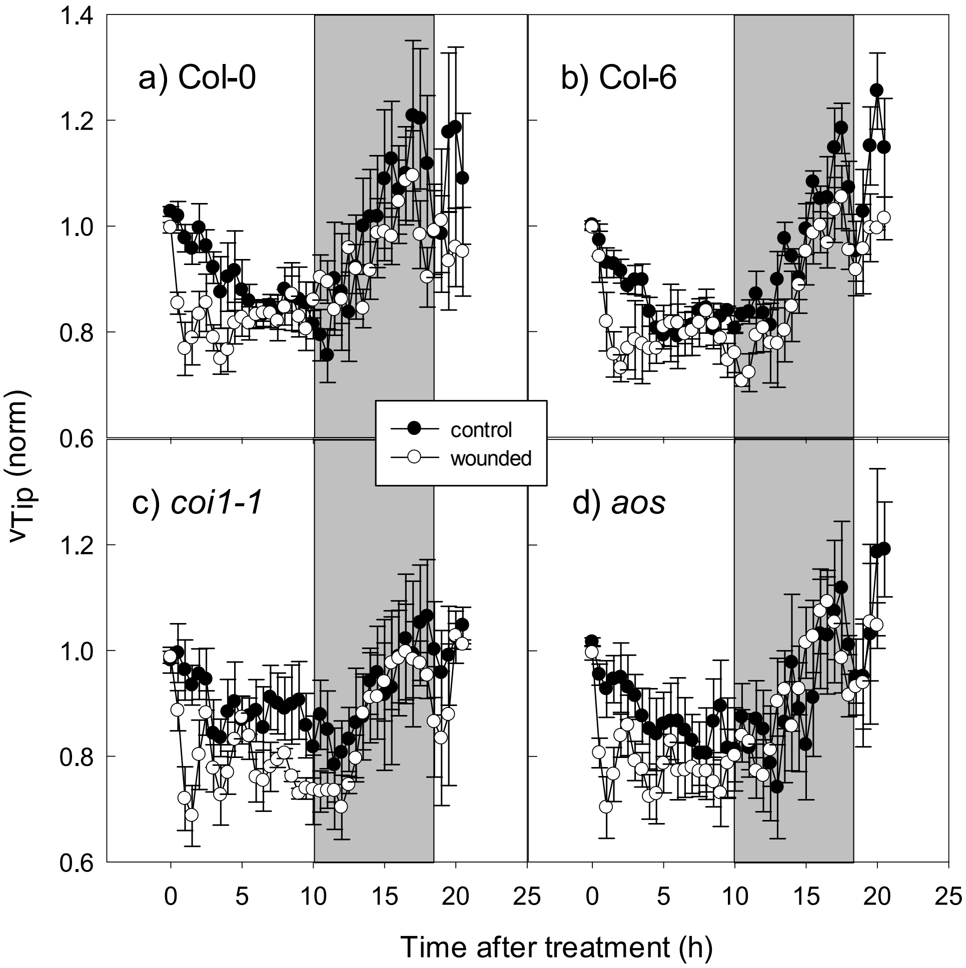

Figure 3 Velocities of the root tip (v_{Tip}), normalized to the time of treatment, of mechanically wounded *A. thaliana* wild types (a) Col-0 and (b) Col-6 and (c) the JA-insensitive mutant *coi1-1* and (d) allene oxide synthase knock-out mutant *aos*. The night period is highlighted in grey. (Mean ± SE, n = 5-8).

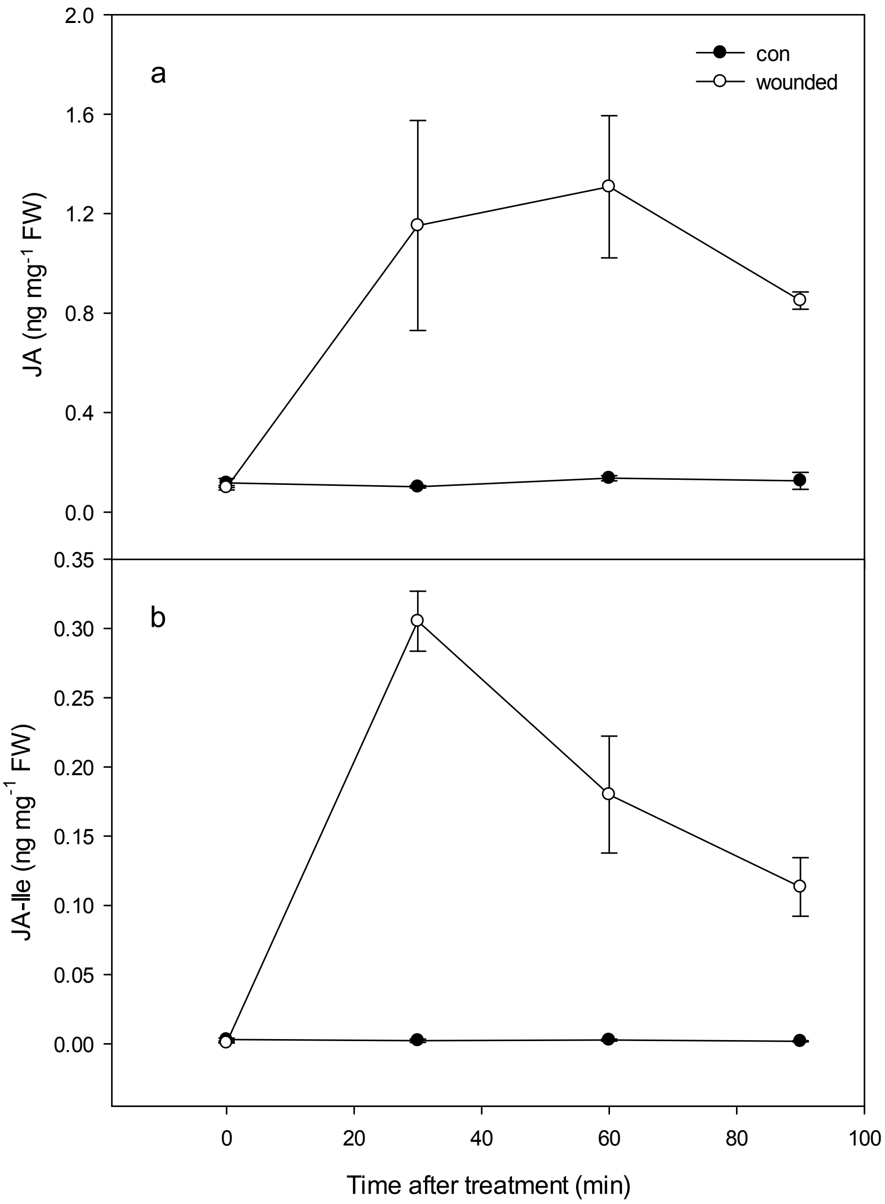

Figure 4 Time course of JA (a) and JA-Ile (b) concentrations following mechanical wounding of *Arabidopsis thaliana* Col-0 seedlings. (Mean ± SE, n = 4-6).

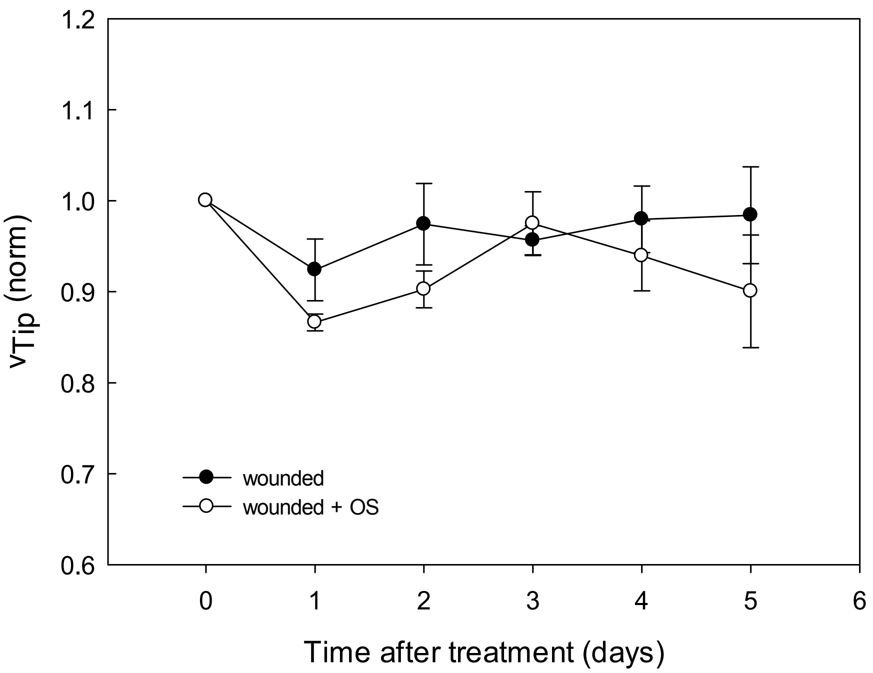

Figure 5 Root growth of *A. thaliana* Col-0 lines following mechanical wounding (wounded) and mechanical wounding and application of oral secretions of *Spodoptera littoralis* (wounded + OS). (Mean ± SE, n = 5-6).

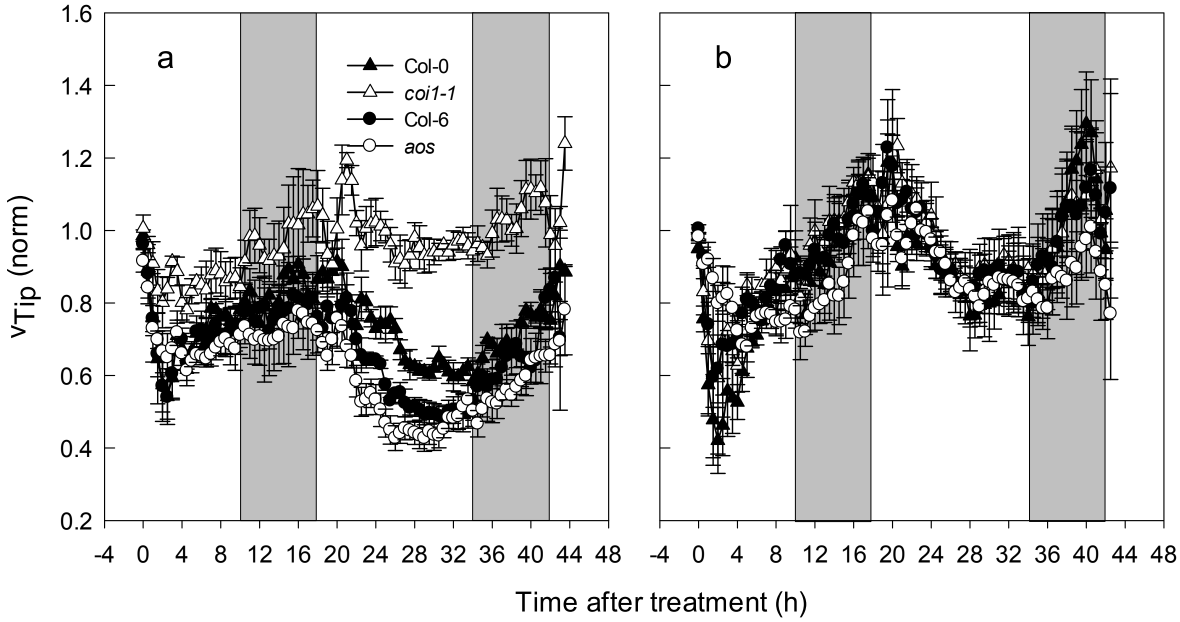

Figure 6 Normalized values of root tip velocities (v_{Tip}) of different *Arabidopsis thaliana* lines (a) after concomitant mechanical wounding and application of *Pseudomonas syringae* pv. tomato DC30000 avrRpt2 and (b) after concomitant mechanical wounding and application of the coronatine-deficient strain *P. syringae* pv. tomato DC30000 NCPPB 1008. The bacteria were applied at concentrations of 1 x 10^7 cfu ml^{-1}. The night period is highlighted in grey. (Mean ± SE, n = 3-5).

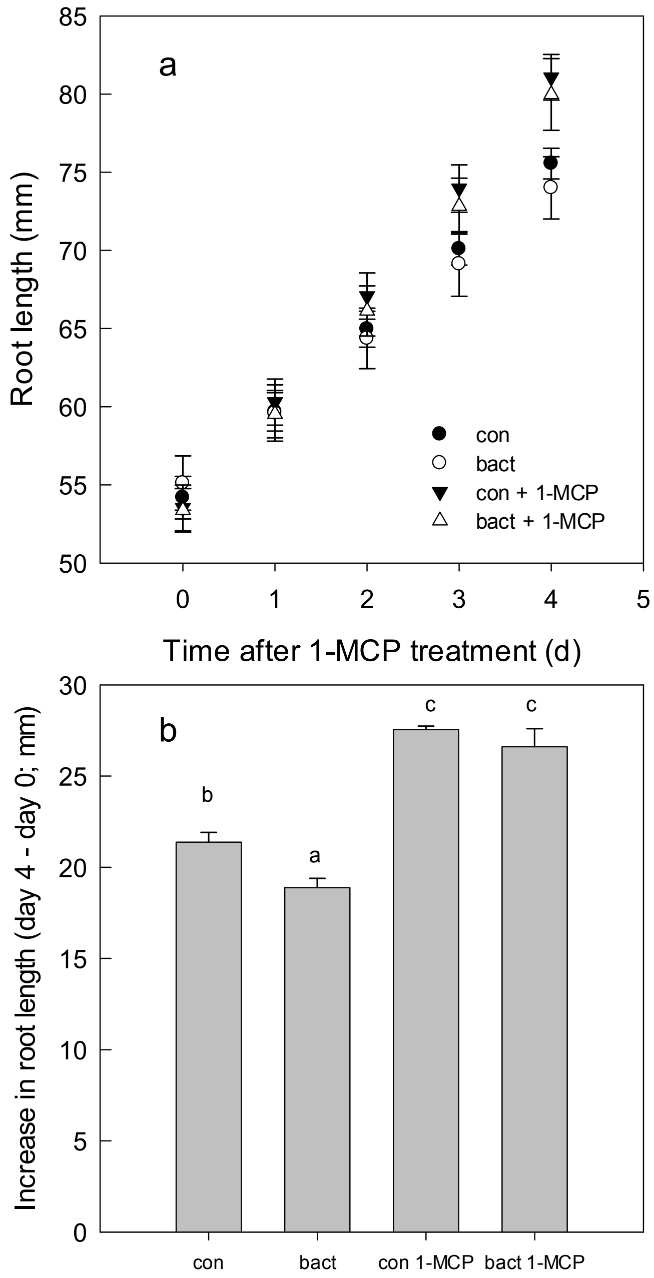

Figure 7 Root growth of untreated *Arabidopsis* seedlings (con), seedlings that were wounded mechanically and treated with *Pseudomonas syringae* pv. tomato DC3000 avrRpt2 at $1x10^7$ cfu ml^{-1} for three days (bact), seedlings treated with 1-methylcyclopropene (con + 1-MCP) and seedlings exposed to 1-MCP for four days and concomitantly wounded and treated with bacteria for three days (bact + 1-MCP). (a) Time course of root length increase; (b) total increase in root length after four days. Different letters indicate significant differences at P < 0.05 level. (Mean ± SE, n = 3-4).

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Statement of authorship

I hereby certify that this dissertation is the result of my own work. No other person's work has been used without due acknowledgement. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.

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