Analysis of defense-related reactions upon herbivore attack in *Ipomoea batatas*

Master’s Thesis
Submitted by

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This thesis was conducted at the Max-Planck-Institute for Chemical Ecology, department of Bioorganic Chemistry, in the working group *Plant defense Physiology* under supervision of PD Dr. Axel Mithöfer in cooperation with the laboratory of Professor Kai-Wun Yeh, Institute of Plant Biology (National Taiwan University).
Für meine Familie; insbesondere meinen Vater.

謝謝我的台灣的朋友們，還有你們與我共享的美妙經歷。我會永遠記得在一起的快樂時光。
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Zusammenfassung

Plants have evolved distinct signaling pathways resulting in direct or indirect defense as a response to biotic and abiotic stress factors. Especially indirect defense mechanisms, comprising emission of volatile organic compounds (VOCs), have gained attention due to their function as signaling compounds for inter-plant communication and attractants for predators during insect attack. High agricultural yield losses caused by pests underline the necessity for inducible strategies to retaliate against herbivore infestation. *Ipomoea batatas*, known as sweet potato, is an easily cultivatable, nutrient-rich tuberous species and the fifth most important crop in the world. Different insect-resistant cultivars of sweet potato are available. Especially *I. batatas* cv. Tainong 57 showed a wound-inducible expression of a trypsin inhibitor, sporamin, thereby serving as a suitable model species for plant-insect interaction studies. Sporamin, a tuber storage protein which is systemically expressed upon wounding was earlier shown to confer insect resistance. Little is known about the regulatory impact of wounding on the defense signaling cascade in sweet potato. Hence, this study addressed defense-related candidate genes and production of phytohormones after mechanical damage inflicted by MecWorm and insect feeding by *Spodoptera littoralis*. A fast induction of defense-related transcription factors could be observed in leaves both locally and systemically, leading to the induction of sporamin after wounding and herbivory. Wounding and herbivory also resulted in a local - but never systemic - production of jasmonates. Additionally, emitted VOCs were analyzed and identified and their putative function as signaling compounds for priming of adjacent plants after wounding was studied. Herbivory and constant mechanical damage resulted in an increased emission and number of different VOCs. Throughout this study, wound-induced VOC blends as well as 4,8-dimethyl-1,3,7-nonatriene (DMNT) alone have been identified as efficient elicitors for defense-related gene upregulation in unwounded sweet potato plants. In contrast, jasmonates could never be induced by airborne DMNT alone. Thus, upon herbivory and wounding a coordinated interaction between jasmonates, regulatory genes, sporamin and VOCs was found. Interestingly, in local and systemic leaves the role of jasmonate seems to be different. However, the most striking result was the finding that the volatile homoterpene DMNT can serve as signaling compound in systemic and conspecific defense activation in sweet potato. This finding highlights the potential of DMNT as an environment-friendly compound used for plant-protection in agriculture.
1 Introduction

1.1 Plant defense mechanisms after herbivory

Plants are sessile organisms which are constantly subjected to many different kinds of biotic as well as abiotic stress. Due to their restricted motility, plants incessantly need to develop new strategies to cope with these increasing environmental challenges. Especially herbivorous insects pose as a major threat based on their variability in ways of attacking the plant. Particularly chewing insects from the orders Lepidoptera and Coleoptera can cause severe damage but simultaneously trigger distinct defense-related signaling pathways in the plant (Howe and Jander 2008; Diezel et al. 2009). In contrast to such drastic impact especially on the leaves, piercing-sucking herbivores like *Tetranychus urticae* cause only minor damage (Van Poecke 2007; Howe and Jander 2008) but nevertheless embody a worthy opponent that needs to be defeated with distinct defense strategies (Leitner et al. 2005). Apart from the mode of feeding, herbivores can also adapt to the defense mechanisms of their favored host plant. By overcoming the plants’ physical and chemical barriers they become so-called specialists. On the other hand there are non-adapted species which are unable to bypass specific plant’s defenses (Stotz et al. 2001; Kliebenstein et al. 2002; Ratzka et al. 2002), called generalists. Since the feeding strategy and the resulting damage are highly fluctuant between herbivorous species, the plant needs to be capable to retaliate with all of its available physico-chemical feasibilities.

1.2 Principles and classification of plant defense

Mechanisms in plant defense are mainly classified as direct or indirect (Mithöfer and Boland 2012). Direct defense strategies comprise establishment of physical barriers by producing morphological features such as thorns, trichomes or increased levels of lignification (Mithöfer and Boland 2012). In addition to these fortifications, plants are also able to produce a variety of secondary metabolites like cyanogenic glycosides, glucosinolates or alkaloids. Another essential aspect in direct defense is the protein-based protection by proteinase inhibitors (PI). PIs can inhibit protease activity in the
insect gut thereby interfering with nutrient utilization or degradation of toxic substances taken up by the feeding herbivore (Mithöfer and Boland 2012).

Indirect defense describes the involvement of additional trophic levels apart from the feeding insect. By production and emission of volatile organic compounds (VOCs) such as terpenoids or green leaf volatiles, predators of the herbivore can be attracted to reduce the infestation (Takabayashi and Dicke 1996; Kessler and Baldwin 2002). Studies with *Medicago truncatula* showed that chewing or other modes of damage caused by different herbivores can result in distinct volatile emission patterns (Leitner *et al.* 2005). Furthermore, VOCs can also prime neighboring plants by giving information about an upcoming threat that the adjacent plant can activate its defense mechanisms (Arimura *et al.* 2000a). Following findings showed that VOCs also serve as semiochemicals whose effect can be seen on different levels in the plant. On a molecular scale, herbivore-induced volatiles were able to activate transcription of genes involved in biosynthesis pathways of ethylene and polyamines whilst simultaneously increasing endogenous jasmonate levels in *Phaseolus lunatus* (Arimura *et al.* 2002).

Another subdivision of defense mechanisms in plants is the distinction between constitutive and induced responses. Constitutive defenses are always present independent of an imminent threat to the plant. Induced defenses are specifically elicited by an aggressor, e.g. herbivore infestation (Mithöfer and Boland 2012). Since there are a tremendous number of possibilities how the plant can upregulate defense mechanisms by involving different genes, molecules and overall protection strategies, the following chapters will give further information on plant-herbivore interaction and inducible indirect defense.

### 1.3 Phytohormones as defense signaling molecules

It is known that plant hormones play an essential role in regulatory processes during plant defense. Especially jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) came into focus as molecules involved in defense against pathogens and herbivore attacks (Bonaventure 2012; Wasternack and Hause 2013). The most prominent compound, JA, is synthesized from linolenic acid via the 13-lipoxygenase pathway (LOX) (Turner *et al.* 2002). Linolenic acid is converted into 12-oxo-phytodienoic acid (OPDA) by lipoxygenase, allene oxide synthase and allene oxide cyclase. OPDA is reduced by the 12-OPDA-reductase (Strassner *et al.* 2002) followed by three rounds of beta-oxidation resulting in JA (Howe and Schilmiller 2002). JA can be further processed into amino acid
conjugates; the one with isoleucine represents the most bioactive form, JA-Ile (Staswick and Tiryaki 2004). Two additional pathways that are branching off the regular JA pathway can result in the production of volatile organic compounds (VOCs). Green leaf volatiles (GLVs) can be produced from hydroperoxylinolenic acid whereas JA itself can be metabolized to cis-jasmone or methylated to form methyl jasmonate (Liechti and Farmer 2006).

It has been demonstrated that JA can regulate herbivory-induced genes (Reymond et al. 2000) in *Arabidopsis thaliana*. In fact, mutants defective in JA synthesis showed impaired expression of insect-inducible genes and were therefore more susceptible to herbivory (Reymond et al. 2000; Li et al. 2004; Paschold et al. 2007).

The active form of JA, jasmonyl-isoleucine (JA-Ile), is produced by JAR1 (Staswick and Tiryaki 2004). JA-Ile possesses the ability to activate complex signaling cascades by inducing the degradation of JAZ proteins (jasmonate-ZIM-domains) to invoke transcription factors like MYC2 (Sheard et al. 2010). This results in the expression of JA-regulated genes (Howe and Jander 2008; Wu and Baldwin 2010), which are mostly active during wounding (Koornneef and Pieterse 2008). The signaling cascade of interest for this study will be outlined in the following chapter 1.5.2. Overall, the effect of plant-produced phytohormones on regulatory genes involved in wound-inducible signaling cascades is still not well known.

### 1.4 *Ipomoea batatas* as a model species

Sweet potato (*Ipomoea batatas* Lam.; Convolvulaceae) is one of the most important staple crops worldwide. Early recordings already indicated its importance as a valuable food resource for many indigenous populations in Central and South America, Ryukyu Island, Africa, the Caribbean, Hawaii and Papua New Guinea since many centuries (Bovell-Benjamin 2007). During the years the production of sweet potato gained more importance, especially in Eastern Asia with China providing nearly 80 percent of the global sweet potato production (Food and Agriculture Organization unpublished). Due to its high starch content and easy cultivation by clonal propagation, it achieved a high nutritional importance especially in undeveloped countries to reduce food shortage and malnutrition (Woolfe 1992). Especially in third-world countries like Africa, sweet potato is a valuable food resource by providing a high yield in unfavourable conditions within 3 to 4 months after planting (Karyeija et al. 1998). Additionally, with its broad genetic base and phenotypic variability, a tremendous number of cultivars has been hybridized to obtain the
maximum yield to meet growing agricultural demands. Especially the development of insect-resistant cultivars attracts attention in agricultural sciences since sweet potato is subjected to a tremendous variety of pests, including slugs, caterpillars and beetles (see Fig. 1). Two of the most common known natural enemies in nature are the sweet potato weevil (*Cyclus formicarius*, (Ray and Ravi 2005)) and the sweet potato whitefly (*Bemisia tabaci*, (Simmons and Abd-Rabou 2007)).

![Herbivores feeding on different Ipomoea batatas cultivars grown in the field in Taiwan.](image)

**Fig. 1** Herbivores feeding on different *Ipomoea batatas* cultivars grown in the field in Taiwan.

In order to prevent agricultural yield loss by pests, the knowledge about the underlying mechanisms of plant-herbivore interaction and possible defenses need to be increased. Even though *Ipomoea batatas* possesses a complex hexaploid non-sequenced genome, the generation of
transgenic overexpression lines in tissue culture has been successfully established (Chen et al. 2016). All the aforementioned factors underline the potential of sweet potato to become an important crop in agriculture to meet global demands for higher yields by exploiting its plasticity and efficiency in defence mechanisms.

1.5 Defense mechanisms after wounding in *Ipomoea batatas*

Plants are exposed to many different forms of biotic as well as abiotic stress, especially when growing outside amidst a variety of herbivores. Therefore, crop plants like sweet potato are increasingly challenged to develop sophisticated defense mechanisms against predators.

1.5.1 Sporamin – a tuber storage protein with trypsin inhibitory activity

Considering putative candidates for defense in *Ipomoea batatas*, a highly abundant storage protein named Sporamin gained importance as protection against herbivores over the years. Sporamin has already been known as a major storage protein in sweet potato tubers (Maeshima et al. 1985) accounting for 60–80% of the total soluble protein content in the tuber. Although expression of Sporamin was mostly present in the tuberous roots, further investigations proved the presence of Sporamin in very low amounts in the aboveground material like stem and leaves in non-stressful conditions (Hattori et al. 1990). After inflicted damage on sweet potato plants, Sporamin is systemically induced in leaves and stems (Yeh et al. 1997a; Wang et al. 2002). Regarding its biological importance, Sporamin is not only serving as a nutritional source but has insect–defense abilities due to its trypsin–inhibitory activity (Yeh et al. 1997b). Analysis of nucleotide sequence homology showed that Sporamin genes can be divided into two subfamilies encoding the corresponding proteins, Sporamin A (31 kDa band in native gel analysis) and B (22 kDa in native gel analysis). The reason for this is not known yet. The overall protein size in denatured conditions was shown to be 25 kDa (Maeshima et al. 1985; Hattori et al. 1989). All deduced amino acid sequences shared four conserved cysteine residues and a negatively charged A4–B1 loop that is conveying the trypsin–inhibitory activity (Yao et al. 2001). Assumptions that the 30-50% similarity of Sporamin to the soybean Kunitz–type trypsin inhibitor (Senthilkumar and Yeh 2012; Lai et al. 2013) plays a role in sweet potato defense mechanisms were confirmed by Yeh et al. (1997b). The impact on
herbivore feeding has been shown by introducing a sweet potato trypsin inhibitor gene in transgenic tobacco plants and letting *Spodoptera litura* larvae feed on it. Infestation of the transformed tobacco lines resulted in severe growth retardation in the larvae, thereby confirming the ability of sweet potato trypsin inhibitors to confer insect resistance by impeding digestion in the insect’s digestive tract (Yeh et al. 1997b; Rajendran et al. 2014).

### 1.5.2 Induced defense signaling cascade in sweet potato

Previous experiments confirmed that a complex signaling cascade can be activated by different modes of damage in sweet potato plants (Wang et al. 2002; Senthilkumar and Yeh 2012; Chen et al. 2016), (Fig. 2). One of the first regulators triggered by stress or pathogens was identified as *WIPK* (wounding-induced protein kinase) (Xu et al. 2014). The kinase itself is most probably activated by a preceding mitogen-associated protein kinase cascade. In a transactivation analysis, WIPK has been shown to positively modulate both *IbNAC1* (*No* apical meristem, *Arabidopsis* transcription activation factor, *Cup*-shaped cotyledon) and *Sporamin* promoter (Lo unpublished). In addition, the transcription factor of JA-responsive genes, MYC2 (Sheard et al. 2010), is considered to be an intermediate regulator during the early wounding response in sweet potato (Chen unpublished). MYC2, also known as *IbbHLH3* in *I. batatas* becomes active by binding as a homodimer to a CACGTG-motif to activate *IbNAC1*. A second transcription factor (JAMs1) is also predicted to regulate the activation of *Sporamin* via *IbNAC1*. JAMs1, also known as *IbHLH4* in sweet potato, is predicted to bind either as a homo- or heterodimer with MYC2 by consequently inhibiting expression of *IbNAC1* (Chen unpublished). Studies have shown that the NAC domain protein, *IbNAC1* can specifically bind to the *Sporamin* Wound-Response *cis*-Element (SWRE) in the *Sporamin* promotor, therefore being crucial for the wounding response in sweet potato (Chen et al. 2016).
Fig. 2 Proposed model of wounding-inducible signaling cascade in *Ipomoea batatas*. In this specific study volatile organic compounds (VOCs), mechanical damage by MecWorm or herbivore infestation were applied to trigger defense responses in the sweet potato leaves. For details and abbreviations see the text (chapter 1.5.2).
1.6 Aims of the study

Despite numerous studies on herbivory-induced defense mechanisms in crop species, less is known so far about the induction of signaling cascades in response to wounding in *Ipomoea batatas*. Especially the distinction between mechanical wounding as a single stimulus and mixed effects due to elicitors secreted during insect feeding on plants is not yet well understood in sweet potato. Previous findings identified *Sporamin* as key defensive compound to confer insect resistance (Yeh *et al.* 1997b). One main goal of this study was the elucidation of genes involved in the wound-inducible signaling cascade in *I. batatas* by quantitative real time PCR towards the activation of *Sporamin* expression. Additionally, the systemic and local expression of *Sporamin* and other regulators in response to mechanical damage and herbivore feeding by the generalist herbivore *Spodoptera littoralis* was to be investigated in this thesis. Apart from gene regulatory processes, jasmonates have also been shown to play a role during insect attack within the sweet potato (Rajendran *et al.* 2014). Therefore, it was of major interest in this study to investigate via LC-MS/MS whether phytohormone and especially jasmonate production was affected by herbivore feeding or mechanical damage only. Apart from within-plant signaling during harmful events, indirect defense mechanisms like the emission of volatile organic compounds (VOCs) in response to herbivory and mechanical damage have not been studied so far in *I. batatas*. Hence, this study also focused on the GC-MS based analysis of VOCs emitted after *Spodoptera littoralis* infestation or continuous damage inflicted by MecWorm. In order to assess a putative biochemical potential of emitted compounds, experiments were conducted exposing unwounded plants to the whole VOCs blend or single compounds released after different wounding stimuli followed by phytohormone and gene expression analyses. Broadly speaking, this thesis focused on inducible (in)direct defense mechanisms in *I. batatas* as response to biotic and abiotic challenges. The identification of coherences between different mechanical damage and herbivory induced signaling pathways that comprise phytohormone production, upregulation of transcription factors and either sporamin generation or the emission of VOCs was the main goal of this study.
2 Experimental procedures

2.1 Plant material and growth conditions

Sweet potato scions (*Ipomoea batatas* Lam.; cv. Tainong 57, see Fig. 3) were grown in round pots with 10 cm diameter under long day conditions (16 h : 8 h, light : dark) at 28°C (day) and 25°C (night) in 70% relative humidity. Illumination in the growth chamber was kept at 100 µmol m$^{-2}$ s$^{-1}$. Experiments conducted at MPI CE in Jena were set up between 9 and 12 o’clock (a.m.). Plants used for studies at NTU (National Taiwan University, Taipei, Taiwan) were cultivated under the same growth conditions at a temperature of 25°C (day) and 20°C (night). In each experiment, plants with six to eight fully expanded leaves were used. Each third fully expanded leaf was locally treated (LW = local wounding) and harvested together with the adjacent fourth leaf (Sys = systemic leaf) using scissors.

![Fig. 3](image)

Fig. 3 Left: *Ipomoea batatas* with its third leaf inserted into the MecWorm apparatus. Right: Sweet potato plants growing in the climate chamber.
2.2 Insect rearing

*Spodoptera littoralis* (Boisd., Lepidoptera, Noctuidae) larvae were hatched from eggs (Bayer Cropscience, Monheim, Germany) and reared on an artificial diet consisting of 500 g hackled beans, 9 g ascorbic acid, 9 g 4-ethylbenzoic acid, 9 g vitamin E Mazola oil mixture (7.1%), 4 ml formaldehyde, 1.2 l water, 1 g sitosterol, 1 g leucine, 10 g AIN-76 vitamin mixture and 200 ml agar-water solution (7.5%). Insects were reared at 23-25°C with a photoperiod of 14 hours. For starving, 3rd to 4th instar larvae were separated into plastic cups 24 hours before each herbivore assay. Experiments conducted at the National Taiwan University applied second- instar *Spodoptera litura* larvae for insect feeding assays. Larvae were provided by Prof. Y. Wu (Department of Entomology, NTU) and separated 24 hours previous to all experiments to ensure starving of the insects.

2.3 Plant treatments

2.3.1 Mechanical wounding by MecWorm

In order to mimic herbivore feeding without elicitors MecWorm (Mithöfer *et al.* 2005), known as the mechanical caterpillar (Fig. 3 left; Fig. 4) was used to inflict continuous mechanical wounding to the third fully developed leaf of each plant. Wounding sites of rectangular shapes were selected with a punching speed of 12 punches per minute lasting for 30, 60 and 180 minutes. For headspace volatile collection the continuous damage has been extended to 18 hours. Additionally the treated leaf was enclosed in a Plexiglas® cabinet of the MecWorm apparatus (see Fig. 4). Wounding areas on the leaf lamina have been arranged without damaging the midrip (see Fig. 4). After treatment, the third fully expanded wounded leaf and the adjacent fourth entirely developed systemic leaf were collected, immediately frozen in liquid nitrogen and stored at -80°C until further processing.
Material and methods

2.3.2 Herbivore infestation

Twenty-four hours prior to herbivore application, 3\textsuperscript{rd} to 4\textsuperscript{th} –instar larvae of \textit{S. littoralis} were separated into plastic cups for starving. Afterwards, the third fully expanded leaf of each plant was infested with one larva of \textit{S. littoralis} and enclosed in a feeding cage (Fig. 5) for 30, 60 or 180 minutes. Tissue samples of the third and fourth fully expanded leaves were collected as previously described. Modifications for volatile collection included that the treated leaf and the feeding herbivore with the surrounding feeding cage were enclosed in odorless PET foil (Toppits, Minden, Germany) over 24 hours. The neighboring fourth fully expanded systemic leaf was also bagged in PET foil for 24 hours. All herbivore experiments were conducted in the growth chamber. Measuring of the treatment period started after the first observed feeding by the herbivore.
Material and methods

Fig. 5 Left: Sweet potato leaf enclosed in a feeding cage during herbivore infestation. Right: Spodoptera littoralis feeding on sweet potato.

2.3.3 Induction by the homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT)

DMNT was provided by Dr. S. Bartram (MPI CE, Jena, Germany). Before use, DMNT was dissolved in pure dichloromethane (1 µg per µl solution) and 20 µg of DMNT were impregnated into a single piece of cotton wool (Fig. 6). The cotton wool was placed without any physical contact centrally between three sweet potato plants in a closed glass container (34 l) for 15, 30 and 60 minutes. In order to investigate concentration-dependent induction of gene expression levels 5, 20 and 60 µg of DMNT solution have been applied over a period of 1 hour. As control equal volumes of pure dichloromethane were incubated on cotton wool and placed centrally between three plants.
Material and methods

For mechanical wounding, approximately 4 fully expanded leaves of two sweet potato plants were wounded by tweezers and placed into an odorless glass container (34 l). The midrip was not wounded during this process. As indicated in Fig. 6, wounded plants were placed in opposite corners inside the glass container with three plants centrally placed between them. Subsequently the glass container was closed and the plants were incubated for 24 hours.

Each third fully developed leaf was collected and RNA was extracted and qPCR performed at NTU.

All samples used for phytohormone extraction after induction by DMNT were collected at MPI CE. In this experiment 1.41 µg of DMNT solution was incubated according to previous experiments, on cotton wool in 2.4 l glass desiccators (DURAN® Vakuum Exsikkator, DURAN GROUP, Wertheim, Germany, see Fig. 7) for 1 h. Leaves were collected as described previously and immediately frozen in liquid nitrogen until further phytohormone extraction.
Material and methods

2.4 RNA extraction and quantitative real time (qRT)-PCR

Collected sweet potato leaves were ground to a fine powder with liquid nitrogen. RNA was extracted using TRIzol® Reagent (Invitrogen™, Darmstadt, Germany) by adding 1 ml reagent to 100 mg of ground leaf tissue. After thorough mixing, all samples were incubated at room temperature for 10 minutes. The following centrifugation steps were performed at 12,000 x g at 4°C. After incubation, samples were centrifuged for 15 minutes and the supernatant was transferred into 200 µl chloroform. Subsequently samples were inverted for 1 minute and centrifuged again for 15 minutes. Addition of 200 µl chloroform to the supernatant was repeated and samples were centrifuged for 15 minutes. The aqueous phase was added to approximately 600 µl isopropanol (1:1, sample: isopropanol) and precipitated overnight at 20°C. For sedimentation, samples were centrifuged for 15 minutes and the supernatant was removed. RNA pellet was washed with 1 ml of cooled 80% ethanol, mixed and centrifuged for 15 minutes. The washing step was repeated once more and the resulting pellet was vacuum-dried in an Eppendorf Concentrator plus (Eppendorf AG, Hamburg, Germany) for 10 minutes at 30°C. Subsequently, the dried pellet was dissolved in 50 µl of preheated water for 15 minutes at 42°C with mixing every 5 minutes. RNA concentration was measured using NanoDrop™ One microvolume UV-Vis spectrophotometer (Thermo Fisher
Material and methods

Scientific™, Schwerte, Germany). First strand cDNA was synthesized from 6 µg of total RNA using RevertAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific™, Schwerte, Germany) with Oligo(dT)₁₈ Primer. Synthesis was conducted according to the manufacturers’ instructions with slight modifications. After mixing 6 µg of template RNA with primer and water, additional incubation at 65°C for 5 minutes with subsequent chilling on ice for 10 minutes was conducted before adding the reaction mix. The sample was incubated at 42°C for 60 minutes. The reaction was stopped at 72°C for 5 minutes. Subsequently cDNA was diluted by addition of 40 µl of water for a total cDNA concentration of 100 ng µl⁻¹.

Real-time qPCR analysis was performed using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, Waldbronn, Germany) and gene-specific primers as previously described by Chen et al. (2016) (listed in Fig. 8), Eurofins Genomics, Ebersberg, Germany). For normalization of gene expression levels, *IbACTIN-2* was used as housekeeping gene.

<table>
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<td>5’ – GCTCCTCTGATAACCTTGGAT – 3’</td>
<td>60,1</td>
</tr>
<tr>
<td><em>IbNAC1</em> F</td>
<td>5’ – CGGCCGGGATACAAATTGTGAAGCTT – 3’</td>
<td>65,0</td>
</tr>
<tr>
<td><em>IbNAC1</em> R</td>
<td>5’ – GAATCGGAAATCCGGGCATCTC – 3’</td>
<td>67,8</td>
</tr>
<tr>
<td>MYC2-1 F</td>
<td>5’ – AGCTCAATTCTTTTATCTCCGGA – 3’</td>
<td>58,9</td>
</tr>
<tr>
<td>MYC2-1 R</td>
<td>5’ – GGACCTTTGTTCATCAAATCTGC – 3’</td>
<td>59,3</td>
</tr>
<tr>
<td>JAMs1-1 F</td>
<td>5’ – ATTCGGAGATTCAAACCACAA – 3’</td>
<td>57,1</td>
</tr>
<tr>
<td>JAMs1-1 R</td>
<td>5’ – TTATAACCCAACAAAGCATGACGC – 3’</td>
<td>59,7</td>
</tr>
<tr>
<td>FPPS F</td>
<td>5’ – ACTGCTTTGACTGCTGCACT – 3’</td>
<td>59,8</td>
</tr>
<tr>
<td>FPPS R</td>
<td>5’ – GCACTGGAACCTTGTGAAGGA – 3’</td>
<td>60,6</td>
</tr>
</tbody>
</table>

*Fig. 8* Oligonucleotide sequences of primers used in quantitative real time (qRT)–PCR.
The master mix was prepared according to the manufacturers’ instructions using 400 nM of each gene-specific primer and 100 ng cDNA per well for a total reaction volume of 25 µl.

Gene amplification was achieved using Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany) with the following program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Amplification</td>
<td>72 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Dissociation</td>
<td>65 °C</td>
<td>5 sec</td>
</tr>
<tr>
<td></td>
<td>95 °C</td>
<td>50 sec</td>
</tr>
</tbody>
</table>

Fluorescence was detected after the amplification phase at 72°C for each cycle and in the final dissociation phase during heating of the DNA from 65°C to 95°C. The second fluorescence detection was used to generate a melting curve. The generated data was processed by using Bio-Rad CFX Manager (Bio-Rad Laboratories GmbH, Munich, Germany).

For analysis, the normalized fold expression was calculated according to the \(\Delta\Delta CT\) method described by Pfaffl (2001) using the formula:

\[
\text{ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}(\text{control} - \text{sample})}}
\]

**Equation 1 Mathematical model to calculate the relative expression ratio in real-time PCR according to Pfaffl (2001).** \(E\) represents the efficiency of the target- \(\text{IbACTIN}-2\) or reference gene. \(CT\) is defined as the threshold cycle in which the detected fluorescence shows a statistically significant increase compared to the background fluorescence. Untreated control plants were defined as expression level 1.

In each experiment at least 5 (up to 16) biological replicates were used. Detected CT -values from technical replicates deviating more than 0.5 from each other were not used for calculation.
The total RNA extracted at NTU was obtained using the method of Chang et al. (1993). Subsequent cDNA synthesis and qPCR was performed as previously described by Chen et al. (2016).

2.5 Phytohormone extraction and quantification

Collected sweet potato leaves were ground to a fine powder in liquid nitrogen and 200 - 250 mg of finely ground leaf material was weighed into an Eppendorf tube (Eppendorf AG, Hamburg, Germany). The extraction and detection was performed as previously described by Vadassery et al. (2012) with minor modifications. For phytohormone extraction, weighed powdery leaf material was mixed with 1.5 ml methanol containing 60 ng D<sub>6</sub>-abscisic acid (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), 60 ng of D<sub>6</sub>-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D<sub>4</sub>-salicylic acid (Sigma-Aldrich, Taufkirchen, Germany) and 12 ng of jasmonic acid-<sup>13</sup>C<sub>6</sub>-isoleucine conjugate as internal standard. After brief mixing, samples shook for 30 minutes at 4°C using a Rotator Mixer RM-Multi 1 (STARLAB GmbH, Hamburg, Germany) using the program 100 rpm: 15 s, 75°: 16 s, 3°: 5 s. Samples were centrifuged afterwards at 13,000 x g at 4°C for 20 minutes, the supernatant was collected and the remaining pellet resuspended in 500 µl methanol. The resuspended pellet was shaken and centrifuged again as previously described. The supernatants were combined and concentrated for 3 hours using Eppendorf Concentrator plus (Eppendorf AG, Hamburg, Germany). The concentrate was resuspended in 500 µl methanol and centrifuged at 16,000 x g at 4°C for 10 minutes. Lastly, 400 µl of supernatant were used for LC-MS/MS measurements.

Phytohormone analysis was performed using Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) with subsequent API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) with a Turbo spray ion source in negative ionization mode. The elution profile was: 0 - 0.5 min, 10% B; 0.5 - 4.0 min, 10 - 90% B; 4.0 - 4.02 min, 90 - 100% B; 4.02 - 4.5 min, 100% B and 4.41 - 7.0 min, 10% B at a flow rate of 1.1 ml min<sup>-1</sup>. Multiple reaction monitoring (MRM) was applied to monitor analyte parent ion→ product ion: m/z 209.1 → 59.0 collision energy (CE) -24 V, declustering potential (DP) -35 V) for jasmonic acid; m/z 215.1 → 56.0 (CE -24, DP -35 V) for D<sub>6</sub>-jasmonic acid, m/z 322.2 → 130.0 (CE -30 V, DP -50 V) for jasmonic acid-isoleucine conjugate, m/z 328.2 → 136.1 (CE -30 V, DP -50 V) for jasmonic acid-<sup>13</sup>C<sub>6</sub>-
isoleucine conjugate, m/z 136.9 → 93.0 (CE -22 V, DP -35 V) for salicylic acid, m/z 140.9→97.0 (CE -22 V, DP -35 V) for Δ4-salicylic acid, m/z 263.0 → 153.2 (CE -22 V, DP – 35 V) for abscisic acid, m/z 269.0 → 159.2 (CE -22 V, DP –35 V) for Δ6-abscisic acid and m/z 290.9 → 165.1 (CE -24 V, DP –45 V) for cis-(+)-12-oxophytodienoic acid (cis–OPDA).

2.6 Volatile collection and GC–MS analysis

Volatile were collected over 24 hours using the closed-loop stripping technique as previously described by Kunert et al. (2009); (see Fig. 9; Fig. 10). Plants were either treated with MecWorm or S. littoralis as described above. Each single third and adjacent neighboring fourth leaf, still connected to the whole sweet potato plant, was enclosed in odorless PET foil bags (Toppits, Minden, Germany) to avoid contamination by soil volatiles. Each third fully developed leaf of the untreated plant was enclosed in odorless PET bags and used as control. Emission patterns of Plexiglas® feeding cages used in herbivore bioassays showed no detectable influence on the induction of volatile release.

Fig. 9 Volatile collection set-up with Spodoptera littoralis trapped in a Plexiglas cage feeding on sweet potato.

For constant air circulation and continuous volatile collection each foil bag was connected to a circulation pump (Fürgut GmbH, Tannheim, Germany) containing a charcoal trap with 1.5 mg
absorption material (CLSA filter, 6 cm long, 0.5 cm diameter, Gränicher & Quartero, Daumazan sur Arize, France, Fig. 10). After collection, volatiles were desorbed with 2 x 20 µl dichloromethane containing 50 µg ml\(^{-1}\) n-bromodecane as internal standard by gentle cooling and heating of the insert with liquid nitrogen.

![Fig. 10](image)

**Fig. 10** Left: Volatile collection using the closed-loop stripping method according to Kunert et al. (2009). Right: Insertion of charcoal trap into the circulation pump.

Analysis was conducted using GC-MS (Finnigan TRACE™ GC 2000, Thermo Fisher Scientific™, Schwerte, Germany) equipped with a Zebron™ ZB -5 column (25 m x 0.25 mm x 0.25 µm, Phenomenex, Aschaffenburg, Germany) and the following temperature profile for separation of VOCs: Initial temperature was set at 45°C for 2 minutes, heating up 10°C min\(^{-1}\) to 200°C and 30°C min\(^{-1}\) to 280°C. Helium was used as carrier gas with a flow rate of 1.5 ml min\(^{-1}\). Split ratio was set at 1: 10 and 1 µl of 40 µl eluate was automatically injected. Injector temperature was set at 220°C.

### 2.6.1 Identification of VOCs

Identification of emitted compounds was performed according to its MS fragmentation pattern and comparison with NIST Mass Spectral Library (NIST 2008; The NIST Mass Spectrometry Data Center,
Gaithersburg, U.S.A.), mainlib (Xcalibur V.2.0.7, Thermo Fisher Scientific™, Schwerte, Germany), MassFinder4 (Dr. D. Hochmuth, Hamburg, Germany) and comparable literature data (Leitner et al. 2005).

### 2.7 Absolute quantification of DMNT

After identification of the homoterpene 4,8-Dimethyl-1,3,7-nonatriene (DMNT) as a putative elicitor for defense, the absolute amount of emitted compound was calculated using a standard curve. Different quantities of DMNT were dissolved in pure dichloromethane to generate solutions with the following concentrations of DMNT: 2.5; 5; 10; 20; 50; 100; 150; 200 and 250 µg ml\(^{-1}\). All dilutions contained 50 µg ml\(^{-1}\) of n-bromodecane as internal standard for comparison with the previous VOCs measurements.

Emitted quantities of DMNT were calculated by division of DMNT peak areas through the respective peak areas of the internal standard. The output value was then inserted into the regression line formula of the measured DMNT standard curve and calculated according to the applied 40 µl elution volume per single leaf.

### 2.8 Statistical analysis

Statistics for experiments comprising RT–qPCR were done using t-test or Wilcoxon-test in RStudio (V. 0.98.1130.0). The test was chosen according to the distribution of the values included in the dataset using Shapiro-Wilk normality test for data exploration. Each experiment was performed with at least five independent biological replicates. Levels of statistical significance are marked as the following: \(p<0.05\) (*); \(p<0.01\) (**) and \(p<0.001\) (***)

Statistical significances of phytohormone contents were tested using Shapiro–Wilk normality test with a subsequent one-way ANOVA (analysis of variance) in SigmaPlot (V 12.1.0). ANOVA was conducted for each single time point during all treatments with at least 8 biological replicates. Dunn’s method was selected for all pairwise multiple comparison procedure. Statistical significance between groups was given when \(p<0.05\).
3 Results

3.1 Induction of defense–related genes by mechanical wounding (MecWorm)

Previous studies have shown that a complex wound-inducible signaling cascade can be activated by different modes of damage in sweet potato plants (see chapter 1.5.2). In a time-course analysis of gene expression levels after continuous mechanical damage by MecWorm (Fig 11), an immediate wounding response could be observed in almost all investigated genes. A strong local as well as a systemic response in expression levels for *IbWIPK* was triggered 30 minutes after wounding (Fig 11). The local response in the directly affected leaf was significantly increased up to 14-fold compared to control plants and thereby reaching its maximum after half an hour of treatment. The higher expression of *IbWIPK* was present, although decreased, after 1 hour and still significantly elevated after 3 hours of wounding (Fig 11). A comparable pattern was observed in the distant systemic leaf with a highly significant rapid 10-fold increased expression of *IbWIPK* after 30 minutes of wounding that returned to the basal expression level after 60 minutes (Fig 11).

*MYC2*, also known as *IbbHLH3* in *I. batatas*, is a key transcription factor in JA-regulated gene expression. Expression of *MYC2* was reaching its maximum rapidly after 30 minutes of damage with a significant 21-fold increase in the wounded leaf. The same observation was made with a highly significant 11-fold upregulation in the unwounded systemic leaves. After 1 hour of treatment expression levels were back to the basal value in both tissues. In a previous study by Chen (unpublished) it was demonstrated that *MYC2* becomes active during early wounding whereas the transcription factor *JAMs1* plays a role during the late response. *JAMs1* expression was significantly elevated in wounded and unwounded leaves after 30 minutes and still significantly detectable after 1 hour. After three hours of treatment no significant difference to the control group could be measured.

*IbNAC1* is induced previous to *Sporamin* after wounding (Chen *et al.* 2016). Indeed, *IbNAC1* undergoes fast activation after 30 minutes of wounding showing highly significant increased expression levels (see Fig. 12). Leaves directly wounded by MecWorm showed a 38-fold increased expression of *IbNAC1*. Unwounded systemic leaves were not as strongly affected as wounded ones,
nevertheless they showed a 13-fold upregulation of \textit{IbNAC1}. After 1 hour, significantly increased \textit{IbNAC1} expression levels were still detectable in both systemic and locally wounded leaves but only at low (2-to 3-fold) expression level. After 3 hours only in locally-punched leaves a still 3-fold increase was detectable. The main target of interest, sporamin, a protein with trypsin inhibitory activity in sweet potato (Rajendran et al. 2014) was shown to increase insect resistance (Yeh et al. 1997b). Measurements of \textit{Sporamin} gene expression after mechanical wounding showed high levels of fluctuation in the control plants as well as in the wounded tissues (see Fig. 12). Almost no significant changes in expression of \textit{Sporamin} could be detected in the mechanically damaged plants. A small but nevertheless significant decrease to a normalized fold expression of 0.5 could be observed after 60 minutes of treatment. The decreased \textit{Sporamin} expression was still prevalent after 3 hours of applied wounding, although not statistically significant. In contrast to the almost unaltered \textit{Sporamin} level in the wounded tissue, the systemic leaf showed a belated response in \textit{Sporamin} expression. Although the results were not significant there was a clear trend. A slight increase after 30 minutes of wounding was detected, followed by an 8-fold increased \textit{Sporamin} expression level after one hour and a remaining elevated level after 3 hours of applied damage.
Fig. 11 Expression of wounding-related genes from *I. batatas* in response to mechanical damage by MecWorm. Changes in the transcript level of *IbWIPK*, *MYC2-1* and *JAMs1-1* were determined in locally wounded leaves (LW) and the adjacent intact systemic leaves (Sys). Locally wounded leaves were constantly wounded by MecWorm. Both leaf types were harvested after 0.5 h, 1 h or 3 h of mechanical damage. Expression levels were determined using qRT-PCR, normalized to *IbACTIN-2* as housekeeping gene and calculated relative to untreated control plants. One-sample Wilcoxon-test or one-sample t-test respectively to the data distribution was selected to calculate statistical significant differences. Bars represent the means ± standard error (SE) with n≥5. Significance levels are indicated by the asterisks (* p<0.05; ** p<0.01; *** p<0.001).
Results

Fig. 12 Expression of wound-responsive *IbNAC1* and trypsin inhibitory *Sporamin* from *I. batatas* in response to mechanical damage by MecWorm. Changes in the transcript level of *IbNAC1* and *Sporamin* were determined in locally wounded leaves (LW) and the adjacent intact systemic leaves (Sys). Locally wounded leaves were constantly wounded by MecWorm. Both leaf types were harvested after 0.5 h, 1 h or 3 h of mechanical damage. Expression levels were determined using qRT-PCR normalized to *IbACTIN-2* as housekeeping gene and calculated relative to untreated control plants. One-sample Wilcoxon-test or one-sample t-test respectively to the data distribution was selected to calculate statistical significant differences. Bars represent the means ± standard error (SE) with n≥5. Significance levels are indicated by the asterisks (*p*<0.05; **p**<0.01; ***p***<0.001).
3.2 Regulation of defense–related genes after feeding by *Spodoptera littoralis*

Applied wounding by MecWorm can be used to demonstrate the effect of continuous mechanical damage to a plant and its response. Hence this kind of treatment omits several factors, e.g. elicitors present in herbivores’ oral secretion, just solely mimicking actual damage by a natural predator. In order to investigate the impact of a generalist herbivore (*S. littoralis*) on the defense regulation in sweet potato, each plant was treated with a single herbivorous larva placed on the third fully expanded leaf (SW, *Spodoptera* wounding), which was allowed to feed for 0.5, 1 and 3 hours. Gene expression levels of the wounded leaf and the adjacent unexposed systemic leaf (Sys) were determined by RT-qPCR. As described above, five genes are promising candidates to play essential roles in the wounding response of *I. batatas*: *IbWIPK, MYC2-1, JAMs1-1, IbNAC1, and Sporamin*.

The investigation of *IbWIPK* showed a highly significant rapid increase in expression levels after feeding for 30 minutes (see Fig. 13). The strongest response to wounding by *S. littoralis* could be measured in the locally treated leaf (SW) with a 31-fold and a less pronounced 5-fold increase of *IbWIPK*-expression in the neighboring systemic leaf. The overall expression pattern with a fast response after 30 minutes and a continuous upregulated expression after 1 hour (9-fold) in the actual wounded leaf (SW) is similar to previous observations during MecWorm treatment. The systemic leaf expression levels return to basal levels after 1 hour of treatment. The overall increase of *IbWIPK* expression is more explicit after herbivore feeding compared to mechanical damage only.

Observation of *MYC2* as one of the regulatory genes in the early wounding response showed a strong increase in expression levels after 30 minutes of treatment (see Fig. 13). The measured *MYC2* expression in the leaf attacked by the larvae rose up to approximately 30-fold, although not statistically significant. Upregulation could still be detected after 1 hour of treatment with a decline to basal activity after three hours. Feeding also strongly affected expression levels in the systemic leaf showing that *MYC2* was significantly triggered after 30 minutes of herbivore treatment with a 34-fold increased gene expression level. In contrast to the prolonged upregulation in the locally wounded leaf, no significant induction of *MYC2* was detected after 1 hour of treatment in the distant leaf.

A fast response of *JAMs1-1* upregulation could be seen in the damaged leaves after 30 minutes. Statistically significant increased expressions were observed after 30 and 60 minutes of treatment.
in the wounded tissue. Nevertheless the 2.5-fold increase in \textit{JAMs1-1} activity at its highest level after 30 minutes was much lower compared to mechanical wounding at the same duration of damage. No clear significant changes in \textit{JAMs1-1} expression could be measured in the systemic leaves.

After an upregulation in expression of regulatory genes like \textit{MYC2} and \textit{JAMs1-1}, \textit{IbNAC1} expression was also significantly increased after 30, 60 and 180 minutes in the leaves directly wounded by \textit{Spodoptera} (see Fig. 14). The response by \textit{IbNAC1} in herbivore–infested leaves is less pronounced with a maximal 7–fold increased expression compared to mechanical wounded leaves. Nevertheless, it was rapidly induced and lasted over three hours. A significant transient wounding response of \textit{IbNAC1} could also be detected in the systemic leaf after 30 minutes, which returned to a basal expression after 1 hour and 3 hours.

After a detectable induction of the previously mentioned wound–responsive genes in \textit{I. batatas}, \textit{Sporamin} showed a tendency but no statistically significant increased expression in leaves exposed to herbivore feeding (see Fig. 14). In contrast to the lacking response in wounded tissue, \textit{Sporamin} expression was significantly enriched after 30 minutes of feeding in the unwounded systemic leaves before it continuously declined to a basic level.
Fig. 13 Expression of wounding-related genes from *I. batatas* in response to herbivore infestation by *Spodoptera littoralis*. Changes in the transcript level of *IbWIPK*, *MYC2-1* and *JAMs1-1* were determined in leaves infested with a single larva of *S. littoralis* (SW) and the adjacent intact systemic leaves (Sys). Both leaf types were harvested after 0.5 h, 1 h or 3 h of herbivore feeding. Expression levels were determined using qRT-PCR, normalized to *IbACTIN-2* as housekeeping gene and calculated relative to untreated control plants. One-sample Wilcoxon-test or one-sample t-test respectively to the data distribution was selected to calculate statistical significant differences. Bars represent the means ± standard error (SE) with n≥5. Significance levels are indicated by the asterisks (* p<0.05; ** p<0.01; *** p<0.001).
Fig. 14 Expression of wound-responsive *IbNAC1* and trypsin inhibitory *sporamin* from *I. batatas* in response to infestation by *Spodoptera littoralis*. Changes in the transcript level of *IbNAC1* and *sporamin* were determined in leaves infested with a single larva of *S. littoralis* (SW) and the adjacent intact systemic leaves (Sys). Both leaf types were harvested after 0.5 h, 1 h or 3 h of herbivore feeding. Expression levels were determined using qRT-PCR, normalized to *IbACTIN*-2 as housekeeping gene and calculated relative to untreated control plants. One-sample Wilcoxon-test or one-sample t-test respectively to the data distribution was selected to calculate statistical significant differences. Bars represent the means ± standard error (SE) with *n*≥5. Significance levels are indicated by the asterisks (*p*<0.05; **p**<0.01; ***p***<0.001).
3.3 Phytohormone levels after mechanical damage by MecWorm

Jasmonic acid (JA) and its conjugates are important signaling molecules in responses to herbivore feeding and therefore play an essential role in plant defense (Schilmiller and Howe 2005). As shown before by (Rajendran et al. 2014), wounding can increase levels of jasmonic acid and salicylic acid (SA) in Ipomoea batatas dramatically in a time–dependent manner. Since there was no further investigation on the systemic response in sweet potato after wounding, besides a deeper analysis of jasmonates, an additional goal of this experiment was to obtain information about other phytohormones involved in plant signaling. Thus JA–precursor molecules, e.g. cis–OPDA and other stress–related hormones (ABA) were investigated in locally wounded and neighboring systemic leaves. First of all, mechanical damage by MecWorm was inflicted over 0.5, 1 and 3 h to determine a time–dependent wounding effect without any additional elicitors.

All measured jasmonates showed a significantly increased content in the locally wounded leaves (LW) during all time points (Fig 15). JA levels showed the highest increase after 30 minutes peaking at 212.46 ng g$^{-1}$ fresh weight with a persistent upregulated JA-level over 1 and 3 hours. The level of the active jasmonate conjugate JA–Ile showed a similar pattern as JA with the highest concentration after 30 minutes followed by a reduced but still higher JA–Ile accumulation in the wounded leaves over time. Degradation products of jasmonates acid like OH–JA, OH–JA–Ile, and COOH–JA–Ile (Fig. 15) were also found to be significantly elevated after 30 minutes and stayed at higher levels during longer wounding periods. Production of jasmonates in the fourth unwounded systemic leaf remained at a basal level. Thus, no systemic response could be detected after mechanical wounding.
Results

Fig. 15 Jasmonate levels after mechanical wounding by MecWorm in *Ipomoea batatas*. Bars show mean (± SE, n≤10) of JA, JA-Ile, OH-JA-Ile, COOH-JA-Ile and OH-JA concentrations after wounding by MecWorm for 0.5, 1, and 3 h. Phytohormone levels were measured in locally wounded leaves (LW) and the adjacent unwounded systemic leaf (Sys). Untreated leaves from undamaged plants were used as controls (C). Statistically significant differences between each treatment group after mechanical damage were analyzed for each time point separately using one-way ANOVA (analysis of variance). Different letters indicate significant differences among groups for p<0.05, determined by Dunn’s test.
In contrast to the rapid increase in jasmonate levels in wounded leaves, its precursor molecule cis–OPDA showed no significant response after 30 minutes of wounding. After 1 hour of inflicted MecWorm damage, cis–OPDA level increased significantly (see Fig. 16) compared to control plants. Interestingly, a systemic as well as a local response of cis–OPDA could be detected after 3 hours of treatment. Significantly higher amounts of cis-OPDA (systemic leaf, 138.23 ng g⁻¹ FW; locally treated leaf, 159.04 ng g⁻¹ FW) were measured in both leaf types. Investigation of the drought–stress related phytohormone abscisic acid (ABA) showed no obvious differences to control plants (Fig. 16) during treatment. The antagonistic phytohormone salicylic acid (SA) only showed a significantly elevated level in the damaged leaf (LW) after 3 hours of treatment.

![Fig. 16 Phytohormone levels after mechanical wounding by MecWorm in Ipomoea batatas. Bars show mean (± SE, n≤10) of ABA, SA and cis-OPDA concentrations after wounding by MecWorm for 0.5, 1, and 3 h. Phytohormone levels were measured in locally wounded leaves (LW) and the adjacent unwounded systemic leaf (Sys). Untreated leaves from undamaged plants were used as controls (C). Statistically significant differences between each treatment group after mechanical damage were analyzed for each time point separately using one-way ANOVA (analysis of variance). Different letters indicate significant differences among groups for p<0.05, determined by Dunn’s test.](image-url)
3.4 Phytohormone levels after feeding by *Spodoptera littoralis*

Previous experiments by Rajendran et al. (2014) showed that mechanical damage and herbivore infestation have different impacts on endogenous JA and SA levels. After inflicting continuous damage with MecWorm on sweet potato leaves (see previous chapter), the effect of herbivory on endogenous phytohormone levels remained to be investigated. Thus *Spodoptera littoralis* was used as a generalist herbivore to infest sweet potato plants which were used for phytohormone extraction and LC–MS measurements. Application of a single larva on the third fully expanded leaf (SW) of each plant lead to an immediate increase in local jasmonate levels (see Fig. 17). Infested leaves showed a rapidly produced amount of JA (260.18 ng g\(^{-1}\) FW) after 30 minutes, which remained significantly elevated compared to control plants during 1 and 3 hours of feeding. The same pattern of phytohormone contents in locally treated leaves became evident for the active jasmonate JA–Ile by reaching its highest produced amount (36.87 ng g\(^{-1}\) FW) after 30 minutes of feeding. Similar to its precursor jasmonic acid, JA–Ile concentrations decreased slightly during 1 hour of feeding and increased again after 3 hours of herbivory. The overall produced amount of active jasmonate is significantly different from control plants during all treatment durations. Other jasmonate catabolites such as OH-JA, OH–JA–Ile and COOH–JA–Ile also showed increased endogenous levels after local feeding that increased over time according to prolonged herbivore infestation (see Fig. 17). In contrast to the strong local response, adjacent systemic leaves showed no significant differences compared to control plants.
Results

Fig. 17 Jasmonate levels after *S. littoralis* herbivory in *Ipomoea batatas*. Bars show mean (± SE, n ≤ 10) of JA, JA-ile, OH-JA-ile, COOH-JA-ile, and OH-JA concentrations after *S. littoralis* feeding for 0.5, 1, and 3 h. Phytohormone levels were measured in local *Spodoptera*–fed leaves (SW) and the adjacent unwounded systemic leaf (Sys). Untreated leaves from undamaged plants were used as controls (C). Statistically significant differences between each treatment group after mechanical damage were analyzed for each time point separately using one–way ANOVA (analysis of variance). Different letters indicate significant differences among groups for p < 0.05, determined by Dunn’s test.
Besides an elevated production of jasmonates, a significant amount of abscisic acid was generated in the damaged leaf after three hours of treatment. Nevertheless ABA, SA and cis–OPDA levels were not significantly affected by herbivore feeding (see Fig. 18). There was also no detectable induced production of stress–related phytohormones in systemic leaves.

Fig. 18 Phytohormone levels after *S. littoralis* herbivory in *Ipomoea batatas*. Bars show mean (± SE, n≤10) of ABA, SA, and cis-OPDA concentrations after *S. littoralis* feeding for 0.5, 1, and 3 h. Phytohormone levels were measured in local *Spodoptera*–fed leaves (SW) and the adjacent unwounded systemic leaf (Sys). Untreated leaves from undamaged plants were used as controls (C). Statistically significant differences between each treatment group after mechanical damage were analyzed for each time point separately using one–way ANOVA (analysis of variance). Different letters indicate significant differences among groups for p<0.05, determined by Dunn’s test.
3.5 Emission of volatile organic compounds (VOCs) after biotic and abiotic damage

Collection of emitted volatiles in unwounded control plants of *Ipomoea batatas* showed a basic composition comprising different classes of hydrocarbons including alkenes, aldehydes and homoterpenes (see Fig. 19 A; Table 1). Mechanical damage for 18 hours revealed an emission of additional aldehydes, aromatics and two kinds of sesquiterpenoids (Fig. 19 B; Table 1). Apart from the previously detected compounds, feeding by the chewing generalist herbivore *Spodoptera littoralis* resulted in a more complex volatile blend (see Fig. 19 C; Table 1). The homoterpene ocimene, hexenyl acetate (ester), pentadecane (alkane) and more hydrocarbon structures were added to the collected volatile mixture. The depicted gas chromatograms (Fig. 19) represent examples for the general pattern of wound-inducible emission in sweet potato. Specific compounds like the homoterpene (E)-4,8-dimethyl–nonatriene (DMNT) were already detected in control plants with an abundance of 13% relative to the internal standard. Application of wounding resulted in a largely increased intensity of emitted DMNT ranging from 136% relative to the internal standard (MecWorm; Fig. 19 B) up to 566% (herbivore feeding; Fig. 19 C). Subsequent to the identification of substances according to their fragmentation patterns (MS), compounds were quantified based on their relative abundance to the internal standard (n-bromodecane) which is shown in table 1. Comparing the emitted blends after wounding showed an overall increased relative amount of released volatile organic compounds after herbivore feeding. Overall, wounding by *Spodoptera littoralis* lead to induction of additional sesquiterpenoids (copaene, farnesene, humulene), alkanes, alkenes, the alcohol geranylgeraniol, indole, *cis*-jasmone and ocimene which were lacking upon mechanical wounding. In general, fewer substances at a lower relative intensity were emitted after MecWorm treatment compared to herbivory. Nevertheless, several aldehydes and a relatively high amount of acetophenone were exclusively emitted after mechanical wounding.
Gas chromatograms of volatiles emitted by *Ipomoea batatas*. (A) Control without wounding. (B) Volatiles induced by mechanical damage (MecWorm) inflicted over 18 hours. (C) Volatiles induced by feeding of *Spodoptera littoralis*. All volatiles were collected over 24 hours and eluted with 50 µl n-bromodecane as internal standard (IS). Asterisks mark contamination by plasticizer or column residuals. All volatiles were identified by retention time of standards. Table 1 indicates identification of compounds.
Table 1 List of compounds identified in volatile blends emitted by *Ipomoea batatas* upon mechanical damage by MecWorm or feeding by *Spodoptera littoralis*. Indicated is the relative intensity (rel.int.) compared to the internal standard: +, rel.int. below 25%; ++, rel.int. between 25 and 50%; ++++, rel.int. above 100%; * compound only found in a single biological replicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. in chromatogram</th>
<th>MecWorm</th>
<th><em>S. littoralis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkanes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heptadecane</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Pentadecane</td>
<td></td>
<td>20</td>
<td>++</td>
</tr>
<tr>
<td>Octyl ether</td>
<td></td>
<td>9</td>
<td>++</td>
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<tr>
<td><strong>Alkenes</strong></td>
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<td></td>
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<tr>
<td>2,6-Dimethyl-1,3,5,7-octatetraene (E)</td>
<td>16</td>
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<td></td>
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<tr>
<td>6-Methyl-5-heptene-2-one</td>
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<td><strong>Aldehydes</strong></td>
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<tr>
<td>Decanal</td>
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</tr>
<tr>
<td>2-Ethyl hexanal*</td>
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<td>+</td>
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<tr>
<td>Nonanal</td>
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<td>4</td>
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<tr>
<td>Octanal *</td>
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<tr>
<td>Acetophenone *</td>
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<tr>
<td>cis -Jasnone *</td>
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<td>19</td>
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<td><strong>Homoterpenes</strong></td>
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<td>+++</td>
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<tr>
<td>3E,7E-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT)</td>
<td>8</td>
<td>+</td>
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</table>
3.6 Absolute quantification of DMNT

Biotic as well as abiotic stress inflicted upon leaves of *Ipomoea batatas* resulted in a strong emission of the nerolidol–derived homoterpene (E)-4,8–dimethyl–nonatriene (DMNT). DMNT is a common plant volatile released after herbivore infestation. As shown by Arimura *et al.* (2002), in Lima bean (*Phaseolus lunatus*) plants it has the ability to induce changes in gene expression levels. In order to determine suitable concentrations for application in further experiments matching natural conditions, an absolute quantification of emitted DMNT has been performed. Increasing concentrations of DMNT dissolved in pure dichloromethane (DCM) with 50 µg ml\(^{-1}\) n-bromodecane as internal standard were used to generate a calibration curve (see Fig 20). The ratio of measured DMNT-peak area divided by the peak area of the internal standard from each experiment was inserted into the formula of the regression line given in Figure 20. Volatiles collected from each single leaf were eluted in a total volume of 40 µl DCM mixed with internal standard, therefore the emitted amount of DMNT refers to DMNT in µg per 40 µl eluate per single leaf. An amount of 2.5 µg ml\(^{-1}\) of DMNT dissolved in DCM was chosen as the lowest detection level. The highest amount of DMNT used for the calibration curve was 250 µg ml\(^{-1}\).
Fig. 20 DMNT–calibration curve used for absolute quantification of (E)-4,8–dimethyl–nonatriene (DMNT) in collected headspace volatile samples. Increasing amounts of DMNT were dissolved in pure dichloromethane with 50 µg n-bromodecane as internal standard. The following concentrations of DMNT were generated: 2.5, 5, 10, 20, 50, 100, 150, 200 and 250 µg ml\(^{-1}\). Analysis was conducted using GC–MS with 1 µl injection volume and three technical replicates per DMNT–concentration.

In the following experiments, VOCs were collected after either mechanical wounding by MecWorm or feeding by the generalist herbivore *S. littoralis* over a period of 24 hours. Because a strikingly increased emission of DMNT was observed during both treatments (Fig. 21), the previously generated standard curve was used to determine the absolute amount of emitted DMNT after both types of wounding (Fig 21). Control values showed that the experimental set-up already triggered the emission of small amounts of DMNT. For MecWorm treatment the fixing of the leaf in the Plexiglas®-cabinet lead to an approximate emission of 0.27 µg DMNT per leaf. The herbivore treatment is more invasive, leading to an emission of 1.01 µg DMNT per leaf since it comprises enclosing the leaf into the feeding cage with additional bagging into plastic foil. Both experimental
Results

Approaches show a significantly induced DMNT emission after handling the plants compared to untouched control plants. However, damaging the leaf with MecWorm over 18 hours resulted in a total emission of 1.81 µg DMNT per leaf, therefore showing a significant 6-fold increase compared to control plants. DMNT emission was even more pronounced in herbivore-infested leaves with a 5-fold increased release of the homoterpene and an overall amount of 5.83 µg DMNT per single leaf, showing a strong induction of VOCs emission by *S. littoralis*. In addition to that, differences between emitted DMNT after mechanical damage and insect-feeding were shown to be significantly different.

![Emission of DMNT after mechanical damage or herbivore feeding.](image)

**Fig. 21 Emission of DMNT after mechanical damage or herbivore feeding.** Volatiles were collected over 24 hours with 18 h mechanical wounding by MecWorm (MW) or 24 h infestation with *Spodoptera littoralis* (SW). Bars represent the mean ± standard error (SE) of emitted DMNT in µg per 40 µl eluate per single leaf. Each experiment was conducted with at least 5 biological replicates. Significance levels are indicated by the asterisks (* p<0.05; ** p<0.01; *** p<0.001). Statistical analyses were performed for each treatment and the respective control using RStudio (V. 0.98.1130.0) with a Shapiro–Wilk normality test and subsequent two-sample t-test or Wilcoxon–test.
3.7 Systemic induction of defense–related genes by the homoterpene DMNT

Due to the highly increased emission after both types of wounding and previous results by Arimura et al. (2002) indicating its ability to induce defense mechanisms in plants, DMNT was selected as a suitable candidate to trigger a systemic response in *Ipomoea batatas* without actual damage to the treated plants. Preceding RT–qPCR experiments by Chen et al. (data not shown) supported the hypothesis of DMNT as a putative elicitor of defense signaling in sweet potato by finding a highly increased expression of farnesyl diphosphate synthase (*FPPS*), which encodes one of the key enzymes in sesquiterpenoid biosynthesis, after mechanical wounding by tweezers.

In order to explore whether DMNT is able to regulate defense–related genes in *Ipomoea batatas*, varying concentrations were applied on cotton and incubated with three sweet potato plants in a glass container. After one hour of incubation a single leaf per plant was harvested, processed as described before and used to perform RT–qPCR. Application of three different concentrations of DMNT in 34–l headspace showed a detectable impact on gene expression levels in early defense–related genes like *MYC2* (Fig. 22). Expression levels of *MYC2* slightly decreased to a normalized fold expression of 0.4 compared to control plants after incubation with 5 µg of DMNT. Contrarily to this observed decrease, application of 20 µg DMNT lead to a 4.4–fold increase of *MYC2* expression levels. The increase of applied DMNT to 60 µg resulted in an elevation of 2.6–fold *MYC2* expression levels.

After an increased expression of the transcription factor *MYC2*, *IbNAC1* expression was investigated after incubation with the previous DMNT concentrations (see Fig. 22). Application of the lowest DMNT concentration showed a slight decrease in *IbNAC1* expression levels (0.9–fold) whereas higher concentrations lead to an increased fold expression of 3.4 (20 µg DMNT) and 4.2 (60 µg DMNT), respectively. Enhancement of gene expression can also be observed in defense–related genes like *Sporamin* and genes involved in JA–synthesis like *LOX2*. As mentioned before, the application of 5 µg DMNT also resulted in a decreased transcript level below 1 compared to control plants treated with DCM. An increased DMNT concentration of 20 µg triggered the strongest observed response in *Sporamin* with a 21–fold upregulation of gene expression. Application of 60 µg DMNT still resulted in a 3.2–fold increased expression of *Sporamin*. Regarding the involvement of DMNT on JA–synthesis, 20 µg of DMNT lead to a 4.4–fold change in the *LOX2* transcript level. This decreased to a 2.3–fold change with an increased concentration of 60 µg DMNT.
Fig. 22 Concentration–dependent expression of wounding-related genes from *I. batatas* in response to systemic induction by DMNT. Three sweet potato plants were incubated in an odorless 34 l glass container with increasing concentrations of DMNT dissolved in pure dichloromethane (DCM). 5, 20 and 60 µg of DMNT were incubated on cotton wool for one hour. Plants incubated with pure dichloromethane were used as control. Changes in the transcript level of *MYC2-1, IbNAC1, Sporamin* and *LOX2* were determined in the third fully expanded leaf of each plant. Expression levels were determined using qRT-PCR normalized to *IbACTIN-2* as housekeeping gene. The gene fold expression change was calculated relative to DCM–treated control plants.
The expression of previously investigated genes involved in defense–regulation in sweet potato is rapidly triggered and changes significantly over time. Hence it was of main interest to determine alterations in gene expression levels during prolonged incubation periods with a constant concentration of DMNT. According to my previous experiments, the application of 20 µg DMNT lead to the most distinctive change in gene expression levels. Thus the aforementioned set–up was modified in a way that three plants were incubated with 20 µg of DMNT in a 34 l glass container for 15, 30 and 60 minutes (see Fig. 23). Rapid changes in transcription levels of MYC2 became evident after 15 minutes of DMNT–incubation with a 6.4–fold increase that was still detectable after 1 hour. An increased IbNAC1 expression also occurred after 15 minutes of treatment whereas the highest transcript level of IbNAC1 was measured after 30 minutes (6.7–fold). IbNAC1 gene expression was still evident after 60 minutes of incubation. The highest systemic response of genes investigated in this experiment became evident for Sporamin. Sporamin transcript levels were 11.2–fold increased after 15 minutes of DMNT treatment therefore showing a fast response of this defense–related gene (see Fig. 23). The most striking induction of Sporamin was detected after 60 minutes with a peaking activity of 21–fold expression. To investigate whether DMNT can also affect JA–synthesis genes to activate additional pathways of defense, LOX2 expression levels were measured. Similar to Sporamin expression patterns, LOX2 transcript levels were elevated after 15 minutes of treatment with the highest accumulation after 1 hour. Incubation for 15 minutes triggered a 2.2–fold enhancement in LOX2 transcripts that constantly increased over time to a maximum of 4.4.
Fig. 23 Expression of defense–related genes in *I. batatas* after systemic induction by DMNT over time. Three sweet potato plants were incubated in an odorless 34 l glass container with 20 µg of DMNT dissolved in pure dichloromethane (DCM) for 15, 30 and 60 minutes. Plants incubated with pure dichloromethane were used as control. Changes in the transcript level of *MYC2-1*, *IbNAC1*, *Sporamin* and *LOX2* were determined in the third fully expanded leaf of each plant. Expression levels were determined using qRT-PCR normalized to *IbACTIN*-2 as housekeeping gene. The gene fold expression change was calculated relative to DCM–treated control plants.
After confirmation that DMNT as a single compound is able to trigger genes in the defense signaling cascade of sweet potato, it was examined whether the whole blend of induced volatiles affected expression levels. Mechanical damage was inflicted by wounding of several leaves on two plants by tweezers with subsequent incubation together with three undamaged plants in a glass container for 24 hours. Plants exposed to mechanical wounding applied by tweezers showed a clear induction of all studied genes: \textit{IbWIPK}, \textit{MYC2-1}, \textit{JAMS-1}, \textit{IbNAC1}, \textit{Sporamin}, and \textit{FPPS} (Fig. 24); however at different levels.
Fig. 24 Expression of defense–related genes in sweet potato after induction by volatiles emitted after mechanical wounding. For mechanical wounding (MW) two sweet potato plants with ≤3 leaves wounded by tweezers were incubated in a 34 l glass container with three unwounded plants for 24 hours. Plants without wounding were used as control. Changes in the transcript level of *IbWIPK*, *MYC2-1*, *JAMS1-1*, *IbNAC1*, *Sporamin* and *FPPS* were determined in the third fully expanded leaf of each plant. Expression levels were determined using qRT-PCR normalized to *IbACTIN*-2 as housekeeping gene. The-gene fold expression change was calculated relative to unwounded control plants.
3.8 Phytohormone levels after systemic induction by DMNT

Investigation of genes involved in synthesis of endogenous jasmonic acid in sweet potato (LOX2) showed increasing expression levels after application of the homoterpene DMNT. Consequently, it needed to be examined if this systemic induction was also directly detectable in the concentrations of jasmonates and other phytohormones. Therefore 1.41 µg of DMNT dissolved in DCM was incubated with a single sweet potato plant in a 2.4 l desiccator for 1 h. Following phytohormone extraction and LC–MS results showed no significant effect of DMNT treatment on jasmonates or stress–related hormones (Fig. 25; Fig. 26).
Fig. 25 Jasmonate levels after systemic induction by DMNT in *Ipomoea batatas*. Bars show mean (± SE, n = 10) of JA, JA-Ile, OH-JA-Ile, COOH–JA–Ile and JA-OH concentrations after application of DMNT over 1 hour. 1.41 µg of DMNT dissolved in DCM was incubated on cotton in a 2.4 l desiccator. Each third fully expanded leaf was used for phytohormone extraction and LC–MS measurements. Leaves from plants incubated with an equal volume of DCM were used as controls (C). Statistically significant differences between control plants and DMNT–treated plants were analyzed using t–test or Wilcoxon–test. Different letters indicate significant differences among groups.
Fig. 26 Phytohormone levels after systemic induction by DMNT in *Ipomoea batatas*. Bars show mean (± SE, n = 10) of ABA, SA and cis–OPDA concentrations after application of DMNT over 1 hour. 1.41 µg of DMNT dissolved in DCM was incubated on cotton in a 2.4 l desiccator. Each third fully expanded leaf was used for phytohormone extraction and LC–MS measurements. Leaves from plants incubated with an equal volume of DCM were used as controls (C). Statistically significant differences between control plants and DMNT–treated plants were analyzed using t–test or Wilcoxon–test. Different letters indicate significant differences among groups.
4 Discussion

4.1 Wounding by MecWorm or Spodoptera littoralis induces a distinct signaling cascade in Ipomoea batatas

Ipomoea batatas is challenged with a plethora of environmental stresses resulting in the initiation of a whole machinery of inducible defense responses. Previous studies have shown that a complex wounding-inducible signaling cascade can be activated by different modes of damage in sweet potato plants (Yeh et al. 1997a; Wang et al. 2002; Rajendran et al. 2014; Chen et al. 2016). A model by Bonaventure (2012) proposed the activation of a mitogen-activated protein kinase (MAPK) cascade by herbivory-associated molecular patterns. Often an activation of MAPK cascades is followed by the induction of wounding-induced protein kinases (WIPK), which are common regulators in stress or pathogen infection responses in plants (Xu et al. 2014). Confirming these predictions, this study showed a strong induction of IbWIPK expression levels in sweet potato after wounding (Fig. 11; Fig. 13). Continuously applied, insect-like mechanical and herbivore-associated damage resulted in a highly increased IbWIPK transcription level after 30 minutes of treatment in local and systemic leaves of the plant, thereby supporting the importance of IbWIPK in the early but not abiding wounding response in sweet potato. Similar observations were made by Lo (unpublished) confirming systemic and local induction of IbWIPK after wounding treatment demonstrating an even earlier response after 15 minutes. Considering the impact of the mode of damage, it was shown that induction after Spodoptera littoralis feeding was more pronounced compared to mechanical damage alone (Fig. 13). Since the induction of IbWIPK was doubled in the locally treated leaf after feeding, elicitors in oral secretion of the herbivore are prone to enhance defense upregulation within the plant (Mithöfer and Boland 2008; Felton et al. 2014). Transactivation analyses conducted by Lo (unpublished) showed the ability of IbWIPK to positively regulate IbNAC1 and Sporamin promoters, thereby connecting the putative wounding-regulated interaction partners.

Considering additional candidate genes supposedly involved in wounding-inducible signaling cascades, studies conducted by Chen (unpublished) suggested IbWIPK to phosphorylate downstream interaction partners MYC2 and JAMs1. MYC2, also known as IbbHLH3 in I. batatas, is a
key transcription factor in JA-regulated gene expression (Rajendran et al. 2014; Chen unpublished). Similar to previously described induction patterns, mechanical damage and herbivore feeding strongly triggered expression of MYC2 in wounded leaves at comparable levels (Fig. 11; Fig. 13). Interestingly, systemic induction of MYC2 was evident in both treatments with the strongest overall response in the unwounded leaf after herbivore infestation. This suggests a brief but fast signaling function of MYC2 in the neighboring leaves, which is nevertheless more persistent in the damaged tissue.

Simultaneous to the wounding-induced upregulation of MYC2 expression, JAMs1, also known as *IbHLH4* in sweet potato, showed an increase in transcription levels after MecWorm or herbivore treatment (Fig. 11; Fig. 13). JAMs1 is predicted to bind either as a homo- or heterodimer to MYC2 by consequently inhibiting expression of *IbNAC1* (Chen unpublished). In this study, a fast accumulation of JAMs1 transcript level was observed simultaneous to MYC2 with a prolonged upregulation of JAMs1 over time (Fig. 11; Fig. 13). This is supporting the hypothesis of JAMs1 serving as a negative regulator of the downstream interaction partner *IbNAC1* by still being active whilst expression levels of the latter already decreased at the same duration of treatment.

MYC2 itself becomes active by binding as a homodimer to a CACGTG-motif to activate *IbNAC1* (Chen unpublished). Mechanical damage lead to a short but drastic increase in *IbNAC1* expression after 30 minutes of treatment in both systemic and wounded leaves (Fig. 12) matching previous findings (Rajendran et al. 2014; Chen et al. 2016). The observation of a comparably fast decline in transcript levels in both tissues indicated *IbNAC1* to play a major role in defense signaling in sweet potato although still functioning as a signaling precursor for sporamin as the actual protein conferring insect resistance (Yeh et al. 1997b). Chen et al. (2016) demonstrated with *IbNAC1* overexpression lines of *Ipomoea batatas* the upregulation of Sporamin; resulting in enhanced trypsin-inhibitory activity causing severe growth retardation in *Spodoptera litura*. Controversely, *Spodoptera littoralis* feeding induced different expression patterns by triggering a rapid systemic induction and a continuous *IbNAC1* upregulation in the wounded leaf over 3 hours (see Fig. 14). This might be a result by previously mentioned herbivore elicitors like proteases or other chemicals in the oral secretion that pose a persistent threat to which the plant responds.

Surprisingly, Sporamin expression levels were highly variable during mechanical wounding and herbivore infestation (Fig. 12; Fig. 14). Control plants already showed induced expression levels of Sporamin without wounding of the plant. Overall Sporamin transcript levels are often found not
significantly induced in wounded leaves in contrast to previous studies (Rajendran et al. 2014; Chen et al. 2016). This effect might be a result of a high sensitivity of sweet potato to external stimuli, which can be seen in the overall fast response in the signaling cascades reported in this study (see Fig. 11-14). An interesting fact is the accumulation of *Sporamin* transcripts in the systemic leaves after mechanical damage and herbivore feeding. Wang et al. (2002) demonstrated the wound-inducibility of *Sporamin* in leaf tissue without giving further information on a systemic response. Experiments by Yeh et al. (1997a) showed that sporamin RNA levels in unwounded leaves were 2 – 3 times higher compared to wounded leaves. The results presented in this thesis support the trend that *Sporamin* expression is increasing in the systemic leaf in a time-dependent manner after mechanical wounding, thereby matching findings by Chen et al. (2016). Interestingly, feeding by *Spodoptera littoralis* resulted in a significantly increased *Sporamin* expression in the systemic leaf only after 30 minutes of treatment (see Fig. 14). These findings support the hypothesis that the wounding-inducible defense signaling cascade in sweet potato - which ultimately protects the plant by the production of sporamin as a trypsin inhibitor against the herbivore - is predominantly systemically triggered. Therefore systemic induction of sporamin can be used to reduce damage caused by herbivores (Yeh et al. 1997b) within the whole plant.

### 4.2 Jasmonates are locally accumulated after different damage patterns

Jasmonates are known to be key regulators in plant defense during herbivore attack (Howe and Jander 2008). The importance of jasmonate accumulation as a response to mechanical wounding and herbivore feeding in sweet potato was demonstrated in this study. Damaging of a single sweet potato leaf resulted in a rapidly triggered accumulation within the same ranges of jasmonic acid and all measured catabolites (see Fig. 15; Fig. 17). It has been shown in preceding observations that JA and other phytohormones can increase within minutes after wounding (Leon et al. 2001; Kessler and Baldwin 2002; Maffei et al. 2007a). Previous findings by Rajendran et al. (2014) reported similar observations with enhanced JA contents after 15 minutes of mechanical damage in *Ipomoea batatas*, consequently showing the sweet potato’s ability to react promptly to wounding stress. Little is known about the production of other jasmonates apart from JA in sweet potato. Therefore this study showed was able to demonstrate a pronounced accumulation of jasmonic acid metabolites and the biologically active form, JA-Ile, after both types of wounding. After the rapidly
enhanced production of JA and JA-Ile in wounded leaves, accumulation of jasmonate metabolites such as OH-JA, OH-JA-Ile and COOH-JA-Ile could be observed in a time-dependent manner with a simultaneous decrease in JA after mechanical damage (see Fig. 15). This confirms the fate of JA and JA-Ile which has been described by Wasternack and Hause (2013). Although the range of accumulated jasmonates was similar after mechanical damage and herbivory, feeding by Spodoptera littoralis triggered a prolonged accumulation of JA, JA-Ile and OH-JA-Ile compared to MecWorm treatment (see Fig. 17). The prolonged response after damage by a chewing herbivore is most probably a result of elicitors in oral secretion of Spodoptera littoralis, which are able to modulate plant defense (Guo et al. 2013). Although the mode of feeding (e.g. chewing) is the same, herbivores secrete different elicitors resulting in differing endogenous JA and SA (salicylic acid) levels in the same plant (Diezel et al. 2009). Salicylic acid is known to act antagonistically to JA, thereby serving as a negative regulator of defense against herbivores in A. thaliana (Cui et al. 2002; Bostock 2005). Rajendran et al. (2014) showed increased accumulation of SA in wounded sweet potato leaves with a continuous response after herbivory. Enhanced levels of SA were only detected locally after 3 hours of continuous mechanical damage by MecWorm (see Fig. 16) with no significant increase during insect feeding (Fig. 18). Differences after mechanical damage could be based on a completely different mode of wounding. This study used MecWorm, the mechanical caterpillar (Mithöfer et al. 2005) to ensure continuous wounding whereas Rajendran et al. (2014) inflicted nonrecurring damage with forceps. Considering stress-related hormones produced during drought stress such as abscisic acid (ABA) (Tuteja 2007), elevated ABA levels could only be measured after prolonged herbivore feeding which is a common response known in plants after infestation by natural enemies (Weldegergis et al. 2015). The levels of jasmonic acid precursor cis-OPDA did not show significant differences compared to control plants during herbivore feeding. Surprisingly, cis-OPDA was accumulated in the mechanically wounded leaf (see Fig. 16) during 1 and 3 hours. Produced amounts of cis-OPDA after 3 hours of wounding were similar in both treatments (approx. 160 ng g⁻¹ FW; see Fig. 16 and 18) with highly variable detected amounts in the control plants. Therefore, statistically significant differences are prone to be caused by handling during the experiments (Almeida-Trapp et al. 2014).

All findings described above were strictly focusing on the local response in the wounded leaf. However, experiments conducted by Koo et al. (2009) reported a wound-induced systemic production of JA and JA-Ile in Arabidopsis thaliana. This study did not show a wound-inducible systemic response in phytohormone levels in Ipomoea batatas. All in all, these findings lead to the
conclusion that accumulation of phytohormones is not essential for systemic induction of defense-associated genes like *Sporamin*. In addition to that, studies by Chen *et al.* (2008) demonstrated additional signal transduction pathways in sweet potato.

### 4.3 Induction of distinct volatile emission patterns after wounding

Even though the mechanism of volatile perception remains so far unknown (Mithöfer and Boland 2012), volatile organic compounds (VOCs) can also have a systemic effect on neighboring plants. This can be a result of serving as a direct elicitor of internal defense or by priming of the adjacent plants in preparation for actual herbivore infestation (Kessler *et al.* 2006). Application of mechanical damage (MecWorm) and herbivore feeding (*Spodoptera littoralis*) on single sweet potato leaves lead to an emission of at least 27 identified compounds (shown in table 1). Comparison of the volatile composition after two different modes of wounding only showed slight differences in terms of volatile bouquet quality but mostly differences in quantities of single compounds (see table 1) (Mithöfer *et al.* 2005; Bricchi *et al.* 2010). Mechanical damage was sufficient to induce emission of a distinct volatile pattern comprising various aldehydes and other classes of hydrocarbons with a relatively low amount of sesquiterpenes. In addition to the sesquiterpenes, homoterpenes like TMTT and DMNT were also present after mechanical wounding. Since volatile compositions are often characteristic according to the causative stimulus (Bricchi *et al.* 2010), feeding by *Spodoptera littoralis* resulted in overall increased emission of volatiles known from control as well as additional ketones, alkanes and sesquiterpenoids (see table 1 and Fig. 19). The overall composition of identified compounds is in accordance with previous findings in *Medicago truncatula* (Leitner *et al.* 2005) and *Phaseolus lunatus* (Dicke *et al.* 1999; Mithöfer *et al.* 2005). Emission of additional compounds after insect feeding is due to the fact that sweet potato appears to be a species that does not respond to MecWorm with the whole set of herbivory-related volatiles (Maffei *et al.* 2007b; Mithöfer and Boland 2008). The increased amount of the individual compounds released after herbivore infestation can be explained by the combination of mechanical wounding with the contribution of HAMPs (herbivory-associated molecular patterns) provided by oral secretion (Mithöfer and Boland 2008). In general, synthesis of VOCs can occur via many different biosynthesis pathways in the plant. Among the largest and structurally diverse groups of plant-derived secondary metabolites are the terpenoids (Stevens 1992). All compounds belonging
to this class originate from isopentenyl diphosphate (IDP), a five-carbon precursor molecule (Gutensohn et al. 2013) which is further processed into precursors of sesquiterpenes, sterols or triterpenes by the mevalonic acid (MVA) pathway in the cytosol, or isoprenes, monoterpenes, diterpenes or carotenoids by the methylerythrol-phosphate (MEP) pathway in the plastids (see Fig. 27). Especially the emission of sesquiterpenes after herbivory in plants plays a key role in defense by providing the sesquiterpene alcohol nerolidol as a precursor for the homoterpene (E)-4,8-dimethyl-nonatriene (DMNT) (Boland et al. 1998). Compounds resulting from this biosynthetic pathway have been repeatedly detected in this study. Absolute quantification of the acyclic homoterpene DMNT showed a tremendous increase in emitted DMNT after wounding especially caused by herbivory (Fig. 21). Emission of DMNT is a common phenomenon after insect feeding, although in species- and herbivore- dependent manner (Arimura et al. 2002; Leitner et al. 2005; Mithöfer et al. 2005). It is known that minor amounts of VOCs can be sufficient for attraction of natural predators (Leitner et al. 2010). Especially DMNT with its many biological functions (Dicke et al. 1999; Tholl et al. 2011) is prone to be a key regulator in wound-inducible signaling within and among sweet potato plants. The high emission levels of DMNT could provide a fast communication between distant leaves on the same plant.
Fig. 27 Biosynthesis of terpenoid plant volatiles. Shown are the methylerythritol pathway (left) and the mevalonate pathway (right) in plants. Abbreviations: MEP methylerythritol pathway; MVA mevalonate pathway; IDP isopentenyl diphosphate; DMADP dimethylallyl diphosphate; GDP geranyl diphosphate; GGDP geranylgeranyl diphosphate; FDP farnesyl diphosphate; DMNT 4,8-dimethylnona-1,3,7-triene. Modified after Bartram et al. (2006).

4.4 Time- and concentration- dependent defense induction by volatiles and DMNT

Plants are able to release VOCs after infestation by predators (Pare and Tumlinson 1999). Volatile blends emitted by herbivore-infested lima bean plants were able to induce expression of genes encoding lipoxygenase (LOX) and farnesyl pyrophosphate synthase (FPS) (Arimura et al. 2000b). Results from this study showed induction of defense-related genes in sweet potato by airborne
volatiles (see chapter 3.7). Whole blend mixtures emitted after mechanical damage triggered the upregulation of defense-related genes as well as genes involved in homoterpene biosynthesis like FPPS (see Fig. 24). Depending on the mode of wounding, differences in the composition of induced volatiles can be observed (Leitner et al. 2005). So far the impact of volatile induction in gene upregulation has only been demonstrated by gel documentation of PCR products, thus no comparable RT-qPCR data is accessible yet. This study showed that all investigated upstream regulators and Sporamin itself were activated by wounding-induced volatiles in Ipomoea batatas (see Fig. 22-24). These findings strongly suggest that priming of unharmed sweet potato plants by its attacked neighboring plants plays an important role in plant defense. The following analysis of DMNT as a key component in defense induction in Ipomoea batatas showed a time- and concentration-dependent regulation (Fig. 22; Fig. 23). The qualitative and quantitative composition of emitted compounds strongly depends on a multitude of different biosynthesis pathways within each plant (Bricchi et al. 2010). An applied concentration of 20 µg DMNT in 34 l headspace was shown to trigger the strongest response of defense-related genes in sweet potato whereas lower concentration had no visible effect (Fig. 22). In fact, higher concentrations still showed induction of transcription factors although at a lower rate. These findings imply that a certain threshold of DMNT has to be reached to induce systemic defense-signaling. Furthermore, the ability of DMNT as a signaling compound appears to reach a saturation maximum at higher concentrations (Fig. 22) which has not been reported yet. Apart from concentration-dependent effects, induction of Sporamin-regulatory transcription factors by DMNT was fluctuating in a time-dependent manner. In support of the proposed signal transduction pathway for sporamin accumulation (Fig. 23), a rapid early induction of MYC2 and IbNAC1 was visible after 15 and 30 minutes, respectively (Fig. 23). Interestingly, Sporamin expression showed the highest overall increase (20-fold) after 60 minutes. thereby confirming that systemic induction in sweet potato possibly follows the activation of upstream transcription factors. Upregulation of LOX2 over time implicated the importance of JA in systemic defense signaling which will be further elaborated in the following excerpt.

4.5 Airborne DMNT does not enhance phytohormone production in sweet potato

In 3.7 it was shown that DMNT systemically induced defense-associated gene expression in distant, unwounded leaves. Especially genes involved in the synthesis of endogenous jasmonates like LOX2
could be upregulated by exposure to airborne DMNT (Fig. 22; Fig. 23). It was already demonstrated in other crop species that green leaf volatiles like hexenyl acetate can prime *Triticum aestivum* against fungal pathogens by upregulation of JA-biosynthesis genes (Ameye *et al.* 2015). Although this study confirmed that priming of regulatory genes by VOCs is possible, the detected amount of JA and its conjugates was not significantly induced in sweet potato after exposure to DMNT (Fig. 25; Fig. 26). Volatiles emitted by *T. urticae*-infested lima bean plants lead to JA accumulation in uninfested plants (Arimura *et al.* 2002). Studies with maize showed that the volatile indole can increase the production of stress hormones like JA-Ile and ABA (Erb *et al.* 2015); nevertheless this effect was not visible in the given study. Scholz *et al.* (2015a) indicated that in spite of jasmonate-induced biosynthetic gene expression, accumulation of jasmonates does not forcibly have to occur. There might be the necessity of post-translational processes (Koo *et al.* 2009) to take place first in order to induce biosynthesis of jasmonates. Since there is no detectable accumulation of jasmonates in unwounded sweet potato leaves with a simultaneous strong induction of *Sporamin*, systemic activation of the defense-signaling cascade described in chapter 1.5.2 appears to be JA-independent. These findings contradict previously proposed models by Rajendran *et al.* (2014) and support the possibility of JA-independent pathways for *Sporamin* induction in unwounded leaf tissues. Alternatively to jasmonate-dependent upregulation of defense-related proteins is the activation by bioactive hydroxyproline-rich glycopeptides (HypSys peptides) reported by Chen *et al.* (2008). These peptides are processed from wound- and jasmonate- inducible precursor proteins with the ability to induce expression of sporamin in *Ipomoea batatas* (Chen *et al.* 2008). Consequently, the activation of *Sporamin* expression in unwounded leaves of sweet potato can be triggered without accumulated JA but by endogenous 1bHypSys peptides. The possibility of JA-independent defense responses has also been observed in *Arabidopsis* plants in which the herbivore-induced accumulation of γ-amino-butyric acid follows the same pattern (Scholz *et al.* 2015b). The occurrence of the reported signaling peptides adds another possibility of defense regulation in sweet potato, thereby underlining the versatility of *Ipomoea batatas* to cope with biotic stress. The generated results indicate that DMNT could direct- or indirectly affect the signaling cascade in sweet potato on the level of the transcription factor MYC2, hence further studies are necessary to validate this hypothesis.
4.6 Conclusion and outlook

The findings presented in this study highlight the complexity of wound-inducible signaling cascades in *Ipomoea batatas*. Giving insight into defense mechanisms during herbivore attack, it could be demonstrated that local upregulation of defense-related genes go hand in hand with jasmonate production and volatile emission. In addition, it was shown that VOCs and especially DMNT serve as potent elicitors for defense upregulation in sweet potato. Strikingly, the occurrence of volatile-induced *Sporamin* during herbivory serves as a promising model for fast communication between distant plant parts, omitting time-consuming signaling along the vascular connections within the plant. However, more research will be needed to decipher the systemic response throughout the plant. Additionally, the biological relevance of priming still has to be confirmed by insect feeding assays after exposure to the previously described volatiles. Apart from DMNT, other single components included in the released volatile blend could also be suitable candidates to trigger defense-related signaling. All in all, it is essential to gain more information on the signaling processes in *Ipomoea batatas* during herbivory to further improve the resistance against insect pests. The generation of improved insect-resistant cultivars will therefore optimize yields of sweet potato tubers to meet worldwide growing agricultural demands achieved by environment-friendly techniques.
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Declaration

I hereby affirm that I have independently written the attached Master’s thesis on the topic:

*Analysis of defense-related reactions upon herbivore attack in *Ipomoea batatas*

and have not used any other aids or sources other than those I have indicated.

For parts that use the wording or meaning coming from other works (including the Internet and other electronic text and data collections), I have identified them in each case by reference to source or the secondary literature.

Furthermore, I hereby affirm that the above mentioned work has not been otherwise submitted as a thesis for a Master’s examination. I further understand the pending completion of the review process I must keep the materials available that can prove this work was written independently.

After the examination process has been completed, the work will be submitted to the Library of the University of Konstanz and catalogued. It will thus be available to the public through viewing and lending. The described data given, such as author, title, etc. are publicly available and may be used by third parties (for example, search engine providers or database operators). As author of the respective work, I do not consent to this procedure.

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