Role of Calmodulin-like Proteins in Calcium-mediated Herbivore Defense Pathways in Arabidopsis.

Dissertation

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Spodoptera littoralis larva feeding on *Arabidopsis thaliana Co-0*

(Foto by Sandra S. Scholz & Monika Heyer)

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Summary

During their lifetime, plants need to adapt to various stimuli originating from the abiotic and biotic environment. One major biotic stress factor is the attack of herbivorous insects feeding on the plant. During the feeding process wounding of plant tissue and contact with elicitors in insect's oral secretion (OS) occurs. The early events upon perception of these stimuli are still poorly understood. Elevations in cytosolic calcium are one of these early events, which activate the downstream signaling network. To reach this a proper decoding of calcium signals by for example different calcium sensor proteins is important. In this study it was demonstrated that in *Arabidopsis thaliana*, several members of the calmodulin-like proteins (CMLs), one group of calcium sensors, are induced upon OS of the generalist herbivore *Spodoptera littoralis*. The expression patterns upon OS treatment can be classified into two groups. While *CMLs 11, 12, 16, 37* show an early and transient expression, the expression of *CMLs 9, 17*, and *23* starts late and shows a sustained level over a longer time.

In herbivory, *CML37* is strongly upregulated upon mechanical wounding, but responds additionally to elicitors in OS and to jasmonic acid (JA)-precursor *cis*-OPDA (*cis*-(+)-12-Oxo-Phytodienoic Acid). Upon stress stimuli, CML37 binds to cytosolic free calcium and undergoes a conformational change characterized by increasing α-helical content and exposure of hydrophobic regions. *Knock-out* mutation of *CML37* increases plants susceptibility to herbivore feeding indicating that CML37 acts as a positive defense regulator. CML37 does not influence the content of glucosinolates or flavonoids, while the elevation of phytohormones *cis*-OPDA and the active conjugate JA-Ile is positively influenced. Here CML37 regulates JA-Ile production by modulating JAR1 activity. Lower JA-Ile levels cause lower expression of anti-insect JA-responsive genes. These results indicate that CML37 is involved in herbivore defense where it regulates the jasmonate pathway. Additionally it was demonstrated that CML37 plays an essential role in the connection of the early event of cytosolic calcium elevations with this jasmonate pathway.

Beside herbivore defense, CML37 is also involved in ABA signaling during drought stress. Mutation of *CML37* results in drastically reduced survival of plants upon different periods of drought treatment, indicating that CML37 acts as a positive regulator in drought-induced ABA

signaling. Summarizing, all these results strongly suggest that calcium sensor proteins of the CML-family are involved in response to different abiotic as well as biotic environmental stimuli.

Plant defense is a complex network of different pathways. Next to the jasmonate pathway, also the accumulation of γ-amino butyric acid (GABA) is calcium-mediated. Upon elevation of cytosolic calcium and binding by calmodulin (CaM), the glutamate decarboxylases (GADs) are activated and GABA produced. It was demonstrated that GABA is accumulated upon wounding of plant tissue by MecWorm treatment, where the accumulation was highest, and *Spodoptera littoralis* feeding. Mutation of *gad12* in Arabidopsis caused very low levels of GABA, while an additional *knock-out* of *gaba-t (pop2-5)* generated a mutant accumulating high levels of GABA over time. Higher level of GABA in the *gad12xgaba-t* plant or in artificial diet significantly reduced growth of *Spodoptera littoralis* larvae. This result indicates that accumulation of GABA upon herbivore feeding acts as a general wounding-induced defense. Local wounding of plant tissue induced an accumulation of GABA in local and also in systemic non-wounded leaves, suggesting a Ca^{2+}-dependent activation of GADs in systemic leaves. GABA elevation was not induced upon coronalon treatment, indicating a JA-independent pathway. Summarizing, the results show that herbivore-induced accumulation of GABA is a wounding-induced, JA-independent and systemic plant defense.

In another set of experiments it was demonstrated that upon wounding, also the JA-precursor OPC-8:0 (analyzed by an 7F-OPC-8:0 analouge) was transported to systemic, non-wounded leaves. Moreover, it was shown that the “jasmonate-induced jasmonates hypothesis” is only valid for the activation of JA-biosynthesis genes after jasmonate application while the level of endogenous jasmonates is not changing.

Zusammenfassung

Als sessile Lebewesen müssen Pflanzen sich ständig an wechselnde Bedingungen ihres abiotischen und biotischen Umfeldes anpassen. Dabei ist der Angriff durch herbivore Insekten ein bedeutender Stressfaktor für die Pflanze. Neben einer massiven Verwundung des Gewebes, kommt die Pflanze beim Angriff von Herbivoren auch mit dessen Reguritat (OS, *oral secretion*) in Berührung, welches eine Vielzahl von Elizitoren enthält. Die frühen Abläufe in Erkennung und Weiterleitung des wahrgenommenen Signals sind noch nicht vollständig untersucht. Die Erhöhung des cytosolischen Calcium Levels spielt dabei jedoch eine wichtige Rolle und ist in der Lage nachfolgende Signalwege zu aktivieren. Um eine, dem Stimulus entsprechende, spezifische Antwort zu erzeugen, muss das entstandene Calcium Signal entschlüsselt werden. Dies geschieht unter anderem durch verschiedene Calcium Sensoren. Die Familie der *Calmodulin-like proteins* (CMLs), den Calmodulinen ähnliche Proteine, sind eine Gruppe dieser Calcium Sensoren in *Arabidopsis thaliana*. Es wurde gezeigt, dass zahlreiche Mitglieder dieser Familie durch das Reguritat von *Spodoptera littoralis* induziert werden. Dabei weisen die Expressionsmuster dieser CMLs verschiedene Verläufe auf. Während die Transkription der *CMLs 11, 12, 16* und *37* schnell hochreguliert wird und anschließend wieder abfällt, startet die Expression von *CML 9, 17* und *23* viel später und hält länger an.

Unter Herbivorbefall ist die Expression von *CML37* stark induziert. Diese wird vorrangig durch die mechanische Verwundung des Pflanzengewebes ausgelöst, aber auch Elizitoren im Reguritat und die JA-Vorstufe *cis*-OPDA können eine Induktion bewirken. Nach erkennen eines Stresssignals bindet CML37 das frei gewordene cytosolische Calcium und durchläuft eine Konformationsänderung bei der sich der α-helikale Gehalt des Proteins erhöht und hydrophobe Bereiche zugänglich werden um mit Interaktionspartnern in Kontakt zu treten. Ein *knock-out* von *CML37* bewirkt eine erhöhte Anfälligkeit von Arabidopsis gegenüber Herbivoren, was für eine Wirkung als positiver Regulator der Herbivorenabwehr spricht. CML37 hat keinen Einfluss auf den Gehalt von Sekundärmetaboliten wie Glucosinolate oder Flavonoide, aber die Akkumulation der Phytohormone *cis*-OPDA und JA-Ile werden positiv beeinflusst. Dabei reguliert CML37 die Produktion von JA-Ile durch Modulierung der Aktivität des beteiligten Enzyms JAR1. Die geringen Mengen an JA-Ile, welches das bioaktive Hormon ist, bewirken wiederum eine geringere Expression von anti-herbivoren Proteinen. Diese Ergebnisse verdeutlichen, dass

CML37 in die Herbivorenabwehr involviert ist und dabei den Jasmonatsignalweg reguliert. Zusätzlich konnte gezeigt werden, dass CML37 somit ein Bindeglied zwischen der Ausschüttung von cytosolischen Calcium und dem Jasmonatsignalweg ist.

Neben der Herbivorenabwehr ist CML37 auch in die Regulation von ABA unter Trockenheitsstress involviert. Mutation von *CML37* bewirkt eine stärkere Anfälligkeit der Pflanzen gegenüber Trockenheit, was nach mehreren Trockenperioden zum Austrocknen der Pflanzen führt. Dies weist darauf hin, dass CML37 auch in der Trockenstress-induzierten Akkumulation von ABA als positiver Regulator wirkt. Zusammengefasst weisen diese Ergebnisse darauf hin, dass Calcium Sensoren der CML Familie in die Reaktion auf und Verarbeitung von abiotischen und biotischen Stressfaktoren involviert sind.

Die Abwehr von Pflanzen ist ein komplexes Netzwerk von verschiedenen Signalwegen. Neben den Jasmonatsignalweg ist auch die Akkumulation von GABA ein Calcium-abhängiger Vorgang. Nach dem Anstieg der freien, cytosolischen Calcium Konzentration und anschließender Bindung des Calciums durch Calmodulin werden die Glutamat-decarboxylasen aktiviert, welche Calmodulin-reguliert sind. Es konnte gezeigt werden, dass die Akkumulation von GABA vor allem durch die vom MecWorm verursachte, mechanische Verwundung der Pflanze induziert wird. Auch Frass durch die Larven von *Spodoptera littoralis* konnte die Akkumulation von GABA bewirken. Die Mutation von *gad12* in Arabidopsis führte zu einer drastischen Reduzierung des GABA-Gehaltes der Pflanzen, während ein zusätzlicher *knock-out* von *gaba-t (pop2-5)* eine Akkumulation von GABA in den Mutanten bewirkte. Der hohe Gehalt von GABA in den *gad12xgab-t* Mutanten und eine artifizielle Ernährung der Larven mit GABA-angereicherten Futter führten zu einer signifikanten Reduzierung der Gewichtszunahme bei *Spodoptera littoralis*. Dieses Ergebnis verdeutlicht, dass die Akkumulation von GABA bei Herbivorenfrass in Arabidopsis eine generelle, durch Verwundung induzierte Abwehrreaktion ist. Eine lokale Verwundung der Pflanze konnte eine Akkumulation von GABA im lokalen sowie in nicht verwundeten, systemischen Blättern induzieren. Dies deutet auf eine durch Calciumsignale in den systemischen Blättern induzierte Aktivierung der GADs hin. Die Produktion von GABA ist unabhängig von Jasmonaten, was durch eine Behandlung mit Coronalon gezeigt werden konnte. Zusammenfassend ist die Akkumulation von GABA eine

durch Verwundung induzierte, JA-unabhängige und systemische Abwehrreaktion gegen Herbivoren.

In weiteren unabhängigen Experimenten wurde die Dynamik der Akkumulation und Weiterleitung der Jasmonat-Vorstufe OPC-8:0 mit Hilfe eines markierten Analoges 7F-OPC-8:0 untersucht. Nach lokaler Verwundung von Arabidopsis Pflanzen, wurde 7F-OPC-8:0 in systemische, nicht verwundete Blätter transportiert. Außerdem konnte in weiteren Experimenten gezeigt werden, dass die „Jasmonat-induzierte Jasmonatproduktion“ nur für die Aktivierung der JA-Biosynthese Gene gilt aber nicht für die Akkumulation von endogenen Jasmonaten.

1 Introduction

1.1 Plant defense strategies against insect herbivory

During their lifespan, plants have to deal with a multitude of stress factors originating from the abiotic as well as the biotic environment. Main abiotic environmental cues influencing the plants performance and fitness include drought and salt stress, ozone and UV-radiation, cold stress and many others (Lawlor, 2011). Biotic stress factors originate from many different groups of organisms like pathogens, nematodes, microorganisms, and also from feeding insects. Given the fact that over 50 % of all insects show herbivorous feeding behavior, plants have to adapt to them by developing and modulating different defense strategies (Schoonhoven *et al.*, 1998; Van Poecke, 2007). Attack of insects, especially with chewing feeding behavior, cause a massive loss of plant tissue and viability leading to low reproduction rate (Stowe *et al.*, 2000). Attack of herbivorous insects combines different stress stimuli inducing plant defense. Perception of herbivory by the plant consists of recognition of wounding of plant tissue and of elicitors provided by the insect's oral secretion (OS) (Maffei *et al.*, 2004; Mithöfer *et al.*, 2005; Mithöfer and Boland, 2008; Wu and Baldwin, 2010).

The plant defense activated upon herbivory, is a complex network of different pathways, which are constitutively expressed or induced upon stimuli perception. Both groups of defense pathways are composed of direct and indirect defenses (Howe and Jander, 2008). Direct defense compounds like glucosinolates or protease inhibitors directly influence the insects performance and feeding behavior, while indirect defenses like emission of volatile organic compounds (VOCs) after herbivore attack function as attractant for parasitic wasps which in turn predate on the attacker (Van Poecke *et al.*, 2001; Van Poecke, 2007). While plants develop new defense compounds or mechanisms to enhance the resistance against herbivores, their attackers find new ways to bypass or detoxify these (Jander, 2014). Generalist herbivores are feeding on many different plant species and have to encounter different defenses, specialist insects are limited to a number of food plants and show a higher level of adaptation to the defense mechanism of these specific plants (Ali and Agrawal, 2012). For example *Manduca sexta* larvae feeding on tobacco plants show a high grade of adaptation to otherwise toxic levels of nicotine (Steppuhn *et al.*, 2004; Pluskota *et al.*, 2007).

This work will focus on the interaction between the mouse-ear cress *Arabidopsis thaliana* (Brassicaceae), a well-known model plant and the generalist herbivore *Spodoptera littoralis* (the Egyptian cotton leaf worm, Lepidoptera). *Spodoptera littoralis* is a major pest of cotton, vegetables, flowers and crop plants and causes high loss of yield in agriculture (*http://www.cabi.org/isc/datasheet/51070*). Larvae of *Spodoptera littoralis* can be kept on a simple artificial diet (Bergomaz and Boppre, 1986), making them a good tool to study herbivory in the lab.

1.1.1 Mechanical defenses

The plant's mechanical defenses are the first layer of defense that a herbivorous insect encounters while feeding on them. In *Arabidopsis thaliana*, the major component contributing to its mechanical defenses are trichomes. These structures on the plant surface, which are formed by epidermal cells, show a high grade of branching. It was shown that trichomes negatively influence the herbivore feeding behavior via its effect on insect mobility (Reymond *et al.*, 2004). Additionally it was shown that in a population of *Arabidopsis lyrata*, plants lacking trichomes are more susceptible to herbivory than plants with higher trichome density (Løe *et al.*, 2007). The plant surface also harbors additional layers of mechanical defense in form of epicuticular waxes which are influencing insect's feeding behavior and egg deposition (Blenn *et al.*, 2012). These mechanical barriers are thus a first line of defense; the major part of the plant's defense against herbivores is, however, made up by different chemical defenses.

1.1.2 Chemical defenses

Arabidopsis thaliana processes a huge arsenal of inducible chemical herbivore defense mechanisms which contribute to direct and indirect defense by influencing the insect's feeding behavior and fitness. One well studied indirect defense of Arabidopsis plants is the emission of volatile organic compounds (VOCs) after herbivore attack (Van Poecke, 2007). Interestingly the composition of VOCs emitted from limabean leaves after *Spodoptera littoralis* feeding and *Cepaea hortensis*, a snail feeding by rasping the plant tissue, was very similar to the VOCs emitted under spider mite infestation. Main components of VOCs are the fatty acid derivative (*Z*)-3-hexenyl acetate (Hex-Ac), the phenolic compound methyl-salicylate (MeSA) and the monoterpene linalool (Dicke *et al.*, 1990; Mithöfer *et al.*, 2005). The blend of volatiles differs in *Pieris rapae* infested and undamaged plants and functions as attractant for parasitic wasps like

C*otesia rubecula*, which are specifically predating on *P. rapae* caterpillars (Van Poecke *et al.*, 2001).

Most defensive compounds produced by plants in response to herbivory belong to the class of secondary metabolites. The primary task of these metabolites is - in contrast to primary metabolites used for growth and biomass production - to defend the plant against herbivorous insects and pathogens (Bennett and Wallsgrove, 1994). Secondary metabolites are both, constitutively stored in different plant tissues and highly induced by herbivore attack (War *et al.*, 2012).

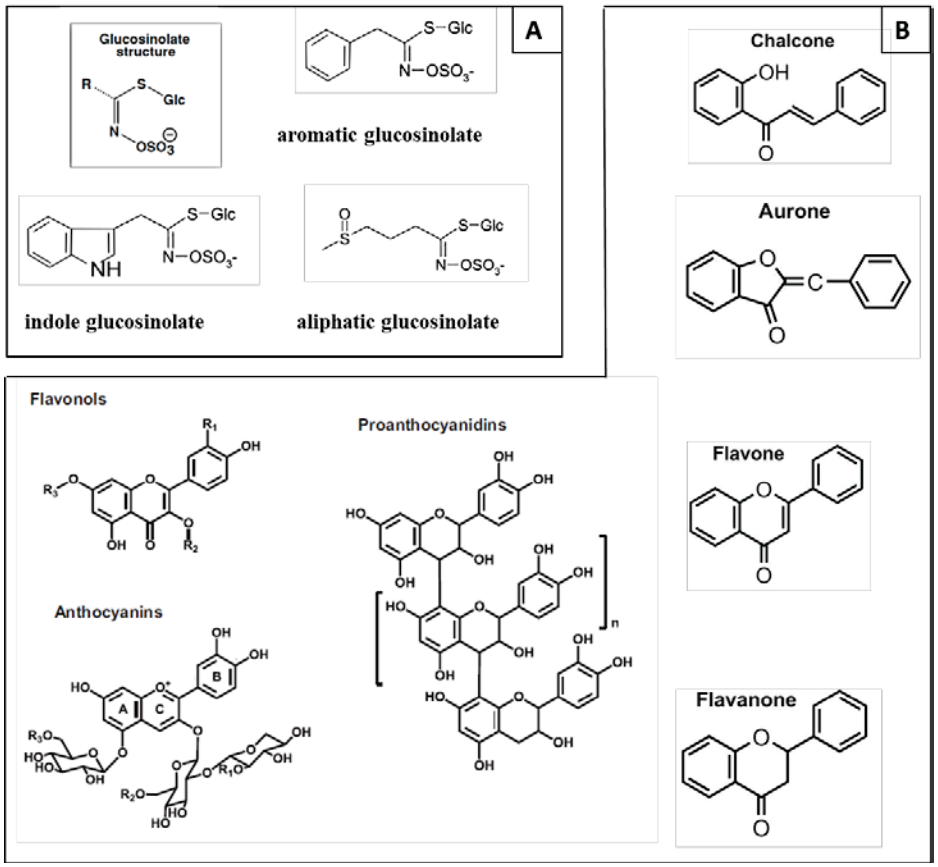

Figure 1. General structures of secondary metabolites in *Arabidopsis thaliana*.

Shown are the structures of glucosinolates (**A**) and flavonoids (**B**) occurring in *A. thaliana*. **A**) Shown are the basic core structure of glucosinolates (encircled) and the major groups of glucosinolates detected in *A. thaliana* (adapted from (Halkier and Gershenzon, 2006; Van Poecke, 2007)). **B**) Shown are the groups of flavonoids detected in *A. thaliana* (adapted from (Falcone Ferreyra *et al.*, 2012; Saito *et al.*, 2013)).

Plants of the family Brassicacae (like Arabidopsis) mainly store glucosinolates which are nitrogen- and sulfur-containing compounds ((Halkier and Gershenzon, 2006), Figure 1A). These can be classified upon their biosyntheses into aliphatic (mainly produced from methionine) and

aromatic (produced from tryptophan, phenylalanine and tyrosine) glucosinolates (Glawischnig *et al.*, 2003; Halkier and Gershenzon, 2006; Bidart-Bouzat and Kliebenstein, 2008). The glucosinolates are not toxic per se, but upon hydrolysis by a myrosinase, toxic products like nitriles, thiocyanates and isothiocyanates are formed. In undamaged Arabidopsis leaves, glucosinolates and myrosinase are stored spatially separated. When an herbivorous insect is chewing on a leaf, both components are mixed together; the toxic degrading products are formed and can act as feeding deterrent (Burow *et al.*, 2006; Wittstock and Burow, 2010; Schramm *et al.*, 2012). Glucosinolates are not uniformly distributed in all leaves of an Arabidopsis plant (Shroff *et al.*, 2008; Shroff *et al.*, 2015) and are induced upon herbivore feeding (Textor and Gershenzon, 2009). Insects that feed on Arabidopsis plants developed different detoxification mechanisms to deactivate the glucosinolate breakdown products. *Spodoptera littoralis* larvae detoxify isothiocyanates by forming conjugates with amino acids or glutathione which are further hydrolyzed (Schramm *et al.*, 2012).

Next to glucosinolates, Arabidopsis plants also produce and store a variety of flavonoids, which are another group of secondary metabolites composed of flavonols, anthocyanins, proanthocyanidins and others (Figure 1B, (Falcone Ferreyra *et al.*, 2012; Saito *et al.*, 2013). Flavanoids are involved in plant defense against UV-B radiation by inhibiting the formation of free radicals as well as the reduction of ROS formed. Besides this, flavonoids are also involved in plant defense against pathogens and herbivores (Verdan *et al.*, 2011). There are indications that induction of the flavonoid biosynthesis pathway by UV light can be inhibited by pathogen-induced defense responses (Logemann and Hahlbrock, 2002).

1.1.3 Anti-herbivore proteins

Many defense compounds like anti-insect proteins produced by the plant act directly on the metabolism or development of feeding insects. So are by the plant produced protease inhibitors (PIs) able to disturb the digestion of ingested food material (Green and Ryan, 1972) and, as a consequence of this, to slow down the development of the insect (Ryan, 1990). Well studied defensive proteins produced by Arabidopsis are the translated products encoded of JA-responsive genes *VSP2, THI2.1* and *PDF1.2*. In previous studies it was demonstrated that *VSP2* (vegetative storage protein 2) is induced by wounding, methyl jasmonate, insect feeding, and phosphate deprivation. The VSP2 protein shows phosphatase activity in acid pH range

corresponding to the pH of insect gut lumen. Here, VSP2 could significantly delay development of the insects and increase their mortality (Berger *et al.*, 1995; Liu *et al.*, 2005). The expression of *VSP2* could also be inhibited by neomycin application in Arabidopsis (Vadassery *et al.*, 2014). Another JA-responsive gene induced by wounding of plant tissue and methyl jasmonate is *THI2.1*, encoding the antimicrobial protein thionin which might also contribute to herbivore defense (Epple *et al.*, 1997; Bohlmann *et al.*, 1998; Vignutelli *et al.*, 1998). PDF1.2, encoding another defensin in Arabidopsis is also activated upon methyl jasmonate and Spodoptera feeding (Manners *et al.*, 1998; De Coninck *et al.*, 2010; Kanchiswamy *et al.*, 2010).

1.1.4 GABA as possible player in plant-herbivore defense

γ-amino butyric acid is well studied as a neurotransmitter in invertebrates. After coupling, GABA-mediated Cl^--channels are opened and the signal is transduced (Bown *et al.*, 2006). In plants, the non-protein amino acid GABA (Figure 2 A) plays a role in regulation of C/N balance and plant growth and development (Palanivelu *et al.*, 2003; Bouche and Fromm, 2004; Mirabella *et al.*, 2008). Beside this, it was hypothesized that GABA has a possible role in plant defense. Excess supply of GABA could lead to hyper activation of the Cl^--channels leading to paralysis of the attacking insect (Bown *et al.*, 2006). So it was shown that high content of GABA in the insect's diet causes developmental restrictions by increasing time to pupation (Bown *et al.*, 2006).

GABA is mainly produced by decarboxylation of L-glutamate catalyzed by glutamate decarboxylases (GADs) in the cytosol (Figure 2 B, (Turano and Fang, 1998; Zik *et al.*, 1998)). The catabolism of GABA into alanine and succinic semialdehyde is localized in the mitochondrial matrix, where a GABA transaminase (GABA-T) removes the amino group of GABA and transfers it onto pyruvate. The succinic semialdehyde than is exported from mitochondria or oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH) (Breitkreuz *et al.*, 2003; Ludewig *et al.*, 2008; Michaeli *et al.*, 2011).

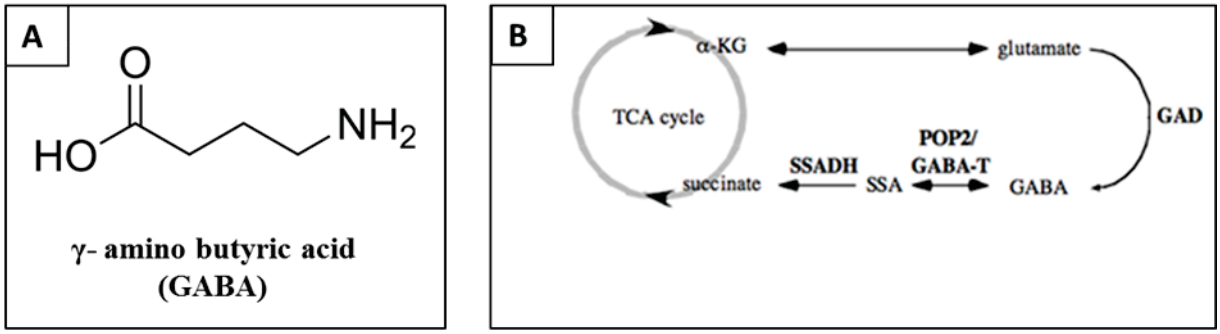

Figure 2. Structure, syntheses and metabolism of γ-amino butyric acid (GABA).

Shown is the molecular structure of the non-protein amino acid GABA (**A**) and the metabolism and catabolism of GABA (**B**). GAD glutamate decarboxylase, GABA-T GABA transaminase, SSA succinic semialdehyde, SSADH succinic semialdehyde dehydrogenase.

Under normal conditions, the activity of GADs and with this the accumulation of GABA, is regulated by Ca^{2+} and calmodulin. Upon stimuli-induced cytosolic calcium elevation, calmodulins (CaMs) bind to calcium, and interact with GADs by coupling to their CaM-binding site (Snedden *et al.*, 1995). Under stress conditions like disruption of plant tissue, GADs are strongly induced by an acidification of the cytosol (Wallace *et al.*, 1984; Carroll *et al.*, 1994; Ramputh and Bown, 1996). This observation combined with the fact that *Choristoneura rosaceana* larvae reared on GABA-containing diet show reduced weight gain, are hints for an involvement in herbivore defense (Ramputh and Bown, 1996; Shelp *et al.*, 1999; Bown *et al.*, 2006). Additionally, it was observed that feeding and even walking behavior of *Heliothis virescens* larvae on *Nicotiana tabacum* leaves increases the content of GABA in the leaf tissue (Bown *et al.*, 2002). Till now, the temporal and spatial accumulation of GABA after herbivore attack is still unknown.

1.2 Plant-herbivore interaction

The recognition of a feeding herbivore starts seconds and minutes after the stimulus is perceived (Figure 3). Each herbivore bears a number of herbivore-associated molecular patterns (HAMPs, see section 1.2.1), which are – as first step in the signaling cascade - recognized by the plant through an array of specialized putative receptors (Mithöfer and Boland, 2008). After the receptor binding, a depolarization of the membrane occurs (see section 1.2.2) which is associated with an influx of calcium ions from external and internal stores into the cytosol ((Maffei *et al.*, 2007; Vadassery *et al.*, 2012a) see section 1.2.3).

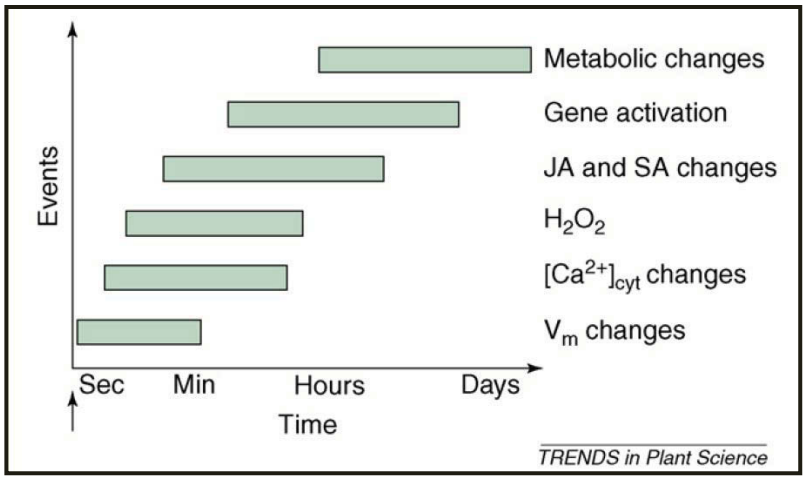

Figure 3. Early events in plant-herbivore interaction (Maffei *et al.*, 2007).

Shown are the first steps of plant herbivore perception, which occurs in the first minutes and hours after attack. After the stimulus perception, the membrane depolarizes and initiates a spike in the cytosolic Ca^{2+} level. This activates a signaling cascade which leads among others to ROS production and the accumulation of phytohormones. These induce the expression of responsive genes and transcripts which in turn can modulate the plants metabolism.

The spikes in cytosolic calcium levels $[Ca^{2+}]_{cyt}$ are decoded by different calcium sensor proteins (see section 1.2.4), which interact with their target proteins to initiate the downstream signaling ((DeFalco *et al.*, 2010), see section 1.2.5, 1.2.6). An accumulation of herbivory- and wounding-related phytohormones like jasmonates, or the production of reactive oxygen species (ROS) are part of this cascade. As a consequence, metabolic changes like production of anti-herbivore peptides (Ryan, 1990; Zavala *et al.*, 2004) or defensive substances like nicotine (Steppuhn *et al.*, 2004) and glucosinolates (Müller *et al.*, 2010) are induced.

1.2.1 Herbivore-associated molecular patterns (HAMPs) and receptors

The plant recognizes attacks by perception of different molecular patterns (MPs), which are associated with the outer surface or released components of the aggressor (Taylor *et al.*, 2004; Ausubel, 2005; Mithöfer and Boland, 2008). It is known that the conserved microbe-specific molecules, referred to as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs), are recognized by pattern recognition receptors (PRRs). MAMPs like flagellin (Flg22), elongation factor Tu (EF-Tu), peptidoglycan (PGN), lipopolysaccharides (LPS), Ax21 (Activator of Xa 21-mediated immunity in rice), fungal chitin, and β-glucans from oomycetes are recognized by plant surface localized PRRs (Jones and Dangl, 2006; Newman *et al.*, 2013; Ranf *et al.*, 2015). Herbivore associated molecular patterns (HAMP) are postulated to be present in insect oral secretions and are of two kinds: (i) chemical elicitors derived from insect oral secretions and oviposition fluids; and (ii) plant-derived self-recognition factors, DAMPs (damage-associated molecular patterns) occurring due to a specific pattern of wounding (Mithöfer and Boland, 2008; Heil, 2009; Heil and Land, 2014). Insect OS contain elicitors,

notable examples are inceptins, which are peptides formed as proteolytic products of plant chloroplastic ATP synthase formed in caterpillar midgut, and fatty acid-amino acid conjugates (FACs) such as volicitin in maize (Alborn *et al.*, 1997).

Upon herbivore attack, the plants encounter two main components of herbivore feeding: the wounding of plant tissue and recognition of elicitors in OS. HAMPs include the oral secretion of the larvae composed of saliva and reguritant, damaged plant parts, ingested and metabolized phytohormones and other components like volicitin, (Alborn *et al.*, 1997; Maffei *et al.*, 2004; Wu and Baldwin, 2010). Recently, a Porin-like protein was identified as elicitor in *Spodoptera littoralis* OS that originated from the insects gut microbiota (Guo *et al.*, 2013). The released quantity of these HAMPs and the leaf area injured may be different for distinct insect feeding styles, which causes a different plant response (Ali and Agrawal, 2012). While insects with piercing sucking mouthparts like aphids cause only small wounds on plant tissue, chewing insects induce a much stronger lesion. The binding of all these HAMPs to unidentified PRRs is the first step of a complex signaling cascade, enabling the plant to react fast and efficient to different environmental stimuli.

1.2.2 Membrane depolarization

Next to the disrupted cells at the brink of the leaf area fed on, neighboring plant cells also respond to environmental stresses by changes in plasma transmembrane potential (Vm, (Ebel and Mithöfer, 1998; Maffei *et al.*, 2004)). For Lima bean (*Phaseolus lunatus*) it was shown, that Vm changes induced by herbivores were much greater compared to these of single wounding and could travel throughout the whole leaf (Maffei *et al.*, 2006; Maffei *et al.*, 2007). Vm changes are followed due to an electrical signal (called action potential) and also by system potentials, which can propagate the signal over longer distances ((Maffei *et al.*, 2007; Zimmermann *et al.*, 2009)). Stress induced Vm changes (depolarization) can also modulate ion fluxes at the plasma membrane by activation of voltage-dependent channels, like Ca^{2+} channels (White, 2000; Maffei *et al.*, 2007). Mousavi et al. (2013) showed in Arabidopsis that for the propagation of electrical signals probably glutamate receptor-like genes are necessary. Finally, the electrical signals are able to induce JA-Ile elevation in systemic leaves (Mousavi *et al.*, 2013).

1.2.3 The second messenger calcium ions (Ca^{2+})

The calcium ion (Ca^{2+}) plays an important role as a second messenger in varied signaling networks of plant cells (Dodd *et al.*, 2010). Plant cells maintain a level of 100-200 nM free cytosolic calcium $[Ca^{2+}]_{cyt}$, the so called Ca^{2+} homeostasis. This incident is due to the fact that high concentrations of cytosolic Ca^{2+} have a cytotoxic effect on phosphate-containing components, including proteins and nucleic acids. To maintain this low level of Ca^{2+} in the cytosol, several active transporters like Ca^{2+}-ATPases (ACAs) located in organelle- and cell membranes pump the Ca^{2+} into the stores (Sze *et al.*, 2000). The Ca^{2+} is stored in high concentrations (10^5 times higher than cytosolic concentration) in different intra- and extracellular stores. While the apoplast serves as external calcium store, different organelles like the vacuole or chloroplasts store Ca^{2+} inside the cell (Knight *et al.*, 1996; Peiter, 2011; Stael *et al.*, 2011). This high gradient of Ca^{2+} concentrations is the basis for a fast response to stress stimuli. Here an influx of Ca^{2+} from the stores into the cytosol induces a calcium signature, whose specific shape, amplitude and duration encode the information perceived (Lecourieux *et al.*, 2006; McAinsh and Pittman, 2009; Dodd *et al.*, 2010).

It has been reported that feeding by *Spodoptera littoralis* on *Phaseolus lunatus* causes a transient increase in cytosolic $[Ca^{2+}]_{cyt}$ in cells adjacent to the insect bite (Maffei *et al.*, 2004). It was shown that application of *Spodoptera littoralis* OS could induce cytosolic Ca^{2+} elevations in *Arabidopsis thaliana* leaf discs and soybean suspension cultures (Maischak *et al.*, 2007; Vadassery *et al.*, 2012a). In *Arabidopsis thaliana* it was demonstrated that antibiotic neomycin selectively blocked the accumulation of OS-induced Ca^{2+} elevation and accumulation of the bioactive JA-Ile, in contrast to JA. Furthermore, neomycin treatment affected the downstream expression of JA-Ile-responsive genes, *VSP2* and *LOX2*, (Vadassery *et al.*, 2014). $Ca^{2+}{}_{cyt}$ elevations in local leaf tissue upon herbivory have also been quantified using Yellow Cameleon reporter (YC 3.6), which allowed a clear distinction between meachnical damage and herbivory and discriminated between two larvae instars (Verrillo *et al.*, 2014). A non-invasive whole plant calcium imaging demonstrated that wounding alone as well as *Spodoptera littoralis* feeding could induce local and also systemic cytosolic calcium elevations in *Arabidopsis thaliana* (Kiep *et al.*, 2015). It has been demonstrated that phytohormones like JA/JA-Ile and OPDA application also induces a specific Ca^{2+} elevation in cytosol and nucleus (Walter *et al.*, 2007; Mazars *et al.*,

2009). To achieve a specific decoding of Ca^{2+} signals both in the nucleus and the cytosol, the plant processes an arsenal of different calcium sensor proteins (DeFalco *et al.*, 2010).

1.2.4 Calcium sensors

In Arabidopsis the most studied groups of calcium sensor proteins are calmodulins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and calcium-dependent protein kinases (CDPKs, now renamed as CPKs), shown in Figure 4 (DeFalco *et al.*, 2010).

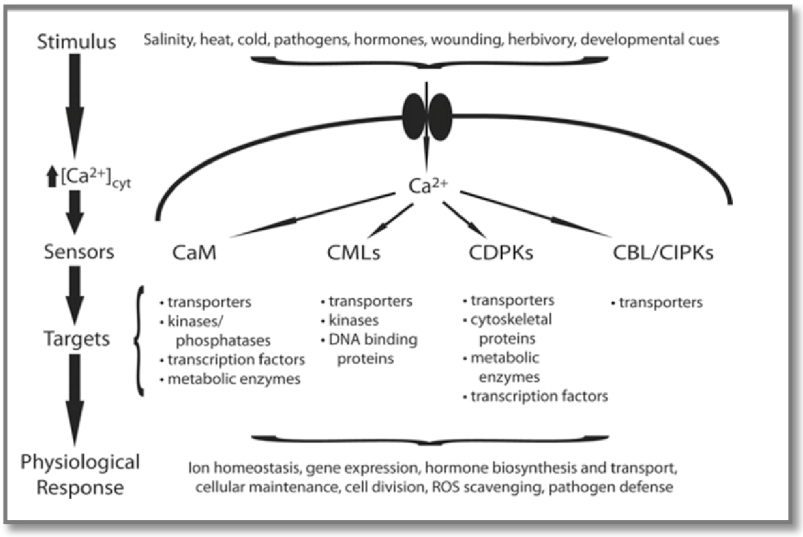

Figure 4. Different classes of calcium sensor proteins activated upon abiotic and biotic stimuli (DeFalco *et al.*, 2010).

Shown are the classes of Ca^{2+} sensor used to decode cytosolic Ca^{2+} spikes induced by diverse stimuli. Here, calcium sensor proteins function as signal relays (CaM/CMLs and CBLs) or primary responders (CDPKs).

In general, calcium sensor proteins found in Arabidopsis can be classified - in sense of mode of action - into two groups: sensor responders and sensor relays (DeFalco *et al.*, 2010). Sensor responders bind the cytosolic free Ca^{2+}, undergo conformational changes and actively regulate downstream signaling by their own enzymatic activity. The family of CPKs, Ca^{2+} sensors involved in e.g. ABA and herbivore defense signaling, belongs to this group (Wu and Baldwin, 2010; Romeis and Herde, 2014). So it was shown, that Arabidopsis *cpk3* and *cpk13* mutants express significantly less JA-responsive genes making them more susceptible to Spodoptera feeding (Kanchiswamy *et al.*, 2010). Silencing of *CPK4* and *CPK5* in *Nicotiana attenuata* plants in contrast caused higher accumulation of JA and reduced growth of *Manduca sexta* larvae (Hettenhausen *et al.*, 2013b; Yang *et al.*, 2014).

Sensor relay proteins in contrast do not contain any enzymatic domain. After binding of calcium and conformational shift, they need to physically interact with target proteins to transfer the signal perceived. CaMs/CMLs and CBLs can be assigned to this group (DeFalco *et al.*, 2010).

CBLs form complexes with CIPKs (CBL-interacting protein kinases) and regulate membrane channels and transporters (Batistic and Kudla, 2004). The function of CBLs is still not well understood since the knowledge about CBL-interacting proteins is limited. First results show that CBLs are involved in salt stress signaling (Batistič and Kudla, 2009). Here, CBL1 and CBL9 are involved in K^+ uptake by activation of a K^+ -transporter under low-K^+ conditions (Xu *et al.*, 2006) and CBL4 (also SOS3) activates an H^+/Na^+ exchanger (also SOS1) under high salt stress (Halfter *et al.*, 2000).

The induction pattern of CAMs and CMLs is better understood (McCormack and Braam, 2003; McCormack *et al.*, 2005). *Arabidopsis thaliana* CAMs, which are very similar to animal CAMs, do not show strong transcript abundance changes in the response to diverse stimuli. Only for *CAM2* (also *TCH1*) it was observed that the expression was induced by touch (Braam and Davis, 1990; Lee *et al.*, 2005). The group of CMLs is involved in the regulation of diverse signaling pathways (McCormack *et al.*, 2005).

1.2.4.1 Calmodulin-like proteins (CMLs)

CMLs are one class of calcium sensor proteins, which act as sensor relays where they are propagating the Ca^{2+} signal. To achieve this, CMLs contain a number of EF-hands (1-6), helix-loop-helix structures, which are responsible for high-affinity cooperative binding of Ca^{2+}. After binding, CMLs undergo a conformational change and can interact with their target proteins (Kawasaki *et al.*, 1998; McCormack and Braam, 2003; Clapham, 2007; Gifford *et al.*, 2007). In Arabidopsis, the class of CMLs consists of 50 members (Figure 5), which show at least 16 % sequence identity to CAMs. Analysis of a neighbor-joining tree, based on amino acid similarities, showed that CMLs cluster in 9 groups (McCormack *et al.*, 2005).

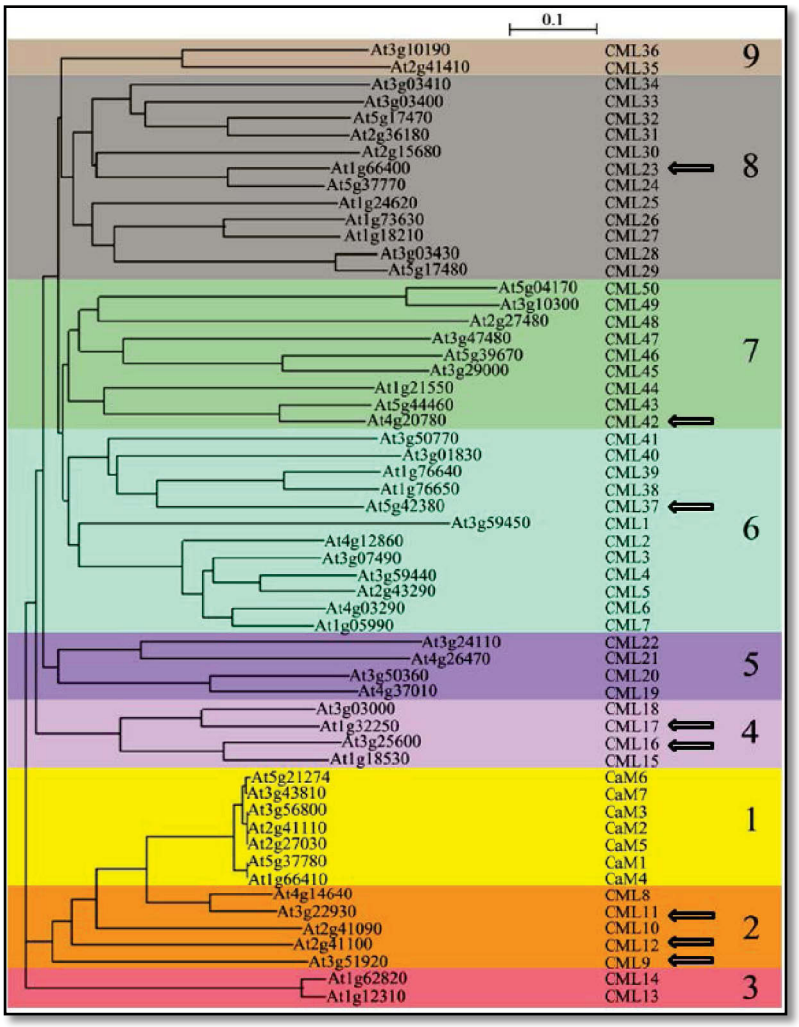

Figure 5. Phylogenetic tree of CAMs and CMLs of *Arabidopsis thaliana* (McCormack *et al.*, 2005).

Shown is a neighbor joining tree, based on amino acid similarities. The seven CAMs and 50 CMLs cluster in 9 different groups. For more details see ((McCormack *et al.*, 2005), adapted). CMLs induced by insect oral secretions are indicated by arrows (Manuscript 1,2 and (Vadassery *et al.*, 2012a)).

While the seven CAM genes in Arabidopsis are very uniformly expressed at a high transcript level, the CMLs show various expression patterns over different tissues and developmental stages of the plant while the transcript levels are quite low. These observed expression patterns do not correlate with the identified CML groups (McCormack *et al.*, 2005). While CMLs like *CML8, 9, 24, 42* are expressed in all major plant organs (Delk *et al.*, 2005; Magnan *et al.*, 2008; Park *et al.*, 2010; Vadassery *et al.*, 2012a), other CMLs show a very specific expression in a single plant organ. So it was shown that in *Arabidopsis thaliana*, *CML43* is only expressed in roots (Bender *et al.*, 2014). Other CMLs show a specific subcellular localisation, for example *CML30* is targeted to mitochondria and *CML3* to peroxisomes (Chigri *et al.*, 2012). *CML39* is mostly expressed during early seedling establishment (Bender *et al.*, 2013) and *CML12* (also *TCH3*) is expressed in growing tissues (Sistrunk *et al.*, 1994). These observations indicate that CMLs might be involved in a tissue- and growth stage-specific decoding of Ca^{2+} signals.

It was demonstrated that the expression of CMLs is induced by diverse abiotic as well as biotic stimuli. So is *CML8* induced by SA and salt stress (Park *et al.*, 2010). CML9 is also induced by SA as well as by infection with *Ps. syringae* and can alter plant responses to ABA and abiotic stress (Magnan *et al.*, 2008; Leba *et al.*, 2012). CML24 modulates ABA level during ion stress, regulates pollen tube growth and can induce changes in flowering time (Delk *et al.*, 2005; Hubbard *et al.*, 2008; Yang *et al.*, 2014). Additionally it was shown that expression of *CML37*, *CML38* and *CML39* are regulated by salt- and drought stress, phytohormones and *P. syringae* infection (Vanderbeld and Snedden, 2007) and CML42 is involved in trichome branching (Dobney *et al.*, 2009). Recently, it was shown that one member of the CML-family, CML42, is involved in *Arabidopsis thaliana* defense against *Spodoptera littoralis* herbivory. CML42 acts as a negative regulator of plant defense against herbivory and affects JA perception of the plant. *CML42* gene expression is herbivore elicitor-specific and is not activated upon mechanical wounding (Vadassery *et al.*, 2012a). It was additionally observed that the gene expression of several CMLs is induced by insect OS (in Manuscript 1, Figure 5, arrows). The exact position of CMLs in the signaling cascade and the further processing of the signal by target proteins are still unknown.

1.2.5 Downstream signaling

The downstream signaling components of plant herbivore defense are not completely known, but it became obvious that several signaling pathways are activated. So are activation of mitogen-activated protein kinases (MAPKs), accumulation of jasmonic acid (JA) and expression of JA-dependent genes, and the production of reactive oxygen species (ROS) involved (Wu and Baldwin, 2010).

The production of reactive oxygen species (ROS), which include Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\cdot}OH$), is well studied as a part of plant response to pathogens (Lamb and Dixon, 1997). In recent studies it became clear that ROS production is also involved in herbivore defense. *Medicago truncatula* plants accumulated ROS only after herbivory while wounding did not induce ROS production (Leitner *et al.*, 2005). In lima bean plants (*Phaseolus lunatus*) it was similarly shown that the production of ROS after herbivory was much higher than that after mechanical wounding alone (Maffei *et*

al., 2006). So showed soybean plants challenged with *Helicoverpa zea* an elevated lipid peroxidation and ˙OH radical formation (Bi and Felton, 1995).

Another early signaling event after herbivore attack is also the activation of MAPKs, which play critical roles in plant resistance to herbivores by reshaping the jasmonate pathway and the transcriptome (Hettenhausen *et al.*, 2015). These activated MAPKs phosphorylate their substrates, which include transcription factors and enzymes (Hazzalin and Mahadevan, 2002). It was shown that FACs, elicitors in insect OS, induce the MAPKs in the wounded leaf of treated *Nicotiana attenuata* plants (Wu *et al.*, 2007). Interestingly, activation of MAPK4 in *Nicotiana attenuata* shows herbivore specific pattern. While OS of *M. sexta* induced MAPK4 and decreased JA accumulation, *Spodoptera littoralis* OS did not induce a change in JA level (Hettenhausen *et al.*, 2013a). In *Arabidopsis thaliana*, grasshopper (*Schistocerca gregaria*) OS was also able to activate MAPKs, MPK3 and MPK6 (Schäfer *et al.*, 2011).

A very powerful tool mediating plant defense are phytohormones, endogenous signaling compounds. Several groups of phytohormones (Figure 6) play important roles in plant growth and development. Next to the regulation and coordination of developmental processes, plant hormones are essential for the adaption to the abiotic and biotic environment (Bari and Jones, 2009).

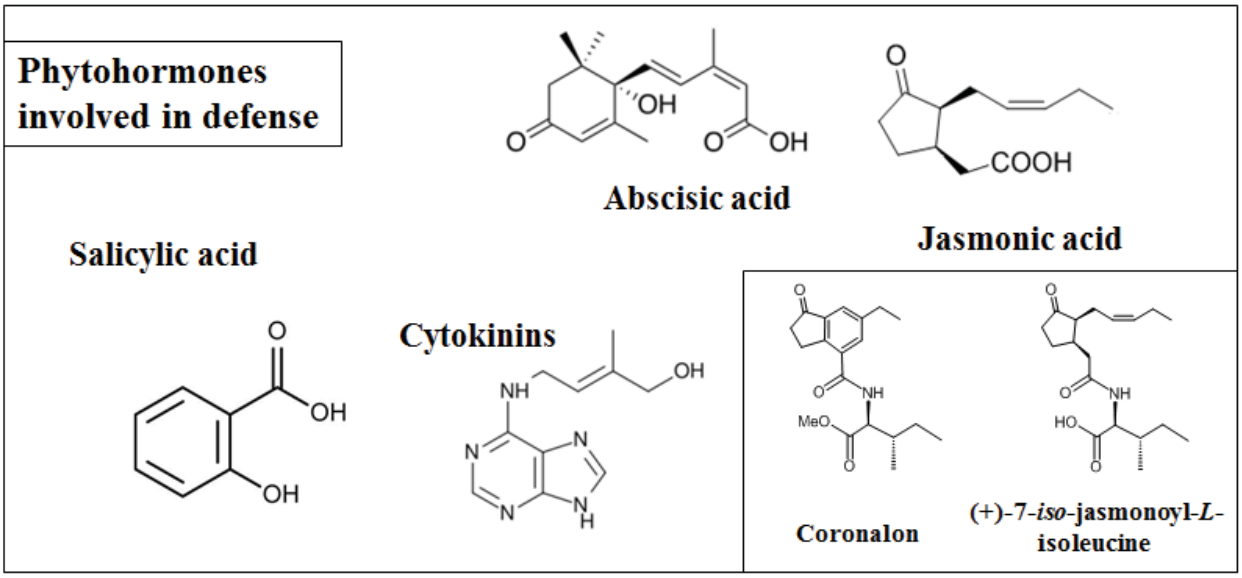

Figure 6. Structure of different phytohormone classes.

Shown are the basic structures of phytohormone classes and the structural JA-Ile mimic coronalon in comparison to JA-Ile itself (inlet).

In plant defense against herbivory, the most important and most studied class of phytohormones is the one of jasmonates (Wasternack, 2007). Jasmonates are lipid-derived molecules originating from plastid membrane-bound α-linolenic acid. The jasmonic acid (JA) biosynthetic pathway is well understood and the enzymes participating in it are well characterized (Vick and Zimmerman, 1984; Schaller and Stintzi, 2009). In the chloroplast, the released α-linolenic acid is metabolized in several steps to form OPC-8:0 followed by *cis*-OPDA, which is catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). After a translocation to the peroxisome, the *cis*-OPDA is further processed to form JA (Schaller and Stintzi, 2009). The active phytohormone JA-Ile is formed by a conjugation of JA and the amino acid isoleucine catalyzed by JASMONATE RESISTANT 1, JAR1 (Staswick and Tiryaki, 2004). Activation of the receptor complex SCF-COI1 by JA-Ile (Figure 7) triggers the degradation of JAZ proteins, the transcriptional repressors of JA responsive genes. This removal of repression leads to activation of the transcription factor MYC2 and the expression of anti-insect JA-responsive genes including *PDF1.2*, *Thi2.1* and *VSP2* (Wasternack and Kombrink, 2010). Mutants of receptor COI1 like *coi1-1* and *coi1-16, jar1* and *jaz1* show higher susceptibility to herbivore feeding (Feys *et al.*, 1994; Chung *et al.*, 2008; Westphal *et al.*, 2008; Chung *et al.*, 2009; Abe *et al.*, 2013).

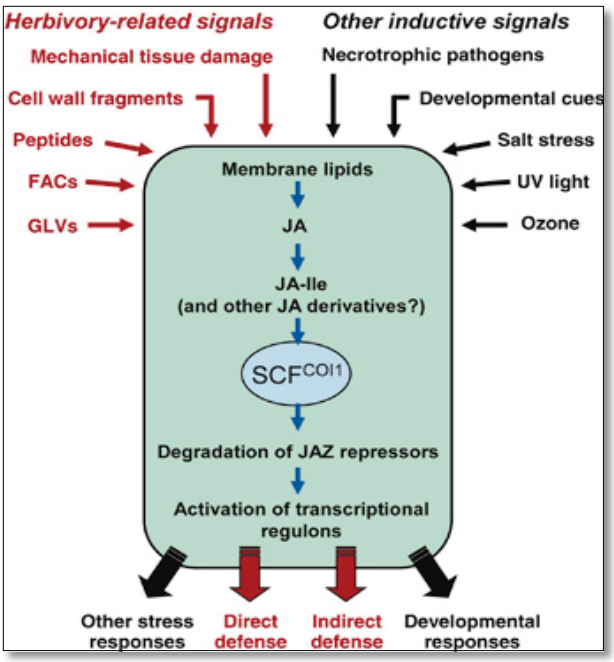

Figure 7. Induction and downstream JA signaling pathway in *Arabidopsis thaliana*.

Accumulation of JA is induced by herbivory and other biotic stimuli as well as by abiotic stimuli. The active jasmonate JA-Ile is produced and interacts with the SCF-COI1 receptor complex. This interaction induces the degradation of JAZ repressor proteins and enables the MYC2-dependent gene expression (from: (Howe and Jander, 2008)).

The production of jasmonates and the subsequent signaling in response to herbivore attack is triggered by wounding and associated osmotic stress as well as by elicitors originating from the insect (Turner *et al.*, 2002; Maffei *et al.*, 2004; Mithöfer *et al.*, 2005). Production and accumulation of jasmonates is a very strong and effective defense reaction against feeding insects, since the response to triggers starts very fast. So it was shown for Arabidopsis leaves that jasmonate accumulation starts already 2-5 minutes after wounding of the plant tissue (Glauser *et al.*, 2008). Additionally it was shown in lima bean leaves that the area fed by *Spodoptera littoralis* larvae, contained a high level of jasmonates, while the surrounding plant tissue showed lower content of jasmonates (Schulze *et al.*, 2007). To further analyze the dynamic and downstream signaling of jasmonates, structural mimics like coronalon (Figure 2 inlet) were applied in previous studies. It was demonstrated that coronalon could successfully induce plant defense reactions like secondary metabolites and the expression of defense-genes (Schüler *et al.*, 2001; Schüler *et al.*, 2004; Pluskota *et al.*, 2007; Nakamura *et al.*, 2014)).

In recent studies, it was shown that also cytokinins (CK), which are involved in resistance to abiotic stress like drought or nutrient availability and senescence signaling, have a possible role in plant herbivore defense. In *Nicotiana attenuata*, CK levels and several genes in the signaling cascade were induced by *Manduca sexta* OS and wounding (Schäfer *et al.*, 2015).

A major player in adaption to abiotic stress stimuli is the sesquiterpenoid abscisic acid (ABA), which is mediating resistance to salt, drought and cold stress by regulation of stomata closure (Zhu, 2002). ABA is also involved in embryo maturation, seed dormancy, germination, cell division and elongation (Finkelstein, 2013). Interestingly it was shown, that ABA is also involved in plant defense signaling. Here the complex interplay between ABA and jasmonic acid (JA)-ethylene signaling pathways can regulate plant defense (Anderson *et al.*, 2004).

1.3 Aim of the work

During their life, plants need to adapt to many different stress factors from the biotic and abiotic environment. The signal transduction pathway connecting the recognition of these environmental cues and the downstream signaling are still poorly understood. One group of proteins induced by various environmental stimuli includes the calmodulin-like proteins, CMLs, which act as Ca^{2+} sensors. CMLs are involved in decoding the important cytosolic Ca^{2+} elevations that originate from diverse stimuli.

The aim of this work was to study the role of Ca^{2+} and different CMLs in Arabidopsis response to herbivory and herbivory-related treatments (Figure 8) as well as in the response to abiotic stress. Therefore, the following aspects were studied in detail:

- analysis of gene expression patterns of different herbivory-induced CMLs in response to *Spodoptera littoralis* oral secretion (OS)
- characterization of a CML knock-out mutant, *cml37*, to study the role and mode of action of *At*CML37 in response to *Spodoptera littoralis* herbivory
- examination of the role of *At*CML37 and *At*CML42 in ABA accumulation in response to drought stress
- analysis of the role, induction and the distribution pattern of the non-protein amino acid GABA in Arabidopsis defense to *Spodoptera littoralis* herbivory and wounding as well as the connection to jasmonate signaling.

Figure 8. Herbivory-related treatments used to study *Arabidopsis thaliana* response to *Spodoptera littoralis* feeding.

Shown are the treatments used to disentangle the response of Arabidopsis plants to Spodoptera larvae. The different approaches used are: mechanical wounding of hole plants, achieved by MecWorm treatment (**A**); application of collected oral secretion of Spodoptera larvae fed on WT plants (**B**); and direct feeding of Spodoptera larvae on potted plants (**C**). All treatments were done for WT and mutant plants in parallel.

2 Manuscript overview

2.1 Manuscript 1

Multiple calmodulin-like proteins in Arabidopsis are induced by insect-derived (*Spodoptera littoralis*) oral secretion

Authors: Jyothilakshmi Vadassery, Sandra S. Scholz, and Axel Mithöfer, (2012).

Status: published, Plant Signaling & Behavior, 7(10), 1277-1280. doi:10.4161/psb.21664.

Summary:

The aim of this study was to investigate the expression profiles of different calmodulin-like proteins (CMLs) in the scope of herbivory. To mimic herbivory, oral secretion of the generalist herbivore *Spodoptera littoralis* was used and applied to wound Arabidopsis plants. It was observed that several CMLs are induced by elicitors in these oral secretions, although the induction patterns were quite different. One group of CMLs showed a very early and transient expression profile (CMLs 11, 12, 16) while the expression of the second group of CMLs (CMLs 9, 17, 23) stared later and showed a sustained peak. This result suggests that a differential expression profile of multiple CMLs serves as a basis for a complex signaling network which enables a specific decoding of calcium signals originating from different stimuli.

JV AM: planed the research, **JV SSS:** conducted and analyzed the research,

JV SSS AM: wrote the paper.

2.2 Manuscript 2

Mutation of the Arabidopsis Calmodulin-like protein CML37 deregulates the jasmonate pathway and enhances susceptibility to herbivory

Authors: Sandra S. Scholz, Jyothilakshmi Vadassery, Monika Heyer, Michael Reichelt, Kyle W. Bender, Wayne A. Snedden, Wilhelm Boland, and Axel Mithöfer, (2014).

Status: published, Molecular Plant, 7 (12), 1712–1726. doi:10.1093/mp/ssu102.

Summary:

The aim of this study was to investigate the role of calcium sensor CML37 in Arabidopsis response to *Spodoptera littoralis* herbivory. The analysis of *CML37* gene induction pattern in wildtype plants revealed that *CML37* is induced by mechanical wounding as well as by elicitors in *S. littoralis* oral secretion. To study the role of CML37 in Arabidopsis, *cml37* mutants were analyzed in the scope of *S. littoralis* herbivory. In feeding assays, it was observed that CML37 acts as a positive defense regulator since *S. littoralis* larvae gained significantly more weight on *cml37* plants. This result was also reflected in the lower accumulation of jasmonate phytohormones and the resulting reduced expression of JA-responsive genes in *cml37*. This observation can be explained by a lower expression of *JAR1* gene as well as a reduced JAR1 enzyme activity in *cml37* mutant plants. The results indicate that CML37 is involved in biotic stress response in Arabidopsis. CML37 is the first CML connecting Ca^{2+} and jasmonate signaling.

SSS JV MH AM: planed the research, **SSS JV MH:** conducted and analyzed the research,

MR: analyzed content of phytohormones and plant secondary metabolites

KWB WS: planed and conducted experiments for analysis of biochemical properties of CML37

SSS JV MH MR AM KWB WS WB: wrote the paper.

2.3 Manuscript 3

Calmodulin-like protein CML37 is a positive regulator of ABA during drought stress in Arabidopsis

Authors: Sandra S. Scholz, Michael Reichelt, Jyothilakshmi Vadassery, and Axel Mithöfer, (2015).

Status: accepted**,** Plant Signaling & Behavior, Volume 10(5), will be published 20th of July.

Summary:

The aim of this study was to investigate the role of calcium sensors CML37 and CML42 in response to abiotic drought stress in Arabidopsis. Interestingly, both CMLs are involved in drought stress response but show antagonistic effects. While *cml37* plants are more susceptible to drought stress treatment and die faster compared to wildtype, *cml42* plants don't show a visible phenotype different from wildtype. Analysis of the drought-related phytohormone ABA revealed that *cml37* plants accumulate significantly less ABA compared to wildtype. In contrast to this, *cml42* plants show in early time points a similar level of ABA like observed in wildtype and in later time points a higher and prolonged ABA accumulation. This result suggests that CML37 is next to biotic stress responses also involved in abiotic stress signaling.

SSS JV AM: planed the research, **SSS:** conducted and analyzed the research

MR: analyzed phytohormones **SSS MR JV AM:** wrote the paper.

2.4 Manuscript 4

Insect herbivory-elicited GABA accumulation in plants is a wound-induced, direct, and jasmonate-independent defense response

Authors: Sandra S. Scholz, Michael Reichelt, Dereje Mekonnen, Frank Ludewig, and Axel Mithöfer, (2015).

Status: submitted 25.06.2015, Plant Cell & Environment.

Summary:

The aim of this study was to investigate the role of the non-protein amino acid GABA (γ-aminobutyric acid) in Arabidopsis response to *Spodoptera littoralis* herbivory. To investigate the possible role of GABA in herbivore defense, which was suggested by previous studies, different GABA mutant lines were analyzed. A *gad12 x pop2-5* mutant line, which accumulates GABA over time, showed significantly lower susceptibility to Spodoptera herbivory. This result indicates that GABA accumulation confers resistance against herbivore feeding. Additionally, GABA shows a concentration-dependent growth inhibition of Spodoptera larvae in a diet-feeding assay. The rapid accumulation of GABA in the plant leaf is induced by tissue damage like MecWorm treatment or Spodoptera feeding, and acidifying of the cytosol. By analyzing the phytohormone and GABA levels in different GABA- (*gad12*; *gad12xpop2-5*) and JA-mutant lines (*jar1*), it became clear that the GABA defense pathway is not dependent on jasmonates and that vice versa jasmonate biosynthesis is also not dependent on the GABA level. Summarizing, the results indicate that the herbivore-induced accumulation of GABA is a general, direct and systemic defense independent of JA.

SSS DM AM FL: planed the research, **SSS DM:** conducted and analyzed the research,

MR FL: analyzed content of phytohormones and GABA,

SSS MR DM AM FL: wrote the paper.

2.5 Manuscript S1

Additional evidence against jasmonate-induced jasmonate induction hypothesis

Authors: Sandra S. Scholz, Michael Reichelt, Wilhelm Boland, and Axel Mithöfer, (2015).

Status: resubmitted 19.06.2015, Plant Science.

Summary:

The aim of this study was to reinvestigate the hypothesis of jasmonate-induced-jasmonate-biosynthesis in *Arabidopsis thaliana* by use of coronalon, a structural mimic of JA-Ile. Coronalon was applied to wounded and unwounded plants to disentangle the influence of tissue damage on the induction of JA biosynthesis genes and accumulation of endogenous jasmonates. It became clear that application of coronalon did neither induce endogenous jasmonate syntheses nor the accumulation of hydroxylated jasmonates. The same pattern was observed for wounded plants, where the coronalon treated plants showed the same level of endogenous jasmonates as the control plants. On the other hand, both treatments were able to induce the expression of JA biosynthesis genes supporting the hypotheses of a post-translational regulation. Summarizing, the results show that JA alone induces JA-biosynthesis genes but no JA accumulation.

SSS AM: planed the research, **SSS:** conducted and analyzed the research

MR: analyzed content of phytohormones, **SSS MR WB AM:** wrote the paper.

2.6 Manuscript S2

Synthesis, biological activity, metabolism and systemic transport of 7-fluoro-OPC-8:0, a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0

Authors: Guillermo H. Jimenez-Aleman*, Sandra S. Scholz*, Monika Heyer, Michael Reichelt, Axel Mithöfer, Wilhelm Boland, (2015).

Status: submitted 12.06.2015, BBA Molecular and Cell Biology of Lipids.

* These authors contributed equally to the work.

Summary:

The aim of this study was to synthesize a labeled OPC-8:0 analogue to study transport mechanisms and metabolism of JA precursors in *Arabidopsis thaliana*. Because F atoms behave very similar to H and are very likely accepted by metabolizing enzymes, a fluorinated OPC was synthesized: F-OPC-8:0. The F-OPC-8:0, applied to *A. thaliana* plants, was metabolized by ß-oxidation to F-OPC-6:0 and F-OPC-4:0. These metabolites, and the active jasmonates produced from them, were able to induce the expression of JA biosynthesis and JA-responsive genes. By analysis of single leaves it was observed, that the applied F-OPC-8:0 was transported to systemic adjacent leaves. The metabolism of F-OPC-8:0 was only detected in the local treated leaf. Summarizing this, we synthesized a fluorinated OPC-8:0 analogue which was biologically active and could show that this JA precursor is transported to systemic non-wounded leaves. Till now, only JA-Ile was known as a transported compound.

GHJA SSS MH AM: planed the research, **GHJA:** carried out the syntheses of 7F-OPC-8:0, **SSS:** conducted the Arabidopsis assays, **MH:** analyzed transport of 7F-OPC-8:0, **GHJA MH SSS:** analyzed the research, **MR:** analyzed content of phytohormones and metabolites, **GHJA SSS MR MH AM WB:** wrote the paper.

3 Manuscripts

Manuscript 1

SHORT COMMUNICATION

Plant Signaling & Behavior 7:10, 1-4; October 2012; © 2012 Landes Bioscience

Multiple calmodulin-like proteins in Arabidopsis are induced by insect-derived (*Spodoptera littoralis*) oral secretion

Jyothilakshmi Vadassery, Sandra S. Scholz and Axel Mithöfer*

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Keywords: Calmodulin-like proteins, *Arabidopsis thaliana*, *Spodoptera littoralis*, herbivory, oral secretions

In plant cells, diverse environmental changes often induce transient elevation in the intracellular calcium concentrations, which are involved in signaling pathways leading to the respective cellular reactions. Therefore, these calcium elevations need to be deciphered into specific downstream responses. Calmodulin-like-proteins (CMLs) are calcium-sensing proteins present only in higher plants. They are involved in signaling processes induced by both abiotic as well as biotic stress factors. However, the role of CMLs in the interaction of plants with herbivorous insects is almost unknown. Here we show that in *Arabidopsis thaliana* a number of *CMLs* genes *(CML9, 11,12,16,17* and *23)* are upregulated due to treatments with oral secretion of larvae of the herbivorous insect *Spodoptera littoralis*. We identified that these genes belong to two groups that respond with different kinetics to the treatment with oral secretion. Our data indicate that signaling networks involving multiple CMLs very likely have important functions in plant defense against insect herbivores, in addition to their involvement in many other stress-induced processes in plants.

Throughout their life, plants are challenged by various abiotic and biotic changes in their environment. Any appropriate reaction to such environmental variations needs the recognition of the respective information, followed by downstream intracellular signaling leading to a specific response. Most of the time, upon perception of stress signals, a transient increase in the cytosolic calcium (Ca^{2+}) concentration can be observed in plant cells.[1,2] However, Ca^{2+} elevations due to stress signals are rather ubiquitous, general responses. One of the determinants of specificity is Ca^{2+} signature, specific to a stimulus, characterized by its duration, amplitude, frequency, and location; the other is presence of Ca^{2+} sensor proteins that contribute to the induction of specific physiological response.[3] In plants, Ca^{2+} sensor proteins are classified as sensor responders, (e.g., calmodulin-dependent protein kinases, CDPKs) and sensor relay proteins. The latter proteins only undergo conformational changes upon Ca^{2+} binding and subsequently interact with target proteins. Calmodulin-like proteins (CMLs) are sensor relay proteins which are unique to plants with 50 members in *Arabidopsis thaliana*.[4] They possess 2 to 6 predicted Ca^{2+}-binding EF hand motifs. With calmodulins, CMLs share at least 15% identity on the amino acid level.[4,5] CMLs are involved in stress perception and plant development. For example, CML24 is known to cause alterations in flowering time, abscisic acid (ABA) level and ion stress;[6,7] *CML37, CML38* and *CML39* transcripts are regulated by abiotic stress (salt and drought), phytohormones (jasmonate and ABA), and biotic stress (phytopathogenic *Pseudomonas syringae*).[8] *CML8* is induced by salicylic acid (SA) and salt stress.[9] CML9 alters plant responses to ABA and abiotic stress and *CML9* gene is induced by infection with *P. syringae*, flg22 elicitor, and SA.[10,11] Only recently it was demonstrated that CML42 represents both a negative regulator of insect herbivory-induced defense, drought induced ABA levels and a positive regulator of UV stress.[12] Moreover, loss of CML42 function leads to aberrant trichomes with increased branching.[13]

We demonstrated that cellular calcium (Ca^{2+}) elevation is an early event in the interaction between *S. littoralis* and *A. thaliana*. Up to now, among the numerous CMLs only one (CML42) has been described to be involved in plant response to insect herbivory.[12] In order to identify new herbivory-related targets in the *CML* gene family, we investigated the transcript level of various *CML* genes in *A. thaliana* upon treatment with oral secretions (OS) from larvae of the generalist herbivore *S. littoralis*. Preliminary microarray analyses using an Affymetrix array revealed that besides the strongly induced *CML42 (At4g20780)*,[12] other *CMLs* were regulated by *S. littoralis* OS application as well. Here, using quantitative real time PCR, we further confirmed and explored the regulation of *CMLs* in more detail.

Therefore, *A. thaliana* leaves were wounded and treated with either water (control) or *S. littoralis* OS and *CMLs* expression was analyzed in these samples. We found that in the 50 member gene family of *CMLs*, mainly *CML9, 11, 12, 16, 17,* and *23* are upregulated by elicitors present in the OS of *S. littoralis*. A further time course experiment of gene expression revealed that expressions could be classified into two main groups: (1) Early

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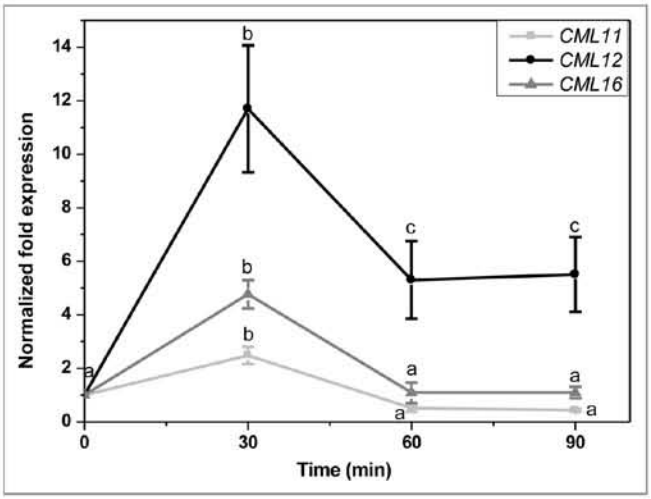

Figure 1. *CML11, CML12,* and *CML16* transcript levels in leaves of *A. thaliana* 30, 60 and 90 min after treatment with *Spodoptera littoralis* oral secretion (OS). Leaves were elicited by pattern wheel wounding and subsequently treating the wound with 20 µL water or 1:1 diluted OS per leaf. Transcript abundance in leaves was determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The fold change was calculated relative to control which was mechanical wounding + H_2O. The graph shows x-fold induction of the mRNA levels by the *S. littoralis* OS relative to the levels in the H_2O treated control leaves. Mean (± SE, n = 5). Different letters indicate significant differences between different time points in a single gene expression (ANOVA; $p < 0.05$). Comparisons between expressions of different genes are not performed.

and transiently expressed *CMLs- CML11, 12,* and *16;* and (2) late and sustained expressed *CMLs- CML9, 17* and *23.*

Group 1 genes (*CML11, 12,* and *16*) showed their highest upregulation 30 min after treatment. Their level of expression decreased at later time points (Fig. 1). In this set, *CML12/TCH3* was the most highly upregulated gene with expression levels reaching 9-fold of the control within 30 min. This level of expression decreased with time to 5-fold. *CML11* and *16* responded to OS treatment with lower expression levels. They also showed maximal upregulation at 30 min but reached basal levels after 60 min. *CML12/TCH3*, encodes a unique calmodulin-like protein, with 6 putative Ca^{2+} binding EF hands as opposed to 4 or fewer EF hands in other CMLs. It is rapidly induced by mechanical stimulation, ethylene, auxin, cold, and extracellular calcium.[14-18] Plants respond to two concomitant stimuli of herbivory: mechanical wounding and recognition of elicitors in OS. The sustained expression of *CML12* could be due to the fact that it is also upregulated by mechanical wounding alone and OS might act to amplify the wound-induced signal. The recently described CML42 also belongs to this group 1.[12] CMLs might also be regulated by jasmonates, apart from direct herbivory signals, due to the jasmonate burst in plants upon *S. littoralis* OS treatment.[12] Stimuli-induced CMLs gene expression[5] upon various stress treatment has revealed the group 1 genes, CML12 and CML16 are not regulated by methyl jasmonate (MeJA). In fact, CML16 expression was downregulated by MeJA.[5] The group 2 genes include *CML9, 17,* and *23.* They are characterized by a sustained expression upon treatment with *S. littoralis* OS. They reached their maximal level of expression at 60 or 90 min after treatment (Fig. 2). Within this group, *CML17* is unique because it was initially downregulated and upregulated only at 60 and 90 min, whereas *CML9* and *23* showed a steady increase over time. In this group both, CML23 and 17 are MeJA-induced genes. This further point to the fact that sustained expression in this group might be due to combined action of both oral secretions and JA burst. CML9 however is an exception and is a MeJA-repressed gene.[5] CML23 and CML24 are known to be potential calcium sensors that have partially overlapping function and regulate nitric oxide accumulation and transition to flowering.[19] However, the overall fold-change of expression in group 2 was lesser than in group 1. We thus identified 6 new target genes in *A. thaliana* which respond to elicitors in *S. littoralis* OS and might be involved in plant defense.

To investigate and finally understand the specific roles of the identified CMLs, in plant herbivore interactions, further functional experiments involving knockout and overexpression of target genes are necessary. However, the fact that at least seven CMLs are regulated on the expression level by application of OS and herbivory suggests a central function for these calcium sensors in plant defense signaling processes.

Materials and Methods

Plant growth and treatment. *Arabidopsis thaliana* seeds (ecotype Columbia) were used for all experiments and grown as described.[12] Experiments with insect oral secretions (OS) were performed according to.[12] Briefly, wounding was done with a pattern wheel (6 vertical motions) on either side of the leaf. OS was collected from 4th instar *Spodoptera littoralis* larvae reared on artificial diet and fed on *A. thaliana* leaves for 24 h prior to OS collection. The harvested OS was centrifuged for 2 min at 13,000 rpm and subsequently diluted 1:1 with water. A total of 20 µl of fresh diluted OS was spread across all the holes on a single leaf. In control plants, water was added. The samples were harvested and stored in liquid nitrogen. Experiments were repeated three times independently.

Expression analysis by Real Time PCR. Leaf material was ground to a fine powder in liquid N_2, and total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturers' protocol. An additional DNase (Turbo DNase, Ambion) treatment was included to eliminate any contaminating DNA. RNA quantity was determined photospectrometrically. Total RNA (1 µg) was converted into single-stranded cDNA using a mix of oligo-dT20 primers using the Omniscript cDNA synthesis kit (Qiagen). Gene-specific primers were designed using the NCBI primer design tool (www.ncbi.nlm.nih.gov/tools/primer-blast). For real time PCR, primers producing 124 to 190 bp amplicons were used. Q-RT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene) using the Brilliant II QPCR SYBR green Mix (Agilent) to monitor double-stranded DNA synthesis in combination with ROX as

a passive reference dye included in the PCR master mix. A dissociation curve analysis was performed for all primer pairs, and all experimental samples yielded a single sharp peak at the amplicon's melting temperature. The mRNA levels for each cDNA probe were normalized with respect to the *RPS18B* mRNA level.[12] Fold induction values of target genes were calculated with the ΔΔCP equation[20] and normalized to the mRNA level of target genes in control leaf, which were defined as 1.0. All of the assays were run in triplicate (biological replication) to control for overall variability. Primer pairs (forward, reverse) used are listed below:

CML9 (At3g51920):
5'-TTG GCA ACG GTG GCA TCA CT-3'
5'-CCA TCG CCA TCA AGG TCG GCT-3'
CML11 (At3g22930):
5'-TCC GCT CAT TGG ATC AGA ACC CT-3'
5'-TCT GCA TCA GTT TCC TGG AGT TGG T-3'
CML12- (At2g41100):
5'-TGG CGG ATA AGC TCA CTG ACG A-3'
5'-TCC GCT TCG TTC ATC AAG TCC TG-3'
CML16 (At3g25600):
5'-GAC GAG CTG GTC GTG GCG AT-3'
5'-TGA CCC AGC AAG TTC CGC CG-3'
CML17 (At1g32250):
5'-CGC CGG CGA AGA GGA CAA CT-3'
5'-ATT CCG CCA CCG TCA AGG CG-3'
CML23 (At1g66400):
5'-CGC TTC ACA AGA AGA AAC CAA AGC A-3'
5'-AGC CGA GAT CCT TCC ATT ACG ATC C-3'
RPS18B (At1g34030):
5'-GTC TCC AAT GCC CTT GAC AT-3'
5'-TCT TTC CTC TGC GAC CAG TT-3'

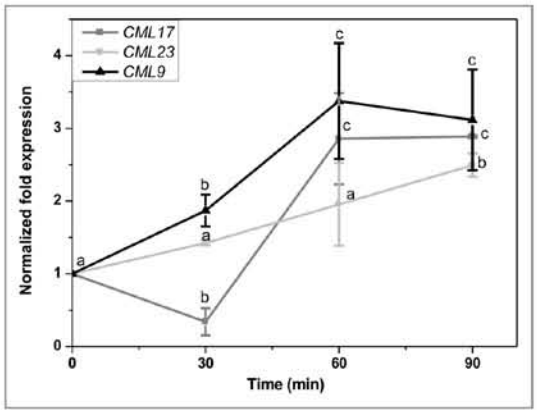

Figure 2. *CML9, CML17,* and *CML23* transcript levels in leaves of *A. thaliana* 30, 60 and 90 min after treatment with *Spodoptera littoralis* oral secretion (OS). Leaves were elicited by pattern wheel wounding and subsequently treating the wound with 20 μL water or 1:1 diluted OS per leaf. Transcript abundance in leaves was determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The fold change was calculated relative to control which was mechanical wounding + H_2O. The graph shows x-fold induction of the mRNA levels by the *S. littoralis* OS relative to the levels in the H_2O treated control leaves. Mean (± SE, n = 3). Different letters indicate significant differences between different time points in a single gene expression (ANOVA; $p < 0.05$). Comparisons between expressions of different genes are not performed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Manuscript 2

Molecular Plant 7, 1712–1726, December 2014 RESEARCH ARTICLE

Mutation of the *Arabidopsis* Calmodulin-Like Protein CML37 Deregulates the Jasmonate Pathway and Enhances Susceptibility to Herbivory

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ABSTRACT Throughout their life, plants are challenged by various abiotic and biotic stress factors. Among those are attacks from herbivorous insects. The molecular mechanisms underlying the detection of herbivores and the subsequent signal transduction are not well understood. As a second messenger, fluxes in intracellular Ca^{2+} levels play a key role in mediating stress response pathways. Ca^{2+} signals are decoded by Ca^{2+} sensor proteins such as calmodulin-like proteins (CMLs). Here, we demonstrate that recombinant CML37 behaves like a Ca^{2+} sensor *in vitro* and, in *Arabidopsis*, *AtCML37* is induced by mechanical wounding as well as by infestation with larvae of the generalist lepidopteran herbivore *Spodoptera littoralis*. Loss of function of *CML37* led to a better feeding performance of larvae suggesting that *CML37* is a positive defense regulator. No herbivory-induced changes in secondary metabolites such as glucosinolates or flavonoids were detected in *cml37* plants, although a significant reduction in the accumulation of jasmonates was observed, due to reduced expression of *JAR1* mRNA and cellular enzyme activity. Consequently, the expression of jasmonate-responsive genes was reduced as well. Summarizing, our results suggest that the Ca^{2+} sensor protein, CML37, functions as a positive regulator in Ca^{2+} signaling during herbivory, connecting Ca^{2+} and jasmonate signaling.

Key words: herbivory; oral secretion; jasmonates; cytosolic calcium; calmodulin-like proteins.

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INTRODUCTION

The environment of a growing plant is hardly stable, forcing the plant to detect and adapt to various abiotic and biotic challenges. The interaction of plants with insects is of great importance given that more than 50% of insect species are herbivores (Schoonhoven et al., 1998; Van Poecke, 2007). Feeding insects first encounter mechanical barriers such as cuticular waxes and trichomes, which are a plant's first line of defense (Eigenbrode and Espelie, 1995; Reymond et al., 2004). Moreover, plants respond actively to insect herbivores with the production of specialized defensive metabolites (Mithöfer and Boland, 2012) and certain phytohormones, jasmonates, that are cyclopentanone compounds derived from linolenic acid via an octadecanoid pathway (Wasternack, 2007; Mithöfer et al., 2009; Wasternack and Kombrink, 2010). Key components of these phytohormones are jasmonic acid (JA) and its active isoleucine (Ile) conjugate, (+)-7-*iso*-Jasmonoyl-*L*-isoleucine (JA-Ile) (Howe and Jander, 2008; Chini et al., 2009; Fonseca et al., 2009), which is catalyzed by the enzyme JASMONATE RESISTANT 1 (JAR1; Staswick and Tiryaki, 2004). The SCF-COI1-JAZ co-receptor of JA-Ile is the important link between

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JA signaling and the ubiquitin/26S proteasome pathway. Activation of the co-receptor by JA-Ile triggers the ubiquitination and degradation of JAZ proteins, which are transcriptional repressors of JA-responsive genes (Chini et al., 2007; Thines et al., 2007). This interaction is potentiated by inositol pentakisphosphate (IP5) (Sheard et al., 2010). Thus, relief of repression leads to activation of the transcription factor MYC2 and the expression of JA-responsive genes (Wasternack and Kombrink, 2010).

The recognition of environmental cues and activation of appropriate downstream signal transduction pathways are essential to achieve stimulus-specific responses. Perception of a feeding herbivore usually involves recognition of HAMPs (herbivore-associated molecular pattern; Mithöfer and Boland, 2008) followed by Ca^{2+} elevation in the cytosol (Maffei et al., 2004). To generate specificity in response, Ca^{2+} signals must be decoded by Ca^{2+} sensor proteins. Typically, these proteins contain a number of paired EF-hand motifs, which have the ability to bind Ca^{2+} (Gifford et al., 2007). Calcium-binding proteins can be divided into two classes. The first is the class of Ca^{2+} responders which are capable of transducing the signal via enzymatic activity. The family of CDPKs (Ca^{2+}-dependent protein kinases) belongs to this group. The second class contains non-catalytic sensor relay proteins that, upon binding Ca^{2+}, undergo a conformational change that enables them to interact with and regulate downstream targets. CaMs (calmodulins), CMLs (calmodulin-like proteins), and CBLs (calcineurin B-like proteins) are the main representatives of this class of Ca^{2+} sensor (DeFalco et al., 2010). Among eukaryotes, CMLs and CBLs occur almost exclusively in plants, underscoring their importance in plant Ca^{2+} signaling.

The family of CMLs contains 50 members in *Arabidopsis*, with sequence similarity between 16% and 74.5% with evolutionarily conserved *At*CaM2 (McCormack and Braam, 2003). CMLs contain one to six EF-hands and are involved in perception of many different stress signals (Bender and Snedden, 2013). *CML8*, *CML37*, *CML38*, and *CML39* are induced by multiple abiotic stresses such as salinity and drought (Vanderbeld and Snedden, 2007; Park et al., 2010); *CML9* is induced by infection with the phytopathogenic bacterium *Pseudomonas syringae* (Leba et al., 2012). Several *CMLs* were also suggested to be induced by herbivore feeding as demonstrated by mimicking herbivory using oral secretion (OS) of the generalist lepidopteran herbivore *Spodoptera littoralis* (Vadassery et al., 2012c). Recently, *Arabidopsis* CML42 was shown to be a negative regulator of herbivore-induced defense and drought stress response (Vadassery et al., 2012b).

In order to gain more insight into the role of CMLs and to understand their functions in plant stress response, we focused on CML37, which was supposed to be inducible by herbivory as well. *CML37* was previously shown to be induced by various abiotic stress treatments although not by the stress-hormone abscisic acid (ABA). Furthermore, *CML37* was activated by avirulent *P. syringae* strain (avrRpt) and wounding; however, salicylic acid (SA) had no effect and methyl jasmonate only a moderate one (McCormack et al., 2005; Vanderbeld and Snedden, 2007). Interestingly, OPDA, a biosynthetic precursor of JA, stimulated *CML37* expression (Taki et al., 2005).

In the present study, we investigated the functional role of *CML37* upon biotic stress in interaction of *Arabidopsis thaliana* and the herbivore *S. littoralis*. Upon herbivory and wounding, *CML37* expression was strongly induced. Use of a loss-of-function approach revealed that CML37 is a positive regulator of plant defenses against herbivory. In *cml37* knockout mutants, attenuated level of JA-Ile as well as reduced expression of JA-responsive genes were observed. This could be causally connected to *JAR1* expression. Collectively, our findings indicate that CML37 plays an important role in biotic stress response.

RESULTS

CML37 Is a Putative Ca^{2+} Sensor

Like conserved CaM, CMLs are characterized by the presence of EF-hand Ca^{2+}-binding motifs, suggesting that these proteins function as Ca^{2+} sensors, but the Ca^{2+}-binding properties of only a few CMLs have been determined empirically (Dobney et al., 2009; Bender et al., 2013). Ca^{2+} sensors undergo characteristic Ca^{2+}-induced conformational changes and shifts in exposed surface hydrophobicity that allow them to interact with their downstream targets. Thus, we examined Ca^{2+}-dependent changes in secondary structure and surface hydrophobicity of recombinant CML37 by circular dichroism (CD) spectroscopy and ANS fluorescence spectroscopy, respectively (Figure 1).

The far-UV CD spectra for CML37 is characteristic of proteins with high α-helical content for both the apo- and Ca^{2+}-bound forms (Figure 1A); the CD spectrum for CML37 shows a large positive band with a λ_{max} of 190 nm and two strong negative bands with local minima at 208 and 225 nm, respectively. Deconvolution of the CD spectrum indicated 42% α-helix for apo-CML37. By comparison, the CD spectrum for Ca^{2+}-CML37 showed an increase in the 190-nm band and a decrease in the 225-nm band—a shift characteristic of increased helical content. Deconvolution of the CD spectrum for Ca^{2+}-CML37 revealed a 19% increase in helical content compared to apo-CML37, demonstrating that CML37 undergoes a Ca^{2+}-dependent conformational change.

Another common feature of Ca^{2+}-sensors is a Ca^{2+}-induced increase in surface-exposed hydrophobicity, which was analyzed using 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence spectroscopy. In the presence of CML37, we observed a pronounced blue-shift and strong increase in the fluorescence emission spectrum for ANS compared to ANS alone (Figure 1B). This change in the emission spectrum was

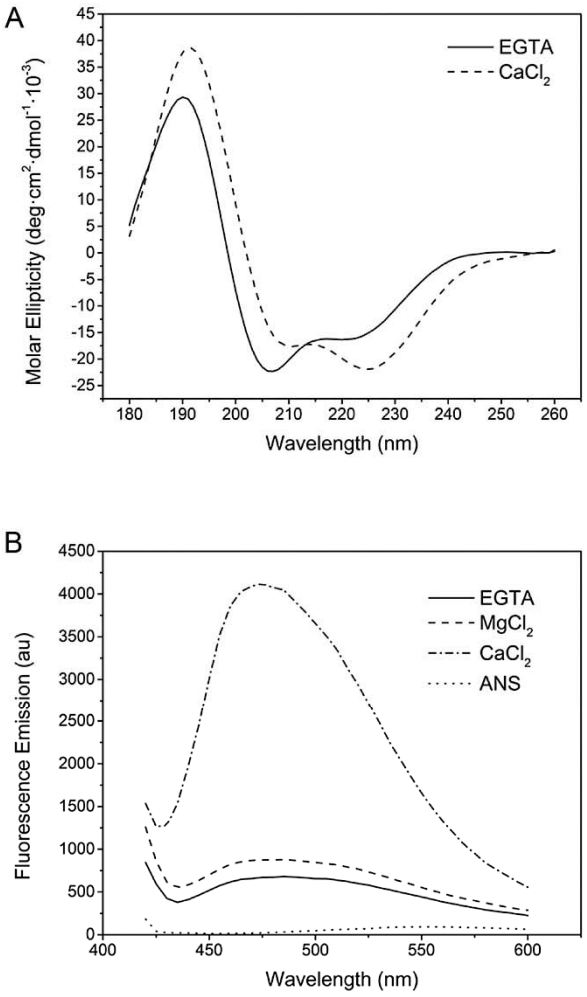

Figure 1 Recombinant CML37 Exhibits Biochemical Properties of a Ca^{2+} Sensor.

(A) Ca^{2+}-induced changes in secondary structure of CML37. Secondary structure of CML37 was monitored using far-UV circular dichroism in the presence (dashed line) or absence (solid line) of saturating $CaCl_2$ (1 mM). Each spectrum is representative of 10 scans. Data are presented in units of molar ellipticity.

(B) 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorescence was used to assess Ca^{2+}-dependent changes in surface hydrophobicity for CML37. Fluorescence emission spectra were recorded for ANS alone (dotted line) and CML37 with ANS following the addition of either $MgCl_2$ (dashed line), EGTA (solid line), or $CaCl_2$ (dash-dot-dash line). Data presented are fluorescence emission in arbitrary units.

observed for both apo-CML37 and CML37 in the presence of $MgCl_2$, indicating that CML37 possesses exposed hydrophobic regions. When CML37 was titrated with $CaCl_2$ in the presence of ANS, a six-fold increase in fluorescence emission was observed, demonstrating that CML37 undergoes a Ca^{2+}-dependent shift in exposed surface hydrophobicity. Taken together, CD analysis and ANS fluorescence spectroscopy show that CML37 changes conformation upon binding Ca^{2+}—a feature consistent with function as a Ca^{2+}-sensor.

Expression of *CML37* Is Induced by Wounding and Herbivory, and Is COI1-Dependent

To identify the Ca^{2+} sensors involved in the interaction between *Arabidopsis* and the generalist insect, *S. littoralis*, Affymetrix arrays were used to monitor the expression of early signaling genes. In one experiment, mechanically wounded leaves with or without *S. littoralis* OS treatment (W+OS, W+W) were compared after 30-min treatment. In a second experiment, genes specifically induced by mechanical larvae, MecWorm (Mithöfer et al., 2005), were identified after 1 and 3 h (unpublished data). Both microarray experiments revealed that a gene encoding *CML37* (At5g42380) was up-regulated. Using quantitative real-time PCR, we corroborated the microarray data and found that *CML37* is strongly induced by feeding activity from the generalist herbivore *S. littoralis* (Figure 2A). Feeding by a caterpillar is a combination of mechanical wounding and contact of the leaf with OS produced by the insect that contains compounds that can induce plant defense (Mithöfer and Boland, 2008). Caterpillar-like wounding employing a mechanical larva, MecWorm, induced *CML37* to the same extent as *S. littoralis* feeding (Figure 2B), whereas treatment with OS showed slower and about 10-fold less pronounced induction (Figure 2C). Foliar application of the phytohormones, OPDA and JA, confirmed previous findings (Taki et al., 2005) that OPDA is the more effective compound for *CML37* induction. Treatment with the JA-Ile analog coronalon (Mithöfer et al., 2004) showed only a weak effect on *CML37* expression (Figure 2D).

The receptor of JA-Ile, COI1, is the link between JA signaling and the ubiquitin/26S proteasome pathway. Loss-of-function mutants of *COI1* are highly insensitive to JA and defective in most JA responses (Feys et al., 1994; Chung et al., 2009). We analyzed the expression of *CML37* in homozygous *coi1-1* plants and wild-type (WT) after different time periods of *S. littoralis* feeding. When compared to WT, *coi1-1* plants failed to induce expression of *CML37* (Figure 2E), indicating that, in the *Arabidopsis–S. littoralis* interaction, *CML37* expression is COI1-dependent.

CML37 Loss-of-Function Plants Are More Susceptible to Generalist Herbivore *Spodoptera littoralis*

In order to analyze whether or not CML37 has an effect on insect herbivory in *Arabidopsis*, we conducted a feeding

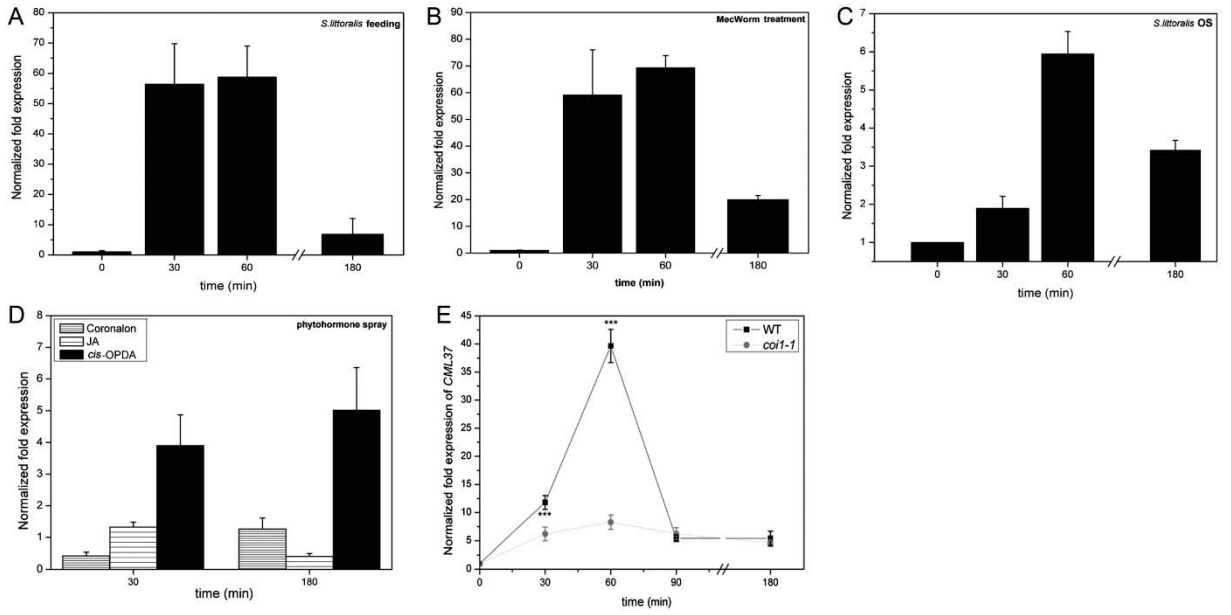

Figure 2 Expression of *CML37* in *Arabidopsis* in Response to Different Herbivory-Related Treatments.

(A) Mean (± SE, $n = 15$) *CML37* transcript levels in *Arabidopsis* leaves after 30, 60, and 180 min of *Spodoptera littoralis* feeding. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. For quantification, undamaged leaves from untreated plants were used as controls. The values represent means of three independent experiments.

(B) Mean (± SE, $n = 15$) *CML37* transcript levels in *Arabidopsis* leaves after 30, 60, and 180 min of MecWorm wounding damage. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. For quantification, undamaged leaves from untreated plants were used as controls. The values represent means of three independent experiments.

(C) Mean (± SE, $n = 15$) *CML37* transcript levels in oral secretion (OS)-treated leaves of *Arabidopsis* after 30, 60, and 180 min of treatment. Leaves were wounded with a pattern wheel followed by application of 20 µl of water or freshly 1:1 diluted OS per leaf. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The expression of *CML37* was calculated relative to control treatments, namely wounding and water application (W+W). The values represent means of three independent experiments.

(D) Mean (± SE, $n = 15$) *CML37* transcript levels in *Arabidopsis* leaves after spraying with different phytohormones followed by incubation for 30 and 180 min. The concentration used was 50 µM for JA (in 0.015% methanol) and coronalon (in 0.1% ethanol) and 10 µM for *cis*-OPDA (in 0.1% DMSO). Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The values represent means of three independent experiments.

(E) Mean expression (± SE, $n = 5$) of *CML37* in Col-0 WT and *coi1-1* plants at 30, 60, 90, and 180 min after *Spodoptera littoralis* feeding. Samples were taken from local *S. littoralis*-fed leaves while undamaged leaves were used as controls for quantification. Statistically significant differences between transcript levels in WT and *coi1-1* plants after treatment were analyzed by *t*-test, *** $P < 0.001$.

assay with *S. littoralis* on different *Arabidopsis* mutant lines. WT plants were used as a negative control, whereas the JA receptor mutant, *coi1-16* plants (Westphal et al., 2008), which are more susceptible to herbivory, were used as a positive control. Two independent T-DNA insertion lines of *CML37* were used for the assay: *cml37-1* with an insertion in the exon of At*CML37* and *cml37-2* with an insertion in the promoter region. After 7 d of treatment, larvae feeding on both *cml37* lines showed a similar and significantly higher weight compared with larvae feeding on Col-0 WT plants (Figure 3). Among treatments, larvae feeding on *coi1-16* plants gained the most weight, as expected. These results suggest that *cml37* loss-of-function mutants are more susceptible to herbivory, and that CML37 acts as a positive defense regulator in the *Arabidopsis–S. littoralis* interaction.

In addition, application of *S. littoralis* OS to *cml37-1* plants expressing the Ca^{2+} sensing cytosolic aequorin showed a similar calcium elevation as found in WT (Supplemental Figure 1). This result suggests that CML37 is located downstream of herbivory-induced cytosolic calcium elevation.

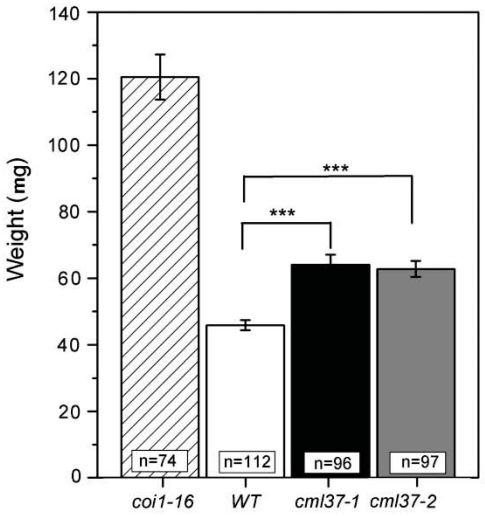

Figure 3 Feeding Assay of *Spodoptera littoralis* Larvae on *Arabidopsis* Wild-Type (WT) and *cml37* Plants.

Larval weight after feeding on Col-0 WT (white), JA receptor mutant *coi1-16* plants, and two independent *cml37* lines (black: line1- SALK_011488C; gray: line2-SALK_017485). *S. littoralis* first instar larvae were pre-weighed and three larvae were placed on each plant. The larval weight (mean ± SE) was measured after 7 d of feeding. The total number of larvae weighed (*N*) is indicated in the bars. Experiments were repeated five times independently. Statistically significant differences between WT and *cml37* plants after feeding were analyzed by *t*-test, *** $P < 0.001$.

Herbivore-Induced Phytohormone Elevation Decreases in *cml37* Plants

Besides cytosolic Ca^{2+} responses, one of the most common plant responses to herbivory is the generation of phytohormones, among which jasmonates are of major importance (Howe and Jander, 2008). We analyzed plant phytohormone levels after *S. littoralis* treatment to test whether they correlated with the increased larval performance on *cml37* lines. Upon *S. littoralis* feeding, both the internal level of *cis*-OPDA and the active jasmonate conjugate, JA-Ile were significantly lower in *cml37-1* and *cml37-2* mutants compared to WT plants (Figure 4 and Supplemental Figure 2). Both *cml37* T-DNA insertion lines showed similar results. The elevation of JA and SA after herbivore feeding was similar in both *cml37* mutant lines and did not change significantly compared to WT (Figure 4 and Supplemental Figure 2) suggesting that *cml37* plants are more susceptible to herbivory due to lower content of in particular JA-Ile.

With respect to the finding that different jasmonates can induce different Ca^{2+} responses (Wasternack, 2007), *cml37-1* mutants were studied concerning their responsiveness to JA and JA-Ile. The *cml37-1* mutants showed an altered elevation of cytosolic calcium after application of JA while application of JA-Ile resulted in cytosolic calcium levels which were comparable to those of WT plants (Figure 5 and Supplemental Figure 1). This result points again at JAR1 as possible target for CML37.

JAR1 Expression and JAR1 Enzyme Activity Are Affected in *cml37-1* Plants

In an attempt to understand the cause of lower jasmonate concentrations, in particular the bioactive JA-Ile, after herbivore attack in *cml37* mutants, we examined the transcript level of the conjugation enzyme, JAR1. In *Nicotiana attenuata*, herbivory can induce JAR genes, as shown for *JAR4* and *JAR6* (Wang et al., 2007). Similarly, in *Arabidopsis JAR1* mRNA is highly induced by wounding (Suza and Staswick, 2008). In *Arabidopsis* WT plants, herbivore feeding elicited a rapid and strong accumulation of *JAR1* transcripts that was observable within 1 h of treatment and was still detectable after 48 h. In contrast, in *cml37-1* mutants, the level of *JAR1* mRNA was drastically reduced compared to WT plants (Figure 6A). A decreased JAR1 enzyme activity was detected in tissue of *S. littoralis*-treated *cml37-1* plants, compared to WT (Figure 6B). JA-Ile is continuously produced in WT plant tissue while, in those of *cml37-1*, the content of JA-Ile stays rather constant.

OPDA- and JA-Responsive Gene Expression Is Affected after Herbivore Feeding in *cml37*

A reduced level of jasmonates would be expected to alter the expression of jasmonate-responsive genes. To address this possibility, we analyzed the activity of *cis*-OPDA-marker genes, *GST1*, *OPR1*, and *OPR3* (Schäfer et al., 2011). *GST1* and *OPR1* showed significantly lower expression in *cml37-1* lines after 24 h of *S. littoralis* feeding when compared to WT plants (Figure 7). *OPR3*, which is involved in JA biosynthesis, was also decreased in *cml37-1* lines, although the difference was not significant.

A very important response influencing the susceptibility of a plant to herbivores is the expression of JA-responsive genes whose products can be toxic to insects (Howe and Jander, 2008; Wu and Baldwin, 2010). Thus, we analyzed the expression of JA-responsive genes *PDF1.2*, *THI2.1*, and *VSP2* after *S. littoralis* feeding. For *cml37-1* plants, all genes showed significantly lower expression after 24 h of feeding compared to WT (Figure 8B), while this effect was not seen at 6-h treatment (except for *VSP2*; Figure 8A). The expression of *MYC2*, whose expression is negatively regulated by JAZ proteins (Chini et al., 2009), was induced after 24 h of insect feeding. However, our data indicate that CML37 contributes to the regulation of certain JA-responsive genes.

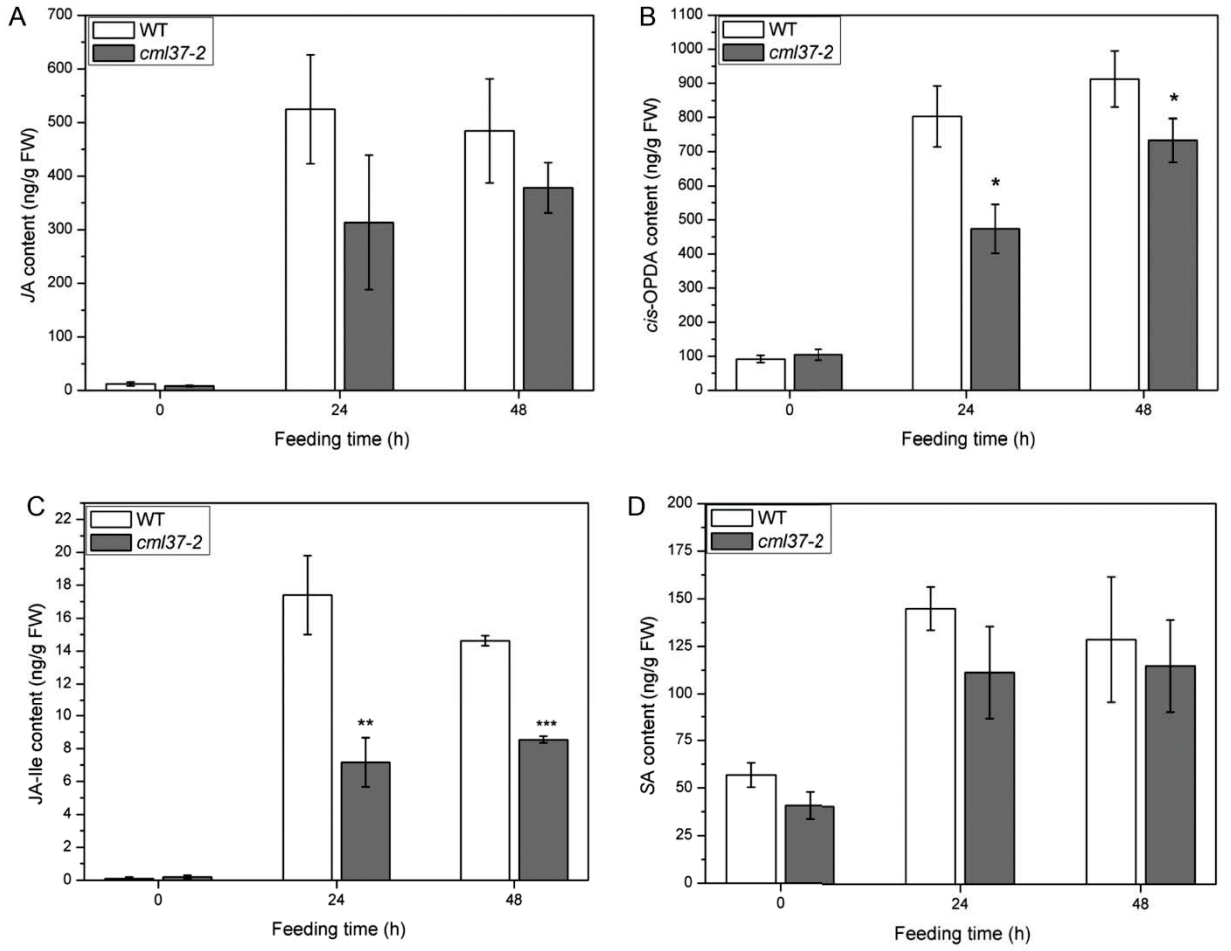

Figure 4 Phytohormone Elevation upon *Spodoptera littoralis* Herbivory in *Arabidopsis* Wild-Type (WT) and *cml37-2* Plants.
Mean (± SE, $n = 20$) levels of JA **(A)**, JA-Ile **(B)**, *cis*-OPDA **(C)**, and SA **(D)** in Col-0 WT (white) and *cml37-2* plants (gray) after *S. littoralis* feeding for 0 (control), 24, and 48 h. Phytohormone levels were measured only from local *S. littoralis*-fed leaves. Untreated leaves from untreated plants were used as controls. Statistically significant differences between phytohormones in WT and *cml37-2* plants after feeding were analyzed by *t*-test for each time point separately, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CML37 Regulates Several *JAZ* Genes upon Herbivory

Recent studies indicated that JAZ proteins act as repressors of JA-responsive genes and that their degradation via the SCF^{Coi1} E3 ubiquitin ligase complex is induced by jasmonate treatment (Chini et al., 2007). Many members of the *JAZ* family are highly induced by mechanical wounding and herbivore feeding (Chung et al., 2008). In order to study a role for CML37 in herbivory-induced *JAZ* family expression, we analyzed *JAZ* transcript levels after *S. littoralis* feeding. The activity of the *JAZ* family varied among members and could be separated into two groups. Relative to WT, one group of *JAZ* genes (*JAZ1*, *JAZ3*, *JAZ5*, and *JAZ6*) in *cml37* mutants was down-regulated after 6 h of *S. littoralis* feeding (Figure 9A), whereas the other group (*JAZ10*, *JAZ12*) was up-regulated (Figure 9B). The expressions of *JAZ2* and *JAZ9* were not changed compared to WT level. It is clear from these data that *cml37-1* mutants are impaired in the regulation of *JAZ* expressions.

Sensitivity to Jasmonates in Root Growth Assays Does Not Change in *cml37-1*

The methyl ester of JA, MeJA, acts as a plant signaling molecule and can affect plant growth and gene expression (Staswick et al., 1992). Previous studies showed that 0.1 μM of MeJA inhibits primary root growth of *A. thaliana*

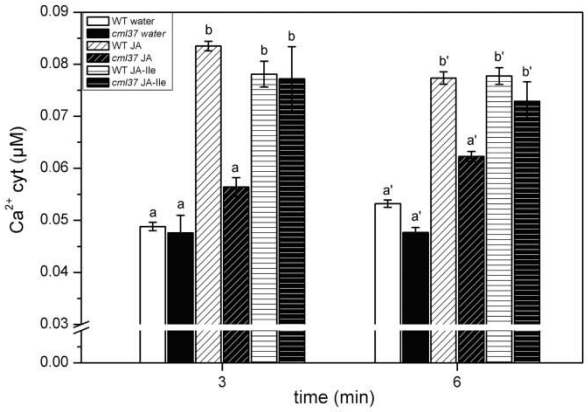

Figure 5 Jasmonate-Induced Changes in Cytosolic Calcium Concentration in *Arabidopsis* Wild-Type (WT) and *cml37-1* Plants Expressing Cytosolic Aequorin.

Mean (± SE, $n = 8$) level of cytosolic calcium concentration in WT (white) and *cml37-1* (black) after application of jasmonates for 3 and 6 min. 40 µl of 500 µM JA (diagonal stripes) or JA-Ile (horizontal stripes) or water as control (blank) were applied to leaf discs of 4–5-week-old aequorin-expressing plants which were equilibrated in 5 µM coelenterazine overnight. Mean $[Ca^{2+}]$cyt was calculated from the relative light units measured in leaf discs at 5-s integration time for 10 min. Different letters indicate significant differences among treatments for each time point separately (ANOVA; $P < 0.05$, SNK).

seedlings up to 50% (Corbineau et al., 1988). Recently, it was found that the *cml42* loss-of-function mutant is more sensitive to MeJA compared to WT (Vadassery et al., 2012b). We therefore analyzed the root growth response of WT, JA-insensitive *coi1-16*, and *jar1*, as well as *cml37-1* to 25 µM MeJA. We observed that *cml37-1* roots showed the same inhibitory response as WT to 25 µM MeJA treatment (Supplemental Figure 3). Thus, we conclude that, in contrast to *cml42*, jasmonate perception in the root growth assay is not influenced by the loss of CML37.

Content of Secondary Metabolites Are Not Affected in *cml37-1* Plants

Arabidopsis possesses a versatile arsenal of secondary metabolites which ensures survival of the plants under various adverse conditions. Secondary metabolites include many different compounds like glucosinolates (GS), terpenoids, or flavonoids (Kliebenstein, 2004). GS are sulfur-rich compounds that produce several different toxic products upon hydrolysis by endogenous thioglucosidases. They exhibit direct toxicity, growth inhibition, or feeding deterrence to a wide range of potential plant enemies such as insects (Halkier and Gershenzon, 2006). We analyzed the total content of GS in *cml37-1* plants after 1 and 7 d of *S. littoralis* feeding. The total content of GS increased after 1 d of insect feeding in both genotypes. When compared to WT, *cml37-1* plants contained the same amount of GS (Supplemental Figure 4A). The most common flavonoid in *Arabidopsis* belongs to the flavonols representing kaempferol glycosides (D'Auria and Gershenzon, 2005). In addition to UV-B protection, flavonoid exudates can elicit an avoidance reaction in herbivores, as shown for *Cistus ladanifer* L. (Treutter, 2005). We determined the content of the three major kaempferol glycosides, namely kaempferol-3-O-[6″O-(rhamnosyl)-glucoside]-7-O-rhamnoside (KRGR), kaempferol-3-O-glucoside-7-O-rhamnoside (KGR), and kaempferol-3,7-O-dirhamnoside (KRR), in *cml37-1* plants upon *S. littoralis* feeding. When compared to WT, *cml37-1* plants contained the same amount of flavonoids (Supplemental Figure 4B).

DISCUSSION

The CML protein family belongs to the group of non-catalytic Ca^{2+} sensor relay proteins. Upon perception of external stimuli, such as abiotic or biotic stress, Ca^{2+} levels are elevated in cells, thereby activating CMLs which can then regulate downstream targets to coordinate a physiological response. Here, we show an analysis of the role of CML37 in *Arabidopsis* plants challenged by herbivorous insect, *S. littoralis*. Our results suggest that CML37 is a Ca^{2+} sensor involved in the regulation of herbivory-induced plant defenses mediated by jasmonates.

CML37 Possesses Biochemical Characteristics of a Ca^{2+} Sensor

Biochemical properties typical of proteins that function as Ca^{2+} sensors include changes in secondary or tertiary structure, and changes in exposed surface hydrophobicity (Dobney et al., 2009; Bender et al., 2013; Bender and Snedden, 2013). These structural changes can be associated with activation of catalytic activity, as is the case for the Ca^{2+} sensor-responder CPKs, or with altered ability to interact with and regulate downstream targets, as is the case for Ca^{2+} sensor relays such as CaM and CMLs. To determine whether CML37 could function as a Ca^{2+} sensor, we analyzed Ca^{2+}-dependent changes in secondary structure and surface hydrophobicity and found that, like other CMLs (Bender et al., 2013), CML37 undergoes typical changes in secondary structure (Figure 1A) and in exposed surface hydrophobicity (Figure 1B). Our analysis indicates that CML37 possesses characteristics associated with Ca^{2+} sensor relay function, but further work is required to determine how the biophysical properties of CML37 mediate its interaction with downstream targets, the identity of which remain unknown at

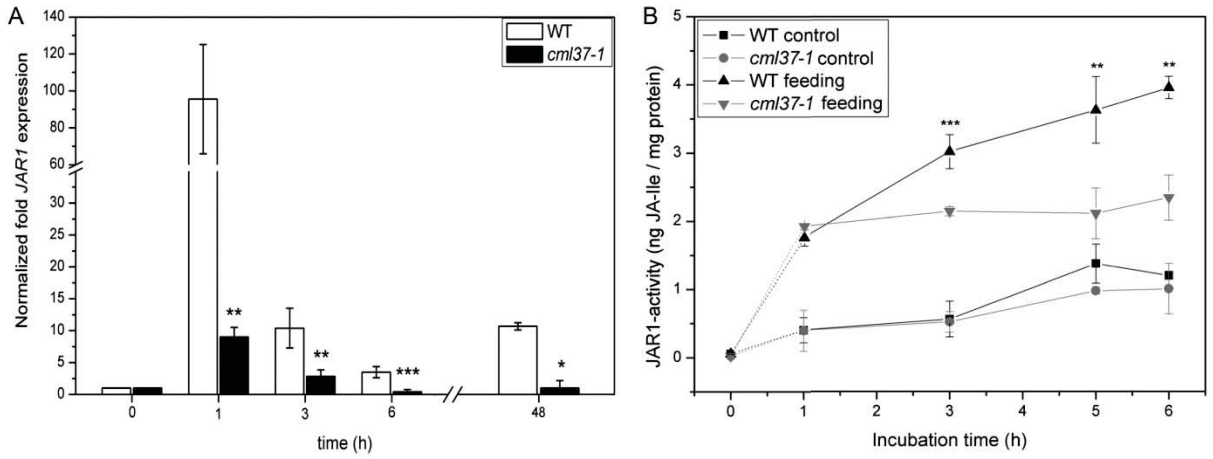

Figure 6 Level of *JAR1* mRNA Expression and Protein Level in *Arabidopsis* Wild-Type (WT) and *cml37-1* Plants after Herbivory.

(A) Mean expression (± SE, $n = 5$) of *JAR1* in Col-0 WT (white) and *cml37-1* plants (black) after *Spodoptera littoralis* feeding for 0, 1, 3, 6, and 48 h. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The expression of *JAR1* was calculated relative to untreated controls (0 h). Statistically significant differences between WT and *cml37-1* plants after treatment were analyzed for each time point separately by *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(B) Mean conjugation activity by formation of JA-Ile (± SE, $n = 5$) by JAR1 in Col-0 WT (black) and *cml37-1* plant protein extract (gray) after *Spodoptera littoralis* feeding for 1 h. Phytohormone levels were measured only from local *S. littoralis*-fed leaves. Untreated leaves from untreated plants were used as controls (0 h). Statistically significant differences between phytohormones in WT and *cml37-1* plants after feeding were analyzed by *t*-test for each time point separately, ** $P < 0.01$, *** $P < 0.001$.

this point. If and how the sub-cellular localization of CML37 changes after receiving a stress signal and binding of Ca^{2+} are unclear and the focus of ongoing work.

CML37 Is Induced by Wounding and OS

In *Arabidopsis*, several members of the CML family are induced by herbivory (Vadassery et al., 2012c). We demonstrated that *CML37* is induced by mechanical wounding, *S. littoralis* feeding, and, to a lesser extent, even by the application of *S. littoralis* OS (Figure 2). It is interesting that herbivory-like mechanical damage has a much higher impact on *CML37* transcript levels than insect-derived OS. After mechanical damage, *CML37* was induced 60-fold whereas application of OS resulted in 6-fold up-regulation. Consequently, upon insect feeding, both components are combined and the induction of *CML37* was 70-fold. These data suggest that the induction of *CML37* is mainly dependent on the wounding process, in contrast to the induction of *CML42*, which was strictly OS-inducible (Vadassery et al., 2012b). Both CML37 and CML42 are localized in the cytosol and the nucleus (Inze et al., 2012; Vadassery et al., 2012b) and display different induction patterns; hence, it is likely that they are involved in distinct tasks in the same compartment. Proteins which physically interact with CML37 are still unknown and will be addressed in ongoing work.

There are additional examples of Ca^{2+}-regulated genes that are wound-dependent. One example is the Ca^{2+}/CaM-binding transcription factor *AtSR1*, which is required for down-regulation of SA levels in plant immune responses (Du et al., 2009). Upon wounding, this activity is changed and the negative impact of SA in both basal and induced JA biosynthesis is abolished (Qiu et al., 2012).

Among the herbivory-responsive *CML*s, the level of induction after application of OS is comparable, in the range of 6–10-fold (Vadassery et al., 2012c). Although elicitors in *S. littoralis* OS remain unidentified, one component in OS able to induce plant defenses are endogenous phytohormones that are ingested by feeding insects. These phytohormones can come into contact with plant tissue due to larval feeding behavior. It was shown that lepidopteran larvae accumulate significant amounts of JA in the salivary glands (Tooker and De Moraes, 2006). It was observed that *cis*-OPDA from *Arabidopsis* leaves is taken up by feeding caterpillars, converted into *iso*-OPDA, and continuously regurgitated while feeding (Vadassery et al., 2012a). However, phytohormones are not the only chemicals that might be responsible for induction of plant defense (Truitt and Pare, 2004; Mithöfer and Boland, 2008). Very recently, it was shown that a channel-forming compound from *S. littoralis* OS is also able to induce the expression of *CML42* in *Arabidopsis* leaves (Guo et al., 2013).

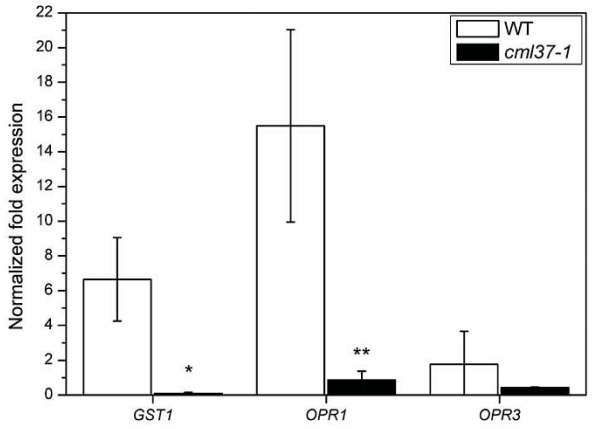

Figure 7 Expression of *cis*-OPDA Marker Genes in *Arabidopsis* Wild-Type (WT) and *cml37-1* Plants upon Herbivory.

Mean (± SE, $n = 10$) transcript levels of *GST1*, *OPR1*, and *OPR3* in *Arabidopsis* leaves in Col-0 WT (white) and *cml37-1* plants (black) after 24 h of *Spodoptera littoralis* feeding. Samples were taken from local *S. littoralis*-fed leaves. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. Undamaged leaves from untreated plants were used as controls (value = 1). Statistically significant differences between transcript levels in WT and *cml37-1* plants after feeding were analyzed by *t*-test for each time point separately, * $P < 0.05$, ** $P < 0.01$.

CML37 Is a Positive Defense Regulator upon *S. littoralis* Herbivory

We demonstrated that performance of *S. littoralis* larvae was significantly better on *cml37* compared to WT plants because the larvae gained more weight on the mutants (Figure 3). This indicates that *CML37* loss-of-function plants are more susceptible to *S. littoralis* herbivory—a fact that was shown for two independent *cml37* lines. Thus, CML37 can be seen as a positive regulator of plant defense in the interaction between *S. littoralis* and *Arabidopsis*. Mechanistically, CML37 seems to participate in jasmonate signaling—more precisely where JA-Ile and *cis*-OPDA act. Upon herbivory, in *cml37* mutants, both of these phytohormones are reduced in concentration (Figure 4 and Supplemental Figure 2). In addition, *cis*-OPDA is not only a precursor of JA and JA-Ile; it also has potential direct toxic effects on insects because of its reactive α,β-unsaturated carbonyl structure (Vollenweider et al., 2000). Some insects are able to use a specific GST to transform or inactivate the plant-derived OPDA in their gut (Dabrowska et al., 2009). Interestingly, it was reported that the level of *cis*-OPDA in damaged plant tissue is much higher than that of JA (Schulze et al., 2007). Thus, we speculate that lower levels of *cis*-OPDA and JA-Ile in *cml37* plants likely underlie their impaired defense against herbivorous insects.

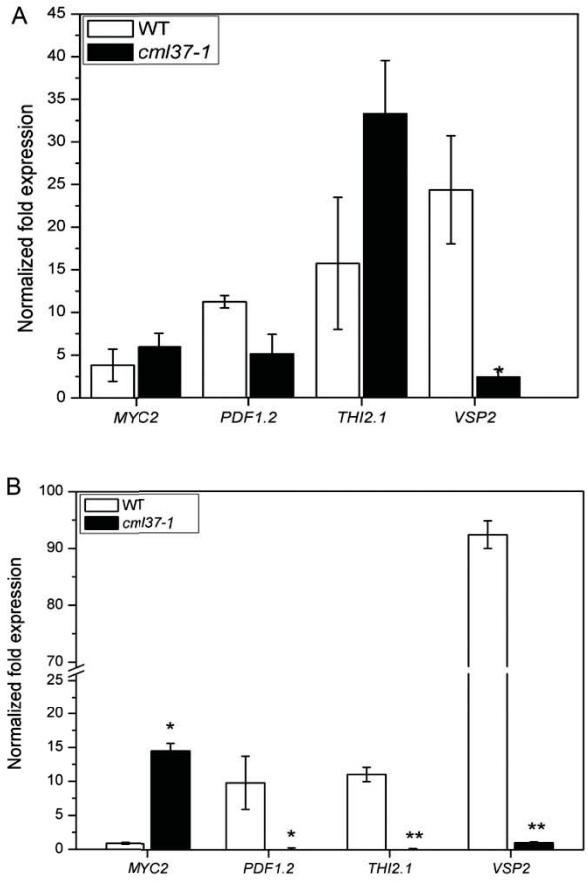

Figure 8 JA-Responsive Genes Expression in *Arabidopsis* Wild-Type (WT) and *cml37-1* Plants upon Herbivory.

Mean expression (± SE, $n = 10$) of *MYC2* and JA-responsive genes *PDF1.2*, *THI2.1*, and *VSP2* in Col-0 WT (white) and *cml37-1* plants (black) after *Spodoptera littoralis* feeding for 6 h **(A)** and 24 h **(B)**. Samples were taken from leaves fed on. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. Undamaged leaves were used as controls for quantification (value = 1). Statistically significant differences between transcript levels in WT and *cml37-1* plants after feeding were analyzed by *t*-test for each gene separately, * $P < 0.05$, ** $P < 0.01$.

We observed that, after *S. littoralis* feeding, the expression of JA-responsive genes *PDF1.2*, *THI2.1*, and *VSP2* was reduced in *cml37-1* plants (Figure 8B) and correlated with the decreased levels of JA-Ile in these plants (Figure 4 and Supplemental Figure 2). In general, the expression of many anti-insect proteins, which are encoded by JA-responsive genes, is tightly regulated by

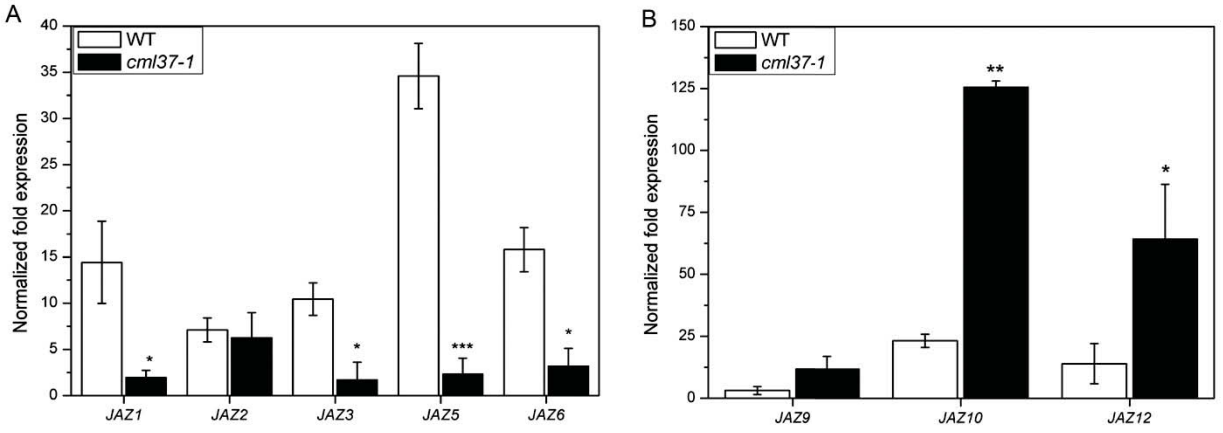

Figure 9 Expression of *JAZ* Genes in *Arabidopsis* Wild-Type (WT) and *cml37-1* Plants upon Herbivory.

Mean expression (± SE, $n = 10$) of *JAZ* genes in Col-0 WT (white) and *cml37-1* plants (black) after *Spodoptera littoralis* feeding for 6 h **(A, B)**. Samples were taken from local *S. littoralis*-fed leaves. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. Undamaged leaves were used as controls for quantification (value = 1). Statistically significant differences between transcript levels in Col-0 and *cml37-1* plants after feeding were analyzed by *t*-test for each gene separately, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the jasmonate signaling pathway (Howe and Jander, 2008). Despite a reduction in JA-responsive gene expression in *cml37-1* mutants, the level of *MYC2* transcripts, the main transcription factor controlling JA-responsive genes (Chini et al., 2009; Montiel et al., 2011), increased upon herbivory (Figure 8B). Moreover, *cml37-1* plants showed remarkably high expression of wounding- and herbivory-inducible *JAZ10* and *JAZ12* genes (Figure 9B), whereas the expression of other *JAZ* genes (*JAZ1*, *3*, *5*, *6*) was reduced or not influenced (Figure 9A). Interestingly, a *jaz10-1* mutant shows enhanced sensitivity towards jasmonates (Grunewald et al., 2009). Thus, we conclude that CML37 negatively regulates *JAZ10* and *JAZ12* expression and, therefore, CML37 is positively regulating the activity of JA-responsive genes whose expression is repressed by JAZ (Figure 8).

Another example of a wound-induced gene in *Arabidopsis* is *JAR1* (Suza and Staswick, 2008). Not surprisingly, *JAR1* transcript levels increased during *S. littoralis* feeding (Figure 6A). In contrast, it was clear that, in *cml37-1* plants, the level of *S. littoralis*-induced *JAR1* transcript was reduced compared with WT, as were levels of JA-Ile (Figures 4 and 6A). These results suggest causality between expression of *JAR1* and the presence of JA-Ile as product of the JAR1 enzyme. Interestingly, the JAR1enzyme activity itself is reduced in *cml37-1* plants after *S. littoralis* feeding, when compared to WT (Figure 6B). Thus, the reduced enzyme activity may be accountable for the reduced accumulation of JA-Ile in the *cml37* mutants.

This observation is supported by the fact that application of JA to *cml37-1* mutants generates a significantly different elevation of cytosolic calcium than in WT (Figure 5 and Supplemental Figure 1). Aequorin-expressing *cml37-1* plants showed a calcium response with the same signature and maximum values as WT, but with a significantly shorter elevation of cytosolic calcium (Figure 5 and Supplemental Figure 1) when treated with JA. This difference was not visible after adding the active JA-Ile conjugate to the *cml37-1* plants. This again indicates that the altered activity of JAR1 in *cml37* plants may be responsible for lower levels of jasmonates as well as for a reduced herbivore defense.

We were also able to show that *cis*-OPDA inducible genes *GST1* and *OPR1* were induced by insect feeding in the WT plant—an effect that was absent from *cml37-1* plants (Figure 7). Inductions of both genes under different stress conditions have previously been reported. It was shown that *GST1* is involved in stress responses induced by H_2O_2 or wounding (Rentel and Knight, 2004) and *OPR1* is induced by wounding, UV, and cold stress (Biesgen and Weiler, 1999). OPR1, in contrast to OPR3, is not active in JA biosynthesis (Stintzi and Browse, 2000). Strikingly, *OPR3* was only slightly increased upon herbivory and no significant difference was observed in the *cml37-1* mutants. These results suggest that CML37 might be involved in the regulation of additional wounding-responsive genes outside of the jasmonate pathway because OPDA cannot be perceived by the SCF–COI1–JAZ-co-receptor complex (Thines et al., 2007; Sheard et al., 2010).

CML37 as Regulator of Plant Stress Responses

In *CML37* loss-of-function mutants, jasmonate levels, in particular JA-Ile, are reduced and, as a consequence,

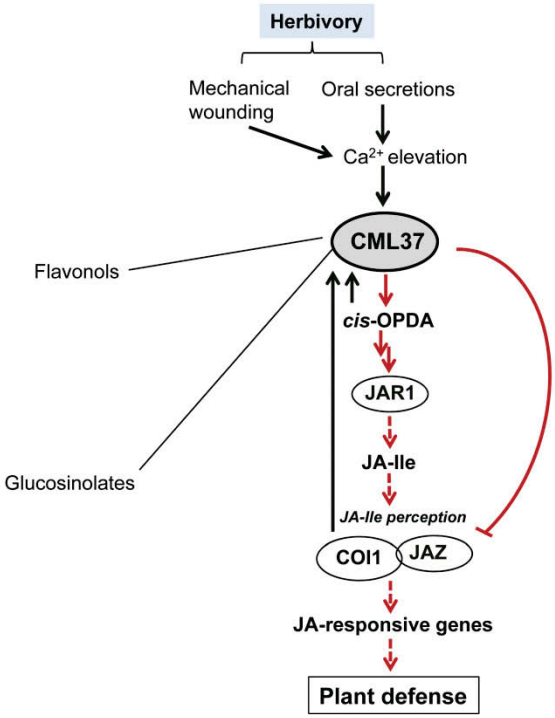

Figure 10 Scheme for Herbivory-Initiated Stress Response Induction in *Arabidopsis thaliana* and the Role of CML37.

CML37 acts as a positive defense regulator in the interaction between *Arabidopsis* and *Spodoptera littoralis*. Wounding and feeding herbivores, respectively, induce *CML37* gene activation and downstream components of the jasmonate pathway eventually leading to plant defenses. *CML37* gene expression is also induced by OPDA and depends on COI1. In wild-type (WT) plants, CML37 leads to increased levels of OPDA (direct effect, continuous red line). Moreover, transcription of *JAR1* is enhanced and, as a consequence, JA-Ile level is increased (indirect effect, dotted red line). While JA-Ile perception by the COI1/JAZ co-receptor is not affected, the increased level of JA-Ile results in an enhanced activation of many downstream defenses. In addition, the expression of certain *JAZ* members (e.g. *JAZ10* and *JAZ12*) that act as repressors of specific JA-responsive genes is decreased in WT plants, further inducing JA-mediated events. Collectively, these effects enhance the ability of plants to respond to herbivory. The content of secondary metabolites—GS and flavonols—is not influenced by CML37.

JA-responsive genes are affected. However, the perception of jasmonates did not change (Supplemental Figure 3): at least in our root growth assay, *cml37-1* seedlings showed the same jasmonate-induced growth-inhibition rate as WT seedlings. This result suggests that the impact of CML loss of function is highly diverse. Whereas mutants of herbivory-linked *cml42* were more sensitive in JA perception and show higher expression of JA-responsive genes (Vadassery et al., 2012b), mutants of wounding-induced *cml37* showed a different pattern. Here, probably due to a down-regulation of *JAR1* and a reduced JAR1 enzyme activity (Figure 6), JA-Ile accumulation rather than perception was affected (Figure 10).

In summary, based upon our study, CML37 can be considered a positive regulator of herbivory-induced defense in *Arabidopsis*, as outlined in Figure 10. This represents a striking contrast to CML42, which functions as a negative regulator of defense (Vadassery et al., 2012b). Moreover, we showed that CML37 very likely acts via JAR1, by promoting its gene expression and, as a consequence thereof, its enzyme activity. This causes a higher level of JA conversion into JA-Ile and the accumulation of the latter. Here, a direct connection between Ca^{2+} and jasmonate signaling is demonstrated. Whether and how these two CML Ca^{2+} sensors might interact during the orchestration of herbivory-induced responses in *Arabidopsis* remains to be elucidated and is the focus of ongoing work.

METHODS

Plant and Insect Materials

Arabidopsis thaliana seeds (*Arabidopsis*, ecotype Columbia, Col-0) and mutant lines with a T-DNA insertion (Alonso et al., 2003) either in the exon of At*CML37* (At5g42380)–SALK_011488C (*cml37-1*) or in the corresponding promoter (SALK_017485, *cml37-2*) were used for insect feeding assays. By use of *CML37* gene-specific primers in RT–PCR, the absence of *CML37* mRNA was confirmed (Supplemental Figures 3 and 4). The exon-insertion line (*cml37-1*) showed a more complete loss of *CML37* transcript and thus was used for further experiments. Homozygous *coi1-1* plants were selected by use of the CAPS marker (Xie et al., 1998). Plants were grown as described (Vadassery et al., 2012b).

Larvae of *S. littoralis* were hatched from eggs and reared on an agar-based optimal diet at 23°C–25°C with 8 h light/16 h dark cycles (Bergomaz and Boppre, 1986). The insect biomass assay was performed using pre-weighed first instar larvae (freshly hatched larvae grown for 3 d in light) to ensure equal starting conditions. For short-term feeding assays, fourth instar *S. littoralis* larvae which were starved 12 h prior to plant feeding were used.

Plant Treatments

Five-week-old plants were used for all experiments. Short-term insect herbivory screens were carried out with three larvae per plant. For experiments with insect OS, wounding was done with a pattern wheel (six vertical motions) on either side of the leaf. The OS was collected from fourth instar *S. littoralis* larvae which were fed on *Arabidopsis* leaves overnight. OS were stored on ice, centrifuged at 13

000 rpm for 2 min, and freshly diluted 1:1 with water; 20 µl of diluted OS was spread across all the holes on a single leaf (W+OS). In control plants, water was added (W+W). Tissue samples were harvested and stored in liquid nitrogen. Mechanical wounding assays were performed with MecWorm (Mithöfer et al., 2005). MecWorm operation was as follows: two, four, or six circles of damaged leaf area in the leaf (r = 1.5 mm) using six punches per minute at defined time points (1, 2, and 3 h). All experiments were repeated three times independently.

For *Arabidopsis* root growth-inhibition assays, seeds were surface-sterilized for 8 min using 50% bleach followed by extensive washing with water. Primary root lengths were measured after plants were grown vertically on MS agar under continuous light at 100 µmol m^{-2} s^{-1} for 14 d after treatment with 25 µM MeJA. WT, JA-insensitive mutants, *coi1-16* and *jar1*, and *cml37* knockout lines were grown on each plate.

Ca^{2+} Measurements

Aequorin-expressing *cml37-1* plants were generated by crossing the *cml37-1* line to a line carrying the cytosolic apo-aequorin gene. F2 progeny from the aequorin x *cml37-1* crosses that were homozygous for the cml37 mutation and that showed a measurable Ca^{2+} discharge using 1 M $CaCl_2$ and 10% ethanol, were used for the experiments. Primers used were the following: for WT fragment CML37-LP (5′-GAGAATATACGCGCGTTATCG-3′) and CML37-RP (5′-TCGTGGTCAACTTGACCTTTC-3′); for T-DNA insertion line LBb1.3 (5′-ATTTTGCCGATTTCGGAAC-3′) and CML37-RP.

Four-to-five-week-old plants were used for all Ca^{2+} measurements. Plants were grown in 10-cm pots under short-day conditions. Leaf discs of the respective plants were taken and equilibrated in 5 µM coelenterazine (PJK, Kleinblittersdorf, Germany) in the dark overnight at 21°C. Measurements were performed according to Vadassery et al. (2012b).

Expression, Purification, and Biophysical Analysis of Recombinant CML37

The *CML37* open reading frame was PCR-amplified using forward (5′-tccatatgactctcgctaagaacc-3′) and reverse (5′-ccatatatatcatctcaacgc-3′) primers to add *Nde*I and *Bam*HI restriction sites, respectively. PCR product was restriction cloned in to *Nde*I–*Bam*HI digested pET5a (Novagen, Darmstadt, Germany) for untagged expression in *Escherichia coli* strain BL21 (DE3) CodonPlus-RIL GroES/EL (Novagen). Purification of recombinant CML37 from *E. coli* inclusion bodies was performed exactly as previously described (Bender et al., 2013). CD and 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence experiments were performed using 10–20 µM CML37 as described before (Bender et al., 2013).

Expression Analysis by Real-Time PCR

Harvested leaf material was stored in liquid nitrogen until use. Tissues samples were homogenized using a Precellys® 24-Dual unit (Peqlab, Erlangen, Germany) with liquid nitrogen cooling. Homogenization parameters were 6500 rpm, 3 × 20 s, and 5-s pauses. Total RNA was isolated using the TRIzol Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. DNA was eliminated by DNase treatment (Turbo DNase; Ambion, Karlsruhe, Germany) and RNA content was determined photospectrometrically. DNA-free total RNA (1 µg) was converted into single-stranded cDNA using a mix of oligo-dT_{20} primers and the Omniscript cDNA synthesis kit (Qiagen, Hilden, Germany). For real-time PCR, gene-specific primers producing 135- to 170-bp amplicons (at exon–exon junction) were designed using the NCBI primer design tool (www.ncbi.nlm.nih.gov/tools/primer-blast). Q-RT–PCR was done in clear optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene, Böblingen, Germany) using the Brilliant II QPCR SYBR green Mix (Agilent, Böblingen, Germany). A dissociation curve analysis was performed for all primer pairs; *RPS18B* was used as endogenous control for all herbivory-related experiments (several genes were tested). Thus, the mRNA levels for each cDNA probe were normalized with respect to the *RPS18B* mRNA level. Fold-induction values of target genes were calculated with the ΔΔCP equation (Pfaffl, 2001) and related to the mRNA level of target genes in control tissue, which were defined as 1.0. All of the assays were run at least in triplicate (biological replication). The primer pairs used are listed in Supplemental Table 1.

Quantification of Metabolites

Jasmonic acid, JA-Ile, *cis*-OPDA, SA, and glucosinolate content in *A. thaliana* leaves was analyzed exactly as described previously (Vadassery et al., 2012b) with the modification that an API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) was used for phytohormone measurements. Flavonoids were analyzed in the flow-through of the DEAE Sepadex A 25 columns used in GS analysis; 100 µl aliquots of the flow-through samples were diluted with 300 µl water and 50 µl of each sample was analyzed by HPLC-UV (Agilent HP1100 Series) instrument equipped with a C-18 reversed phase column (Nucleodur Sphinx RP, 250 × 4.6 mm, 5 µm particle size; Macherey-Nagel, Düren, Germany). The mobile phase consisted with of 0.2% formic acid (v/v) (solvent A) and acetonitrile (solvent B) used in gradient mode at a flow rate of 1 ml min^{-1} at 25°C. The gradient was as follows: 100% A (5 min), 0%–45% B (15 min), 45%–100% (0.1 min), 100% B (1.9 min), and 100% A (3.9 min). The eluent was monitored

by a photodiode array detector at 330 nm. Kaempferol glycosides were quantified based on an external standard curve of an authentic standard of Kaempferol 3,7-dirhamnoside (High-Purity Compound Standard GmbH, Cunnerdorf, Germany) applying a relative molar response factor of 1.0. The compounds were identified based on UV visible absorption and on mass spectra from LC–MS analysis on a Bruker Esquire 6000 IonTrap mass spectrometer (LC conditions were the same as for HPLC-UV analysis) in comparison to the identified metabolites in the literature (Tohge et al., 2005).

JAR1 Conjugation Assay

Four-to-five-week-old plants were used for the assay. Three larvae of *S. littoralis* (fourth instar) were placed on plants for a feeding period of 1 h. Tissue of leaves fed on (100 mg) was collected in 500 µl lysis buffer (50 mM Tris–HCl pH 7.5; 0.5 % Triton X-100; 100 mM NaCl; 1 mM DTT; 10% Glycerol; 2 mM EDTA; cOmplete EDTA-free® Protease Inhibitor Cocktail, Roche, Basel, Switzerland), homogenized, and incubated on ice for 1 h. After centrifugation for 5 min, 1 ml of supernatant was used for the assay; protein concentration was determined by Bradford assay. Conjugation assay composition was adapted according to Staswick and Tiryaki (2004) and contained 3 mM ATP, 1 mM JA, and 1 mM Ile. Samples were incubated at 28°C and reaction was stopped by adding 200 µl of 0.25 M HCl. Samples were treated with 1 vol. methanol containing 8 µl ml^{-1} internal standard (see above) and analyzed by LC–MS. Content of JA-Ile was calculated per mg protein (Bradford).

Statistical Analysis

All statistical tests used are indicated in the corresponding figure legends. Statistical differences between different groups were examined by either *t*-test in SigmaStat 2.03 or by one-way ANOVA and posthoc SNK test (root growth assay) in SPSS Statistics 17.0.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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SUPPLEMENTAL DATA

Table S1. Primers used for RT-PCR.

Target (Atg number)	Sequence
***RPS18B* (*At1g 34030*)**	5'- GTCTCCAATGCCCTTGACAT -3'
	5'- TCTTTCCTCTGCGACCAGTT -3'
Actin2 (At3g18780)	5'- AGTGGTCGTACAACCGGTATTGTGCT -3'
	5'- TCCCGCTCTGCTGTTGTGGTG-3'
CML37 (At5g 42380)	5′- GGTGGAGGAAGTGGTGAAGA - 3′
	5′- GTAAACTCGCCGCCGTAATA - 3`
OPR1 (At1g 76680)	5′- TGTGTCCTTGTTGTTGCAGGTTTTG - 3′
	5′- TCCAACACGGTCTGGTCCGA - 3′
OPR3 (At2g 06050)	5′-CCTTCTTCCAGATCGGCGGAGACAT -3'
	5′-GGCGCCAGAACCACTCGATGA -3'
GST1 (At1g 02930)	5'- GCCTTTCATCCTTCGCAACCCCT -3'
	5'- TCGCCATGTCCTTGCCAGTTGA -3'
JAR1 (At2g 46370)	5'- TCCGTTTCGTCTGATCGGGATGT -3'
	5'- AGCTTCTTCAGGGTCAGTAGCGT -3'
MYC 2 (At1g 32640)	5'- CGGAGATCGAGTTCGCCGCC -3'
	5'- AATCCCGCACCGCAAGCGAA -3'
JAZ1 (AT1G19180)	5'- CGCGAGCAAAGGCACCGCTA -3'
	5'- TCCAAGAACCGGTGAAGTGAAGC -3'
JAZ2 (AT1G74950)	5'- CCCGGCCTCTTTAGCCTGCG -3'
	5'- ACCGTGAACTGAGCCAAGCTG -3'
JAZ3 (AT3G17860)	5'- AGAGATTCAGCTCCCAACAGAGGAA -3'
	5'- TGGAAACCCGGCATCGACATGG -3'
JAZ5 (AT1G17380)	5'- GCTAAGGCACAAGCGCCGGA -3'
	5'- GCATCGCATTTTGTTTCCCTGGTGG -3'

JAZ6 (AT1G72450)	5'- TGTCAACGGGACAAGCGCCG -3'
	5'- TCCGGTGCTACTTTTGCCGGT -3'
JAZ9 (AT1G70700)	5'- ATGCGCCGGGAACGGTTTGA -3'
	5'- GCAGCAACGGGTGTGTCCCT -3'
JAZ10 (At5g 13220)	5'- TCGAGAAGCGCAAGGAGAGATTAGT -3'
	5'- AGCAACGACGAAGAAGGCTTCAA -3'
JAZ12 (AT5G20900)	5'- ATGAGCCACGCGCTTCCGTT -3'
	5'- ACCGTTGGCTCAGCGGTTTGA -3'
PDF1.2 (At5g44420)	5'- CTGCTTTCGACGCACCGGCA -3'
	5'- GTTGCATGATCCATGTTTGGCTCCT -3'
Thi2.1 (At1g72260)	5'- CGCCATTCTCGAAAACTCAGCTGA -3'
	5'- GTTTAGGCGGCCCAGGTGGG -3'
VSP2 (At5g24770)	5'- ACGACTCCAAAACCGTGTGCAA -3'
	5'- CGGGTCGGTCTTCTCTGTTCCGT -3'

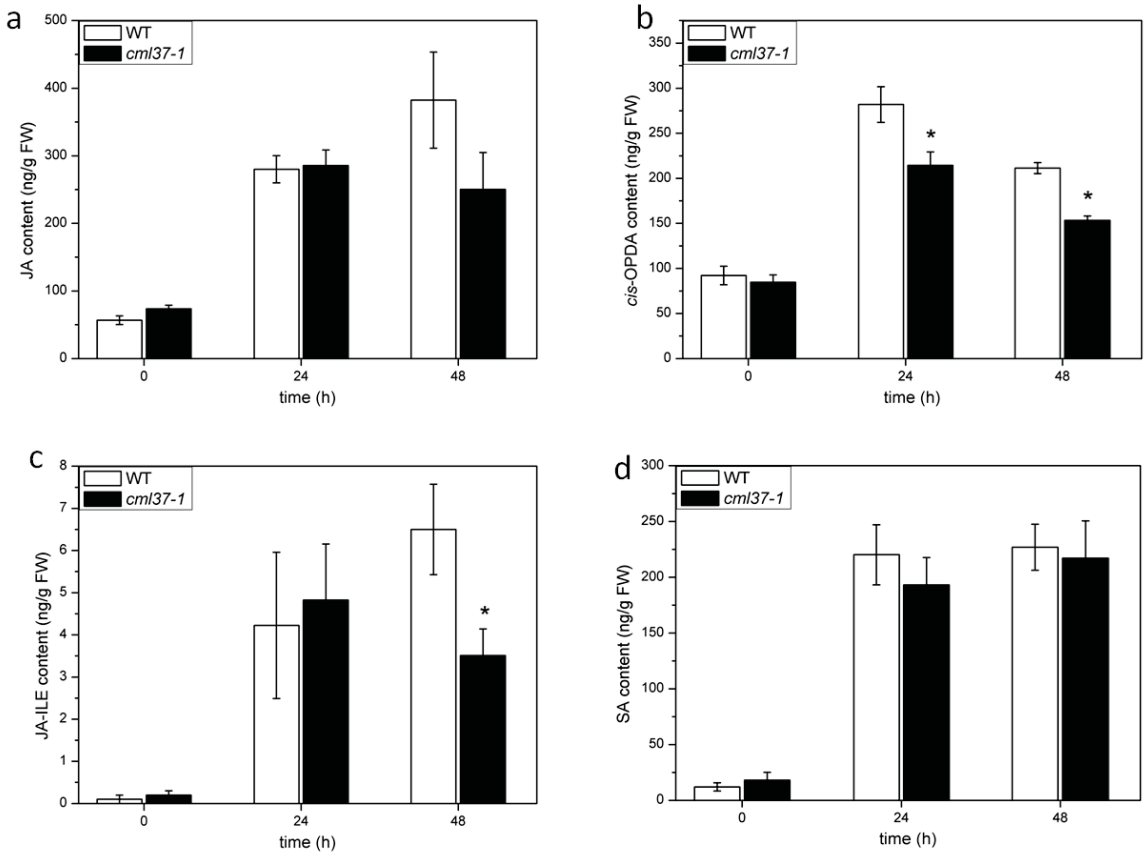

Figure S1. Phytohormone elevation upon *Spodoptera littoralis* herbivory in *cml37-1* line (SALK_011488C).

Mean (± SE, n=20) levels of JA (**a**), (+) JA-Ile (**b**), *cis*-OPDA (**c**) and SA (**d)** in Col-0 WT (white) and *cml37-1* plants (black) after *S. littoralis* feeding for 24 and 48 h. The phytohormone levels were measured from local *S. littoralis* fed leaves. Untreated leaves were used as controls. Statistically significant differences between phytohormones in Col-0 and *cml37* plants after feeding were analysed by t-test, *p=<0.05.

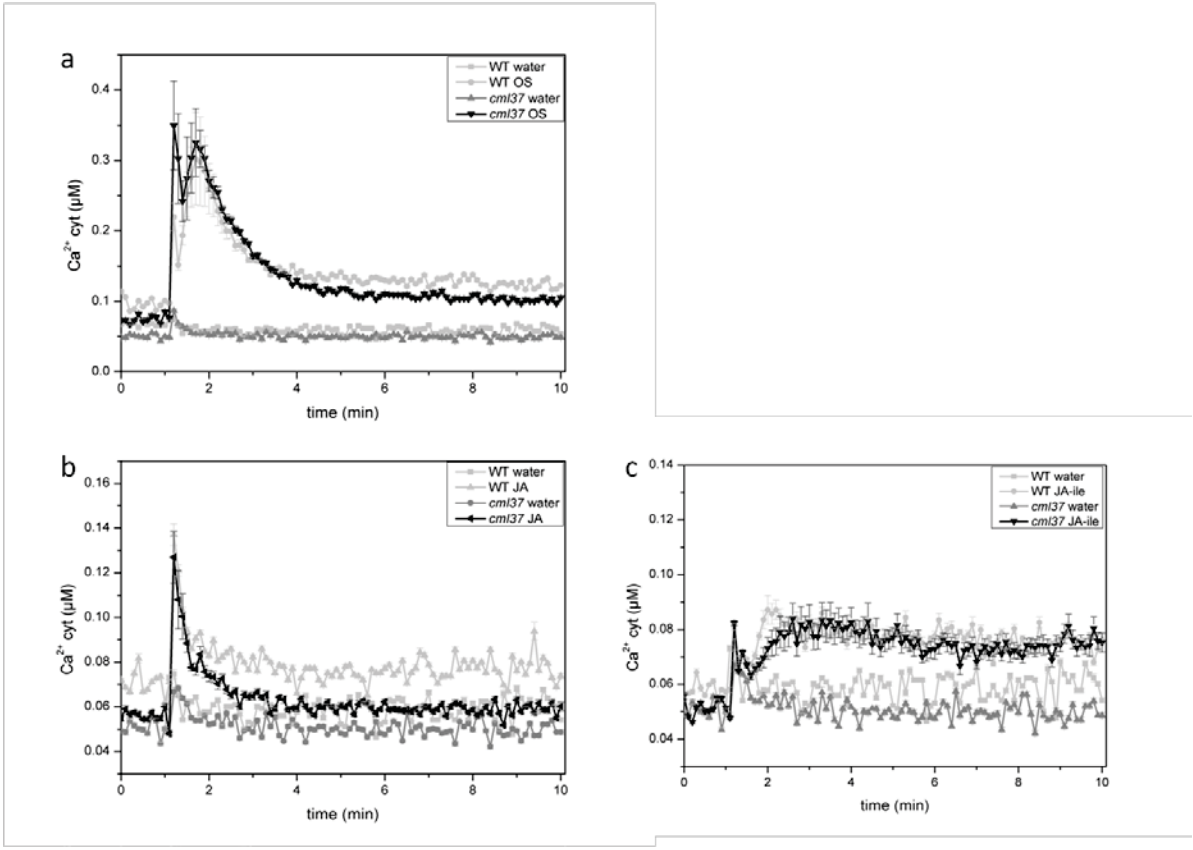

Figure S2. *S. littoralis* OS- and jasmonate-induced changes in cytosolic calcium concentration (Ca^{2+}cyt) in Arabidopsis WT and *cml37-1*plants carrying the cytosolic aequorin.

a) Mean (± SE, n=8) level of cytosolic calcium concentration in Col-0 WT (light grey) and *cml37-1* plants (dark grey, black) after application of OS. 40 µL of *S. littoralis* OS (1:1 diluted) or water (control) were applied to equilibrated leaf discs of 4-5 week old aequorin expressing plants.

b) Mean (± SE, n=8) level of cytosolic calcium concentration in Col-0 WT (light grey) and *cml37-1* plants (dark grey, black) after application of JA. 40 µL of a 500 µM JA solution or water (control) were applied to equilibrated leaf discs of 4-5 week old aequorin expressing plants.

c) Mean (± SE, n=8) level of cytosolic calcium concentration in Col-0 WT (light grey) and *cml37-1* plants (dark grey, black) after application of JA-Ile. 40 µL of a 500 µM JA-Ile solution or water (control) were applied to equilibrated leaf discs of 4-5 week old aequorin expressing plants.

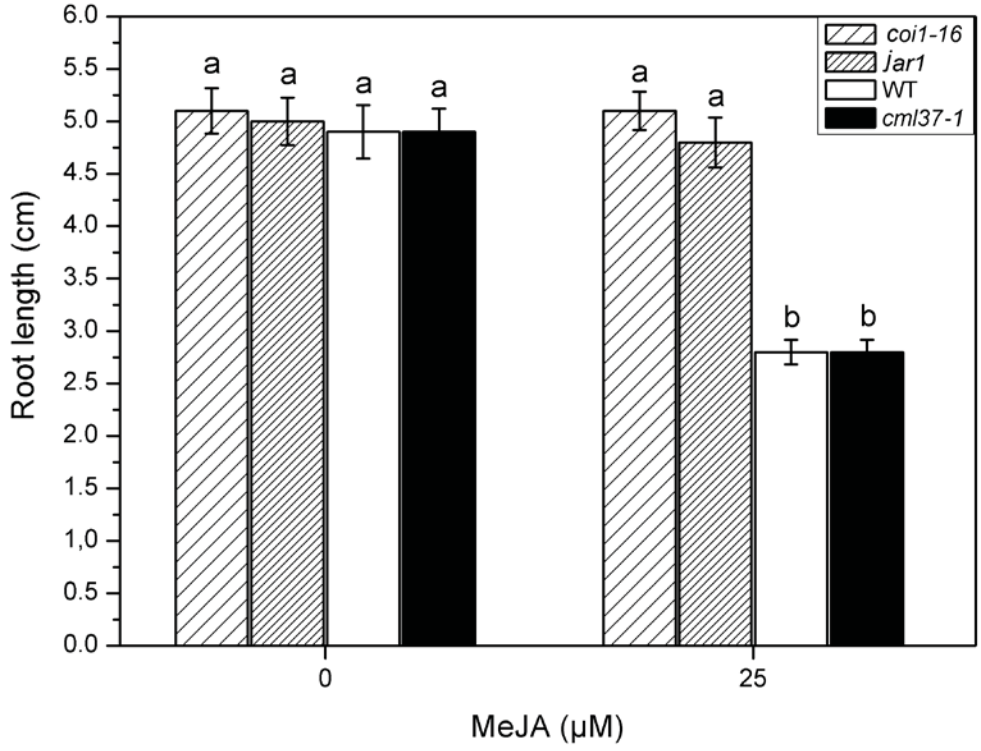

Figure S3. Root growth assay in Arabidopsis wild type (WT) and *cml37-1* plants treated with MeJA.

Mean root length of Col-0 WT (white), *coi1-16*, *jar1* and *cml37-1* seedlings (black) after 14 days of vertical growth on 25 µM MeJA. JA mutants *coi1-16* and *jar1* were used as positive controls. Statistically significant differences between plants were analysed by One Way ANOVA and Student-Newman-Keuls-Test (P = <0.05).

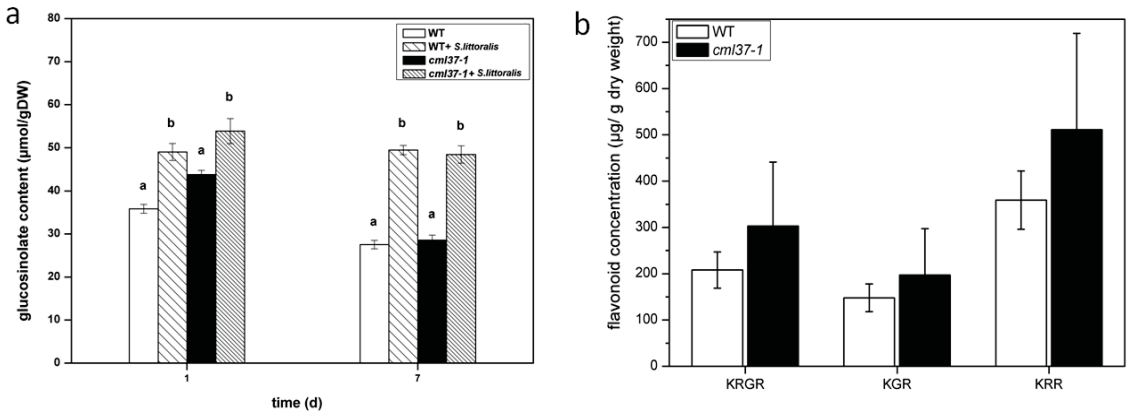

Figure S4. Content of glucosinolates and flavonoids in Arabidopsis wild type (WT) and *cml37-1* plants upon herbivory.

a) Mean (± SE, n=10) levels of glucosinolates in Col-0 WT (white) and *cml37-1* (black) plants after *Spodoptera littoralis* feeding for 1 and 7 days. Untreated leaves were used as control and the experiment was repeated independently. Statistically significant differences between WT and *cml37* plants were analysed by One Way ANOVA and Student-Newman-Keuls-Test. Groups were tested for each time point separately (P = <0.05).

b) Mean (± SE, n=6) levels of kaempferol- glycosides in unwounded Col-0 WT (white) and *cml37-1* plants (black). No statistical difference detected. KRGR: kaempferol 3-O-[6”-O-(rhamnosyl)glucoside] 7-O-rhamnoside, KGR: kaempferol 3-O-glucoside 7-O-rhamnoside, KRR: kaempferol 3,7-O-dirhamnosid. Statistically significant differences between flavonoids levels in WT and *cml37-1* plants were analysed by Mann-Whitney Rank Sum Test for each flavonoid separately.

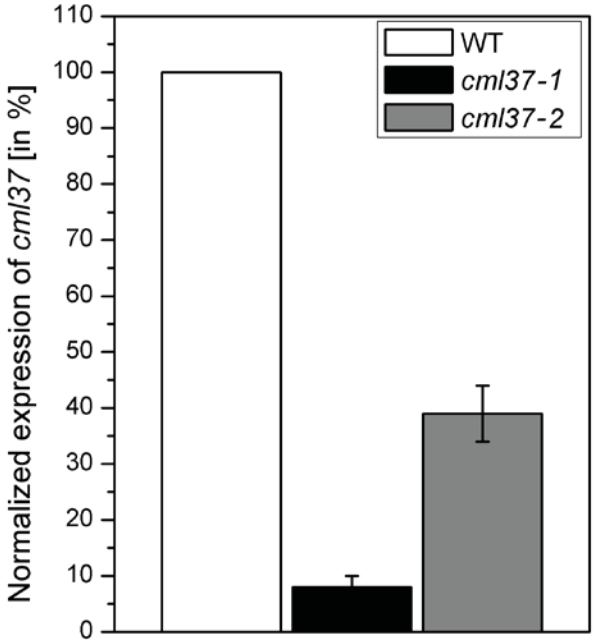

Figure S5. Level of *CML37* expression in independent *cml37* lines.

Mean expression (± SE) of *CML37* in Col-0 WT (white) and *cml37* plants (black, grey) after *Spodoptera littoralis* feeding for 1 h. *cml37* plants of independent t-DNA lines were used: *cml37-1*: SALK_011488C; *cml37- 2*: SALK_017485. Transcript levels were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The expression of *CML37* was calculated relative to WT control.

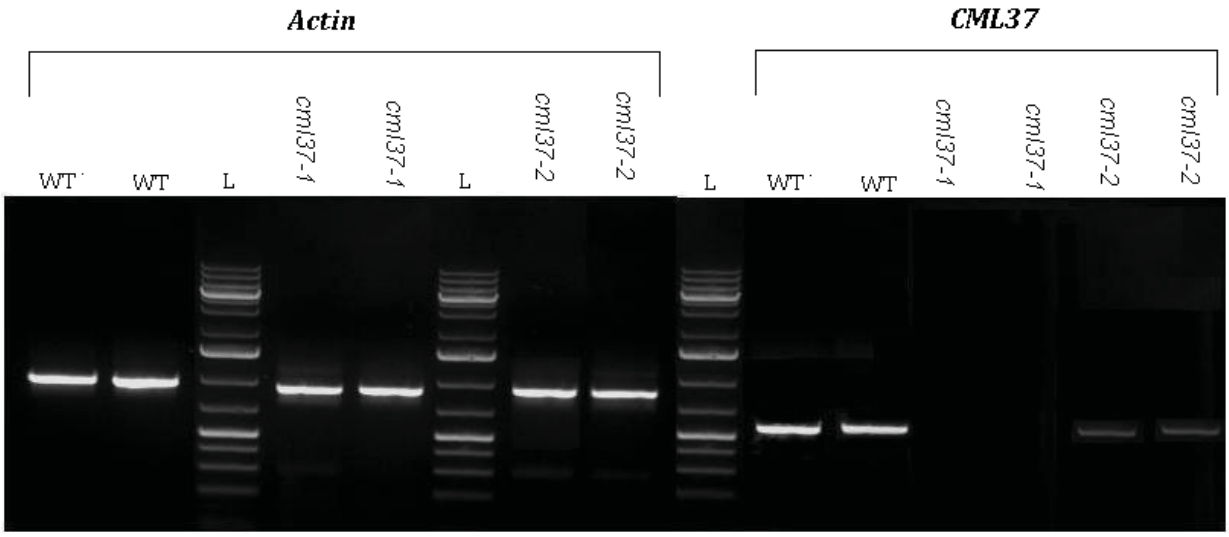

Figure S6. Identification of cml37-knock-out lines by RT-PCR.

Expression of *CML37* in Col-0 WT and *cml37* plants was determined after *Spodoptera littoralis* feeding for 1 h by use of RT-PCR. Equal amount of PCR product was loaded on a 0.8% agarose gel, Actin was used as control. L ladder, *cml37-1* SALK_011488C, *cml37-2* SALK_017485.

Manuscript 3

SHORT COMMUNICATION

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Calmodulin-like protein CML37 is a positive regulator of ABA during drought stress in Arabidopsis

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Keywords: *Arabidopsis thaliana*, abscisic acid, CML37, CML42, calmodulin-like proteins, drought stress

Plants need to adapt to various stress factors originating from the environment. Signal transduction pathways connecting the recognition of environmental cues and the initiation of appropriate downstream responses in plants often involve intracellular Ca^{2+} concentration changes. These changes must be deciphered into specific cellular signals. Calmodulin-like proteins, CMLs, act as Ca^{2+} sensors in plants and are known to be involved in various stress reactions. Here, we show that in Arabidopsis 2 different CMLs, *At*CML37 and *At*CML42 are antagonistically involved in drought stress response. Whereas a CML37 knock-out line, *cml37*, was highly susceptible to drought stress, CML42 knockout line, *cml42*, showed no obvious effect compared to wild type (WT) plants. Accordingly, the analysis of the phytohormone abscisic acid (ABA) revealed a significant reduction of ABA upon drought stress in *cml37* plants, while in *cml42* plants an increase of ABA was detected. Summarizing, our results show that both CML37 and CML42 are involved in drought stress response but show antagonistic effects.

During their life, plants are challenged by a multitude of different stresses. Thus, in order to survive, plants have to deal with and adapt to all forms of stress, biotic as well as abiotic. The signal transduction pathway connecting the recognition of such environmental cues and the downstream responses are still poorly understood. However, it is well accepted that Ca^{2+} is one of the major second messengers involved in many different signaling pathways.[1] Changes in intracellular Ca^{2+} concentrations due to different stimuli are described as calcium signatures.[2] However, Ca^{2+} signatures are not sufficient to explain specificity of the particular responses. Therefore, signaling components that can decipher and transduce the Ca^{2+} signal to further downstream signaling components are necessary. Such candidates for Ca^{2+}-binding proteins are the so-called calmodulin-like proteins, CMLs, which act as Ca^{2+} sensors.[3] CMLs are unique to plants with 50 members in *Arabidopsis thaliana* and they do not exhibit enzymatic activities but undergo conformational changes upon Ca^{2+} binding, which enables subsequent interaction with target proteins that, upon binding, will be modulated in their activities.[4]

CMLs are involved in plant development and responses to various stress factors from the abiotic and the biotic environment. For instance, CML24 can cause changes in abscisic acid (ABA) level during ion stress as well as changes in flowering time; it also regulates pollen tube growth by modulating the actin cytoskeleton and controlling the cytosolic Ca^{2+} concentration.[5-7]

CML39 functions during early seedling establishment and activates defense responses.[8] *CML9* gene is induced by *P. syringae* infection, flg22 elicitor and salicylic acid (SA) and it alters plant responses to ABA and abiotic stress;[9,10] *CML8* is also induced by SA and salt stress.[11] Transcripts of CML37, CML38 and CML39 are regulated by salt- and drought stress, some phytohormones (jasmonate and ABA), and biotic stress (phytopathogenic *Pseudomonas syringae*).[12] Recently, several CMLs were identified that are regulated by herbivory or herbivore-related signals present in insect-derived oral secretions.[13-15] For 2 particular calmodulin-like proteins, CML37 and CML42, it was demonstrated that they have antagonistic effects. CML42 represents a negative regulator of insect herbivory-induced defense, while CML37 is quite the opposite, a positive regulator of insect herbivory-induced defense.[13,15] CML37 was shown to connect Ca^{2+} and jasmonate signaling by affecting the synthesis of jasmonic acid-isoleucine conjugate.[15] All those examples demonstrate that almost all *CML* genes studied so far are involved in more than one stress-responsive pathway, including biotic and abiotic stress, as well as developmental stimuli. These findings make them ideal candidates in plants to study cross talk between different pathways on the one hand and specificity in the activation of pathways on the other hand.

Here, we decided to further investigate properties of the antagonists CML37 (At5g42380) and CM42 (At4g20780). Based on an experiment that suggested an involvement of

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CML42 in drought stress,[13] we chose this particular stress factor again for our investigation. We observed that *cml37* plants were much more susceptible for drought stress than WT and *cml42* plants (**Fig. 1**). While *cml37* plants were already completely dried out, *cml42* and WT plants survived the treatment. In plants, drought stress is always combined with osmotic stress, which regulates ABA biosynthesis.[16] Thus, the level of ABA was determined in WT, *cml37* and *cml42* plants after one week of drought stress and after a second cycle of drought stress, again for one week (**Fig. 2**). The content of ABA in *cml37* plants exposed to dryness was significantly lower when compared to WT (**Fig. 2**). While WT plants contained an average of 200 ng ABA (g FW)$^{-1}$ after one week, and 100 ng ABA (g FW)$^{-1}$ after 2 weeks of drought stress, *cml37* plants could only reach level of about 40 ng ABA (g FW)$^{-1}$ at both time points. In contrast, *cml42* plants accumulated about 250 ng ABA (g FW)$^{-1}$ already after the first week and, in contrast to the WT, retained this high level in the second week.

The accumulation of ABA plays an important role in drought and osmotic stress response. ABA promotes the closure of stomata and affects drought-related gene expression which mediates a higher drought tolerance and resistance.[17] Thus, the phenotypical results shown in **Figure 1** can be directly explained by a much lower accumulation of ABA in the *cml37* mutant.

Hence, we can state that CML37 is a positive, while CML42 is a negative regulator of ABA accumulation that is induced by drought stress. These results also reflect and confirm the striking antagonism found for these particular CMLs in insect herbivory resistance.[13,15] Further experiments with over-expressing CML37 and CML42 lines, as well as double knock out lines will be carried out in the near future in order to understand how such an

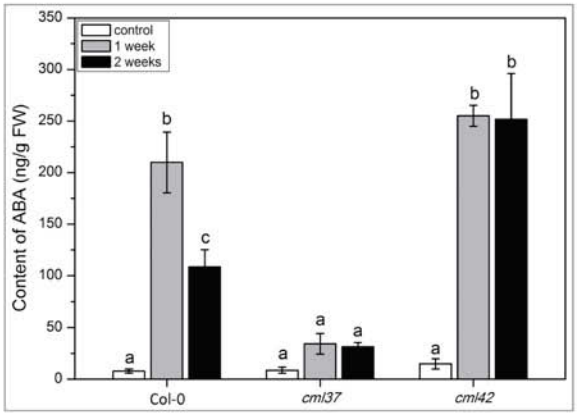

Figure 2. ABA level in Arabidopsis WT (*Col-0*), *cml37* and *cml42* knock-out plants upon drought treatment. Mean content (± SE, n = 6) of ABA in Col-0, *cml37* and *cml42* plants. ABA content was analyzed in untreated plants (white), and plants subjected to 1 week (gray) and 2 weeks of drought treatment (black). ABA was measured from the whole plant rosette. Statistically significant differences between treatments were analyzed by One Way ANOVA ($P < 0.05$, SNK test).

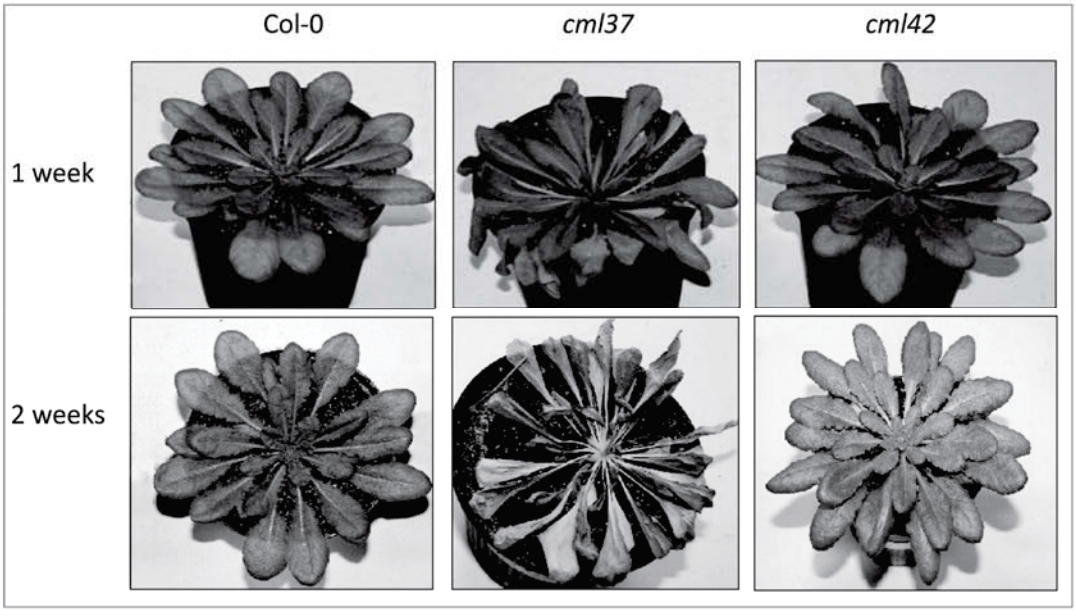

Figure 1. Drought phenotype of Arabidopsis WT (*Col-0*), *cml37* and *cml42* knockout plants. Drought phenotype of WT *Col-0* (left), *cml37* (middle) and *cml42* plants each after 1 (top line) or 2 weeks (bottom line) of drought treatment. The plants shown represent the typical phenotype observed in multiple replicates.

antagonistic interaction is realized on the cellular and molecular level.

Materials & Methods

Plant growth and drought treatment

Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments and plants were grown as described before.[13] CML knock-out lines *cml37* (SALK_011488C) and *cml42* (SALK_041400C) were obtained from the SALK Institute and selected for homozygosity.[13,15,18] For all experiments 4 week old plants, grown under short-day conditions, were used. Drought treatment was applied for 1 or 2 weeks. In particular, plants were not watered for 1 week (normally daily) till substrate was completely dry, before plants were harvested for ABA analysis. In parallel, after 1 week of drought stress plants were fully watered (till the pot was fully soaked with water), and subjected to a second week of drought stress before ABA analysis was performed. To minimize experimental variation, WT and mutants were placed in the same tray.

Phytohormone analysis

For abscisic acid phytohormone extraction 250 mg of fresh plant material was used. Leaf material was weighed and frozen in liquid nitrogen. D_6-abscisic acid (Santa Cruz Biotechnology, Heidelberg, Germany) was used as internal standard. Samples were homogenized for 1 minute at 1000 rpm in the Genogrinder 2010 (Spex Sample Prep, Stanmore, UK) and extracted and analyzed as described before using an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany) equipped with a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm, Agilent) for chromatography and an API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source that was operated in the negative ionization mode for mass spectrometry.[13]

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Manuscript 4

Running title: Insect-induced GABA accumulation

Insect herbivory-elicited GABA accumulation in plants is a wound-induced, direct, systemic and jasmonate-independent defense response

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Significance statement

Here a systematic study with *Arabidopsis thaliana* wild-type and mutant plants that are either impaired in γ-aminobutyric acid (GABA) synthesis or constitutively accumulating GABA is presented. Results indicate that during insect herbivore attack the glutamate decarboxylase-dependent generation and accumulation of the non-proteinogenic amino acid GABA is a wound-induced, direct and systemic defensive reaction. The GABA-based defense mechanism can be compared with other tissue-disruption mediated defenses such as glucosinolate accumulation.

Abstract

The four-carbon non-proteinogenic amino acid γ-aminobutyric acid (GABA) is present in all organisms analyzed so far. In invertebrates GABA acts as neurotransmitter; in plants different functions are still under discussion. Among others, its involvement in abiotic stress reactions and as defensive compound against feeding insects is suggested. GABA is synthesized from glutamate by glutamate decarboxylases (GAD) and degraded by GABA-transaminases. Here, in *Arabidopsis thaliana, gad12* double mutants showing reduced GABA contents as well as GABA-enriched triple mutants (*gad12 x pop2-5*) were generated and employed for a systematic study of GABA induction, accumulation and related effects in Arabidopsis leaves upon herbivory. Results demonstrate that GABA accumulation is stimulated by mechanical, insect feeding-like wounding by a robotic caterpillar, MecWorm, as well as by real insect (*Spodoptera littoralis*) herbivory. Higher GABA level in plant tissue in turn affects the performance of feeding larvae as well as higher levels of GABA in insect diet. GABA enrichment occurs not only in the challenged but also in adjacent leaf. This induced defense response is neither depending on jasmonates, phytohormones typically involved in defense reactions against herbivores, nor is jasmonate induction depending on the presence of GABA. Thus, in plants the rapid accumulation of GABA very likely represents a general, direct and systemic defense reaction against insect herbivores.

Keyword index: γ-aminobutyric acid, *Arabidopsis thaliana*, herbivory, *Spodoptera littoralis*, MecWorm, jasmonate, wounding, plant defense.

INTRODUCTION

The four carbon non-proteinogenic amino acid γ-aminobutyric acid (GABA) is widespread in animals, plants and microorganisms. GABA is mainly synthesized by decarboxylation of L-glutamate in the cytosol. The reaction is carried out by glutamate decarboxylases (GAD, EC 4.1.1.15). In Arabidopsis, five genes encoding GADs exist. It is suggested that GAD activity is regulated by pH and Ca^{2+}/calmodulin (Bown *et al.* 2006; Carroll *et al.* 1994; Snedden *et al.* 1995). At neutral pH, GAD activity depends on Ca^{2+}/calmodulin; however, any acidification of the cytosol, for example by wounding-mediated disruption of the vacuole, can stimulate GAD activity independent on Ca^{2+}/calmodulin. In addition, GABA can also be produced from polyamines (Shelp *et al.* 2012). Catabolism of GABA is localized in the mitochondrial matrix. A GABP (GABA permease) transporter encoded by a single copy gene in Arabidopsis has been described to import GABA into mitochondria (Michaeli *et al.* 2011). However, the lack of a prominent phenotype of the loss-of-function *gabp* mutant argues for the presence of other transporters capable of importing GABA into mitochondria. Once in the mitochondrial matrix, a transaminase reaction catalyzed by a GABA transaminase (GABA-T) moves the amino group of GABA onto pyruvate yielding alanine and in parallel succinic semialdehyde (SSA). In Arabidopsis, GABA-T is also encoded by a single copy gene. Disruption of the *GABA-T* gene leads to strong GABA accumulation. In the vegetative growth phase, no prominent phenotype of *gaba-t* mutants can be observed, however, fertility is decreased in the mutant due to impaired pollen tube growth (Palanivelu *et al.* 2003; Renault *et al.* 2011; Yu *et al.* 2014). SSA is either exported from mitochondria by a yet unknown transporter and further metabolized

(Breitkreuz *et al.* 2003), or is oxidized to succinate, a tricarboxylic acid (TCA) cycle intermediate, by succinic semialdehyde dehydrogenase (SSADH). Disruption of the single copy *SSADH* gene leads to a severe phenotype. It has been shown that accumulation of SSA is causative for the production of leaf necrosis and impaired growth of *ssadh* mutants due to the production of reactive oxygen species (Bouche *et al.* 2003; Fait *et al.* 2005; Ludewig *et al.* 2008).

So far, GABA has been found in all plant species investigated (Shelp *et al.* 2009). It has been mostly considered as a metabolite somehow involved in the control of C/N balance and in anaplerotic alimentation of the Krebs cycle (Fait *et al.* 2008). Beyond, function of GABA in plants is far from being revealed. Several findings started a discussion about functions of GABA as a signaling compound in plant growth and development (Bouche *et al.* 2004). For example, in *Arabidopsis thaliana* it was shown that pollen tube-growth in pistils as well as hypocotyl- and root-growth depend on controlled low GABA levels (~1 nmol g^{-1} DW) (Palanivelu *et al.* 2003; Renault *et al.* 2011). Again in Arabidopsis, low GABA levels are important and a prerequisite for *E*-2-hexenal-induced root growth inhibition (Mirabella *et al.* 2008). In all these cases it was demonstrated that mutations in the same gene (*GABA-T*) encoding a γ-amino butyric acid transaminase, caused enhanced GABA levels in the resulting *pop2* and *her1* mutant plants (Mirabella *et al.* 2008; Palanivelu *et al.* 2003; Renault *et al.* 2011). The increased endogenous concentration of GABA seems to be the reason for impaired cell elongation in the mutants and the corresponding phenotypes (Renault *et al.* 2011). Other studies demonstrated that GABA is involved in the differentiation of the vascular system in pine (*Pinus pinaster*) seedlings (Molina-Rueda *et al.* 2015). Shelp *et al.*

(2006) also suggested that GABA might be involved in the communication between plants and other organisms such as fungi, bacteria and certain invertebrates (Shelp *et al.* 2006).

For many years it is known that GABA accumulates in plants upon various abiotic stress challenges such as mechanical stimulation and tissue damage, salt and cold stress (Kinnersley *et al.* 2000; Ramputh *et al.* 1996; Renault *et al.* 2010; Shelp *et al.* 1999; Wallace *et al.* 1984). GABA is also suggested to be involved in plant defense against herbivorous insects (Bown *et al.* 2006; Huang *et al.* 2011; Mithöfer *et al.* 2012). This hypothesis is based on several facts and observations: (i) GABA is known as an inhibitory neuromuscular transmitter acting at GABA-gated chloride channels in invertebrates, including insects, where it could affect normal development when ingested by feeding (Bown *et al.* 2006; Shelp *et al.* 2009). Thus, the presence of GABA might deter feeding of herbivorous insect as shown for *Choristoneura rosaceana* (oblique-banded leafroller) larvae raised on synthetic diet (Ramputh *et al.* 1996). (ii) Leaf tissues of soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*) that were only slightly wounded by crawling insect species (*C. rosaceana* and the tobacco budworm, *Heliothis virescens*, respectively) showed 4- to 12-fold enhanced GABA accumulation within 5 to 10 min (Bown *et al.* 2002). (iii) Transgenic *N. tabacum* plants with elevated GABA levels due to constitutive transgenic expression of a GAD enzyme were more resistant to both *H. virescens* larvae and *Meloidogyne hapla*, the root-knot nematode (Bown *et al.* 2006; MacGregor *et al.* 2003; McLean *et al.* 2003).

In 2006, Alan W. Bown and colleagues postulated in an opinion article "…that wounding stimulates gamma-aminobutyrate (GABA) accumulation in plants, which in turn deters

herbivory by invertebrate pests" (Bown *et al.* 2006). Nearly a decade later, there is still a lack of experimental proof concerning the herbivory-related stimulus that is necessary and sufficient to induce GABA accumulation in plant leaves and whether this GABA contributes to the plants' defense. Here, we address these questions systematically. Moreover, many herbivory- or wounding-related defense responses in plants are strongly depending on and mediated by the well-studied jasmonates, fatty acid-derived phytohormones (Mithöfer *et al.* 2009; Wasternack 2007). Thus, we also examined whether the induced defense of GABA accumulation is a jasmonate-regulated process.

Materials and methods

Plant and insect material, growth and plant treatment

4-5 week old *Arabidopsis thaliana* plants (wild-type: ecotype Col-0; mutants: *gad12*, *gad12 x pop2-5*, *jar1*) were used for all experiments. All plants were grown as described elsewhere (Vadassery *et al.* 2012). Larvae of generalist herbivore *Spodoptera littoralis* were hatched from eggs and reared on an agar-based optimal diet at 23–25°C with 8 h light/ 16 h dark cycles (Bergomaz *et al.* 1986). For 7 d feeding assay, 1st instar larvae were used (they were kept in light for 3 d after hatching). The larvae were pre-weighed to ensure equal starting conditions for all experiments. For short term feeding assays (3 h), 4th instar *S. littoralis* larvae which were starved overnight prior to plant feeding were used. For coronalon treatment the plant was sprayed with 1 ml of a 50 µM solution (0.1% ethanol, equivalent to 50 nmol) or solvent control and incubated with a cover to prevent evaporation.

Mechanical wounding was done like described earlier using MecWorm (Scholz *et al.* 2014). To discriminate between a local and a systemic accumulation of GABA, leaves of plants were counted according to (Farmer *et al.* 2013). Leaf number 8 was treated with MecWorm for 1.5 h; leaf 8 (local) as well as different systemic leaves (5, 9, and 11) were harvested.

S. littoralis growth inhibition assay with GABA

To determine growth effects of GABA on *S. littoralis*, 2nd instar larvae were reared on artificial diet (see above) containing defined amounts of GABA (solved in water). A 0.5 M GABA stock solution was diluted several times; 100 µL of each were dropped on weighed pieces (1 g) of the artificial diet to get final concentrations of 0, 0.01, 0.1, 0.5 and 1 µmol GABA (g diet)$^{-1}$. All insects were kept separated. The food was renewed every second day while the GABA concentration was maintained. The larval weight was determined before the experiment was started (day 0) and after 7 days of feeding. To calculate the growth inhibition, the measured increase in weight at different GABA concentrations was correlated with the control (no GABA, set to 100%).

Generation of single, double and triple mutants

The seeds of the single mutants *gad1* (At5g17330; SALK_017810), *gad2* (At1g65960; GK_474E05) and *pop2-5* (At3g22200; GK_157D10) were obtained from the respective stock centers. F2 plants were screened for homozygousity by genotyping. For that, genomic DNA extraction from the individual plants was carried out as follows. Leaf samples were collected in 1.5 mL Eppendorf tubes containing 2-3 glass beads of 2 mm

in size and snap-frozen in liquid nitrogen. The samples were crushed to powder using a tissue lyzer (Qiagen, Cat No 85220) for three minutes at a frequency of 20 s^{-1}. Then, 200 μL of extraction buffer (0.2 M Tris HCl pH 7.5, 25 mM EDTA, 0.5% SDS and 250 mM NaCl) was added and homogenized. The mixture was spun down for one minute at 14,000 rpm, and 150 μL of the supernatant was transferred into new tubes. Next, an equal volume of 100% isopropanol was added, mixed and incubated at room temperature for five minutes. Finally, the mixture was spun down at 14,000 rpm for five minutes, and the pellet was dissolved in 100 μL ddH_2O. PCR analysis was performed using 2 μL of the DNA extract. For the generation of the *gad12* double mutant, the respective single mutants were crossed by emasculating the mother plant followed by pollination with the pollen from the male parent. For the isolation of homozygous double mutants, a similar procedure was followed as for the single mutants. The triple mutant was generated by crossing the homozygous *gad12* double mutant with the homozygous *pop2-5* mutant. The screening procedure was carried out as described above.

RNA extraction, cDNA synthesis and RT-PCRs for mutant characterization

Leaf samples (~100-200 mg) were collected from Arabidopsis plants and snap-frozen in liquid nitrogen. RNA extraction was carried out as described before with minor modifications (Logemann *et al.* 1987). Briefly, frozen tissue was crushed to powder using a pre-cooled electrical drill machine. Immediately, 1 mL of Z6 buffer (8 M guanidinium hydrochloride, 20 mM MES, 20 mM EDTA, pH 7.0) containing 0.7% (v/v) β-mercaptoethanol was added and homogenized by vortexing. Next, 500 μL PCI (phenol:

chloroform: isoamylalcohol 25:24:1) was added and mixed by inverting the tube 10-15 times. After incubation for three minutes at room temperature, samples were spun down for ten minutes at 4°C with 14,000 rpm. The aqueous phase (700 μL) was transferred to a new tube and 1/20 volumes acetic acid (1 M) and 0.7 volumes ethanol (100%) was added, mixed and incubated at room temperature for ten minutes. The mix was spun down with 14,000 rpm for ten minutes at 4°C. The pellet was then washed first with 500 μL of sodium acetate pH 5.0 followed by a second wash with 500 μL 70% ethanol. Finally, the pellet was air-dried and dissolved in 100 μL of RNase-free distilled water. Prior to cDNA synthesis the total RNA was treated with DNase (Promega) for one hour at 37°C. The concentration of RNA was quantified using a NanoDrop (NanoDrop 1000 V.3.8), and the integrity of the RNA was verified on a 1% agarose gel. The cDNA was synthesized from 1.5 μg of total RNA in 20 μL of total reaction mixture according to the manufacturer's protocol (Bioscript). The synthesized cDNA was diluted three times and the expression of the target genes was analyzed using qRT-PCR. Used primers are listed in Table S1. The primers discriminating between the *GAD* paralogs have previously been reported except for *GAD5* (Renault *et al.* 2010).

Quantification of phytohormones

For quantification of phytohormones 250 mg of sample was weighed and frozen in liquid nitrogen. The extraction procedure and determination of JA and JA-Ile was carried out as described before (Vadassery *et al.* 2012).

Quantification of γ–aminobutyric acid (GABA)

Approximately 250 mg of fresh leaves were weight. The γ–aminobutyric acid (GABA) was extracted with 2 mL of methanol and the resulting extract was diluted in a ratio of 1:20 (v:v) in water containing the U-^{13}C, ^{15}N labelled amino acid mix (algal amino acids ^{13}C, ^{15}N, Isotec, Miamisburg, USA, at a concentration of 10 µg of the mix per ml). GABA in the diluted extracts was directly analyzed by LC-MS/MS. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-1 min, 3%B in A; 1-2.7 min, 3-100% B in A; 2.7-3 min 100% B and 3.1-6 min 3% B in A. The mobile phase flow rate was 1.1 mL/min. The column temperature was maintained at 25 °C. The liquid chromatography was coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source operated in positive ionization mode. The instrument parameters were optimized by infusion experiments with pure standards. The ionspray voltage was maintained at 5500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: GABA (*m/z* 104.1 →87.1; DP 51, CE 17), U-^{13}C, ^{15}N-Ala (*m/z* 94.1 →47.1; DP 51, CE 17). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. GABA in the sample was quantified using U-^{13}C, ^{15}N-Ala applying a response factor of 1.0.

RESULTS AND DISCUSSION

Two of five *GAD* genes are mainly expressed in shoots and roots

In *Arabidopsis thaliana*, five *GAD* genes have been identified (Shelp *et al.* 1999). Here, we analyzed the relative expression of all five *GAD* paralogs in shoots and roots of wild-type plants. *GAD1* transcripts were mainly detected in roots (Figure 1a) and *GAD2* transcripts were abundantly detected in shoots and in considerable amounts in roots (Figure 1a), observations in line with previous findings (Turano *et al.* 1998; Zik *et al.* 1998). *GAD4,* on the other hand, exhibited a weak expression in shoots and an even weaker expression in roots (Figure 1). *GAD4* expression was also detected in flowers and siliques (Figure S1). The transcripts of *GAD3* and *GAD5* were neither detectable in shoots nor in roots. However, the transcript of *GAD3* could be detected in young siliques (Figure S1), and *GAD5* transcripts were detected in flowers (Figure S1). Indeed, strong expression of *GAD5* in gametes of *Arabidopsis thaliana* has been reported in publically available expression resources (Hruz *et al.* 2008; Winter *et al.* 2007).

A *gad12* double mutant contains low GABA amounts in shoots and roots

Next, we asked whether a simultaneous knock out of *GAD1* and *GAD2* would lead to major changes in the GABA pools of shoots and roots. To test that, we generated a *gad12* double mutant by crossing single *gad1* and *gad2* T-DNA insertion mutants (Figure S2a, b) and confirmed the absence of full-length transcripts (Figure S2c). However, a truncated *GAD2* transcript that consisted of exon 1, exon 2 and a part of exon 6, which is unable to encode a functional GAD, could be detected (Figure S2c,d). GADs belong to the pyridoxal phosphate-dependent aspartate aminotransferase super-

family of proteins (Marchler-Bauer *et al.* 2011). The residues important for binding of pyridoxal phosphate in *A. thaliana* GAD2 protein have been predicted to be Ser-125, Ser-126, Ile-129, Ile-208, Asp-243, Ser-246, Ser-273 and Lys-276 in the native protein. Moreover, Lys-276 is predicted to be involved in catalytic activity (Marchler-Bauer *et al.* 2011). These co-factor binding and catalytically active residues are encoded by bases located in exons 3, 4 and 5 of the native transcript. However, in the truncated version of the *GAD2* transcript, those exons were absent, and hence the protein very unlikely remains any decarboxylase activity. Furthermore, a premature stop codon has been detected close to the junction between the 2nd and the 6th exon to further shorten the unlikely functional protein (Figure S2d).

The *gad12* double mutant revealed a 20-fold reduction of GABA, compared to the wild type (Figure 2). Despite reports indicating the possible synthesis of GABA from the degradation of polyamines (Bouchereau *et al.* 1999; Fait *et al.* 2008; Shelp *et al.* 2012), GABA in *A. thaliana* seems to be mainly produced from the decarboxylation of glutamate by the activity of GADs. However, GABA contents of *gad12* double mutants were not below the detection limit, either because of the above mentioned degradation of polyamines fueling GABA synthesis or because of low expression of *GAD4* (Figure 1). To examine whether an additional compensatory expression of *GAD* paralogs in *gad12* mutants occurred, the transcript levels of *GAD4* were analyzed in both shoots and roots and compared to the wild type. *GAD4* transcripts were found to be up-regulated (Figure 1b) and might be sufficient to explain the presence of GABA in the double mutant.

An additional knock-out of *GABA-T* gene in the *gad12* double mutant caused higher GABA contents in shoots and roots

The GABA content of plant organs is not only determined by its synthesis. Its degradation by GABA-T activity also affects the accumulation of GABA, as was also discussed by Renault et al. (Renault *et al.* 2010). We assumed that the low GABA contents in *gad12* mutants would be elevated when breakdown of GABA is prevented due to the absence of GABA-T activity. Hence, we created a triple mutant by crossing the *gad12* double mutant to a *gaba-t* (*pop2-5*) mutant. *Pop2* mutants were previously shown to accumulate high GABA contents in *A. thaliana* (Ludewig *et al.* 2008; Palanivelu *et al.* 2003; Renault *et al.* 2011). Homozygous knock-outs of all three genes of the triple mutant were verified by PCR (Figure S3). The *gad12 x pop2-5* triple mutant contained seven times more GABA than the wild-type and half as much compared with the *pop2-5* single mutant (just given for comparison) (Figure 2). It is likely that the triple mutant slowly accumulates GABA with time because of the low GABA synthesis rate due to the absence of the most prominent GAD activities. In contrast, accumulation of GABA might be more rapid in *pop2-5* single mutants, i.e. the proportion of GABA contents between wild-type, *gad12 x pop2-5* triple and *pop2-5* single mutants might not be constant with time but changes with developmental stages.

Triple mutant plants are less susceptible to *Spodoptera littoralis* feeding

Due to the finding that higher GABA levels can affect insects (Bown *et al.* 2006; MacGregor *et al.* 2003; Ramputh *et al.* 1996) the influence of different endogenous GABA contents *in planta* was investigated in parallel in an insect herbivore feeding

assay. In contrast to former experiments (Bown *et al.* 2006; MacGregor *et al.* 2003), we did not look for feeding preferences but for insect performance on different mutant lines. Therefore, we carried out a bio-assay employing the different plant lines available, i.e. wild type, g*ad12,* and g*ad12 x pop2-5* plants, and herbivorous larvae of the generalist lepidopteran species *Spodoptera littoralis*. While *S. littoralis* larvae feeding on *gad12* mutant plants showed the same increase in body weight as on wild type, the larvae feeding on g*ad12 x pop2-5* plants gained significantly less weight (Figure 3). The constitutive accumulation of GABA over time in this mutant (Figure 2) might contribute to the enhanced resistance against *S. littoralis* feeding. Interestingly, lower GABA level in the *gad12* mutant did not result in an altered feeding behavior of *S. littoralis* larvae compared to the wild type (Figure 3) suggesting that this insect species can tolerate some basic level of the defensive compound GABA. To follow up this idea, 2nd instar *S. littoralis* larvae were reared on artificial diet containing different amounts of GABA (Figure 4). Concentrations were chosen between 0 and 1 µmol GABA (g diet)$^{-1}$; these concentrations covered the GABA levels determined for the investigated WT and mutant lines (Figure 2). Interestingly, lower concentrations of GABA between 0 and 0.08 µmol g^{-1}, which resembled the constitutive GABA content in *A. thaliana* Col-0 wild type plants, did not significantly affect *S. littoralis* larvae growth (Figure 4). The increase in larval weight is just reduced about 5% compared to water treatment. This observation suggests that *S. littoralis* indeed has certain tolerance to GABA in the food source. A significant decrease in growth was observed for a GABA concentration of 1 µmol g^{-1}; here the larvae gained 23% less weight compared to the control (Figure 4). Thus, these results can explain the *S. littoralis* feeding behavior on the different GABA mutant lines

where the GABA content of both wild type and the *gad12* mutant did not cause any growth inhibition (Figure 3) but, in contrast, the GABA content of the g*ad12 x pop2-5* triple mutant induced an extrapolated decrease in growth of about 15%. However, this alone cannot explain the results shown in Figure 3, but an increased GABA level very likely contributes to the whole array of defenses against *S. littoralis*.

A similar finding for a species-specific tolerance has been described for *S. littoralis* that fed on *Nicotiana attenuata* mutant plants (irMPK4 x irCOI1), where a jasmonate-independent defense pathway could not inhibit growth of *S. littoralis* larvae in contrast to larvae of *Manduca sexta* (Hettenhausen *et al.* 2013).

Spodoptera littoralis feeding- and wounding-induced jasmonate induction is not affected in GABA mutants

Knowing that many plant defense reactions against herbivorous insects are regulated by jasmonates (Mithöfer *et al.* 2009; Wasternack 2007) we decided to further investigate the contribution and involvement of this phytohormone class on GABA accumulation. Thus, the levels of jasmonic acid (JA) and its bioactive derivative, (+)-7-*iso*-jasmonoyl-*L*-isoleucine (JA-Ile) (Fonseca *et al.* 2009), were determined in Arabidopsis wild type and the GABA mutant plants upon herbivore treatment.

As shown in figure 5a, the different basic GABA concentrations present in the three plant lines are obvious. In wild type and in the triple mutant GABA level increased over time. The concentrations of JA and JA-Ile also clearly increased due to larvae feeding but no significant differences were detectable between wild-type and the two mutant

lines (Figures 5b, c). Obviously, the different levels of GABA did not affect the jasmonate level.

Insect herbivory is a combination of two events, first of all wounding of plant tissues and second the introduction of insect-derived compounds that come in contact with the tissues during the feeding process (Mithöfer *et al.* 2008). Using a robotic caterpillar, MecWorm, we are able to mimic the behavior of a feeding *S. littoralis* larva in order to investigate the impact of the isolated wounding process without the contribution of insect-derived compounds (Mithöfer *et al.* 2005). As shown in figure 6, MecWorm treatment alone caused the accumulation of GABA in wild-type plants. Wounding disrupts cell structure and releases the acidic vacuole content. As shown for carrot suspension cells, acidic pH values stimulate GAD activity *in vivo*, and as a consequence thereof, the generation and accumulation of GABA (Carroll *et al.* 1994). Compared with insect feeding (Figure 5a), MecWorm wounding caused about eight-fold higher GABA accumulation in wild-type plants due to the facts that more leaf material was wounded and, in addition, the leaf material was not fed up by the insects. As expected, in the knock out plant *gad12* no GABA accumulated (Figure 6a). Jasmonate levels increased significantly upon wounding; however, the amount of JA and JA-Ile in the controls and in the treated plants was similar, independent on the plant lines (Figures 6b, c).

Wounding induces GABA accumulation in adjacent leaves

An interesting feature of the GABA-forming GAD enzyme is its activation at acidic conditions whereas under neutral conditions the activity depends on Ca^{2+}/calmodulin (Bown *et al.* 2006; Snedden *et al.* 1995). Thus, wounding and the accompanying

acidification of the cytosol can explain GABA accumulation in the treated, local leaf. Unfortunately, data for Ca^{2+}/calmodulin activation of GAD is only based on *in vitro* studies. Knowing that wounding and herbivory can also stimulate a systemic increase of the cytosolic Ca^{2+} concentration (Kiep *et al.* 2015), the systemic accumulation of GABA was investigated upon wounding of a defined leaf with MecWorm. As shown in Figure 7, mechanical damage of leaf 8 did not only cause a significant increase of GABA concentration in the local leaf but also in the adjacent leaf 5, which is directly connected to leaf 8 (Farmer *et al.* 2013). Although no response was detected in other leaves, this result strongly suggests that the induced Ca^{2+} increase in non-wounded tissue can trigger the activity of GAD, supporting *in vivo* the statement of Snedden and colleagues (1995) that systemic GABA synthesis might depend on Ca^{2+} signaling.

GABA elevation is jasmonate independent

To further investigate whether the accumulation of GABA might be induced by jasmonates, we treated Arabidopsis wild-type plants with the synthetic JA-Ile mimic coronalon that has been shown to induce all typical JA-Ile effects (Nakamura *et al.* 2014; Schüler *et al.* 2004; Svoboda *et al.* 2010). As can be seen in figure 8, no changes in GABA concentration were detectable within three hours after treatment with 50 µM coronalon, while JA-biosynthesis and JA-responsive genes were induced indicating a sufficient concentration of coronalon (Figure S4). This result is clearly in contrast to the results obtained in wild-type plants where GABA accumulation was detected upon herbivory (Figure 5a) or mechanical wounding (Figure 6a) within the same period, indicating that GABA accumulation is not jasmonate dependent. In order to support this

result, we performed an additional experiment where wild-type Arabidopsis and a jasmonate signaling mutant, *jar1* that is unable to generate JA-Ile (Staswick *et al.* 2002), were treated with *S. littoralis* larvae. Whereas in wild-type and *jar1* control plants the level of GABA was the same, after 3 h of feeding in wild-type as well as in *jar1* plants a significant higher concentration of GABA was detected compared to the respective controls (Figure 9). This was an expected result because the feeding process causes GABA accumulation (Figure 5a). More interesting is the finding that in *jar1* plants a significant increase of GABA could be measured compared with wild type plants (Figure 9). On the one hand this shows again that jasmonate-based signaling is not involved in GABA accumulation and on the other hand that on defense-impaired *jar1* plants more GABA could be generated very likely because the larvae fed more.

CONCLUSIONS

The non-proteinogenic amino acid γ-aminobutyric acid, GABA, is widespread in eukaryotes including invertebrates, where it activates Cl^- -channels at neuromuscular junctions. For plants, various physiological role(s) for GABA are still under discussion. Here, experimental evidence based on GABA-reduced and GABA-enriched *Arabidopsis thaliana* mutants demonstrates that wounding of plant tissue and cell disruption caused by feeding insects is sufficient to induce a rapid, jasmonate-independent GABA synthesis and accumulation. When ingested the enhanced GABA levels become toxic for the insect. Similar to the tissue- and cell disruption-mediated formation of toxic isothiocyanates from glucosinolates and hydrogen cyanide (HCN) from cyanogenic glucosides (Mithöfer *et al.* 2012), respectively, GABA formation from glutamate also

represents a first line of general and rapid defense against invertebrate pests. Thus, GABA contributes to the arsenal of direct and local plant defenses.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Table S1. List of primers used for RT-PCR.

Figure S1. *GAD3-5* expression in different Arabidopsis tissues.

Figure S2. Molecular characterization of *gad1* and *gad2* T-DNA insertion mutants.

Figure S3. Genotyping of *gad12 x pop2-5* triple mutants.

Figure S4. Induction of JA-biosynthesis and JA-responsive genes upon coronalon spray.

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Figure legends

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Figure 6. GABA and Jasmonate levels upon MecWorm treatment in Arabidopsis wild-type (WT), *gad12* and *gad12* x *pop2-5* plants. Mean (± SE, n=6) levels of GABA (a), JA (b) and JA-Ile (c) were determined in control plants (white) and 3 h after treatment (black). Hormone and GABA levels were measured only from treated leaves. Untreated leaves from untreated plants were used as controls. Statistically significant

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Figure 7. Accumulation of GABA in individual Arabidopsis leaves after MecWorm treatment. Mean (± SE, n=5) levels of GABA were determined in individual leaves of untreated control plants and plants after treatment for 1.5 h with MecWorm. In treated plants, leaf 8 was subjected to mechanical damage and systemic leaves 5, 9 and 11, and treated leaf 8 were analyzed for GABA level. Statistically significant differences between the GABA level in the same leaf of the control and treated plant were analyzed by t-test (for each leaf separately, $p< 0.05$, Mann-Whitney-U test), **P=<0.01.

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Figure S1. *GAD3-5* expression in different Arabidopsis tissues. Leaves, flowers and young siliques of wild-type and *gad12* plants were investigated, all grown under greenhouse conditions. Specific primers amplifying the respective genes were used.

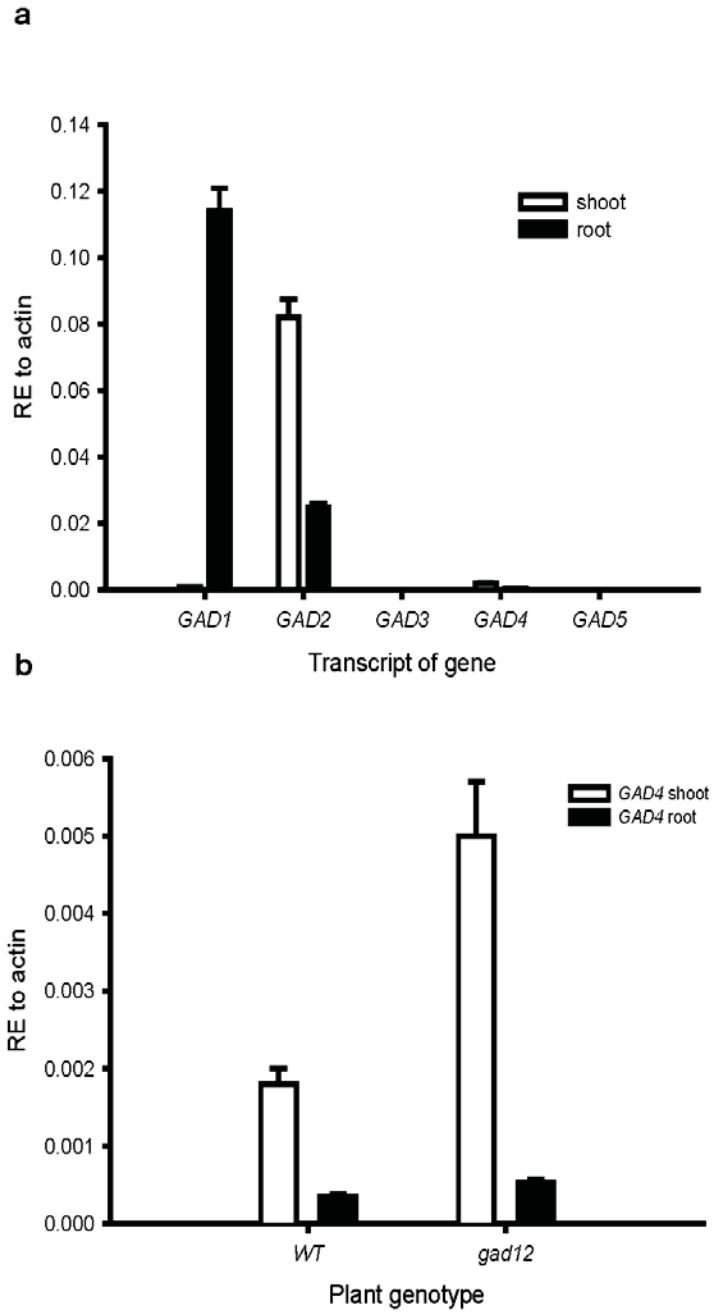
Figure S2. Molecular characterization of *gad1* and *gad2* T-DNA insertion mutants. (**a**) Schematic representation of the T-DNA insertions in *GAD1* and *GAD2* genes. (**b**) Screening of *gad1* and *gad2* mutants with gene- and T-DNA-specific primer combinations. F, R and LB represent gene-specific forward, reverse and T-DNA-specific primers, respectively. (**c**) Transcript analysis of *GAD1* and *GAD2* genes from wild-type (Wt) and *gad12* plants in shoots and roots. (**d**) Sequence of the truncated *GAD2* transcript. Sequences in blue, brown and purple represent exon 1, exon 2 and exon 6, respectively. Sequences in red are of unknown origin, probably intron, but inserted between exon 2 and exon 6. Sequences in bold black represent a stop codon due to a frame shift.

Figure S3. Genotyping of *gad12 x pop2-5* triple mutants. For the analysis T-DNA- and gene-specific primers were used. F, R and LB represent gene-specific forward, reverse and T-DNA-specific primers, respectively.

Figure S4. Induction of JA-biosynthesis and JA-responsive genes upon coronalon spray.

Normalized fold expression (± SE, n=6) of *LOX2, AOS*, *JAR1, JAZ10* and *VSP2* after 1 (white) and 3 h (black) of coronalon treatment. Plants were sprayed with 1 ml of 50 µM coronalon (50 nmol). Expression was normalized to the plant *RPS18* mRNA level. For control, plants were sprayed with the same volume of water, its expression level was set to 1.

Figure 1



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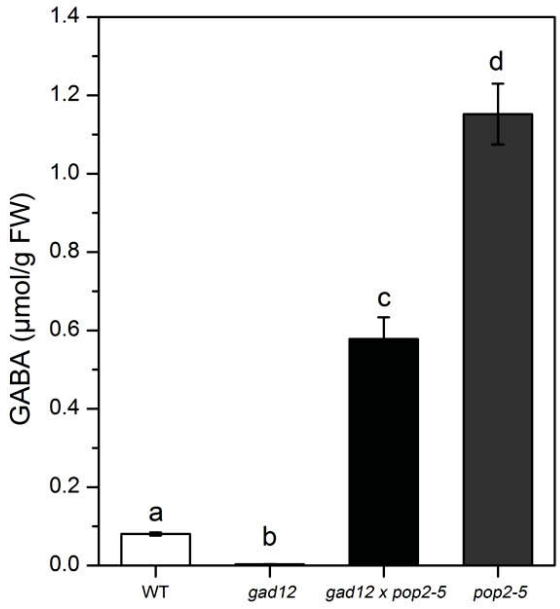

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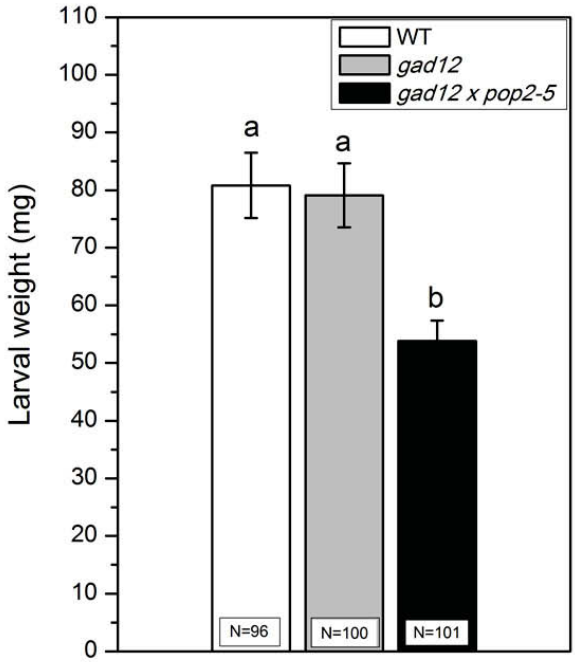

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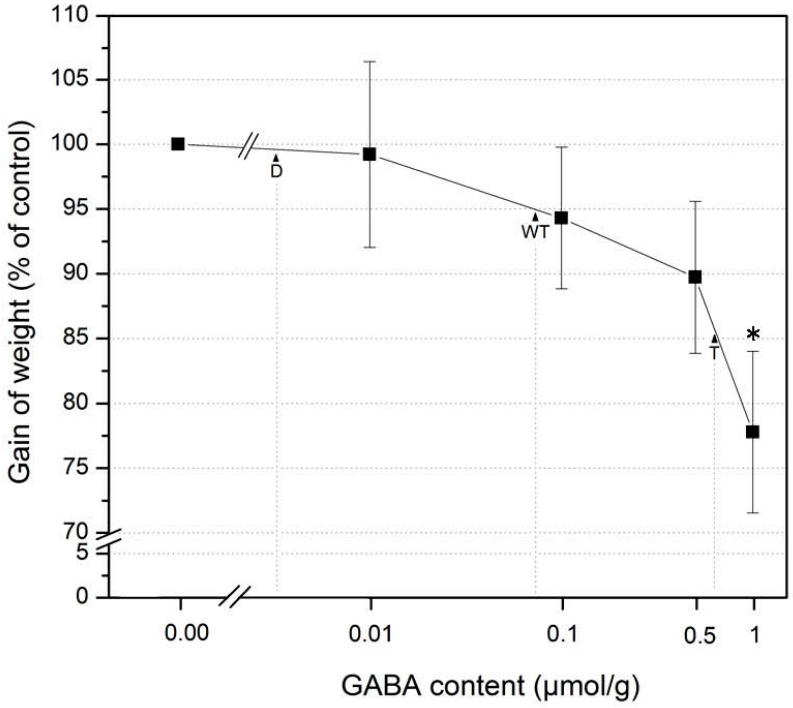

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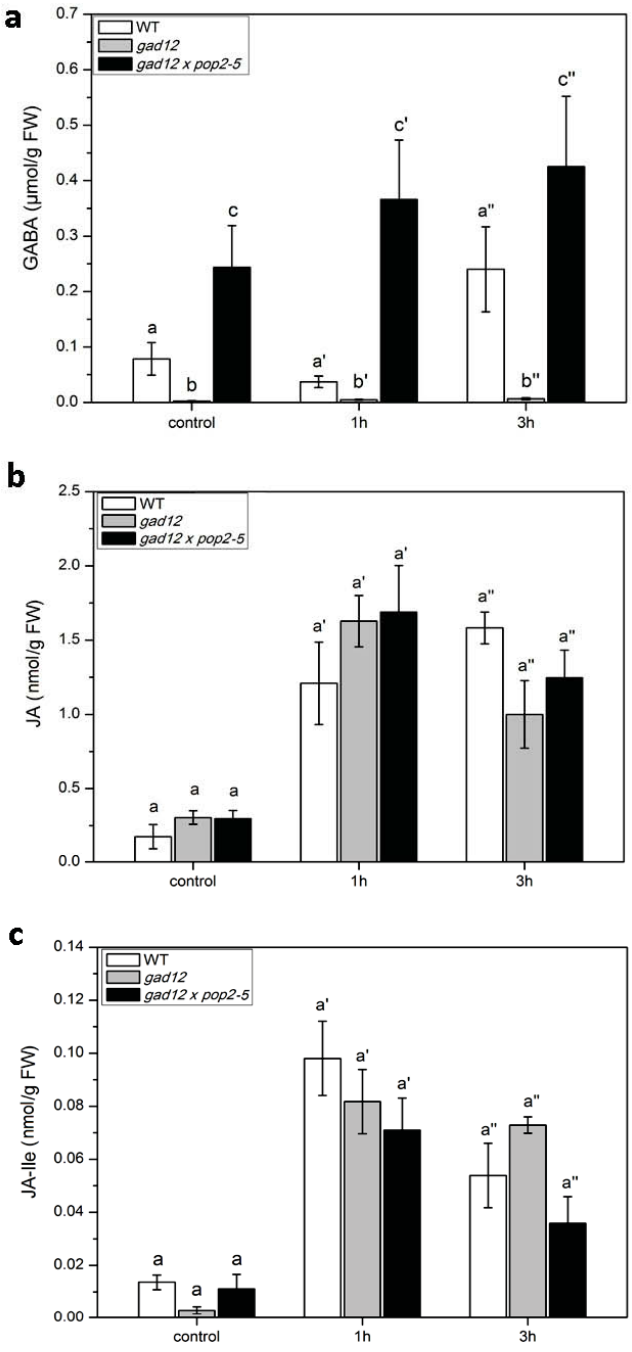

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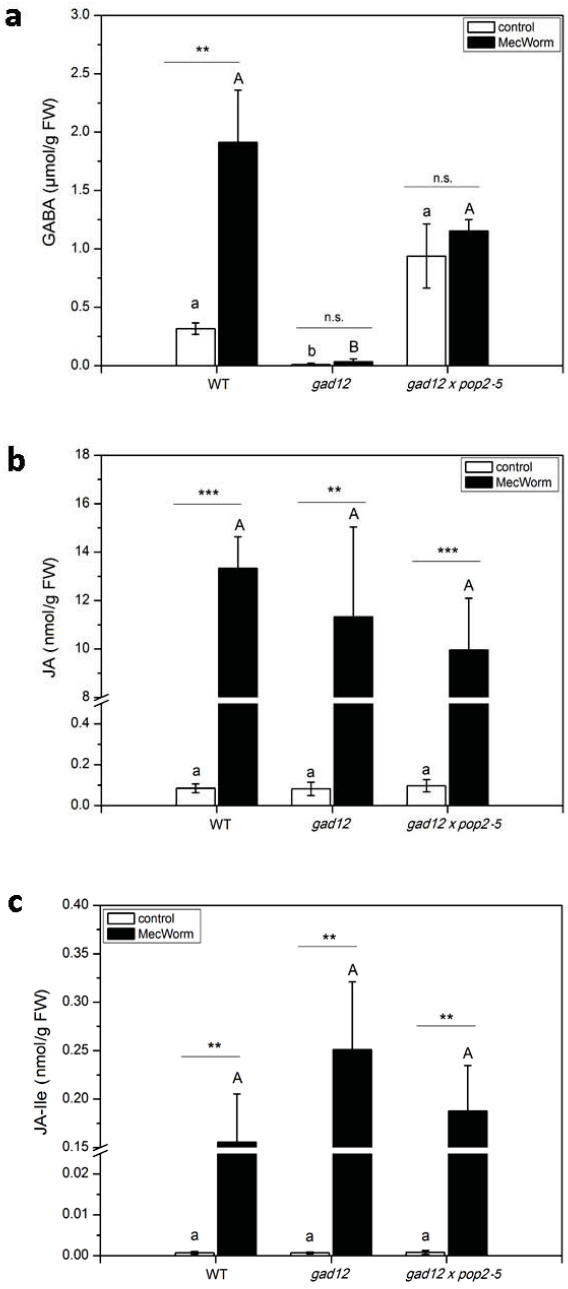

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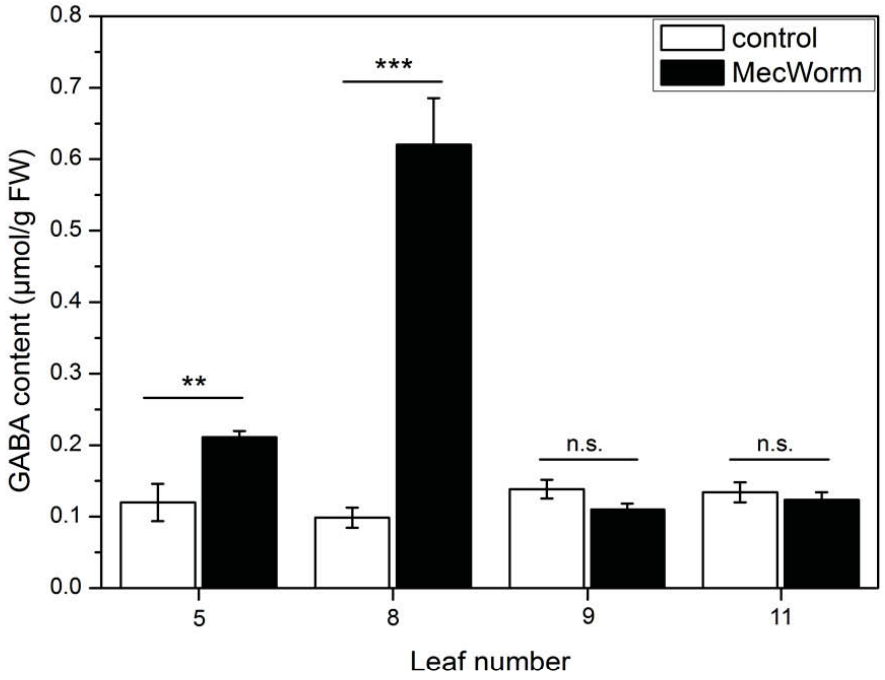

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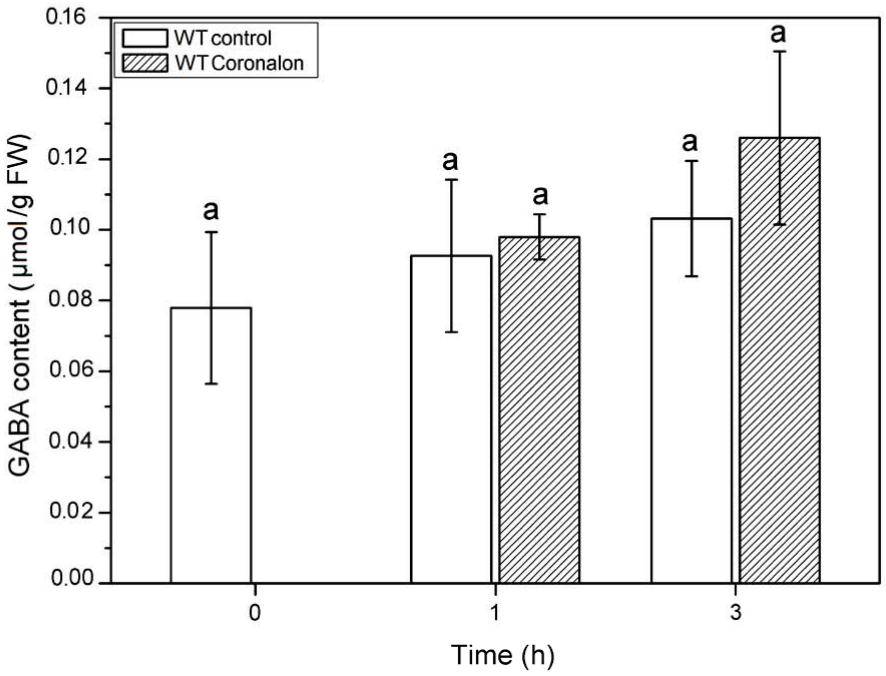

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Figure 9

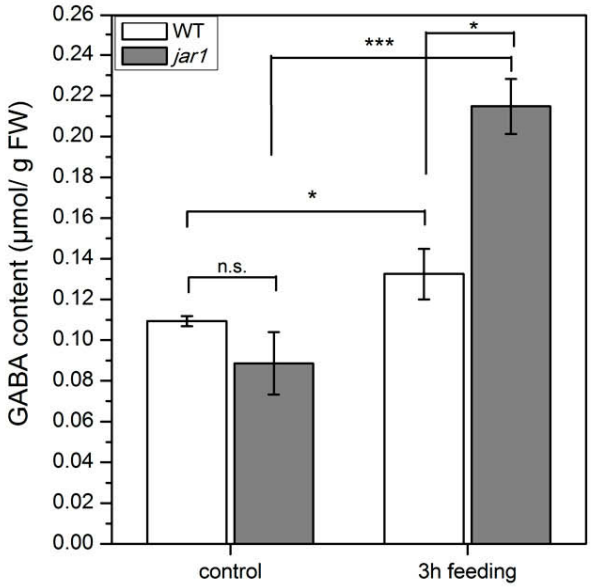

Figure 9. Accumulation of GABA after *S. littoralis* feeding in wild-type (WT) and *jar1* plants. Mean (± SE, n=6) levels of GABA were determined after a feeding period of 3 h. Hormone and GABA levels were measured only from local *S. littoralis*-fed leaves. Untreated leaves from untreated plants were used as controls. Statistically significant differences between the treatments were analyzed by t-test, *P=<0.05, ***P = <0.001.

Table S1. List of primers used for RT-PCR.

Gene	Primer sequence	Orientation	Function
GAD1	CTTGAACGATCTCTTGGTCG	Forward	RT-PCR
	CGCTGTTAGATTCACTCTTCTC	Reverse	RT-PCR
GAD2	CTGTCTGCACCATGTTCGG	Forward	RT-PCR
	CACACCATTCATCTTCTTCC	Reverse	RT-PCR
GAD3	GCACATTTTTCCCTTTACTTTTCTTTAGC	Forward	RT-PCR
	GCTACTAACGGAACGCCG	Reverse	RT-PCR
GAD4	CATTTCAAACCCAAAAATCAAAGTTCG	Forward	RT-PCR
	GCAAATTGTGTTCTTGTTGG	Reverse	RT-PCR
GAD5	GCAAGGTACTTTGAGGTAGAGC	Forward	RT-PCR
	CCAATACTTAGTGATATCCTCC	Reverse	RT-PCR
GAD1	GGCAAGTGGAGGATTCATTG	Forward	qRT-PCR
	TTTCTCCAGATCACCCAACC	Reverse	qRT-PCR
GAD2	GAGAAATTCGCTCGGTACTTCGAG	Forward	qRT-PCR
	GTGTTCTCGTCTACCATTTCTGCTG	Reverse	qRT-PCR
GAD3	CTTTAGGTGACGGTGAAGCCG	Forward	qRT-PCR
	TGGCTCCGGTTACAATATTAGGT	Reverse	qRT-PCR
GAD4	GCTGATTCGTCTTGGATTCG	Forward	qRT-PCR
	AAACGCCACTAACGGAACAC	Reverse	qRT-PCR
GAD5	CAGGATTGCACATCTTGCTG	Forward	qRT-PCR
	CCACAAGGCGTTTCCAATAC	Reverse	qRT-PCR
RPS18	GTCTCCAATGCCCTTGACAT	Forward	qRT-PCR
	TCTTTCCTCTGCGACCAGTT	Reverse	qRT-PCR
VSP2	ACGACTCCAAAACCGTGTGCAA	Forward	qRT-PCR
	CGGGTCGGTCTTCTCTGTTCCGT	Reverse	qRT-PCR
AOS	AAGCCACGCGGCGTTTA	Forward	qRT-PCR
	GGAGTCTCCGTCTCCGGTCCA	Reverse	qRT-PCR
LOX2	ACGCTCGTGCACGCCAAAGT	Forward	qRT-PCR
	CCTCAGCCAACCCCCTTTTGA	Reverse	qRT-PCR
JAR1	TCCGTTTCGTCTGATCGGGATGT	Forward	qRT-PCR
	AGCTTCTTCAGGGTCAGTAGCGT	Reverse	qRT-PCR
JAZ10	TCGAGAAGCGCAAGGAGAGATTAGT	Forward	qRT-PCR
	AGCAACGACGAAGAAGGCTTCAA	Reverse	qRT-PCR

Fig. S1. *GAD3-5* expression in different Arabidopsis tissues.

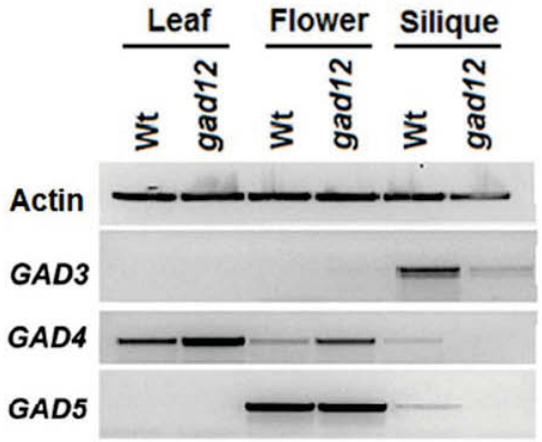

Figure S1. *GAD3-5* expression in different Arabidopsis tissues. Leaves, flowers and young siliques of wild-type and *gad12* plants were investigated, all grown under greenhouse conditions. Specific primers amplifying the respective genes were used.

Fig. S2. Molecular characterization of *gad1* and *gad2* T-DNA insertion mutants.

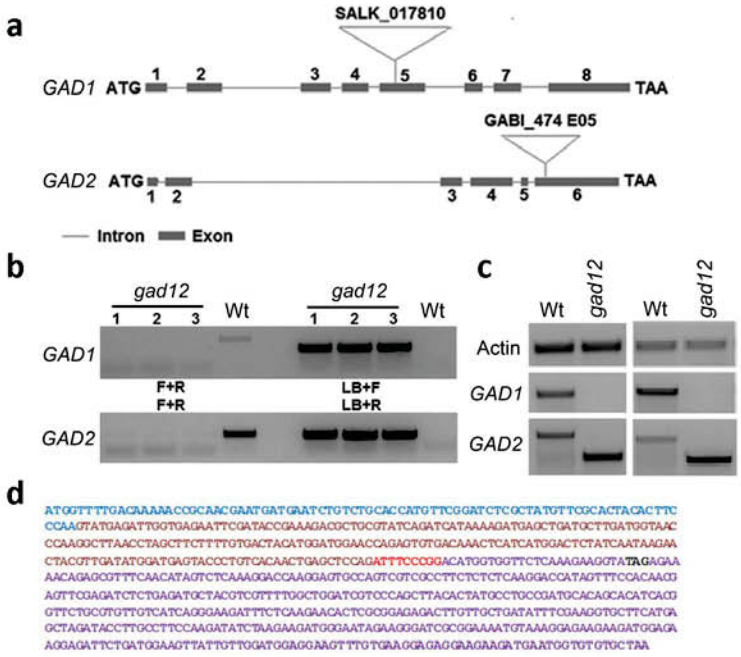

Figure S2. Molecular characterization of *gad1* and *gad2* T-DNA insertion mutants. (**A**) Schematic representation of the T-DNA insertions in *GAD1* and *GAD2* genes. (**B**) Screening of *gad1* and *gad2* mutants with gene- and T-DNA-specific primer combinations. F, R and LB represent gene-specific forward, reverse and T-DNA-specific primers, respectively. (**C**) Transcript analysis of *GAD1* and *GAD2* genes from wild-type (Wt) and *gad12* plants in shoots and roots. (**D**) Sequence of the truncated *GAD2* transcript. Sequences in blue, brown and purple represent exon 1, exon 2 and exon 6, respectively. Sequences in red are of unknown origin, probably intron, but inserted between exon 2 and exon 6. Sequences in bold black represent a stop codon due to a frame shift.

Fig. S3. Genotyping of *gad12 x pop2-5* triple mutants.

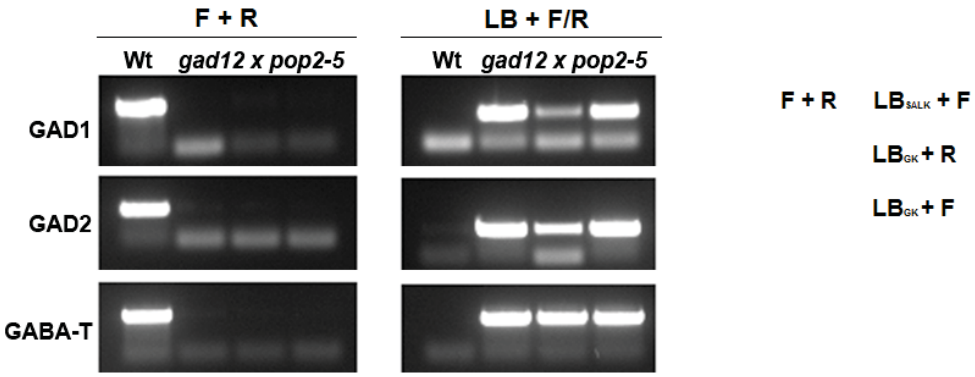

Figure S3. Genotyping of *gad12 x pop2-5* triple mutants. For the analysis T-DNA- and gene-specific primers were used. F, R and LB represent gene-specific forward, reverse and T-DNA-specific primers, respectively.

Fig. S4. Induction of JA-biosynthesis and JA-responsive genes upon coronalon spray.

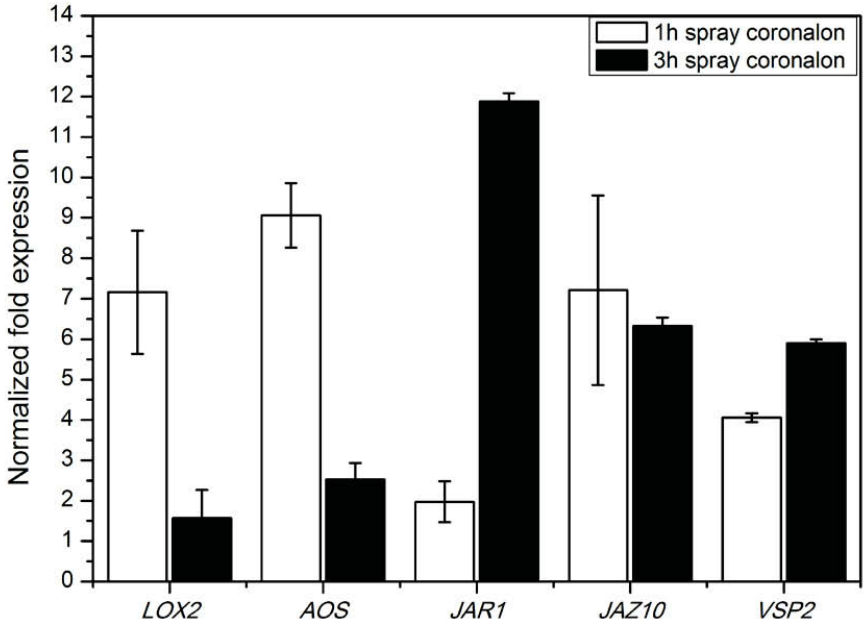

Fig. S4. Induction of JA-biosynthesis and JA-responsive genes upon coronalon spray.

Normalized fold expression (± SE, n=6) of *LOX2, AOS*, *JAR1, JAZ10* and *VSP2* after 1 (white) and 3 h (black) of coronalon treatment. Plants were sprayed with 1 ml of 50 µM coronalon (50 nmol). Expression was normalized to the plant *RPS18* mRNA level. For control, plants were sprayed with the same volume of water, its expression level was set to 1.

4 Unpublished results

4.1 Supplemental experiment for manuscript 2 (Scholz *et al.*, 2014)

The JAR1 enzyme is encoded by a gene belonging to the *GH3* gene family (Staswick *et al.*, 2002; Staswick and Tiryaki, 2004). Genes of this family encode for different enzymes which catalyze the conjugation of amino acids to IAA, JA or SA (Staswick *et al.*, 2005; Terol *et al.*, 2006). To exclude that other enzymes encoded by the *GH3* gene family are able to conjugate Ile with JA in the JAR1 conjugation assay, the assay was redone with the *jar1* mutant included in the setup (Figure S 1).

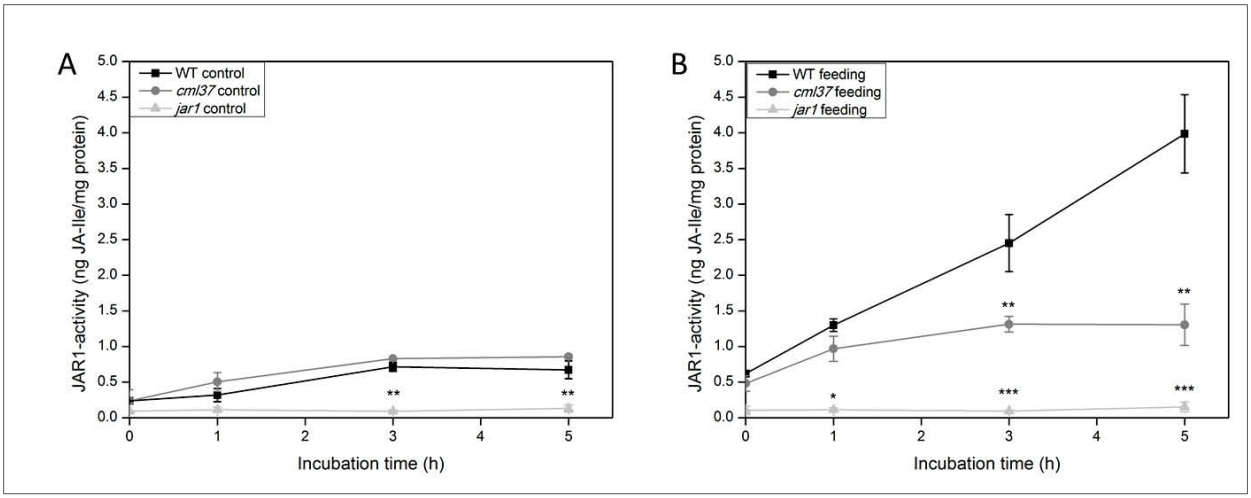

Figure S 1. Level of JAR1 activity in Arabidopsis Wild-Type (WT), cml37 and jar1 plants after herbivory.

Mean conjugation activity by formation of JA-Ile (± SE, n = 6) by JAR1 in Col-0 WT (black), *cml37* (dark gray) and *jar1* (light gray) plant protein extract after *Spodoptera littoralis* feeding for 1 h. Phytohormone levels were measured only from local *S. littoralis*-fed leaves. Untreated leaves from untreated plants were used as controls (0 h). Statistically significant differences between phytohormones in WT and *cml37* plants and between WT and *jar1* plants after feeding were analyzed by t-test for each time point separately, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In the *jar1* mutant, no production of JA-Ile was observed over the whole time span (0-5 h, Figure S 1B). This indicates that, in the used plant protein extracts, no other enzyme is capable of conjugating Ile with JA. So this result supports the hypothesis that the observed reduced production of JA-Ile in *cml37* mutant over time corresponds to a reduced activity of JAR1 (Scholz *et al.*, 2014).

4.2 Supplemental experiment for manuscript 4 (Scholz *et al.*, 2015, Plant Cell & Environment, submitted)

In previous experiments, it was observed that *cml37* mutants show additionally to a reduced elevation of phytohormones also an altered accumulation of GABA after *Spodoptera littoralis* feeding. To study this observation, the content of GABA was determined in *cml37* plants treated with MecWorm (Figure S 2A), mechanical wounding followed by application of oral secretion (MW+OS, Figure S 2B) and *Spodoptera littoralis* feeding (Figure S 2C).

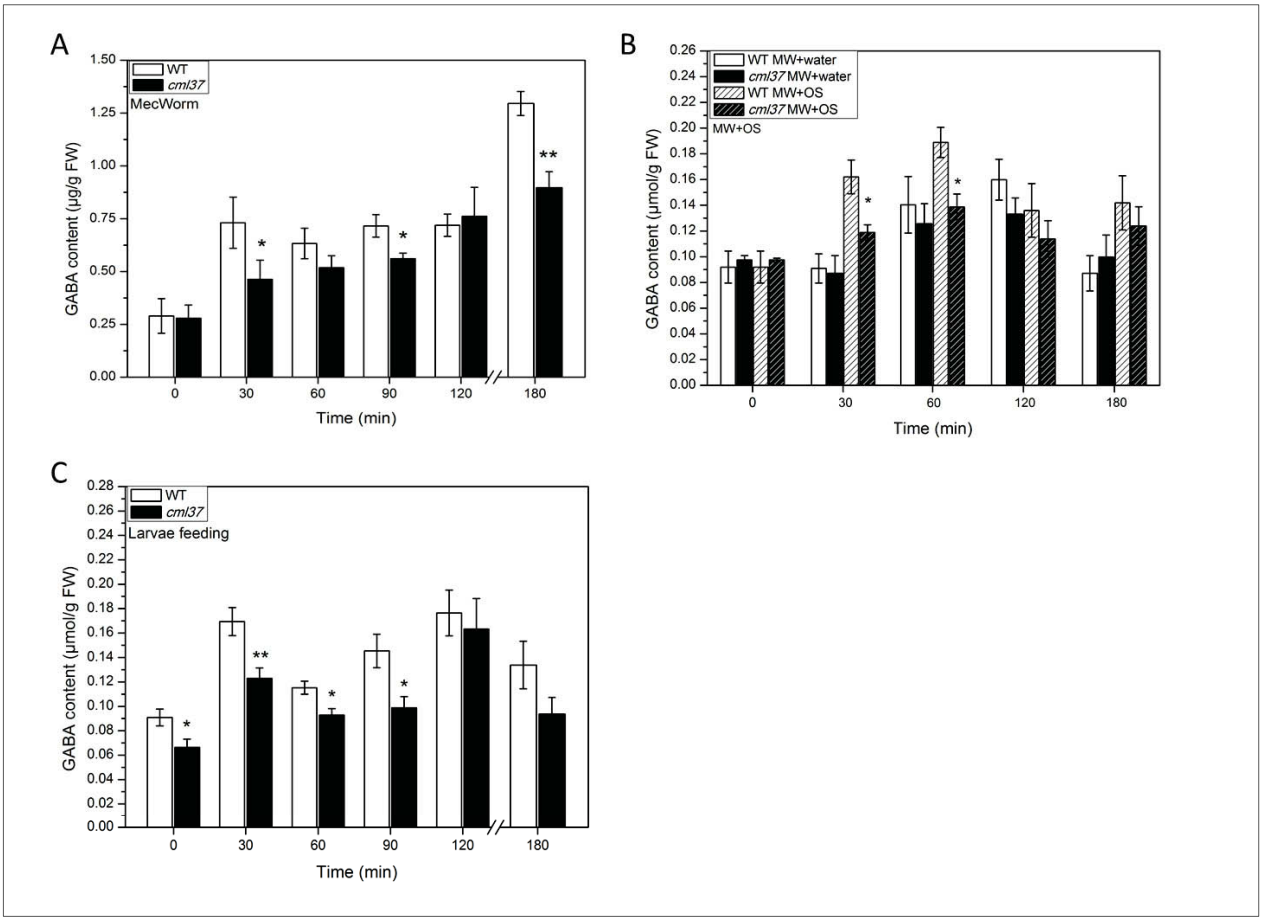

Figure S 2. Accumulation of GABA in Arabidopsis Wild-Type (WT) and cml37 plants in response to different herbivory-related treatments.

Mean content of GABA (± SE, n = 4-8) in Col-0 WT (white) and *cml37* (black) plants after MecWorm treatment (**A**), MW+OS (**B**) and *Spodoptera littoralis* feeding (**C**) for 0-3 h. GABA levels were measured only from local treated or *S. littoralis*-fed leaves. Untreated leaves from untreated plants were used as controls (0 h). Statistically significant differences between GABA levels in WT and *cml37* plants after the respective treatment were analyzed by t-test for each time point separately, * $P < 0.05$, ** $P < 0.01$.

A reduced accumulation of GABA in tissue of *cml37* plants was observed in response to either wounding of the plant by MecWorm (Figure S 2A) or the feeding insect (Figure S 2C) and

application of elicitors in oral secretion (Figure S 2B). This observation corresponds to the results obtained for the *S. littoralis* feeding assay in *cml37* mutant lines, where they gain more weight compared to wild type plants (Scholz *et al.*, 2014). The result suggests that a lower level of GABA in *cml37* plants is another fact triggering the significantly higher increase of *S. littoralis* larval weight.

5 Discussion

To survive and reproduce successfully as sessile organisms, plants must adapt to drastically changes in their environment. This includes challenges of abiotic conditions related to the habitat, like drought or salt stress, temperatures and radiation. Additionally, the plant needs to adjust and react to a multitude of stimuli from the biotic environment, including pathogens and nematodes or fungi. The attack of herbivorous insects can cause a severe loss of plant biomass resulting in decreased plant viability (Stowe *et al.*, 2000). In the herbivore feeding process, the damage of plant tissue is diverse - caused by different insect feeding strategies. While piercing-sucking insects induce only a small lesion, chewing insects consume huge parts of leaves. The wounding of plant tissue is one of the major stress signals perceived and transduced by the plant. A mechanical wounding alone is able to induce several components of plant defense. So it was shown that the robotic larva MecWorm, mimicking the insect feeding dynamic, could induce a volatile emission and ROS production in lima bean which was similar to the one observed after herbivore feeding. At the same time, MecWorm treatment could neither induce membrane depolarization nor the change of cytosolic calcium level, which are able to induce more downstream signaling pathways (Mithöfer *et al.*, 2005; Bricchi *et al.*, 2010).

While feeding on the plant tissue, insects simultaneously provide a complex mixture of HAMPs, which act as additional elicitors of plant defense mechanisms (Alborn *et al.*, 1997; Maischak *et al.*, 2007; Mithöfer and Boland, 2008; Mithöfer and Boland, 2012; Guo *et al.*, 2013). So it was shown that Spodoptera OS could successfully induce membrane depolarization, elevations in cytosolic calcium level, also in systemic leaves, and the expression of elicitor-regulated genes (Maffei *et al.*, 2004; Maischak *et al.*, 2007; Vadassery *et al.*, 2012a; Guo *et al.*, 2013; Kiep *et al.*, 2015). These results indicate that full activation of plant defense upon herbivory is dependent on the perception of wound- and elicitor-induced signals. Different herbivory-related treatments (see Figure 8) are applied to disentangle the complex signaling process.

To defend against attacking insects, the plant has developed a complex network of herbivore defense pathways. Since the production of defensive compounds, which primarily belong to the class of secondary metabolites, is very costly for the plant, the defensive network includes constitutive as well as inducible defense pathways (War *et al.*, 2012). Several important

components of these defense pathways are well studied, but the activation, regulation and interaction between different pathways is still not completely known (Wu and Baldwin, 2010). The best-studied defense pathway responding to herbivory and wounding is the jasmonate pathway. Key components of jasmonate phytohormones are JA and its bioactive isoleucine conjugate JA-Ile (Wasternack, 2007; Howe and Jander, 2008; Chini *et al.*, 2009; Fonseca *et al.*, 2009b; Mithöfer *et al.*, 2009; Wasternack and Kombrink, 2010). By interaction with the SCF-COI1-receptor complex, downstream signaling components like JA-responsive gene expression is induced (Devoto *et al.*, 2002; Thines *et al.*, 2007; Fonseca *et al.*, 2009a). Next to the induction of jasmonate phytohormones, secondary metabolites such as glucosinolates, alkaloids or flavonoids play a role in herbivore defense (Glawischnig *et al.*, 2003; Steppuhn *et al.*, 2004; Halkier and Gershenzon, 2006; Maffei *et al.*, 2006; Falcone Ferreyra *et al.*, 2012; Mithöfer and Boland, 2012). Also for other compounds like the non-protein amino acid, GABA, a role in plant defense was postulated (Bown *et al.*, 2006).

By studying the complex network of plant defense, it became clear that many defense pathways are calcium-mediated and that a stimulus-elicited elevation of cytosolic calcium is an early event inducing downstream signaling components (Maffei *et al.*, 2004; Maffei *et al.*, 2007; Vadassery *et al.*, 2012a). Upon herbivore feeding, stress stimuli like wounding or OS as well as water loss-induced drought stress are able to initiate membrane depolarization and influx of Ca^{2+} into the cytosol, whereby the Ca^{2+} can originate from different external and internal stores (Maffei *et al.*, 2004; Mithöfer and Boland, 2008; Mazars *et al.*, 2009; Dodd *et al.*, 2010; Kiep *et al.*, 2015). This elevation in cytosolic calcium level carries the information of the stimulus perceived (coded in e.g. amplitude or duration) and is further processed and decoded by different calcium sensor proteins to induce the downstream signaling components (DeFalco *et al.*, 2010; Dodd *et al.*, 2010).

The aim of this study was to investigate the role of calmodulin-like proteins (CMLs), one group of calcium sensor proteins, in calcium-mediated herbivore defense pathways in the interaction of *Arabidopsis thaliana* and the generalist herbivore *Spodoptera littoralis*. To understand the mode of action, the induction pattern of different CMLs was characterized under herbivore infestation. To gain insight into the position of CMLs in the signaling cascade and to investigate if a connection between the early signaling event calcium and the induction of defense metabolites

can be detected, *knock-out* mutants of a highly herbivory-inducible CML (CML37, At5g42380) were analyzed for its capability to modulate different defense pathways in Arabidopsis.

Several calmodulin-like proteins are induced upon herbivory

Several studies have outlines the significance of calcium sensors (of the sensor responder group) in herbivore defense signaling. So it was shown that several CPKs in the model plants *Arabidopsis thaliana* and *Nicotiana attenuata* are involved in herbivore defense by regulating the JA pathway. Here the accumulation of JA and the expression of several JA-responsive genes were altered in mutant lines (Kanchiswamy *et al.*, 2010; Wu and Baldwin, 2010; Hettenhausen *et al.*, 2013b; Romeis and Herde, 2014). For members of the sensor relay proteins a role in herbivore defense was not known but assumed. For CML42, which is involved in trichome branching in Arabidopsis, a negative regulation of *Spodoptera littoralis* feeding was demonstrated (Dobney *et al.*, 2009; Vadassery *et al.*, 2012a; Guo *et al.*, 2013). Different members of the CML family such as *CML37, 38, 39, 40* were known to respond to mechanical wounding of the plant (McCormack *et al.*, 2005).

A first microarray experiment, where Arabidopsis plants were wounded and challenged with insect OS, showed that several other members of the CML family were induced upon treatment (Manuscript 1,(Vadassery *et al.*, 2012c)). To gain insight into the level and dynamics of CML expression upon herbivory-related treatments, the expression patterns of different CML genes were analyzed with real-time PCR over the first 90 minutes. While *CML11, 12, 16* and 42 show an early and transient expression (Figure 1, Manuscript 1, (Vadassery *et al.*, 2012a)), *CML9, 17* and *23* are expressed later and for a longer time span (Figure 2, Manuscript 1). Strikingly, the level of expression is very low for the tested CMLs, except for *CML12* which shows an increase in transcript level of about 10-fold (Figure 1, Manuscript 1). This observation matches previous results where *CML12* was shown to be highly induced by touch. Here, a fast and strong initial peak and a transient expression of the respective gene was observed (Braam and Davis, 1990). The rather low expression of other CMLs in our study is also not surprising, since it was shown that CMLs in general show only very low changes in transcript level after various stress treatments (McCormack and Braam, 2003; McCormack *et al.*, 2005). CML9 is mainly involved in pathogen defense and is induced upon SA and *Ps. syringae* treatment. Here CML9 acts in regulating plant immunity by a flagellin-dependent signaling pathway (Leba *et al.*, 2012). This

function is activated upon infestation with the pathogen and could require a more prolonged expression to sufficiently induce plant pathogen defense.

Interestingly *CML37*, another CML family member induced upon insect OS, shows also a 6-fold higher transcript level compared to control treatment (Figure 2C, Manuscript 2, (Scholz *et al.*, 2014)). The expression pattern shows a fast peak followed by a decline in transcript level after 180 min. This dynamic suggests wounding as a major stress stimulus for induction of *CML37*. MecWorm experiments confirmed that *CML37* is highly induced upon mechanical damage alone, where the expression was up to 70-fold induced (Figure 2B, Manuscript 2). In real herbivore feeding experiments, where wounding of the plant tissue and OS are both present, a similar expression level was observed (Figure 2A, Manuscript 2). This indicates that *CML37* is a highly wound-regulated gene like *CML39* or *41*, whereas *CML42* was shown to be specific to elicitors in OS and was not induced upon mechanical wounding (McCormack *et al.*, 2005; Vadassery *et al.*, 2012a). These results indicate that several members of the CML family of calcium sensors are clearly induced upon herbivory and herbivory-related treatments.These differences in expression pattern make CMLs good candidates for regulators of different herbivore-induced defense pathways. Additionally, the results strongly suggest that cytosolic calcium elevations and their specific decoding are an important step in herbivore defense signaling.

Calmodulin-like proteins 37 and 42 show antagonistic regulation upon herbivory

In a previous study *cml42* mutants were analyzed for their performance under herbivore infestation and the ability to induce herbivore defense pathways was quantified (Vadassery *et al.*, 2012a). It was shown that CML42 acts as a negative regulator of plant defense by interfering with COI1-mediated JA sensitivity and is thereby decreasing the expression of JA-responsive genes. In *cml42* mutants *Spodoptera littoralis* larvae gained significantly less weight compared to wildtype plants (Vadassery *et al.*, 2012a). Different from this, *Spodoptera littoralis* larvae gained significantly more weight on *cml37* mutant lines indicating that CML37 acts as a positive defense regulator (Figure 3, Manuscript 2), though both CML37 and 42 were upregulated upon Spodoptera feeding. This indicates that CML37 and 42 show an antagonistic regulation of plant herbivore defense. Combined with the fact that both CMLs are located in the same cell compartments – nucleus and cytosol (Inze *et al.*, 2012; Vadassery *et al.*, 2012a) – this interplay

could be a basis for "fine-tuning" of plant defense pathways. For CML37 several interactions with other calcium sensors like CAMs, CMLs and CPKs was predicted, based on computational calculation (Supplement, Figure S3, Table S1). The mode of action as sensor relay protein requires an interaction with downstream targets to transduce the signal decoded (DeFalco *et al.*, 2010).

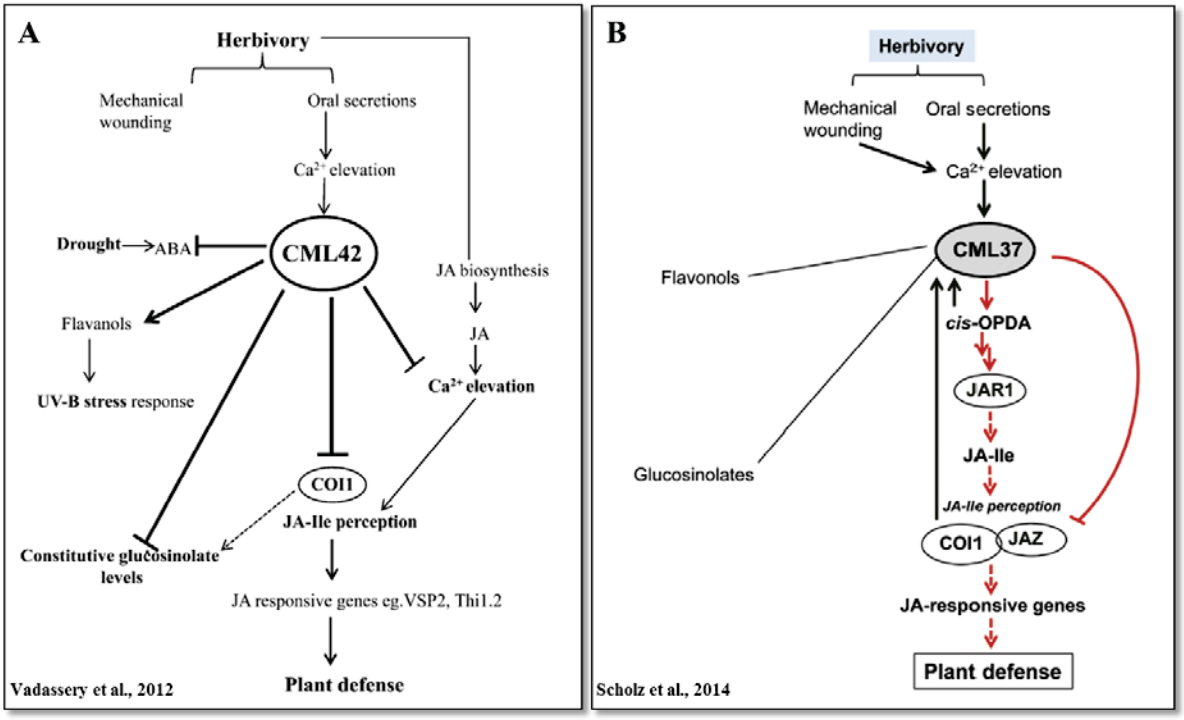

Figure 9. Scheme for herbivory-initiated stress response induction in *Arabidopsis thaliana* and the roles of CML42 and CML37.

Shown are the summarized stress responses of (**A**) CML42 to herbivory and abiotic stress (Vadassery *et al.*, 2012a) and (**B**) CML37 to herbivory (Scholz *et al.*, 2014). Different thickness, ends and color of lines indicate different regulations by the respective CML: (**A**) dotted black line: from literature, black line: not regulated, bold black line with arrow: positive regulated, bold black line with block: negative regulated; (**B**) black line: not regulated, bold black line with arrow: positive regulated, red line: direct regulation by CML37, dotted red line: indirect regulation by CML37. For more details see original publications.

The antagonistic effect on *Spodoptera littoralis* performance feeding on Arabidopsis *cml37* and *cml42* mutant plants is also reflected in the regulation of individual defense pathways (Figure 9). While *cml37* plants do not show a change in level and composition of secondary metabolites like glucosinolates and flavonoids, *cml42* plants constitutively store more aliphatic glucosinolates while the content of kaempferol glycosides is decreased (Figure S4, Manuscript 2, (Vadassery *et al.*, 2012a)). *CML42* expression is independent of herbivory-induced jasmonate accumulation, while *CML37* is significantly induced by JA precursor *cis*-OPDA (Figure 2D, Manuscript 2,

(Taki *et al.*, 2005; Vadassery *et al.*, 2012a)). The incident that *CML37* is induced by *cis*-OPDA, whose production and accumulation is promoted by wounding of plant tissue (Koo *et al.*, 2009), points to an important role of CML37 in regulation of the jasmonate pathway.

The jasmonate pathway finally leads to the expression of anti-herbivore compounds encoded by JA-responsive genes (Berger *et al.*, 1995; Bohlmann *et al.*, 1998; Wasternack and Kombrink, 2010; Wu and Baldwin, 2010). The transcription of these genes is catalyzed by the transcription factor MYC2 which is activated upon release of JAZ repressor proteins when JA-Ile binds the receptor COI1 (Xie *et al.*, 1998; Devoto *et al.*, 2002; Thines *et al.*, 2007; Chini *et al.*, 2009). An antagonistic regulation was also observed for the influence of COI1 on CML expression. Here *CML42* up regulation is negatively regulated by COI1; *CML37* expression is again contrarily regulated. In *coi1-1* mutants, CML37 transcript is significantly less abundant than in WT plants indicating that the presence of COI positively regulates its expression (Figure 2E, Manuscript 2, (Vadassery *et al.*, 2012a)). Additionally, in *cml37* the expression of several *JAZ* genes (including wound-induced *JAZ10*) is increased, leading to a stronger repression of MYC2 (Figure 9, Manuscript 2, (Chung *et al.*, 2008)). These observations are reflected in the expression of JA-responsive genes, which are positively regulated by CML37 whereas the presence of CML42 results in decreased expression levels. In *cml37*, the expression of OPDA-responsive genes (Taki *et al.*, 2005; Schäfer *et al.*, 2011) showed the same pattern (Figure 7 and 8, Manuscript 2). Summarizing all these observations, CML37 and CML42 clearly show an antagonistic regulation upon *Spodoptera littoralis* herbivory. CML37 strongly enhances plant defense by increase of JA-responsive gene expression, while CML42 works against this. In previous studies it was shown that the level of JA-responsive genes has a strong effect on herbivore feeding (Kanchiswamy *et al.*, 2010; War *et al.*, 2012), *knock-out* of CDPKs 4 and 5 in *Nicotiana attenuata* also caused alterations in anti-herbivore compounds influencing herbivore performance (Hettenhausen *et al.*, 2013b). A *cml37xcml42* double knock-out mutant, which was selected for homozygousity and will be available for further experiments, will help to gain more insight into the interplay of these antagonistic operating CMLs. A pathway regulation by both positive and negative regulators can help to balance the expression of genes and is the basis for a fast adaptation to a changing environment. It was shown that regulation of transcription in many eukaryotic systems involves both positive and negative regulatory elements, so are for example WRKY transcription factors active as positive or negative elements (Johnston *et al.*, 1987; Tsuda and Somssich, 2015).

Calmodulin-like protein 37 regulates jasmonate accumulation by regulating JAR1 activity

Jasmonate phytohormones are accumulated upon wounding of plant tissue. Genes involved in JA biosynthesis are known to be induced by jasmonates, raising the hypotheses that a positive feedback loop or jasmonate-induced-jasmonate-biosynthesis exists (Wasternack *et al.*, 2006; Wasternack, 2007; Koo *et al.*, 2009; Wasternack and Kombrink, 2010). For tomato (*Solanum lycopersicum*), lima bean, and *Nicotiana attenuata* leaves it has been shown that such a positive feedback loop does not exist for JA accumulation (Koch *et al.*, 1999; Miersch and Wasternack, 2000; Pluskota *et al.*, 2007). By use of coronalon, a structural mimic of the active JA isoleucine conjugate which was able to induce expression of JA-responsive genes (Figure 2 and A1, Manuscript S1), it was observed that no accumulation of endogenous jasmonic acid, JA-Ile, nor of their hydroxylated metabolites took place (Figure 3 and 5, Manuscript S1). Despite of this, application of coronalon without wounding was able to induce the expression of JA-biosynthesis genes (Figure 2 and A1, Manuscript S1), indicating a feedback loop on JA-biosynthesis enzymes. Simultaneously application of wounding and coronalon showed a much stronger effect on gene expression, confirming the importance of tissue damage as major trigger inducing jasmonate biosynthesis.

In an additional study investigating the transport of jasmonates in the plant, it was shown that 7F-OPC-8:0, an analogue of the JA precursor OPC-8:0, was transported into unwounded systemic leaves (Figure 6, Manuscript S2). This indicates that even precursors of jasmonates, produced upon wounding in the local leaf could be transported to non-wounded systemic leaves to initiate a defense response. Such a reaction was shown in tomato and tobacco plants, where JA-Ile was transported to non-treated leaves (Sato *et al.*, 2009; Sato *et al.*, 2011), while in *Nicotiana attenuata* no transport was observed (Paschold *et al.*, 2008).

A continuous wounding of plant tissue upon herbivore feeding is causing an accumulation of jasmonates (Turner *et al.*, 2002; Devoto and Turner, 2005; Wasternack and Hause, 2013). In *cml42*, the biosynthesis and accumulation of jasmonates upon herbivory were not affected (Vadassery *et al.*, 2012a). Interestingly *cml37* mutant plants showed additional to low JA-responsive gene expression a decrease in accumulation of jasmonates after 48h of Spodoptera feeding. The accumulation of *cis*-OPDA and JA-Ile were significantly reduced compared to wildtype plants (Figure 4 and S2, Manuscript 2). Surprisingly this effect was more pronounced

and earlier visible in the *cml37-2* line, which still has 40% of *CML37* expression (Figure S5, Manuscript 2). The basis for this observation is still not clear. For sure, the decreased elevation of phytohormones is another factor contributing to enhanced susceptibility of *cml37* plants to herbivore feeding. Lack of ability to produce phytohormones upon attack facilitate herbivore feeding, as shown for Arabidopsis *opr3* mutants (Stintzi and Browse, 2000) or *Nicotiana attenuata lox* mutants (Halitschke and Baldwin, 2003). The level of *cis*-OPDA itself, although it is just a precursor of JA, has also an influence on plant defense. This effect is caused by its reactive electrophile structure. When ingested by the feeding herbivore, *cis*-OPDA is isomerized in the insect gut to form the less toxic *iso*-OPDA (Dabrowska *et al.*, 2009; Vadassery *et al.*, 2012b). Several plant genes like *OPR1* and *GST1* are also specifically regulated by OPDA and are referred to as OPDA-responsive genes (Taki *et al.*, 2005; Schäfer *et al.*, 2011).

cml37 mutants show a reduced accumulation of JA-Ile upon herbivore feeding (Figure 4 and S2, Manuscript 2). The respective enzyme conjugating jasmonic acid with isoleucine is JAR1 (Staswick *et al.*, 2002; Suza and Staswick, 2008). In real-time experiments after herbivore feeding, *cml37* plants showed a significant reduced accumulation *of JAR1* mRNA while non-treated plants showed the same level like wildtype plants (Figure 6A, Manuscript 2). This suggests a positive regulation by CML37. This result is only based on transcriptional data and the impact of decrease in mRNA level is not clear. To analyze if the reduced mRNA level is reflected in JAR1 activity on protein level, a JAR1 conjugation assay was performed (Staswick and Tiryaki, 2004). Here the result could be confirmed; the level of produced JA-Ile in *cml37* plant extracts was significantly lower than the one in wildtype plants (Figure 6B, Manuscript 2). To exclude that other enzymes encoded by the *GH3* gene family (Staswick *et al.*, 2005; Terol *et al.*, 2006) are able to conjugate Ile with JA in the JAR1 conjugation assay, the assay was redone with the *jar1* mutant included in the setup (Figure S1, Unpublished results). In *jar1*, no accumulation of JA-Ile was observed, indicating that only activity of JAR1 was measured. Taken together, a reduced JAR1 activity was demonstrated for *cml37* plants. This probably causes lower production of JA-Ile and with this a reduced plant herbivore defense. Whether this reduced JAR1 activity is caused by a lower JAR1 protein level or by post-translational modifications is not clear and needs to be studied. In *Nicotiana attenuata* plants it was similarly shown that silencing of two *JAR1* homologs resulted in decreased herbivore performance (Wang *et al.*, 2007).

Calmodulin-like protein 37 connects Ca^{2+} signaling with jasmonate pathway

Summarizing the results obtained, CML37 regulates the jasmonate pathway by modulating JAR1 activity (Scholz *et al.*, 2014). To investigate if CML37 could be a possible link between the early event calcium and the jasmonate pathway, the biochemical properties of CML37 protein were analyzed. To fulfill the function as calcium sensor relay protein, CML37 needs to successfully bind calcium followed by a conformational change (DeFalco *et al.*, 2010). In previous studies, these properties were analyzed for CML 39, 42 and 43 where all these CMLs showed a real calcium sensor activity (Dobney *et al.*, 2009; Bender *et al.*, 2013; Bender *et al.*, 2014). Also CML37 positively passed the analysis (Figure1, Manuscript 2). It was shown that upon Ca^{2+} binding, CML37 undergoes a conformational change indicated by an increase in helical content about 19% compared to apo-CML37 (Figure1A, Manuscript 2). Also in 8-anilinonaphthalene-1-sulfonic acid (ANS)-fluorescence spectroscopy a pronounced blue-shift and strong increase in fluorescence was observed in the presence of CML37, indicating an increase in surface-exposed hydrophobicity due to a conformational change (Figure1B, Manuscript 2). These results demonstrate that the possible calcium sensor function, predicted by structure analysis of CML37, can be confirmed. Upon Ca^{2+} binding, CML37 undergoes a conformational change enabling an interaction with downstream target proteins. Possible targets of CML37 are not known till now; a computational study revealed a large number of possible partners (Figure S3, Table S1, Supplement). In co-immunoprecipitation analysis, several possible candidates were identified as potential interacting partners of CML37 (data not shown), these results still need to be confirmed in future studies.

It was shown that upon herbivore feeding, different components in OS trigger the elevation of cytosolic calcium (Maffei *et al.*, 2004; Maffei *et al.*, 2007; Maischak *et al.*, 2007; Mithöfer *et al.*, 2009; Vadassery *et al.*, 2012a). Wounding of the plant tissue, both mechanically and by a feeding insect, is able to induce a systemic cytosolic calcium elevation in non-wounded leaves. Interestingly application of OS on mechanical-wounded leaves could decrease the intensity of the induced systemic cytosolic calcium elevation (Kiep *et al.*, 2015). This observation indicates that insect OS contains elicitors which facilitate a bypass of plant defense mechanisms.

To identify the position of CML37 in the signaling cascade, the elicited calcium signal upon different stimuli were analyzed in *cml37* and wildtype plants. Here OS and different jasmonates

were tested in aequorin-expressing Arabidopsis plants that were generated for the questions (Figure 5 and S2, Manuscript 2), (Knight *et al.*, 1997). Jasmonates are induced by wounding and are one component of OS able trigger cytosolic calcium elevations (Vadassery *et al.*, 2012a; Vadassery *et al.*, 2012b). The application of *Spodoptera littoralis* OS showed similar cytosolic calcium elevations in both wildtype and *cml37* plants (Figure S2a, Manuscript 2). This suggests a position for CML37 downstream of OS-induced Ca^{2+} signals in the signaling cascade. While the application of the active jasmonate JA-Ile induced again similar cytosolic calcium elevations in wildtype and *cml37* plants (Figure 5 and S2c, Manuscript 2), JA was not able to induce a cytosolic calcium elevation in *cml37* plants (Figure 5 and S2b, Manuscript 2). This again indicates that an altered activity of JAR1 in *cml37* plants may be responsible for lower levels of jasmonates as well as for a reduced herbivore defense. With this we could show for the first time that a calcium sensor - CML37 - is a crucial signaling component connecting Ca^{2+} and JA signaling. In *cml42* plants, JA-induced Ca^{2+} elevations were more sensitive - indicated by a higher maximum peak height - but showed the same trend like observed for wildtype plants (Vadassery *et al.*, 2012a). These results indicate that the early signaling event calcium plays a crucial role in activation of jasmonate-based herbivore defense.

Calmodulin-like proteins are involved in ABA signaling

CMLs are also induced by various stress factors originating from the abiotic environment (McCormack *et al.*, 2005). So it was shown that CML37 is induced by salt, cold, ozone or drought stress (Vanderbeld and Snedden, 2007). Drought stress is also occurring during herbivore attack, since wounding of the plant tissue and disruption of plant cells causes water loss and associated osmotic pressure (Zhu, 2002; Aldea *et al.*, 2005). Interestingly, insect OS is able to suppress feeding-induced water loss of Arabidopsis host plants (Consales *et al.*, 2011). The phytohormone ABA is a major signaling component mediating drought stress in plants (Zeevaart and Creelman, 1988). It was shown that several CMLs are involved in ABA signaling; CML9 plays an essential role in modulating responses to salt and drought stress as well as ABA (Magnan *et al.*, 2008). Also CML42, a regulator of herbivory, is involved in drought-induced ABA signaling (Figure 9). Here, *cml42* plants show enhanced accumulation of ABA after different periods of drought treatment (Vadassery *et al.*, 2012a). *cml37* plants subjected to drought stress, show a drastically reduced accumulation of ABA (at both time points) compared

to wildtype and *cml42* plants (Figure 2, Manuscript 3). This low level of ABA can be related to a high water loss of the plant due to reduced closure of stomata (Leckie *et al.*, 1998). Drought-stressed *cml37* plants, accumulating lower levels of ABA, show a clear phenotype indicating water loss. While wildtype and *cml42* plants are still vital after 2 weeks of drought, all *cml37* plants are dried out (Figure 1, Manuscript 3). This indicates an again antagonistic regulation by CML42 and CML37: the signaling of ABA during drought. Keeping in mind that more and more evidence occurs that ABA shows activity in modulating herbivore defense (Atkinson and Urwin, 2012; Dinh *et al.*, 2013; Vos *et al.*, 2013), the low accumulation of ABA in *cml37* plants could influence herbivore defense. To study this question the expression of CML37 should be analyzed in different ABA mutant lines to detect possible cross-effects.

Plant defense is composed of different Ca^{2+}- mediated pathways

As discussed above, calcium plays an important role in activation of different plant defense-related pathways like the jasmonate pathway. Recent studies suggest that also the accumulation of γ-amino butyric acid (GABA) in plants might act as defense mechanism against herbivores (Bown *et al.*, 2006). The production of GABA from glutamate is a Ca^{2+}-mediated reaction since the catalyzing enzymes GADs (glutamate decarboxylases, Figure 1 and 2, Manuscript 4) are calmodulin-regulated (Snedden *et al.*, 1995; Zik *et al.*, 1998; Snedden and Fromm, 1999). Upon calcium-binding under neutral pH GADs are activated and GABA is produced. Acidifying of the cytosol caused by tissue damage and rupture of vacuoles mimicked by MecWorm treatment, can induce a calcium-independent activation of GADs ((Figure 6a, Manuscript 4, (Carroll *et al.*, 1994). During *Spodoptera littoralis* feeding and associated leaf wounding, GABA accumulates in the respective plant tissue (Figure 5a, Manuscript 4). Using different GABA mutant lines it was shown that *Spodoptera littoralis* larvae gain significantly less weight in the mutant line which is accumulating high levels of GABA during time (Figure 2 and 3, Manuscript 4). This indicates a feeding-deterrent effect of GABA which was already postulated for *Choristoneura rosaceana* larvae, reared on GABA-containing diet, that showed reduced body weight and prolonged time to pupation (Ramputh and Bown, 1996). Rearing of *Spodoptera littoralis* larvae on GABA-containing artificial diet showed that a concentration of 1 μmol g^{-1} GABA reduced larval growth significantly (Figure 4, Manuscript 4). This observation is explainable by the properties of GABA, which is acting as neurotransmitter activating GABA-inducible Cl^--

channels. An increased level of GABA in the insect leads to hyper-activation of these channels eventually initiating paralysis (Bown *et al.*, 2006). The accumulation of jasmonates upon herbivore feeding was not altered in different GABA mutant lines (Figure 5 and 6, Manuscript 4) and coronalon application did not induce GABA synthesis (Figure 8, Manuscript 4). These results show that GABA is a jasmonate-independent defense pathway. Interestingly, accumulation of GABA is not restricted to the local treated leaf as GABA was detected in systemic adjacent leaves (Figure 7, Manuscript 4). This suggests that GABA acts as systemic defense against herbivores. Knowing that wounding and herbivory can stimulate a systemic increase of cytosolic Ca^{2+} concentration also in non-wounded leaves (Kiep *et al.*, 2015), this Ca^{2+} increase could trigger the activity of GADs and induce systemic GABA synthesis demonstrated *in vitro* (Snedden *et al.*, 1995). Summarizing these results, insect herbivory-elicited GABA accumulation in plants is a wound-induced, direct, systemic and jasmonate-independent defense response mediated by calcium.

Interestingly, in *cml37* mutant plants, the GABA accumulation upon MecWorm treatment and *Spodoptera littoralis* feeding is reduced in local leaf, while application of OS did not show significant differences (Figure S2, Unpublished results). This suggests that the effect is caused upon mechanical wounding of *cml37* plant tissue. The reason for this observation is not known and further studies are necessary. However, the preliminary results already indicate that CML37 might also play a role in herbivory-induced GABA accumulation.

Outlook

The induction pattern of CML37 upon herbivory is still not completely studied, since only the response to insects with chewing feeding behavior was investigated. Given the fact that *CML37* is induced by both mechanical wounding and OS, the expression pattern will be analyzed in plants infested with insects causing only low tissue damage: spider mites (collaboration TU Dresden). Here the influence of mechanical wounding is reduced. Additionally, the response to spider mites will be studied in different CML *knock-out* plants, including *cml37, cml42* and *cml9*. These studies can provide more information on regulation of CML proteins upon a different stress composition. Here, also a double *knock-out* mutant *cml37xcml42* will be analyzed. The generated double *knock-out* mutant *cml37xcml42* will be tested in insect feeding assays with *Spodoptera littoralis* larvae and will be characterized for the ability to induce

different herbivore defense components including jasmonates and glucosinolates. The study of this mutant will help to understand the antagonistic regulation of these two CMLs.

To complete the knowledge about the mode of action of CML37 and 42, interacting partners of both proteins will be further analyzed by co-immunoprecipitation assays. Here stable transgenic lines, carrying a tagged CML-protein (CML37-GFP and CML42-Flag) are used. First experiments generated some candidates, which need to be proven by further experiments. For both CMLs, possible interacting proteins of the ACA family (Ca^{2+}-dependent ATPases) were identified. Proteins of this family are transmembrane proteins responsible for active transport of Ca^{2+} ions from the cytosol into different Ca^{2+} stores (Sze *et al.*, 2000). For CML37, ACA4 (Geisler *et al.*, 2000) and ACA11 (Lee *et al.*, 2007), two proteins localized in the vacuole membrane, were repeatedly found as possible partners. ACA4 was also a candidate found in Co-IP with CML42 as target protein, next to ACA1 (Malmström *et al.*, 1997), which is located in the envelope of chloroplasts (experiment by A. Yilamujiang, (Yilamujiang, 2012)). Thus, all these possible interacting partners are involved in recovering the Ca^{2+} homeostasis after the stimulus was perceived.

All these experiments will help to get insight into the complex regulation and interaction of different CMLs upon abiotic and biotic stress. The plant pocesses 50 different CML proteins expressed in different plant tissues and during different developmental stages. These studies of single cml *knock-outs* under different conditions can be a useful tool to understand the plants need to express this huge amount of calcium sensors to guarantee a proper decoding of environmental stimuli and to achieve a proper adaptation to its environment.

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7 Supplement

Manuscript S1

Additional evidence against jasmonate-induced jasmonate induction hypothesis

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Abstract

Jasmonates are phytohormones involved in development and stress reactions. The most prominent jasmonate is jasmonic acid, however, the bioactive jasmonate is (+)-7-*iso*-jasmonoyl-*L*-isoleucine (JA-Ile). Biosynthesis of jasmonates is long time known; compartmentalization, enzymes and corresponding genes are well studied. Because all genes encoding these biosynthetic enzymes are jasmonate inducible, a hypothesis of jasmonate-induced-jasmonate-biosynthesis is widely accepted. Here, this hypothesis was revisited by employing the synthetic JA-Ile mimic coronalon to intact and wounded leaves, which excludes structural cross-contamination with endogenous jasmonates. At an effective concentration that induced various jasmonate-responsive genes in Arabidopsis, neither accumulation of endogenous jasmonic acid, JA-Ile, nor of their hydroxylated metabolites was detected. Results indicate that in spite of jasmonate-induced biosynthetic gene expression, no jasmonate biosynthesis/accumulation takes place supporting a post-translational regulation.

Keywords:

Jasmonates; coronalon; wounding; JA-Ile; jasmonate responsive genes.

1. Introduction

In higher plants, low molecular lipid-derived signal molecules are ubiquitously distributed and involved in many developmental processes as well as in many different stress-related physiological responses [1-3]. Fatty acid-derived octadecanoids such as *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA) and jasmonic acid (JA) are well known examples for the so-called jasmonates, an important class of oxylipin phytohormones. The biosynthesis of octadecanoids, starting from α-linolenic acid, was first reported by Vick and Zimmermann [4]. Today, the whole biosynthetic pathway of jasmonates is elucidated in detail including the identification and cloning of all enzymes involved, as described in many reviews [1,2,5,6]. Briefly, biosynthesis of jasmonic acid (JA) takes place in three different cell compartments. In the chloroplast, α-linolenic acid is released from membranes, activated by a 13-lipoxygenase (13-LOX) to a hydroperoxyoctadecatrienoic acid, which is further converted to an unstable epoxide by action of a 13-allene oxide synthase (13-AOS); followed by an allene oxide cyclase (AOC) catalyzed cyclization to *cis*-OPDA. After transport of *cis*-OPDA into peroxisomes the cyclopentenone ring is reduced by a *cis*-OPDA reductase 3 (OPR3) and subsequently the carboxylic acid side chain is shortened by β-oxidation to generate (+)-7-*iso*-JA, which is again released into the cytosol and epimerizes to the less active (−)-JA. It is worth to mention that the expression of all genes for JA biosynthesis is inducible by jasmonate treatment [7-9], suggesting a jasmonate-induced-jasmonate-biosynthesis. However, only in 2004 it became clear that not JA itself but its isoleucine conjugate represents the active phytohormone [10]. This conjugation is catalyzed by JASMONATE RESISTANT 1 (JAR1) using (+)-7-*iso*-JA as the JA substrate [10]. As the endogenous bioactive jasmonate (+)-7-*iso*-jasmonoyl-*L*-isoleucine (JA-Ile, Fig. 1) was identified [11]. Strikingly, JA-Ile is the only jasmonate that interacts with the corresponding SCF^{COI1}-JAZ co-receptor complex thereby initiating the jasmonate depending responses in a plant cell [12,13]. In detail, upon JA-Ile binding, the COI1-JA-Ile subunit of the SCF^{COI1} complex (acting as an E3 ubiquitin ligase) interacts with JAZ proteins and forms the whole co-receptor complex [14]; JAZ proteins, acting as repressors of jasmonate signaling, are subsequently ubiquitinated and targeted for 26S proteasome-mediated degradation [2]. This activates transcription factors such as MYC2, subsequently the expression of JA-responsive genes and, as a consequence thereof, the onset of defense reactions [2].

Interestingly, coronatine, a bacterial phytotoxin from *Pseudomonas syringae* consisting of the polyketide coronafacic acid and the rare cyclopropyl amino acid, coronamic acid [15], is also able to induce typical jasmonate-induced responses. The thereby suggested interaction between coronatine and the JA-Ile co-receptor complex has already been shown, demonstrating high affinity binding [14]. Because the synthesis of coronatine is complex and tedious, alternative compounds exhibiting the same biological

activities have been designed and synthesized as structural mimics of coronatine, namely the 6-substituted 1-oxoindanoyl isoleucine conjugates [16-18]. In particular, a 6-ethyl-indanoyl-isoleucine conjugate (2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester), coronalon (Fig. 1), has been widely tested and established as efficient mimic of various jasmonate-induced responses in plants; among others, induction of secondary metabolites, volatiles and defense-related genes in various plant species, induction of intracellular calcium transients, pest resistance in field studies, root growth inhibition [16,17,19-21], for reviews: [22,23]. Only recently, based on modeling studies it was predicted for lima bean (*Phaseolus lunatus*) that coronalon can directly interact with the COI1-JAZ co-receptor as well [18].

In the present work we use the JA-Ile mimic coronalon to re-investigate the hypothesis of jasmonate-induced jasmonate accumulation in plant cells. By studying the effects of exogenous application of jasmonates - or their structural mimics like coronalon - it clearly has to be distinguished between a feedback loop in terms of activation of JA biosynthesis genes and expression of JA-responsive genes on one side, and accumulation of endogenous jasmonates on the other side. This work was motivated by the fact that very many reviews on jasmonate biosynthesis suggest a jasmonate-induced-jasmonate-biosynthesis regulation of JA biosynthesis and accumulation [1,2,24-26] although for tomato (*Solanum lycopersicum*), lima bean, and *Nicotiana attenuata* leaves it has been shown that such a positive feedback loop does not exist for endogenous JA accumulation [27-29]. In addition, for *Arabidopsis thaliana* leaves it has been demonstrated that coronatine application cannot induce JA-Ile accumulation in contrast to wounding [30]. Thus, in this study not only both jasmonates, JA and the bioactive JA-Ile, but also their first degradation metabolites are investigated in order to discover potential differences in JA versus JA-Ile accumulation. Moreover, in addition to earlier studies, the effect of wounding in combination with exogenous jasmonate application was studied to address the possibility of additive or synergistic effects with respect to jasmonate accumulation.

2. Material and Methods

2.1 Plant material and treatment

Arabidopsis thaliana ecotype Columbia was used for all experiments and plants were grown as described before [31]. Four to five week old plants, grown under short-day conditions, were used. For wounding, each side of the leaf was treated with a pattern wheel (six vertical motions) followed by an immediate application of 10 µL of 50 µM of coronalon or solvent control (0.1 % ethanol) on each side (20 µL per leaf). For coronalon spray treatment, plants were sprayed with 1 mL of 50 µM coronalon or

solvent control. All plants were incubated for the indicated time points. To minimize evaporation of the applied solutions, plants were incubated with a translucent cover. Coronalon was synthesized as described [18]. For double treatment with coronalon and α-linolenic acid (LA; 18:3), plants were pretreated for 1 h with 500 µM LA spray followed by coronalon application and subsequent incubation (with cover) for 1 and 3 h, respectively.

2.2 RNA Extraction and Q-RT-PCR

For RNA extraction 100 mg of fresh plant material was used. Samples were homogenized for 1 minute at 1000 rpm in the Genogrinder 2010 (Spex Sample Prep, Stanmore, UK) and extracted following the protocol described before [32]. Q-RT–PCR was carried out in 96-well plates on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA) by the use of Brilliant II QPCR SYBR green Mix (Agilent, Böblingen, Germany). The obtained mRNA levels of the genes of interest were normalized to the *RPS18B* mRNA level in each cDNA sample and the dissociation curve was analyzed for all primer pairs. In the Bio-Rad CFX Manager Software (3.1), expression levels of genes of interest were calculated by use of the Normalized Expression (ΔΔCq). The primer pairs used are listed in supplementary materials (Table A1).

2.3 Phytohormone analysis

For jasmonate phytohormones and derivatives extraction (jasmonic acid, JA; (+)-7-*iso*-jasmonoyl-*L*-isoleucine, JA-Ile; OH-JA; 12-OH-JA-Ile), 250 mg of fresh plant material was used. Samples were homogenized for 1 minute at 1000 rpm in the Genogrinder 2010 (see 2.2), extracted and analyzed according to [31]. Briefly, for JA and OH-JA (here we could not discriminate 11-OH-JA and 12-OH-JA), 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid and for JA-Ile and 12-OH-JA-Ile 15 ng of jasmonic acid-[$^{13}C_6$]isoleucine conjugate were used as internal standards. For chromatography an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany) equipped with a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent) was used. For mass spectrometry, in the negative ionization mode, an API 5000 tandem mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbospray ion source was employed in MRM modus. Concentration of OH-JA was calculated using 9,10-D_2-9,10-

dihydrojasmonic acid applying a response factor of 1.0; 12-OH-JA-Ile was quantified using jasmonic acid-[$^{13}C_6$]isoleucine applying a response factor of 1.0.

3. Results and Discussion

As previously described, JA biosynthesis genes are induced by jasmonates [7,8]. This led to the claim of a jasmonate-induced jasmonate accumulation or a positive feedback loop in jasmonate biosynthesis, respectively [1,2,24-26]. Strikingly, although it was demonstrated for tomato leaves by feeding deuterated precursor and for lima bean, *N attenuata* and Arabidopsis leaves by treating with jasmonate mimics that jasmonate biosynthesis and accumulation was not induced by jasmonates [27-30], these findings are often ignored and it was not distinguished between biosynthesis gene induction and jasmonate biosynthesis. Therefore, we decided to reinvestigate the hypothesis of jasmonate-induced-jasmonate-biosynthesis using coronalon instead of labeled jasmonate precursor. The high biological activity at low concentrations makes the JA-Ile mimic coronalon a valuable and versatile signaling compound for the induction and examination of jasmonate depending responses in plants [17,22]. These features predestine coronalon for studies where endogenous level of jasmonates, in particular of JA-Ile, is investigated although simultaneously the plant is challenged with exogenously applied jasmonates. Thus, due to the different chemical structures (Fig. 1), the risk of endogenous JA-Ile level contamination with exogenous JA-Ile and incorrect measurements can easily be ruled out. Recently, a similar approach to directly assess the effect of jasmonates without potential secondary effects associated with tissue damage was performed using coronatine [33].

In order to ensure that the coronalon treatment was sufficient to induce jasmonate-responsive gene expression we chose two different approaches; first, spraying Arabidopsis leaves with 1 mL coronalon (50 nmol) and second, wounding of a leaf with a pattern wheel and immediate application with in total 20 µL of a 50 µM coronalon (1 nmol) solution directly on the small wounds. As shown in Figure 2A, two genes involved in jasmonate biosynthesis encoding LOX2 [34] and AOS [35] were upregulated already one hour after coronalon spray compared to the spray control. After three hours the induction was lower but still detectable. Moreover, the gene for a jasmonate signaling protein, JASMONATE-ZIM DOMAIN 10 (JAZ10) [36] (Fig. 2B), was inducible and detectable after coronalon spray at both time points as well as the defense-related gene encoding vegetative storage protein 2 (VSP2) [37] (Fig 2C). The wounding approach showed similar results although it is obvious that gene inductions were much stronger (Fig. 2, A1). In particular *JAZ10* but also *VSP2* expression levels were drastically enhanced (Fig. 2). Here, it is worth mentioning that the induction kinetics of early biosynthetic genes is different compared with the

late-response gene *VSP2*. Whereas *LOX2* and *AOS* show higher induction after one hour of treatment, *VSP2* has its maximum after three hours, representing the expected kinetics (Fig. 2). Based on these results we can conclude that both treatments are effective and sufficient to initiate typical jasmonate responses in terms of gene expression indicating the practicability of the experimental approach.

Next, the jasmonate phytohormone content of Arabidopsis leaves which were treated in the same way was determined. In contrast to previous studies where either JA or JA-Ile [28-30] was determined, we analyzed both jasmonates as well as their inactivation products in parallel. As can be seen in Figure 3, the accumulation pattern of both active compounds was similar; only the amount of JA was higher than that of JA-Ile, as known from numerous studies performed before. Spaying with coronalon neither induced biosynthesis and accumulation of JA nor of JA-Ile within three hours, compared to the controls (Fig. 3) and in agreement with findings from Koo et al., 2009 [30]. In contrast to this observation, higher levels of JA and JA-Ile were determined upon wounding and wounding plus coronalon application, both after one and three hours (Fig. 3). This increase of JA and JA-Ile was not unexpected because many examples demonstrated a burst of JA and JA-Ile upon wounding alone [38-42], which was also independent on the presence of exogenously applied jasmonates emphasizing the importance of the wounding trauma [30,43].

Consequently, for injured plants, no significant difference in jasmonate levels was found between coronalon-treated and water-treated plants (Fig. 3). On the one hand this result indicates that wounding alone was responsible and sufficient for JA and JA-Ile accumulation and on the other hand that even an additional challenge with another jasmonate could not induce higher levels of the respective compounds. Neither an additive nor a synergistic effect was found. Even three hours of coronalon treatment could not increase JA and JA-Ile contents, actually the amount declined, although these last results were not significant. This excludes the possibility that exogenously applied jasmonate-mimic is taken up only slowly and exhibits its activities with delay. The observed gene induction after one hour also supports this point of view (Fig. 2). In addition, a rapid appearance of jasmonates within minutes upon wounding was shown in local and interconnected leaves in Arabidopsis [40,44]. Strikingly, this increase of jasmonate levels was detectable prior to jasmonate biosynthesis gene expression, suggesting that, with respect to endogenous jasmonate accumulation, there is no feedback loop necessary. This shows the plants' ability to form jasmonates without preceding gene expression. In earlier studies it was shown that enzymes of jasmonate biosynthesis such as LOX and AOS are expressed abundantly in Arabidopsis as well as other species [45,46]. Thus, in case of wounding and subsequent release of, for example, LA as biosynthetic jasmonate precursor, it is conceivable that the presence of substrates could result in a fast accumulation of jasmonates, even before expression of jasmonate biosynthesis genes was significantly induced [1,46,47]. In order to test this possibility, treatment of plants with LA first followed by coronalon was performed

with non-wounded leaves. As shown in Figure 4, the presence of LA as biosynthetic precursor was not sufficient to generate a significant increase of JA or JA-Ile, respectively. This can be explained with slow uptake of the exogenously applied LA that has to enter the plastids to come in contact with LOX which starts the initial reaction of jasmonate biosynthesis. This explanation is in agreement with previous studies in tomato plants, where accumulation of JA after treatment with LA was shown only after 24 h [48,49]. Complementary results were observed upon LA treatment of rice plants. Here an increase in JA and JA-Ile production was observed already after 10 min [50]. However, in this particular experiment a LA concentration of 50 mM was used, which is quite high and exceeds physiological concentrations in vivo. This might result in artificial JA accumulation. However, another explanation is that substrate availability alone is not sufficient and further, yet unknown regulation mechanisms and wounding-related signals are necessary.

To further test whether the metabolism of induced jasmonates is extremely fast and the accumulation was not detected before JA and JA-Ile are converted into inactive forms, derivatives of both compounds were examined. As known for JA-Ile, the ω-oxidation pathway catalyzed by CYP94B3 and CYP93C1, in which JA-Ile is converted to 12-OH-JA-Ile and then further oxidized to 12-carboxy-JA-Ile, is a major route for catabolism of the bioactive hormone [51,52]. The occurrence of OH-JA forms in plant tissue is also well described [53]. As can be seen in Figure 5, the pattern of OH-derivatives resembles the patterns which have been detected for JA and JA-Ile before (Fig. 3). No increase of OH-JA or 12-OH-JA-Ile was measured upon treatment with coronalon indicating that no higher non-hydroxylated substrate levels were present. The same holds true if LA was supplied alone or in combination with coronalon (Fig. A2).

It appears clear that in Arabidopsis, *N. attenuata* and lima bean as well as in tomato [28-30] a positive feedback loop for jasmonate-induced jasmonate biosynthesis and accumulation does not exist. This result seems somewhat surprising because induction of jasmonate biosynthesis genes was repeatedly demonstrated [7-9]. Thus, it is tempting to suggest that the jasmonate biosynthesis might be regulated post-translational [30] and enzymes are kept inactive in healthy plants, requiring post-translational events for their activation. Evidences for post-translational regulation processes of biosynthetic enzyme activities are already described. For example, in tomato the activity of OPR3 seems to result from a monomer/dimer equilibrium including self-inhibition mediated by dimerization [54]. For the enzyme AOC homo- and hetero-dimerization was observed, which led at least partially to altered enzyme activity [55]. Moreover, substrate availability and tissue specificity might play an additional role in the non-genomic regulation of jasmonate synthesis [2]. Such post-translational regulation scenarios could also explain why transgenic plants, which overexpress jasmonate biosynthetic enzymes, have similar JA levels compared to wild-type plants but show increased JA accumulation upon wounding [46].

More studies addressing crystal structures and the biochemistry of the biosynthetic enzymes will provide deeper insights into processes of post-translational modifications and will help to decode the regulation of jasmonate biosynthesis. Beyond these aspects, the jasmonate pathway represents a striking example that gene expression studies are important tools to understand the regulation of enzymes or pathways but without corresponding determination of the final products the interpretation of such data is difficult. Here, the often postulated positive feedback regulation for endogenous jasmonate biosynthesis held true for the expression of biosynthetic genes but not for the biosynthesis and accumulation of the phytohormones JA or JA-Ile.

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Appendix:

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Figure legends

Figure 1. Structures of (+)-7-*iso*-jasmonoyl-*L*-isoleucine (JA-Ile) and 6-ethyl indanoyl isoleucine (coronalon).

Figure 2. Expression of JA-responsive and JA-biosynthesis genes after coronalon treatment.

Normalized fold expression (± SE, n=6) of *AOS* and *LOX2* (**A**), *JAZ10* (**B**) and *VSP2* (**C**) after 1 and 3 h of coronalon treatment. Gene expression was determined without (**grey**) or with (**black**) wounding prior to treatment. Expression was normalized to the plant *RPS18* mRNA level. For control, the respective treatment with water (spraying water; wounding and immediate water application) was used and its expression level set = 1.

Figure 3. Elevation of jasmonates after coronalon treatment.

Mean content (± SE, n=6) of JA (**A**) and JA-Ile (**B**) after 1 and 3 h of coronalon treatment. Phytohormone content was determined without (**white stripes and grey**) or with (horizontal **stripes and black**) wounding prior to treatment. Untreated plants were used as control (0h). No statistical significant differences between treatment with/without coronalon for each time point were detected (t-test, $p<0.05$).

Figure 4. Elevation of jasmonates after combined linolenic acid (18:3) and coronalon treatment.

Mean content (± SE, n=6) of JA (**A**), JA-Ile (**B**), after 1 and 3 h of 18:3 and coronalon treatment. Jasmonates content was determined without wounding prior to spray. Pretreatment with 18:3 was done

for 1 h. Untreated plants were used as control (0h). No statistical significant differences between the treatments were detected for each time point separately (One Way-ANOVA, SNK-test, $p<0.05$).

Figure 5. Elevation of jasmonate-metabolites after coronalon treatment.

Mean content (± SE, n=6) of OH-JA (**A**) and 12-OH-JA-Ile (**B**) after 1 and 3 h of coronalon treatment. Phytohormone contents were determined without (**white stripes and grey**) or with (**horizontal stripes and black**) wounding prior to treatment. Untreated plants were used as control (0h). No statistical significant differences between treatment with/without coronalon for each time point were detected (t-test, $p<0.05$).

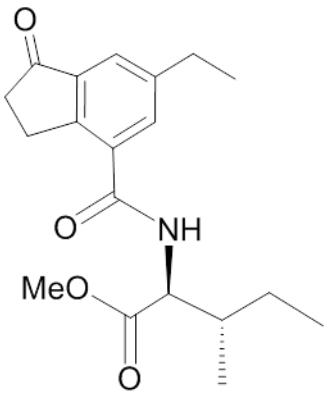
Figure A1. Expression of JA-responsive and JA-biosynthesis genes after control treatment.

Normalized fold expression (± SE, n=6) of *AOS* and *LOX2* (**A**), *JAZ10* (**B**) and *VSP2* (**C**) after 1 and 3 h of water treatment. Gene expression was determined without (**white and diagonal stripes**) or with (**horizontal stripes and squares**) wounding prior to treatment. Expression was normalized to the plant *RPS18* mRNA level. Untreated plants were used as control (expression level = 1).

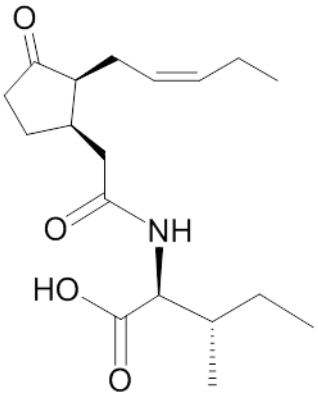
Figure A2. Elevation of jasmonate-metabolites after combined linolenic acid (18:3) and coronalon treatment.

Mean content (± SE, n=6) of OH-JA (**A**), 12-OH-JA-Ile (**B**), after 1 and 3 h of 18:3 and coronalon treatment. Jasmonates content was determined without wounding prior to spray. Pretreatment with 18:3 was done for 1 h. Untreated plants were used as control (0h) No statistical significant differences between the treatments were detected for each time point separately (One Way-ANOVA, SNK-test, $p<0.05$).

Figure 1.



coronalon



(+)-7-*iso*-jasmonoyl-*L*-isoleucine

Figure 2.

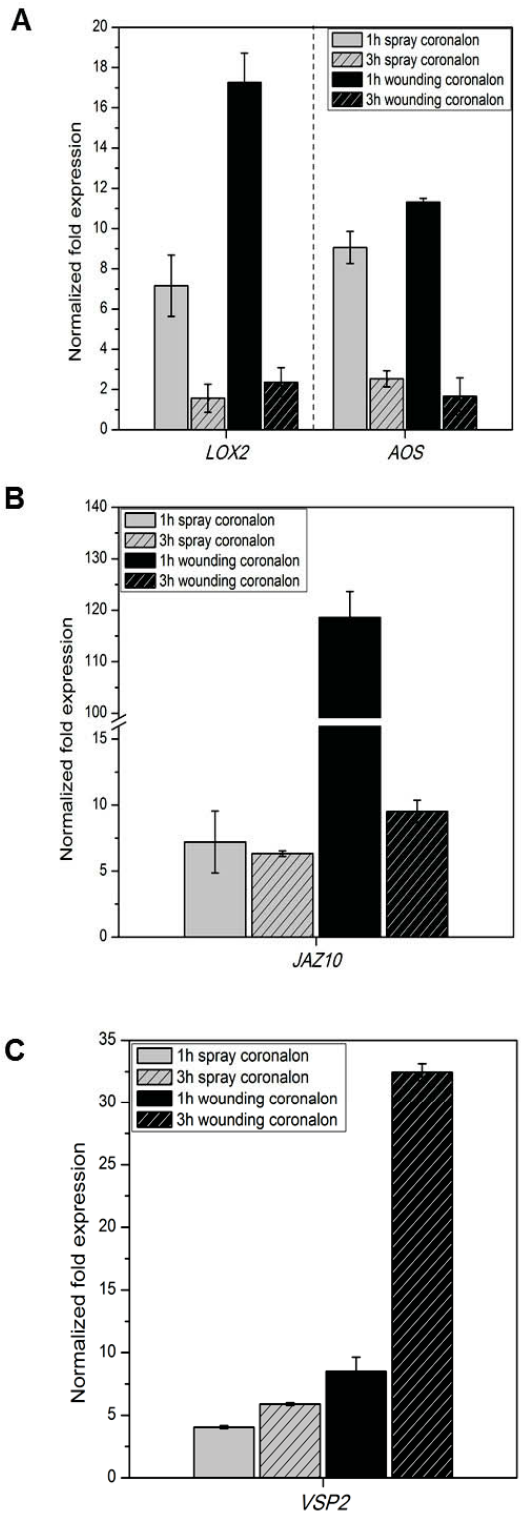

A
1h spray coronalon
3h spray coronalon
1h wounding coronalon
3h wounding coronalon
Normalized fold expression
LOX2
AOS
B
1h spray coronalon
3h spray coronalon
1h wounding coronalon
3h wounding coronalon
Normalized fold expression
JAZ10
C
1h spray coronalon
3h spray coronalon
1h wounding coronalon
3h wounding coronalon
Normalized fold expression
VSP2

Figure 3.

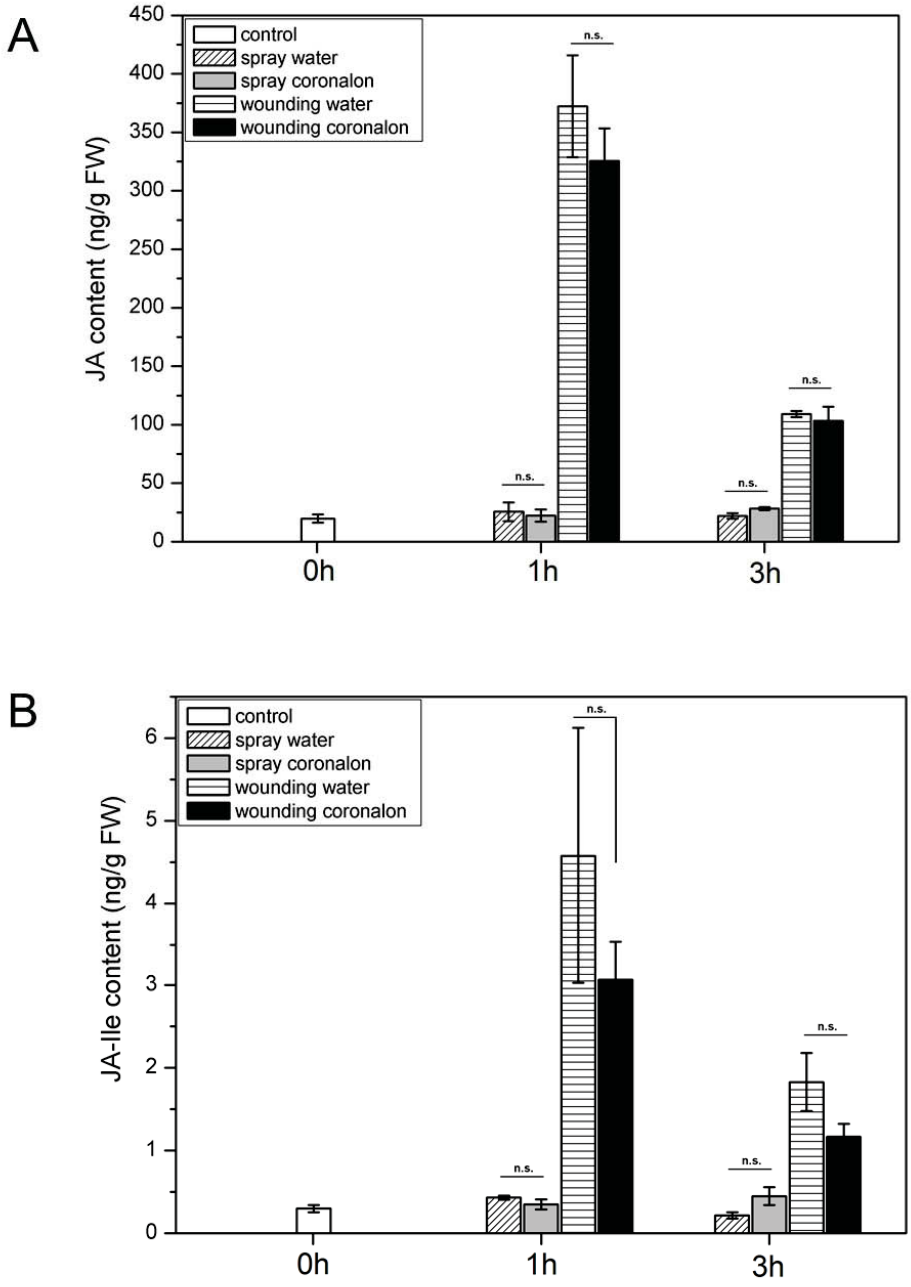

A
control
spray water
spray coronalon
wounding water
wounding coronalon
JA content (ng/g FW)
450
400
350
300
250
200
150
100
50
0
n.s.
0h
1h
3h
B
JA-Ile content (ng/g FW)
6
5
4
3
2
1
0

Figure 4.

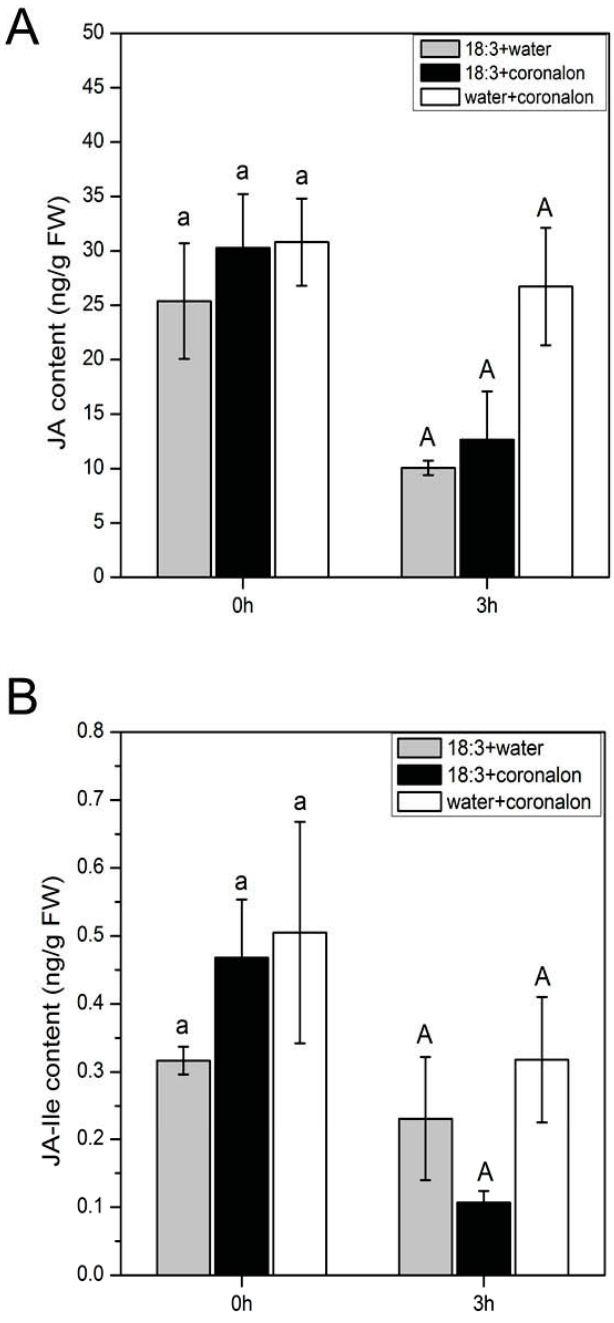

A
JA content (ng/g FW)
18:3+water
18:3+coronalon
water+coronalon
0h
3h
B
JA-Ile content (ng/g FW)
18:3+water
18:3+coronalon
water+coronalon
0h
3h

Figure 5.

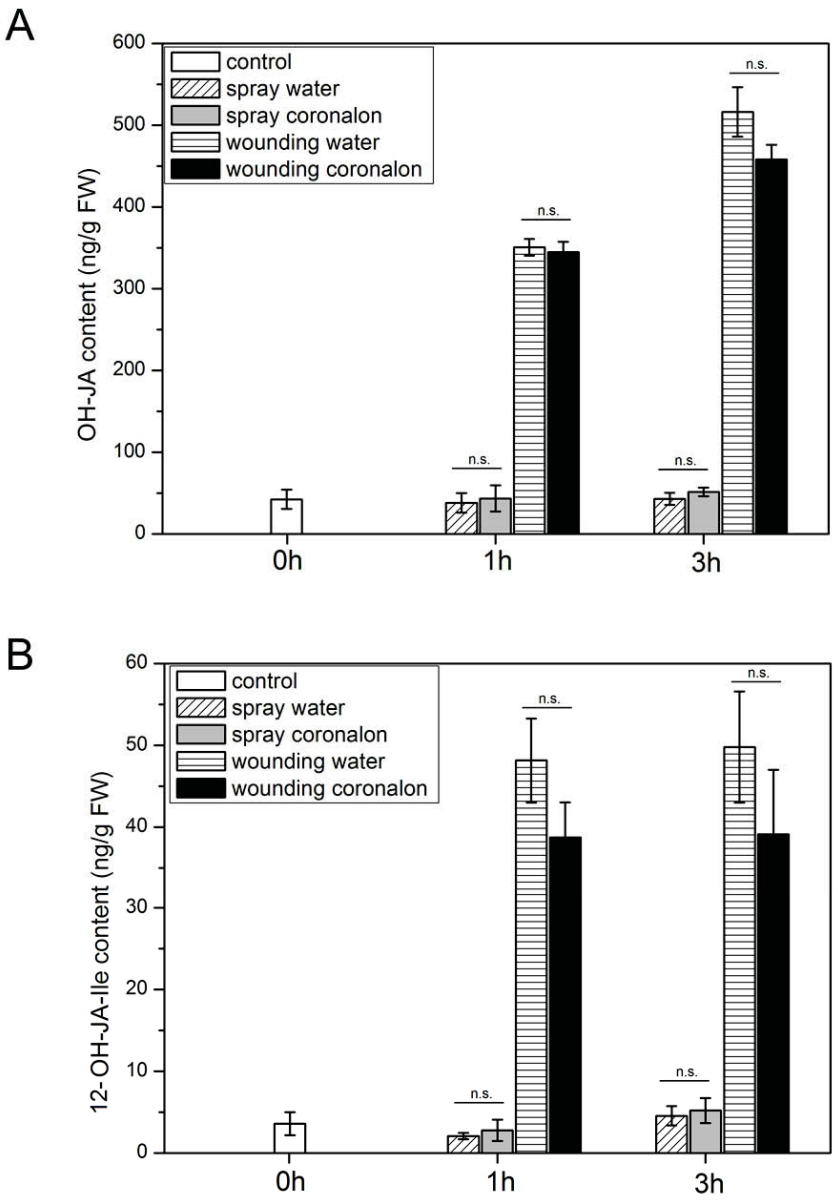

A
OH-JA content (ng/g FW)
600
500
400
300
200
100
0
control
spray water
spray coronalon
wounding water
wounding coronalon
n.s.
n.s.
n.s.
n.s.
0h
1h
3h
B
12-OH-JA-Ile content (ng/g FW)
60
50
40
30
20
10
0
control
spray water
spray coronalon
wounding water
wounding coronalon
n.s.
n.s.
n.s.
n.s.
0h
1h
3h

Table A1. Primers used for RT-PCR

Target (Atg number)	Sequence
RPS18B (At1g 34030)	**5'- GTCTCCAATGCCCTTGACAT -3'**
	5'- TCTTTCCTCTGCGACCAGTT -3'
JAZ10 (At5g 13220)	**5'- TCGAGAAGCGCAAGGAGAGATTAGT -3'**
	5'- AGCAACGACGAAGAAGGCTTCAA - 3'
VSP2 (At5g 24770)	**5'- ACGACTCCAAAACCGTGTGCAA -3'**
	5'- CGGGTCGGTCTTCTCTGTTCCGT -3'
AOS (At5g 42650)	**5'- AAGCCACGCGGCGTTTA -3'**
	5'- GGAGTCTCCGTCTCCGGTCCA -3'
LOX2 (At3g 45140)	**5'- ACGCTCGTGCACGCCAAAGT -3'**
	5'- CCTCAGCCAACCCCCTTTTGA -3'

Figure A1.

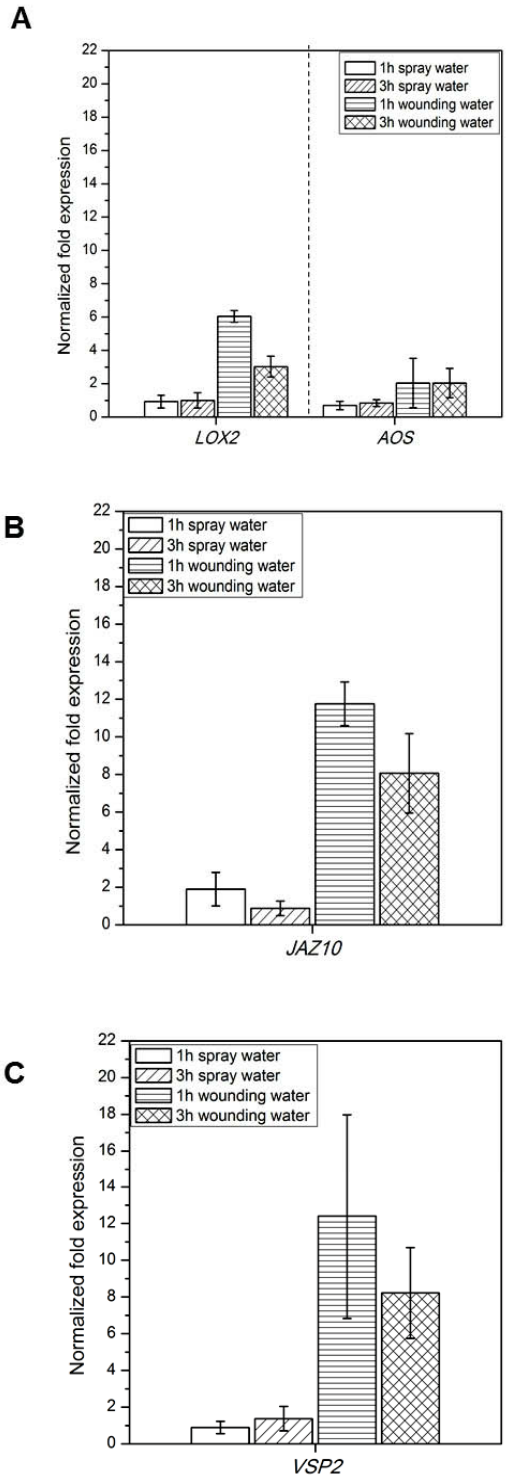
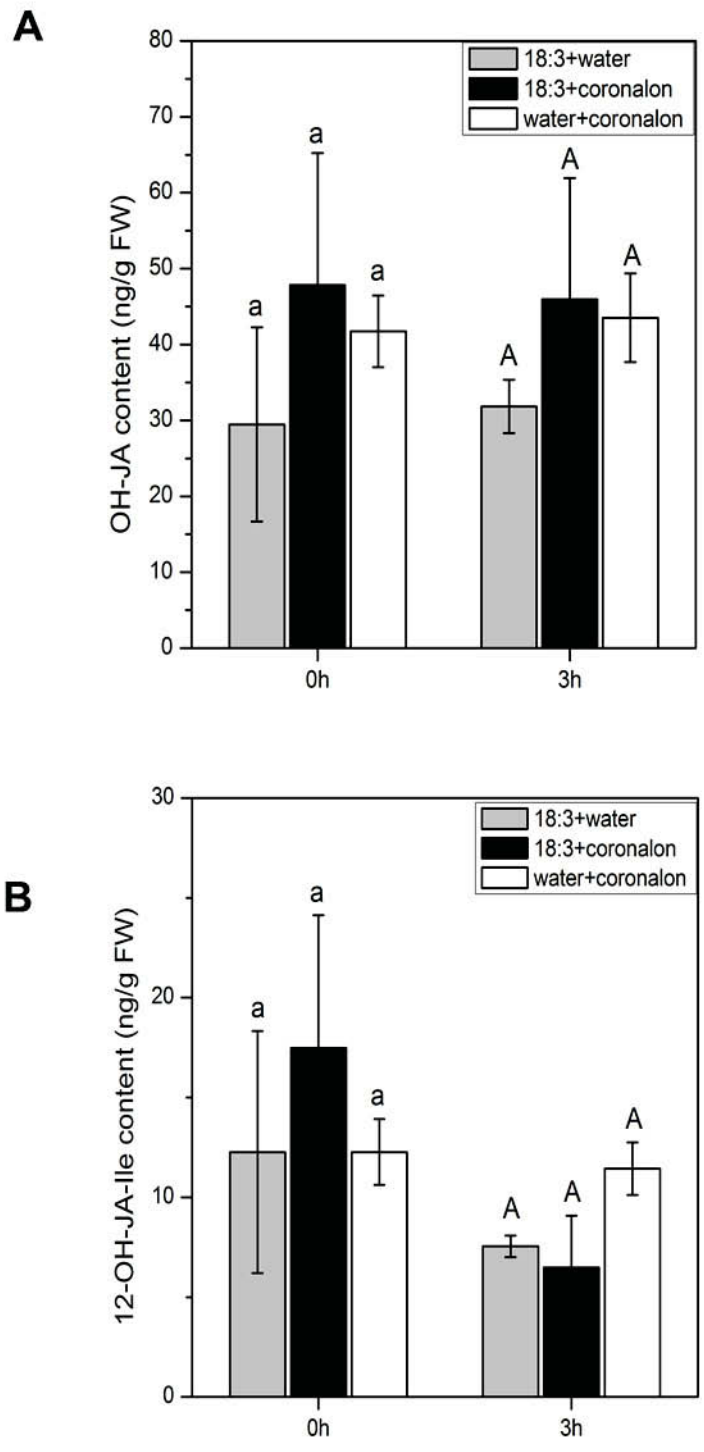

A
1h spray water
3h spray water
1h wounding water
3h wounding water
Normalized fold expression
LOX2
AOS
B
1h spray water
3h spray water
1h wounding water
3h wounding water
Normalized fold expression
JAZ10
C
1h spray water
3h spray water
1h wounding water
3h wounding water
Normalized fold expression
VSP2

Figure A2.


A
OH-JA content (ng/g FW)
18:3+water
18:3+coronalon
water+coronalon
0h
3h
B
12-OH-JA-Ile content (ng/g FW)
18:3+water
18:3+coronalon
water+coronalon
0h
3h

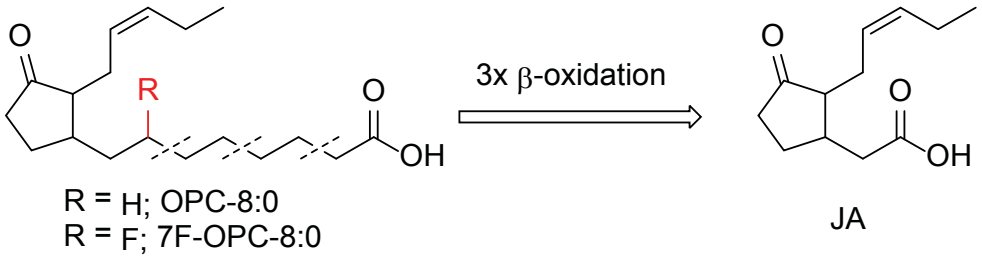
Manuscript S2

Graphical Abstract

Synthesis, biological activity, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0

Guillermo H. Jimenez-Aleman, Sandra S. Scholz, Monika Heyer, Michael Reichelt, Axel Mithöfer, and Wilhelm Boland

Fluorinated oxylipins as probes to study metabolism and signal transport in plants



Synthesis, biological activity, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0

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Abstract

Jasmonates are fatty acid derivatives that mediate many developmental processes and stress responses in plants. Synthetic jasmonate derivatives (commonly isotopically labeled), which mimic the action of the endogenous compounds are often employed as internal standards or probes to study metabolic processes. However, tools to evaluate jasmonates' spatial and temporal distribution are yet lacking. In this study we explore whether a fluorinated jasmonate could mimic the action of the endogenous compound and therefore, be employed as molecular probe to study metabolic processes. We describe the synthesis, the metabolism and the biological activity of (*Z*)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (7F-OPC-8:0), a fluorinated analogue of the JA-precursor OPC-8:0. Like endogenous jasmonates, 7F-OPC-8:0 induces the transcription of marker jasmonate responsive genes (JRG) and the accumulation of jasmonates after exogenous application to *Arabidopsis thaliana* plants. By using UHPLC-MS/MS, we could show that 7F-OPC-8:0 is metabolized *in vivo* similarly to the endogenous OPC-8:0. Furthermore, the fluorinated analogue was successfully employed as a probe to show its translocation to undamaged systemic leaves when it was applied to wounded leaves. This result suggests that OPC-8:0 –and maybe other oxylipins– may contribute to the mobile signal which triggers systemic defense responses in plants. We highlight the potential of fluorinated oxylipins to study the mode of action of lipid-derived molecules *in planta*, either by conventional analytical methods or fluorine-based detection techniques.

Keywords

Arabidopsis thaliana; fatty acids metabolism; jasmonate; JA biosynthesis; UHPLC-MS; systemic response; signal translocation.

Highlights

- The synthesis of 7F-OPC-8:0, a fluorinated analogue of OPC-8:0 is described
- 7F-OPC-8:0 is metabolized similarly to the endogenous jasmonate OPC-8:0
- Jasmonate responsive genes and jasmonates accumulation are induced by 7F-OPC-8:0
- 7F-OPC-8:0 and its β-oxidation products can be easily detected by LC-MS/MS
- 7F-OPC-8:0 is systemically translocated in the plant after wounding stress

Abbreviations: JA, jasmonic acid; *cis*-OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JA-Ile, JA-L-isoleucine conjugate; 7F-OPC-8:0, (*Z*)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid; OPC-8:0, 8-((1*S*,2*S*)-3-oxo-2-((*Z*)-pent-2-en-1-yl)cyclopentyl)octanoic acid; JRG, jasmonate responsive genes; FA, fatty acids; LOX, 13-lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, *cis*-OPDA reductase 3; OPC-6:0, (*Z*)-6-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)hexanoic acid; OPC-4:0, (*Z*)-4-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)butanoic acid; ACX, acyl-CoA oxidase; MFP, multifunctional protein; KAT, L-3-ketoacyl-CoA thiolase; PET, positron emission tomography; MeJA, methyl jasmonate; 5F-OPC-6:0, (*Z*)-5-fluoro-6-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)hexanoic acid; 3F-OPC-4:0, (*Z*)-3-fluoro-4-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)butanoic acid; DAST, diethylaminosulfur trifluoride; PCC, pyridinium chlorochromate; PPTS, pyridinium *p*-toluenesulfonate; VSP2, vegetative storage protein 2; JAZ, jasmonate-ZIM-domain protein; GST1, glutathione-S-transferase 1; SCF^{COI1}, SKP, Cullin, COI1 receptor complex; MRM, multiple reaction monitoring; HRMS, high resolution mass spectrometry.

1. Introduction

Oxylipins are a diverse group of lipid-derived signaling compounds that are present throughout the plant kingdom [1]. They are generated following oxidation of polyunsaturated fatty acids (FA) such as linoleic acid, linolenic acid, and hexadecatrienoic acid [2, 3]. Jasmonates (JAs) are among the best characterized FA derivatives [4]. These metabolites mediate many developmental processes and stress responses in plants, including leave senescence, mechano-sensitive signal transduction, secondary metabolism and plant responses to wounding or herbivory [4-7]. Jasmonic acid (JA) is probably the most studied member of the JAs' family [4].

The JA biosynthetic pathway is well understood and many of the involved enzymes are well characterized [8, 9]. It starts in the plastid with the release of linolenic and hexadecatrienoic acids from the plastidic glycerolipids. A 13-lipoxygenase (LOX) is capable of oxidizing linolenic acid to 13-hydroperoxy linolenic acid (13-HPOT), which can be metabolized to different classes of oxylipins (Fig.1) [10]. The conversion of 13-HPOT to 12,13-epoxyoctadecatrienoic acid (12,13-EOT) by an allene oxide synthase (AOS) is the main transformation of 13-HPOT. The allene oxide cyclase (AOC) acts on 12,13-EOT to produce *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA) stereospecifically, which is the first jasmonate having the cyclopentanone ring and remarkable bioactivity. Further conversion of *cis*-OPDA implies its translocation from the chloroplasts to the peroxisomes. There, *cis*-OPDA reductase 3 (OPR3) reduces *cis*-OPDA to 8-((1*S*,2*S*)-3-oxo-2-((*Z*)-pent-2-en-1-yl)cyclopentyl)octanoic acid (OPC-8:0), lacking the highly reactive α,β-unsaturated keto group. Three rounds of β-oxidations are required for shortening the carboxyl side chain of OPC-8:0 producing OPC-6:0, OPC-4:0 and finally JA in that order (Fig. 1). The final product of the β-oxidations is (3*R*,7*S*)-JA (OPC-2:0), that can epimerize to the more

stable isomer (3*R*,7*R*)-JA [4, 11]. Both isomers co-exist *in planta* and we refer to them simply as JA.

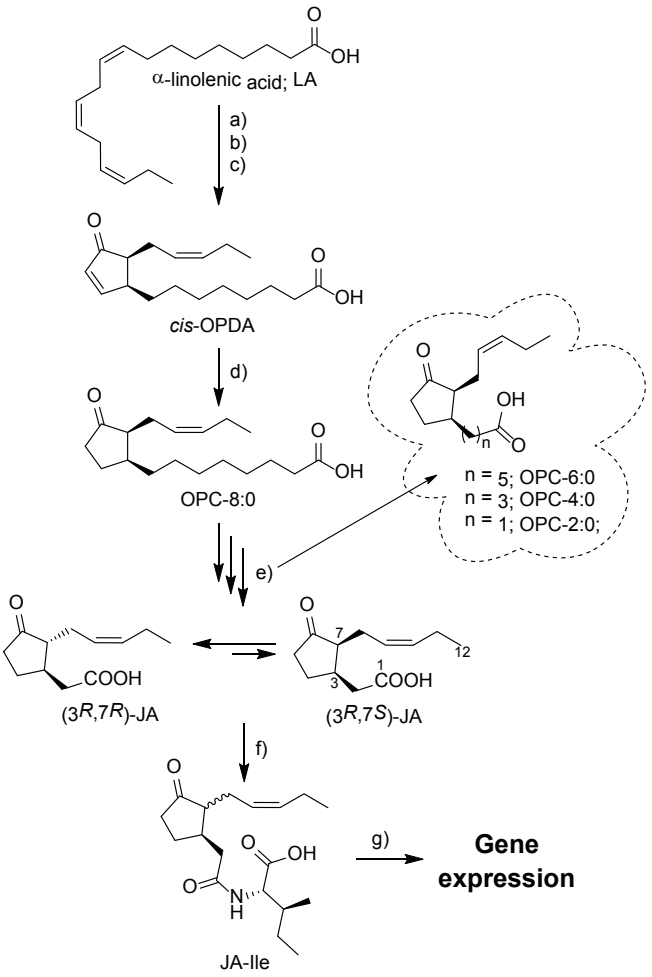

Fig. 1. Simplified scheme of the biosynthesis and signaling of jasmonates. In the plastids, LA is converted into *cis*-OPDA by the sequential action of LOX (a), AOS (b), and AOC (c). (3*R*,7*S*)-JA is formed in the peroxisomes by OPR3 (d) acting on *cis*-OPDA followed by three cycles of β-oxidation (e). (3*R*,7*S*)-JA can epimerize to the more stable isomer (3*R*,7*R*)-JA. In the cytosol, JA is conjugated to L-isoleucine (Ile) by jasmonic acid-amido synthetase (JAR1) to form the bioactive jasmonate JA-Ile (f), which can be subsequently perceived by the SCF^{COI1} co-receptor complex in the nucleus (g). This last process leads the expression of JRG and jasmonates induced responses. See text for detailed explanation and abbreviations. The compounds are shown in the stereochemistry occurring *in planta*.

Synthetic derivatives of jasmonates have been very helpful to elucidate the structural requirements for bioactivity, the biosynthetic and metabolic pathway of jasmonates [12-17]. These compounds, in particular isotopically labeled ones, have proven their utility to study transport phenomena in diverse plant species by different techniques (e.g., LC-MS and PET) [18]. For instance, after feeding wounded leaves with deuterium-labeled JA, [^{2}H]JA was translocated to systemic leaves and metabolized there to its ω-hydroxylated form 12-OH-JA [19]. It was later demonstrated that JA-Ile had a higher mobility than JA despite its lower polarity, and application of [^{2}H]JA-Ile to wounded leaves leads to a higher accumulation of JA and JA-Ile in distal leaves compared with control plants [20]. The translocation of methyl jasmonate (MeJA, **1**) was investigated by PET employing [^{11}C]MeJA as a tracer [21]. In this study it was claimed that **1** moves in both the phloem and xylem. However, it was shown later that the ester group (carrying the [^{11}C]) of MeJA (**1**) can be cleaved *in vivo* [22]. Therefore, further studies are required revisiting this topic.

Besides isotopically labeled compounds, fluorinated analogues have been widely employed to study biological processes. For example fluorinated FAs, provided very useful information on the structure-activity relationship, biosynthetic pathways, biological activities and metabolism of the target molecules [23, 24]. Fluorinated derivatives of abscisic acid helped to gain insights into the biological activity and mechanism of activation and shutdown of this phytohormone [25]. Moreover, fluorine is a monoisotopic element (100% natural isotopic abundance) with a high gyromagnetic ratio (γ = 40.05 MHz/T). These properties make fluorinated molecules very interesting probes to be use in techniques like in HRMS and NMR. Interestingly, the fluorine chemistry of jasmonates remains little explored, although a few studies have dealt with fluorinated jasmonates. These compounds have shown different biological properties such as

tuber-inducing effect in potato [26], anti-tumor action [27], and selective induction of plant secondary metabolites [28]. However, to the best of our knowledge, there are no physiological studies of fluorinated jasmonates described in the literature.

Herein we cover the biological activity of a fluorinated derivative of the JA-precursor OPC-8:0, its metabolic fate the plant and the possibility of using this molecule as a probe to follow signal-trafficking *in planta*. We describe the synthesis of 7F-OPC-8:0 (**10**), explored its biological activity by mean of gene expression assays and jasmonate induced profiles after exogenous application to *Arabidopsis thaliana* plants. Furthermore, a UHPLC-MS/MS method was developed to identify 7F-OPC-8:0 (**10**) and its metabolic derivatives in plant leaf extracts. This method was further employed to study whether **10** could be systemically translocated in the plant.

2. Results and discussion

*2.1. Synthesis and characterization of 7F-OPC-8:0 (**10**)*

7F-OPC-8:0 (**10**) was prepared as a mixture of isomers starting from commercially available MeJA (**1**). The synthesis was carried out according to the procedure depicted in Fig. 2. The fluorine atom was introduced at position C7 due to three main reasons (i) the replacement of a hydrogen atom by fluorine (similar atomic radius) should not cause steric hindrance or stereochemical restrictions in metabolic processes, (ii) to assures the tracking 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**) or 3F-OPC-4:0 (**12**), and no other JA derivatives when using fluorine-based imagine techniques such as PET and MRI, and (iii) the easy chemistry required for the introduction of the fluorine in that particular position.

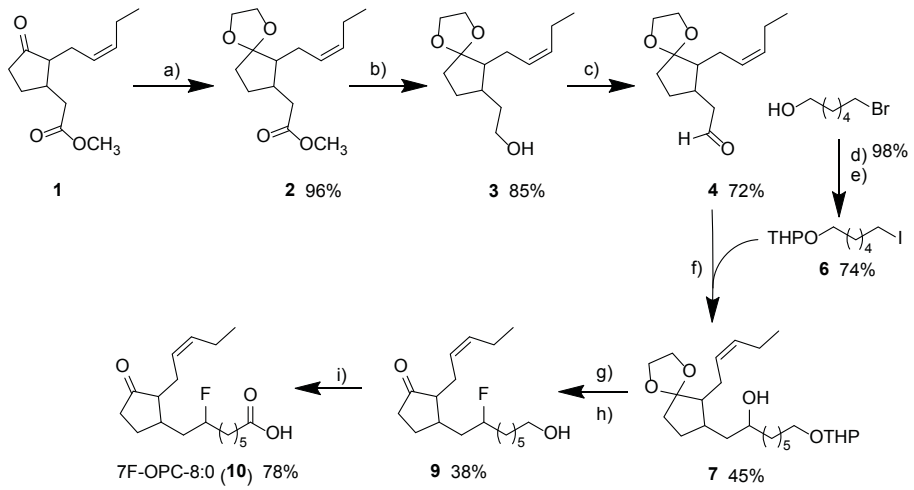

Fig. 2. Synthesis of 7F-OPC-8:0 (**10**). Reagents and conditions: a) 1,2-ethanediol/C_6H_6/*p*-TsOH, reflux; b) $LiAlH_4/Et_2O$; c) PCC/CH_2Cl_2/AcONa, 4 Å molecular sieves; d) NaI/Me_2CO; e) CH_2Cl_2/THP/*p*-TsOH, room temp.; f) *n*-pentane/Et_2O (3:2)/*t*-BuLi, -78 °C; g) DAST/CH_2Cl_2, -78 °C; h) Me_2CO/EtOH/H_2O (1:1:1)/PPTS; i) Jones reagent (4 M). For abbreviations see the text below.

The synthesis proceeded smoothly with moderate to high yields. Protection of the carbonyl group of the cyclopentane ring of **1**, followed by reduction of the ester group of **2** with $LiAlH_4$ in Et_2O, and oxidation of the alcohol **3** with pyridinium chlorochromate (PCC) afforded the aldehyde **4** in excellent yield (Fig. 2, steps a-c). Elongation of the side chain of **4** was carried out by both Grignard reaction and via the organolithium reagent derived from **6**. The second strategy was more efficient and provided cleaner products. Treatment of the alcohol **7** with diethylaminosulfur trifluoride (DAST) successfully afforded the fluorinated derivative **8**, which was deprotected without previous purification. Deprotection of both, the carbonyl and hydroxyl group of **8** was achieved in one single step by stirring **8** in a solution of Me_2CO:EtOH:water (1:1:1) containing pyridinium *p*-toluenesulfonate (PPTS). The fluorine containing alcohol **9** was obtained in 38% over two steps. Finally, treatment of **9** with Jones reagent harbored the

fluorinated analogue 7F-OPC-8:0 (**10**) (78%, mixture of isomers). As summary, **10** was obtained from MeJA (**1**) in six major transformations and 8% overall yield.

*2.2. 7F-OPC-8:0 (**10**) induces the expression of JA-responsive genes (VSP2, OPR3, JAZ1) and cis-OPDA-responsive genes (GST1, OPR1)*

The biological activity of 7F-OPC-8:0 (**10**) was evaluated through gene expression assays and its capability of induce accumulation of jasmonates in *A. thaliana* plants after exogenous application of the compound. Jasmonates coordinate the plant responses to biotic and abiotic challenges by the induction of JRG expression, which is mediated by the SCF^{COI1}-JAZ co-receptor complex [29]. The activation of JRG is a typical plant response to herbivory [30-32] and mechanical wounding [33]. Activation of such genes also occurs after exogenous application of several endogenous jasmonates [31, 34]. To evaluate the biological activity of 7F-OPC-8:0 (**10**), *A. thaliana* plants were sprayed with this compound and the JRG expression was monitored. For this purpose two genes, strongly induced by JA were chosen: *VSP2* and *OPR3* [35]. Both genes are highly induced by 7F-OPC-8:0 (**10**) compared to solvent control, with a maximum peak at 30 min after treatment (Fig. 3 A,B). This result was consistent with similar analyses carried out with endogenous jasmonates [36]. Additionally, the gene coding for the transcriptional repressor of JA-signaling, JAZ1 [37], was induced after treatment with 7F-OPC-8:0 (**10**) (Fig. S1, SI).

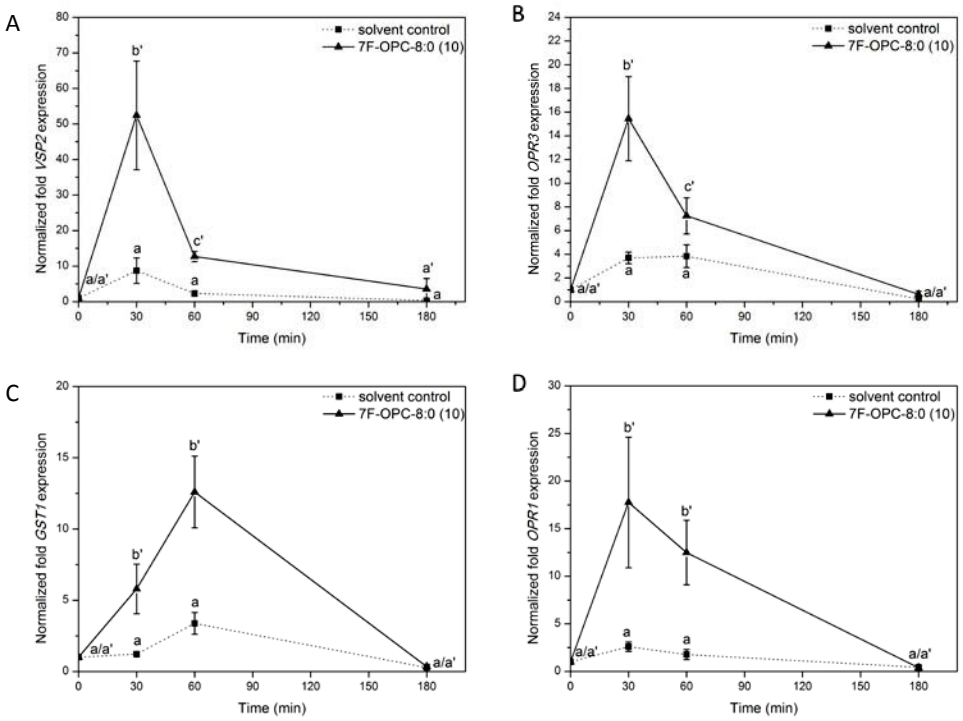

Fig. 3. Mean expression (± s.e., n=5) of JA- (A, B) and *cis*-OPDA-responsive genes (C, D) in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Expression of *VSP2* (A), *OPR3* (B), *GST1* (C) and *OPR1* (D) was analyzed after 30, 60 and 180 min. All samples were normalized to *RPS18B* level and untreated plants were used as control (expression level = 1). Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

On the other hand, some genes show a specific induction by the JA precursor *cis*-OPDA and are classified as *cis*-OPDA-responsive genes [35]. Two of which – *OPR1* and *GST1* – have been used as markers for *cis*-OPDA-responsive gene expression after wounding [38]. These genes are also highly upregulated after plant treatment with 7F-OPC-8:0 (**10**) (Fig.3 C,D). These results suggest that 7F-OPC-8:0 (**10**) induces not only JA-responsive genes, but also genes responding specifically to *cis*-OPDA.

*2.3. 7F-OPC-8:0 (**10**) treatment-dependent increase of endogenous jasmonates levels, including cis-OPDA levels*

The activation of JRG is usually preceded by a transient increase in the internal levels of endogenous jasmonates [5]. As mentioned, compound **10** is capable of activating a subset of JRG (see section 2.2). Accordingly, we expected that after plant treatment with 7F-OPC-8:0 (**10**), the jasmonates profile would be similar to the profile observed after simulated herbivory, wounding or exogenous application of jasmonates. Figure 4 shows the jasmonates profile for a time course experiment for *A. thaliana* plants treated with **10**. The concentrations measured for JA, JA-Ile, 11/12-OH-JA[1] and *cis*-OPDA showed the same trend (e.g. the JA/JA-Ile burst) observed in plants after simulated herbivory, JA treatment, or mechanical wounding.

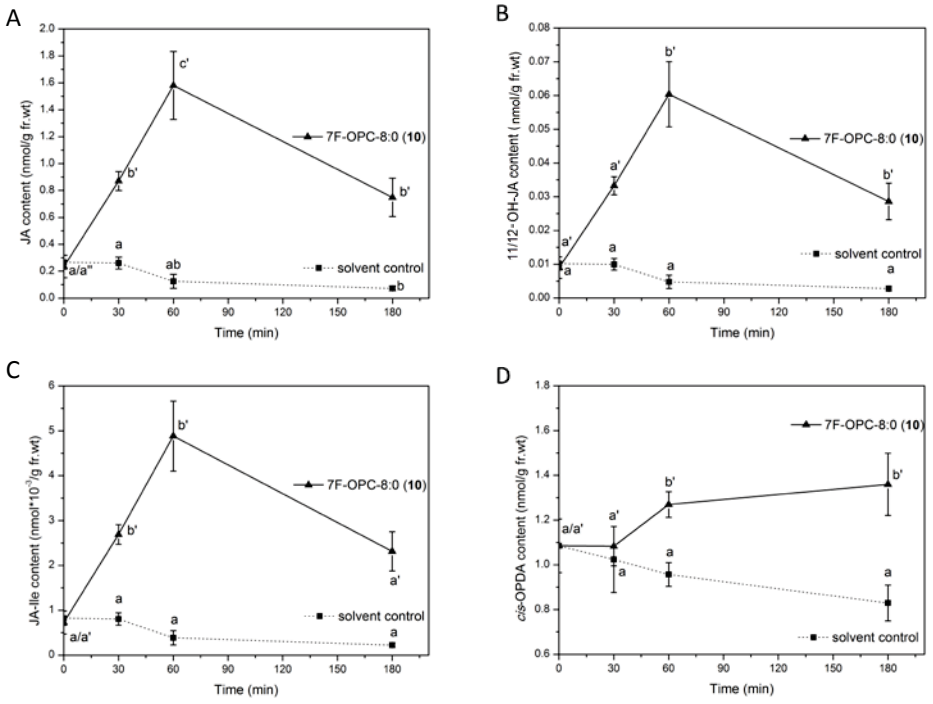

Fig. 4. Mean content (± s.e., n=5) of jasmonate profiles in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. The content of JA (A), 11/12-OH-JA (B), JA-Ile (C) and *cis*-OPDA (D) was determined after 30, 60 and 180 min. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

[1] The analytical method does not distinguish between 11-OH-JA and 12-OH-JA

This finding suggests that JAs downstream of OPC-8:0 in the metabolic pathway (Fig.1) increase their level due to the *in vivo* metabolized 7F-OPC-8:0 (**10**). This suggestion agrees with that one postulated by Miersch and Wasternack for tomato plants [34]. These authors treated tomato leaves with deuterium labeled OPC-8:0 and found that increasing JA and MeJA (**1**) levels were merely due to the metabolism of the deuterated applied compound, which is in line with our findings. In the same study, it was shown that the biosynthesis of jasmonates is not induced by exogenous treatment with jasmonates. Notwithstanding, we also found somewhat higher concentrations of *cis*-OPDA (1.2 fold) in plants treated with **10** compared to control plants after 1h (Fig. 4D), although these differences were significant. A possible explanation to this observation is that, *in A. thaliana*, *cis*-OPDA can be produced from storage sources such as arabidopsides. These molecules contain *cis*-OPDA linked through an ester bond to a glycerol moiety [39]. Accumulation of arabidopsides has been reported during hypersensitive response and after wounding [40] in *A. thaliana*. The cleavage of the ester bonds of arabidopsides leads to an increase in free *cis*-OPDA, indicating a function for arabidopsides as storage of signal compounds that can prolong the JA signaling [41]. To explore the possibility that arabidopsides could be the source for the increase in *cis*-OPDA level in our experiments, we analyzed the content of arabidopside A and B in the leaf extracts. A pronounced depletion of the content of arabidopsides A and B was observed at the same time frame in which the increase of *cis*-OPDA level occurred (Fig. S2, SI). This supports that arabidopsides may represent the source for the increase in the *cis*-OPDA. Whether a similar phenomenon is characteristic for the exogenous application of endogenous jasmonates needs further investigation. Until here, our data suggest that 7F-OPC-8:0 (**10**) can be metabolized by the plant like a true mimic of the JA precursor OPC-8:0.

*2.4. 7F-OPC-8:0 (**10**) is metabolized in vivo similar to the endogenous OPC-8:0*

Next, we addressed the question whether 7F-OPC-8:0 (**10**) can be metabolized by the plant like a true mimic of OPC-8:0, and may represent the source for the increased levels of jasmonates downstream to OPC-8:0 in the JA-biosynthetic pathway (Fig. 4). In other words, we investigated whether **10** could undergo β-oxidations to produce JA.

A LC-MS/MS method was developed to identify **10** and the products resulting from its first two β-oxidations namely 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**). After the third β-oxidation the fluorine atom is lost. First, synthetic **10** was employed to fine tune the method in negative ionization mode on a Triple-Quadrupole mass spectrometer. The fragmentation pattern of **10** revealed that the molecular ion $[M-H]^-$, together with an intense peak resulting from a HF loss ($[M-H-20]^-$) are the most reliable fragments (Fig. S3, SI). We were able to identify two signals corresponding to **11** and **12** in the samples of treated plants, by setting the quadrupole 1 (Q1) to $[M-H]^-$ and the quadrupole 3 (Q3) to $[M-H-20]^-$ in MRM mode. The identity of both peaks was corroborated by means of HRMS (Fig. S4, SI). The concentrations found for **11** and **12** showed a similar profile to that observed for other jasmonates in this study (Fig.5).

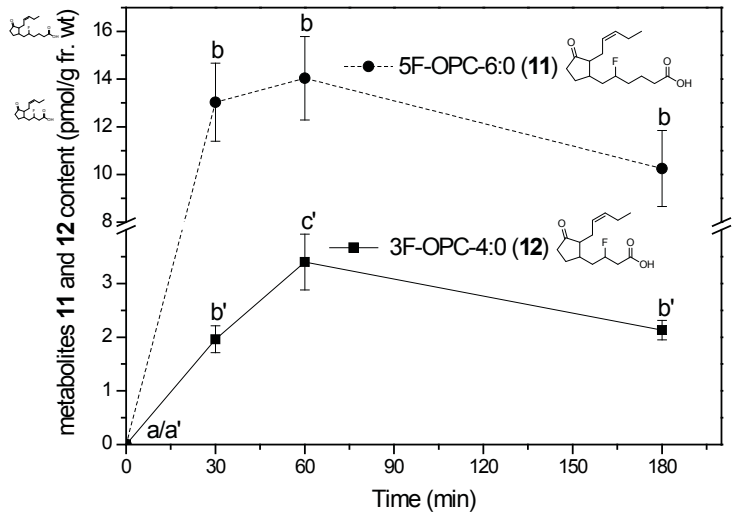

Fig. 5. Mean content (± s.e., n=5) of the 7F-OPC-8:0 (**10**) derived metabolites 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) in *A. thaliana* Col-0 leaves after treatment with **10**. The content of 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) was determined after 30, 60 and 180 min. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p<0.05$, SNK test).

Our results show that 7F-OPC-8:0 (**10**) undergoes at least the first two β-oxidation steps similar to the endogenous OPC-8:0 in the JA biosynthetic pathway. The presence of the fluorine atom does not hamper the oxidative degradation. The first step of the β-oxidation mechanism is the generation of an enoyl-CoA substrate, which is carried out by the acyl-CoA oxidase (ACX) family of enzymes in *A. thaliana* [42]. This comprises the concerted abstraction of the pro-*R*-α-hydrogen as a proton (H^+) along with the elimination of the corresponding pro-*R*-β-hydrogen to the N-5 position of a flavin moiety as a hydride (H^-) equivalent [42]. As we employed a mixture of isomers of **10** in the bioassays, the last β-oxidation step might be inhibited by one of the enantiomers of 3F-OPC-4:0 (**12**). However, we did not detect over-accumulation of this particular metabolite beyond the pattern observed for other jasmonates (Fig. 5). Furthermore, we

could determine that the content of the endogenous β-oxidation product OPC-4:0 remains at its constitutive levels in treated plants (Fig. S5, SI). Altogether, these data suggest that the exogenously applied 7F-OPC-8:0 (**10**) serves as biosynthetic precursor for the observed increase of the endogenous jasmonates levels (Fig. 4), i.e. no induced biosynthesis of endogenous JA, which is in agreement with previous studies [34].

2.5. 7F-OPC-8:0 (10) is systemically translocated in the plant

In response to wounding, plants accumulate jasmonates not only in wounded leaves but also in undamaged systemic leaves [5, 20]. Currently it is not clear if this accumulation results from the direct transport, the *de novo* synthesis of the phytohormones or a combination of both events initiated by upstream signals [6]. Likewise, it is not well understood whether jasmonates including early precursors like OPC-8:0, may function as systemic signals in the plant.

We employed 7F-OPC-8:0 (**10**) as a probe to explore the possibility of this molecule being translocated and therefore involved in systemic signaling events in *A. thaliana* plants. The vascular connections between leaves are defined in *A. thaliana* plants [43]. Following the nomenclature of Farmer et al. [44], plants were mechanically wounded (pattern wheel) at leaf 8 of the *A. thaliana* rosette and **10** was immediately applied to the wounds. The contents of **10** and its derived metabolites 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) were determined in both damaged local and undamaged systemic leaves (Fig. 6).

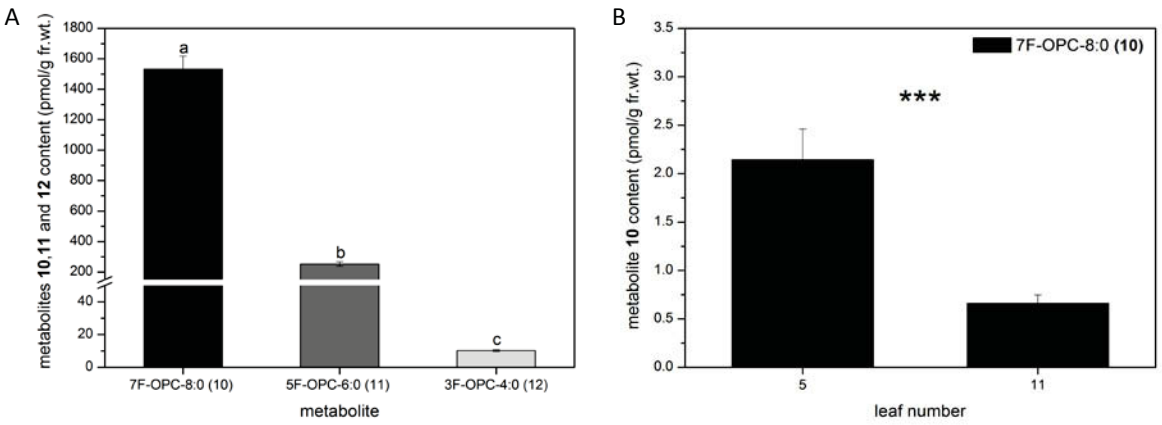

Fig. 6. Mean content (± s.e., n=11) of 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) in *A. thaliana* Col-0 leaves after wounding and treatment with **10** for 60 min. (**A**) Content of **10**, **11** and **12** in the treated local leaf 8. Statistically significant differences between the content of the metabolites were analyzed by One-Way ANOVA ($p<0.05$, SNK test). (**B**) Content of **10** in systemically connected leaves 5 and 11. Metabolites **11** and **12** were not detected in leaves 5 and 11. Statistically significant differences between content of **10** in different leaves were analyzed by Mann-Whitney Rank Sum Test ($p<0.05$, *** $p<0.001$).

In correspondence with previous results (Fig.5, section 2.4), **10** and its degradation products **11** and **12** could be measured in the treated leaf 8 (Fig. 6A). Interestingly, the level of **11** and **12** was higher in the wounded leaf 8 than after application to an undamaged leaf. This could be explained by two reasons. On the one hand, the wounding effect can trigger the JA-biosynthesis in the damaged leaves [5], and consequently activate the jasmonate's metabolic machinery contributing to the metabolism of **10**. On the other hand, compound **10** could be assimilated easier through the wounds than when it is sprayed to unwounded tissue. 7F-OPC-8:0 (**10**) was detected both in younger (leaf 11) and older leaves (leaf 5), which are connected to leaf 8 via contact parastichies [43]. Leaves 5 and 11 were reported to react systemically when leaf 8 was wounded or feed by an insect, even though they don't share a direct vascular connection with

leaf 8 [45, 46]. The content of **10** in leaf 5 was significantly higher than in leaf 11. This is not surprising as some differences have been reported in the systemic response of these leaves [45]. The concentrations of **10** found in leaves 5 and 11 are in the same order of magnitude of those reported for JA-Ile systemically transported to distal leaves after wounding [5]. These results indicate that not only jasmonates but also their precursor OPC-8:0 is transported throughout the plant after wounding.

Interestingly, metabolites **11** and **12** were not detected in systemic leaves. A poor detection limit of the method employed could explain this; the levels of **11** and **12** in systemic leaves at the measured time point may be too low for detection. Therefore, neither a translocation of these compounds produced in leaf 8, nor a local synthesis from the translocated **10** can be ruled out. Further investigations are needed to clarify these questions. Based on these results, we conclude that the accumulation of jasmonates in systemic leaves is not only due to *de novo* synthesis of the phytohormones, but also an effect of the transport of JAs and precursors to the systemic undamaged tissue. Our data indicate that transport of OPC-8:0 occurs into older and younger leaves suggesting the action of this molecules as a systemic signal in a bidirectional way [43].

3. Conclusions

We developed a short synthesis of 7F-OPC-8:0 (**10**) – a fluorinated analogue of the JA precursor OPC-8:0 – with good overall yield. This compound was shown to be biologically active concerning the induction of marker JRG and accumulation of endogenous jasmonates in *A. thaliana* leaves. Furthermore, we were able to detect metabolites **11** and **12** derived from the β-oxidations of **10** in leaves extracts. As it has been demonstrated that application of jasmonates do not induce JA-biosynthesis, this suggests that externally applied jasmonates and analogs are

metabolized to downstream JAs activating gene expression. Moreover, it has been demonstrated that the fluorinated analogue **10** can be employed as a true mimic of the endogenous jasmonate OPC-8:0 in *A. thaliana* plants. We successfully employed 7F-OPC-8:0 (**10**) to show its translocation from damaged leaves to undamaged systemic leaves. This suggests that the JA precursors can also contribute to propagate systemic signals which induce defense responses of the plant in distal tissues to damaged area. Our results reveal the potential of the fluorine chemistry to study jasmonates – and optionally other phytohormones or plant lipid derivatives – metabolism and signaling. Plants are the energy source of many herbivorous organisms, therefore fluorinated jasmonates may be employed to study the metabolic fate of the fluorinated molecule in feeding organisms or even in tri-trophic interactions. Availability of compound **10** will allow the replacement of the fluorine atom by its radioactive isotope ^{18}F to study transport phenomena in real time employing PET.

4. Material and methods

4.1. General material and methods

All chemicals were obtained from commercial suppliers. If necessary, solvents were purified prior to use. Thin layer chromatography was performed on silica gel 60 F_{254} on aluminum plates (Merck) and visualized with potassium permanganate staining. Flash chromatography was performed on silica gel 60 (40-63 μm) from Merck. Proportions of the employed solvents are referred to volume (v/v) if not mentioned otherwise.

GC-MS spectra were recorded on a ThermoQuest CE Instruments GC 2000 Series coupled to a ThermoQuest Finnigan Trace MS mass spectrometer; GC column HP-5MS capillary column (15 m × 0.25 mm ID with 0.25 μm film thickness, Phenomenex). Injection port: 250 °C; Split flow: 15 ml min^{-1} with split ratio of 1:10; Temperature program: 60 °C (2 min) at 15 °C min^{-1} to 280 °C (5 min). Helium at

1.5 ml min^{-1} served as carrier gas. The ionization method was electron impact (70 eV) in positive mode (EI^+). GC-MS for control of the chemical reactions was carried out on Hewlett Packard Series II, equipped with a Phenomenex Zebron ZB-5ms (30 m x 0.25 mm, 0.25 μm) column (conditions as described above for the Trace MS, but in split-less mode). HRMS (ESI^-) for compound **10** was performed on a Bruker Daltonics - maXis Ultra High ResolutionTOF instrument.

NMR spectra were recorded at 300K either on a Bruker DRX500 spectrometer (operating frequency 500 MHz for ^1H and 125 MHz for ^{13}C) or a Bruker Avance 400 NMR spectrometer (operating frequency 400 MHz for ^1H and 100 MHz for ^{13}C). ^1H NMR chemical shifts were referenced relative to the TMS signal. As compounds are mostly mixture of isomers, MS and NMR data are reported for the major isomer only.

4.2. Synthetic procedures

*4.2.1. Methyl (Z)-2-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)acetate (**2**):*

A 50 ml round-bottomed flask was charged with commercially available MeJA (**1**) (2.461 g, 11 mmol), 1,2-ethanediol (0.749 g, 12.1 mmol), dry C_6H_6 (10 ml), and *p*-TsOH (0.07 g, catalyst). The flask was attached to a Dean-Stark trap, refluxed for 4 h and worked-up. The crude product **2** (6.118 g; 96.7 %) was employed in the next reaction without purification. GC-MS (EI^+): *m/z*(%): 41.18(18), 55.03(32), 67.00(38), 85.94(51), 99.00(100), 153.07(64), 195.08(55), 268.22 $[M]^+$ (36).

*4.2.2. (Z)-2-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)ethan-1-ol (**3**):*

The synthesis was carried as follow. A 100 ml three-necked flask under Ar atmosphere was charged with $LiAlH_4$ (1.082 g, 28.5 mmol), dry Et_2O (45 ml) and **2** (6.118 g, 22.8 mmol) dissolved in dry Et_2O (10 ml) was added dropwise. After the addition was complete, the mixture

was further stirred for 1.5 h. The reaction mixture was worked-up and evaporation of solvents afforded crude **3** (4.687 g; 85%) which was sufficiently pure for further transformation. GC-MS (EI^+): *m/z*(%): 55.07(41), 99.20(100), 153.20(35), 195.27(47), 240.31 $[M]^+$ (30).

*4.2.3. (Z)-2-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)acetaldehyde (**4**):*

A 250 ml three-necked flask, equipped with a magnetic stirring bar and pressure-equalizing funnel, was purged with argon and charged with dry CH_2Cl_2 (80 ml), finely powdered PCC (11.780 g, 54.6 mmol, 1.5 equiv.), AcONa (0.440 g), and 15 g of 4 Å molecular sieves in powder. Compound **3** (8.760 g, 36.4 mmol) dissolved in CH_2Cl_2 (20 ml) was added dropwise to the reaction mixture which was stirred for 4 h (room temp.), and then filtered through a pad of Florisil. The filtrate was concentrated on a rotary evaporator, and the residual oil was purified by flash chromatography on silica gel (EtOAc/*n*-hexane, 1:4) to give **4** (6.270 g, 72%) as a colorless oil. GC-MS (EI^+): *m/z*(%): 55.12(48), 99.27(100), 153.43(31), 194.60(38), 195.61(45), 238.63 $[M]^+$ (8). ^1H NMR (500MHz, $CDCl_3$): δ 9.72 (s, 1H), 5.30-5.40 (m, 2H), 3.82-3.93 (m, 4H), 2.54-2.71 (m, 1H), 2.35 (ddd, *J*=16.7, 9.5, 2.4 Hz, 1H), 2.12-2.26 (m, 2H), 2.01-2.10 (m, 3H), 1.92-1.99 (m, 1H), 1.61-1.83 (m, 3H), 1.19-1.33 (m, 1H), 0.94 ppm (t, *J*=7.6 Hz, 3H); ^{13}C NMR ($CDCl_3$, 126MHz): d = 202.2, 132.4, 127.5, 117.4, 64.7, 64.2, 51.3, 49.9, 37.1, 35.2, 28.1, 26.5, 20.5, 14.1 ppm

*4.2.4. 2-((6-iodohexyl)oxy)tetrahydro-2H-pyran (**6**):*

6-iodohexan-1-ol (**5**) was prepared as described in Ng and Fromherz [47]. Compound **5** was obtained as thick yellow oil (2.478 g, 98%) and directly employed in the next reaction. The tetrahydropyranyl ether of **5** was prepared by stirring a solution of **5** (2.478 g, 10.86 mmol) and

2,3-dihydropyran (4.579 g, 54.44 mmol; 5 equiv.) in CH_2Cl_2 (50 ml, room temp.) was added *p*-TsOH (0.025 g), the mixture stirred for 2 h, and then worked-up. The remaining faintly yellow oil was chromatographed on silica gel (*n*-hexane-EtOAc, 9:1) to afford pure **6** (2.502 g, 74%). GC-MS (EI^+): *m/z*(%): 41.34(58), 55.03(99), 83.01(60), 84.74(100), 168.96(15), 311.10(20), 312.22 $[M]^+$ (4).

4.2.5. (Z)-1-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)-8-((tetrahydro-2H-pyran-2-yl)oxy)octan-2-ol (7):

An oven-dried 50 ml flask was charged with **6** (0.500 g, 1.6 mmol) and dry *n*-pentane/ Et_2O (16 ml, 3:2) under argon atmosphere to give an approximately 0.1 M solution. All additions were performed by using argon-flushed syringes and a positive pressure of argon was maintained within the flask during all subsequent operations. The flask was cooled to -78 °C with a dry ice-acetone bath and *t*-BuLi (2.2 ml, 1.6 M in *n*-pentane, ca. 2.2 equiv.) was then added dropwise via syringe. Stirring was continued at -78 °C for additional 5 min following the addition, the cooling bath was then removed, and the mixture was allowed to warm and stand at room temperature for 1 h to consume unreacted *t*-BuLi. Afterwards, aldehyde **4** (0.515 g, 1.5 equiv.) was added dropwise and the reaction mixture was worked up. Flash chromatography (*n*-hexane/Et_2O, 1:1) afforded **7** (0.305 g, 45%). GC-MS (EI^+): *m/z*(%): 41.20(15), 54.98(27), 85.02(100), 99.04(93), 153.03(14), 195.02(19), 239.18(5), 339.26(8), 424.35 $[M]^+$ (0.5).

*4.2.6. (Z)-7-(2-fluoro-8-((tetrahydro-2H-pyran-2-yl)oxy)octyl)-6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonane (**8**):*

To a solution of DAST (0.090 ml, 0.65 mmol, 1.2 equiv.) in dry CH_2Cl_2 (0.26 ml) at -78 °C was added under Ar a solution of the alcohol **7** (0.207 g, 0.49 mmol) in dry CH_2Cl_2 (0.1 ml) via argon flushed syringe. The solution was stirred at -78 °C for 2 h and 3 h after removal of the cooling bath. The reaction mixture was then quenched with saturated K_2CO_3 and the aqueous phase extracted with Et_2O (3×10 ml). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product **8** was submitted to deprotection without further purification.

*4.2.7. (Z)-3-(2-fluoro-8-hydroxyoctyl)-2-(pent-2-en-1-yl)cyclopentan-1-one (**9**):*

Deprotection of **8** was achieved in one step by stirring the compound in a mixture of Me_2CO/EtOH/water (1:1:1) containing PPTS (0.010 g, 10% of the alcohol). Flash chromatography on silica gel (Et_2O/*n*-pentane, 3:2) afforded the desired product **9** (0.055 g, 38 %, two steps). TLC R_f 0.16. GC-MS (EI^+) **9**-TFA derivative: *m/z*(%): 40.68(30), 54.60(26%), 66.72(19), 82.81(100), 94.80(19), 108.82(19), 123.87(22), 151.00(23), 326.31(3), 394.34 $[M]^+$ (1). ^{1}H NMR (500MHz, $CDCl_3$): δ 5.30-5.43 (m, 1H), 5.12-5.26 (m, 1H), 3.63-3.71 (m, 1H), 3.57 (t, *J*=6.5 Hz, 2H), 2.23-2.36 (m, 3H), 2.06-2.23 (m, 2H), 1.95-2.05 (m, 3H), 1.73 (m, 2H), 1.45-1.55 (m, 3H), 1.22-1.44 (m, 10H), 0.89 ppm (t, *J*=7.5 Hz, 3H); ^{13}C NMR (126MHz, $CDCl_3$): δ 219.5, 132.6, 124.4, 68.3, 61.9, 54.1, 41.5, 37.5, 37.0, 36.7, 31.6, 28.4, 26.1, 24.7, 24.6, 24.3, 19.6, 13.2 ppm.

*4.2.8. (Z)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (7-F-OPC, **10**):*

Jones reagent (4 M) was added to a solution of **9** (0.055 g, 0.18 mmol) in Me_2CO (2 ml) at 0 °C until the color of the reagent persisted. After 30 min at 0 °C, excess of the reagent was quenched by addition of 2-propanol. The resulting mixture was filtered through a pad of Celite by elution with Et_2O and washed several times with brine. The organic solution was dried over $MgSO_4$, concentrated under reduced pressure and the remaining oil purified by flash chromatography (CH_2Cl_2-Me_2CO, 1:1). 7-F-OPC (**10**) was obtained in 78 % yield (0.044 g). HRMS (ESI$^-$-TOF): *m/z* = 311.2042 [M-H] (calc. for $C_{18}H_{28}FO_3$, 311.2023) ^{1}H NMR (400MHz, $CDCl_3$): δ 5.36-5.54 (m, 1H), 5.17-5.33 (m, 1H), 4.44-4.74 (m, 1H), 2.37 (m, 6H), 1.95-2.19 (m, 5H), 1.58-1.93 (m, 5H), 1.30-1.56 (m, 6H), 0.96 ppm (t, *J*=7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 101MHz): δ 213.3, 178.0, 133.9, 125.2, 92.5, 54.9, 40.2, 39.0, 38.1, 38.0, 35.6, 33.6, 28.9, 27.1, 25.5, 24.5, 20.6, 14.1 ppm

4.3. Plant material and treatments

Arabidopsis thaliana ecotype Columbia was used for all experiments and plants were grown as described [30]. Four to five week old plants, grown under short-day conditions were sprayed with 0.75 ml (50 µM) of 7F-OPC-8:0 (**10**) or solvent control (0.125 % ethanol) and incubated for the indicated time periods. For investigating the systemic translocation of 7F-OPC-8:0 (**10**), the leaves of five week old plants were numbered according to Farmer et al. [44]. Plants were wounded at leaf 8 with a pattern wheel parallel to the midrib as described [30]. A total amount of 20 µl of 50 µM 7F-OPC-8:0 (**10**) was applied to the wounds. Leaf 5, 8 and 11 of each plant were harvested 60 min after treatment.

4.4. RNA-isolation and RT-PCR

For RNA isolation, 1 leaf (~ 100 mg) was harvested and stored in liquid nitrogen until use. Samples were homogenized with a Genogrinder 2010 (Spex Sample Prep, Stanmore, UK) for 1 min at 1000 rpm. RNA extraction and cDNA synthesis was performed as described before [32]. Q-RT-PCR was carried out in 96-well plates on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA) by the use of Brilliant II QPCR SYBR green Mix (Agilent, Böblingen, Germany). Analysis of dissociation curve was performed for all primer pairs and *RPS18B* was used as endogenous control for all experiments. The obtained mRNA levels of the genes of interest were normalized to the *RPS18B* mRNA level in each cDNA probe. Expression levels were calculated by use of the Normalized Expression (ΔΔCq) in Bio-Rad CFX Manager Software (3.1). Untreated plants were used as control (expression level = 1). The primer pairs used are listed in supplementary materials (Table S1).

4.5. Quantification of phytohormones

Analysis of phytohormones followed previously described methods with some modifications [30]. Finely ground leaf material (250 mg) was extracted with 1.5 ml of methanol containing 60 ng of [$^{2}H_{6}$]JA, and 12 ng of JA-[$^{13}C_{6}$]Ile conjugate as internal standards. The homogenate was mixed for 30 min and centrifuged at 13000 rpm for 20 min at 4 ºC and the supernatant was collected. The homogenate was re-extracted with 500 µl methanol, mixed and centrifuged and the supernatants were pooled. The combined extracts were evaporated under reduced pressure at 30 ºC and dissolved in 500 µl methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent). Water and acetonitrile containing formic acid (0.05%) were employed as mobile phases A and B respectively. The elution profile was: 0-0.5 min, 5%

B; 0.5-9.5 min, 5-42% B; 9.5-9.51 min 42-100% B; 9.51-12 min 100% B and 12.1-15 min 5% B. The mobile phase flow rate was 1.1 ml min^{-1}. The column temperature was maintained at 25 ºC. An API 5000 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards if available. The ion spray voltage was maintained at -4500 eV. The turbo gas temperature was set at 700 ºC. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: *m/z* 209.1 →59.0 (CE -24 V; DP -35 V) for jasmonic acid; *m/z* 215.1 →56.0 (CE -24 V; DP -35 V) for [$^2H_6$]JA; *m/z* 322.2 →130.1 (CE -30V; DP -50V) for JA-Ile; *m/z* 328.2 →136.1 (CE -30V; DP -50V) for JA-[$^{13}C_6$]Ile conjugate ; and m/z 290.9 → 165.1 (CE -24 V; DP -45 V) for *cis*-OPDA. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of *cis*-OPDA, [$^2H_6$]JA was used as the internal standard applying an experimentally determined response factor of 0.5.

*4.6. Quantification of 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**), 3F-OPC-4:0 (**12**) and endogenous OPC-4:0*

For the analysis of the fluorinated jasmonate 7F-OPC-8:0 (**10**) and its β-oxidation products, 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**), the same extracts as for phytohormone quantification were used. In the systemic transport study, single leaf extraction was performed. The whole leaf material was used and extracted with 1 ml of MeOH containing 40 ng of [$^2H_6$]JA, and 8 ng of

JA-[$^{13}C_6$]Ile conjugate as internal standards. Following the protocol mentioned above the combined, evaporated extract was dissolved in 200 µl MeOH . The following MRMs were added to the LC-MS/MS method described above: analyte parent ion → product ion: *m/z* 311.0 →291.0 (collision energy (CE)-20 V; declustering potential (DP) -100 V) for 7F-PC-8:0 (**10**); *m/z* 283.0 →263.0 (CE -20 V; DP -100 V) for 5F-OPC-6:0 (**11**); *m/z* 255.0 →235.0 (CE -20 V; DP -100 V) for 3F-OPC-4:0 (**12**); *m/z* 237.0 → 59.0 (CE -22 V; DP -120 V) for OPC-4:0. For all four compounds, [$^2H_6$]JA was used as the internal standard applying a theoretical response factor of 0.5.

The identity of compounds **11** and **12** was corroborated by LC-HRMS. MS analysis was carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Measurement conditions: ESI negative ionization mode; capillary temperature 275 C, capillary voltage 35 V; full-scan mass spectra, mass range of *m/z* 100 – 1000; mass resolution of m/Δm 30000. The software XCALIBUR (Thermo Fisher Scientific, Waltham, MA, USA) was employed for data interpretation. LC was performed on UltraMate 3000 (Thermo Fisher Scientific, Bremen, Germany) equipment. Separation was achieved with an Acclaim RSLC C18 column (2.2µm, 2.1 x 150mm; Thermo Fisher Scientific, Bremen, Germany). Formic acid (0.1%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-15 min, 1-100% B; 15-18 min, 100% B; 18-18.1 min 100-1% B; 18.1-24 min, 1% B. The mobile phase flow rate was 0.3 ml min^{-1}. The column temperature was maintained at 25 ºC.

4.7. Relative quantification of Arabidopside A and Arabidopside B

For the quantification of arabidopside A and arabidopside B the same extract as for phytohormone analysis were used. Samples were analyzed by LC-MS/MS as for phytohormone analysis (see above) with the following modifications: chromatographic gradient was: 0-0.5 min, 10% B; 0.5-4 min, 10-90% B; 4-7 min 90-100% B; 7-7.5 min 100% B and 7.5-10 min 10% B.

An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in negative ionization mode. The following MRMs were used: analyte parent ion → product ion: *m/z* 773.5 →291.0 (collision energy (CE)-36 V; declustering potential (DP) -30 V) for Arabidopside A; *m/z* 801.5 →291.0 (CE -36 V; DP -30 V) for Arabidopside B. Relative quantification is presented as normalized peak area in relation to the internal standard [$^2H_6$]JA.

Footnotes

Electronic Supplementary Information available: [Fig. S1-S5, primer list (table S1) and copy of NMR spectra of important compounds].

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Synthesis, biological activity, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0

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Supplementary Material

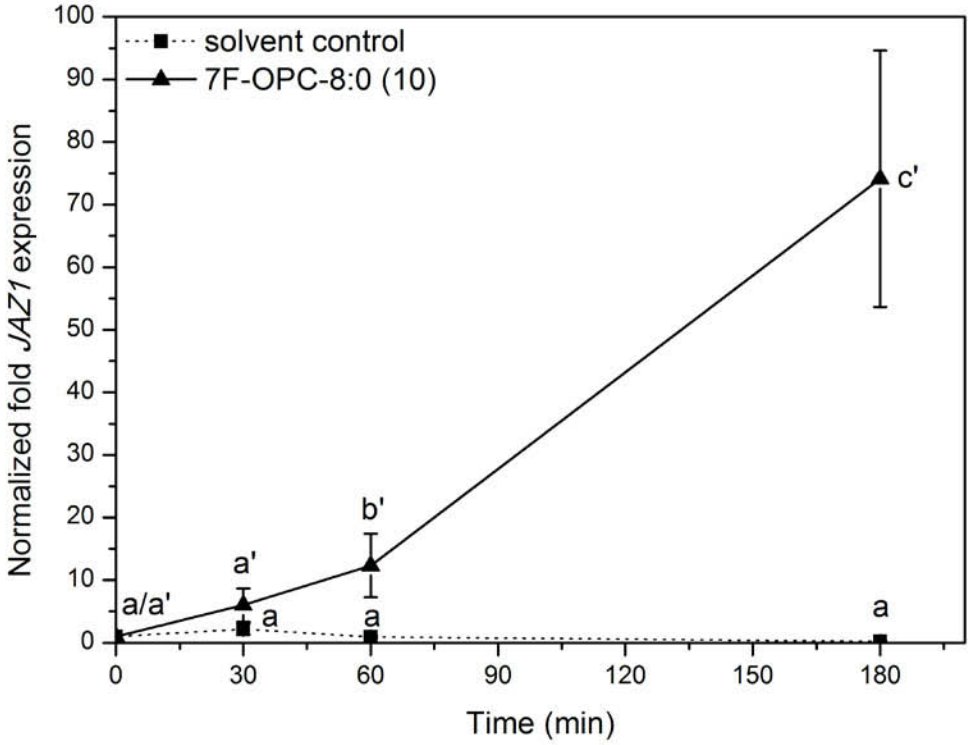


Fig. S1 Mean expression (± SE, n=5) of *JAZ1* in *Arabidopsis Col-0* after treatment with 7F-OPC-8:0 (**10**) or solvent control. Expression was analyzed after 30, 60 and 180 min. All samples were normalized to the *RPS18B* level and untreated plants were used as control. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

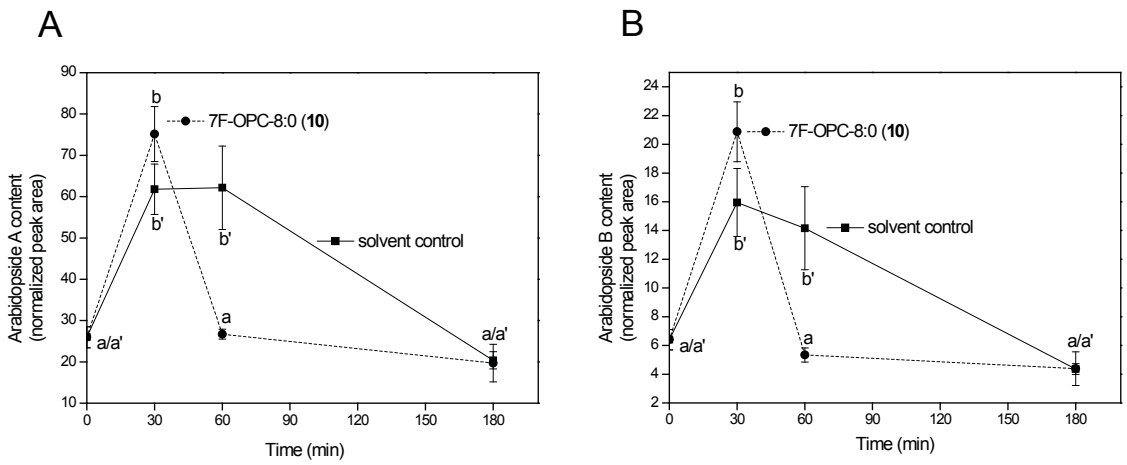


Fig. S2 Mean relative content (± SE, n=5) of arabidopside A (A) and arabidopside B (B) in *Arabidopsis* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Measurements at 30, 60 and 180 min. Peak area was normalized to the IS $[^2H]_6$JA. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

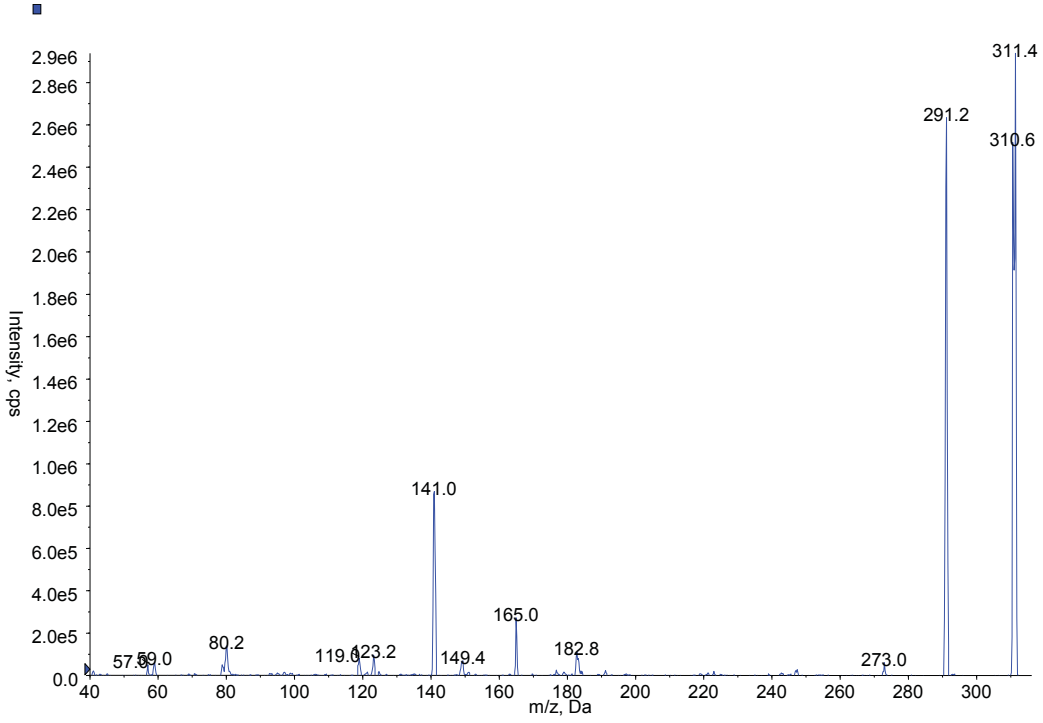


Fig. S3 MS2 spectrum of 7F-OPC-8:0 **(10)**. The fragmentation pattern of **10** reveals the molecular base peak $[M-H]^-$ (311.4 *m/z*) and a peak produced by the loss of HF ($[M-H-20]^-$, 291.2 *m/z*) as the most abundant fragments.

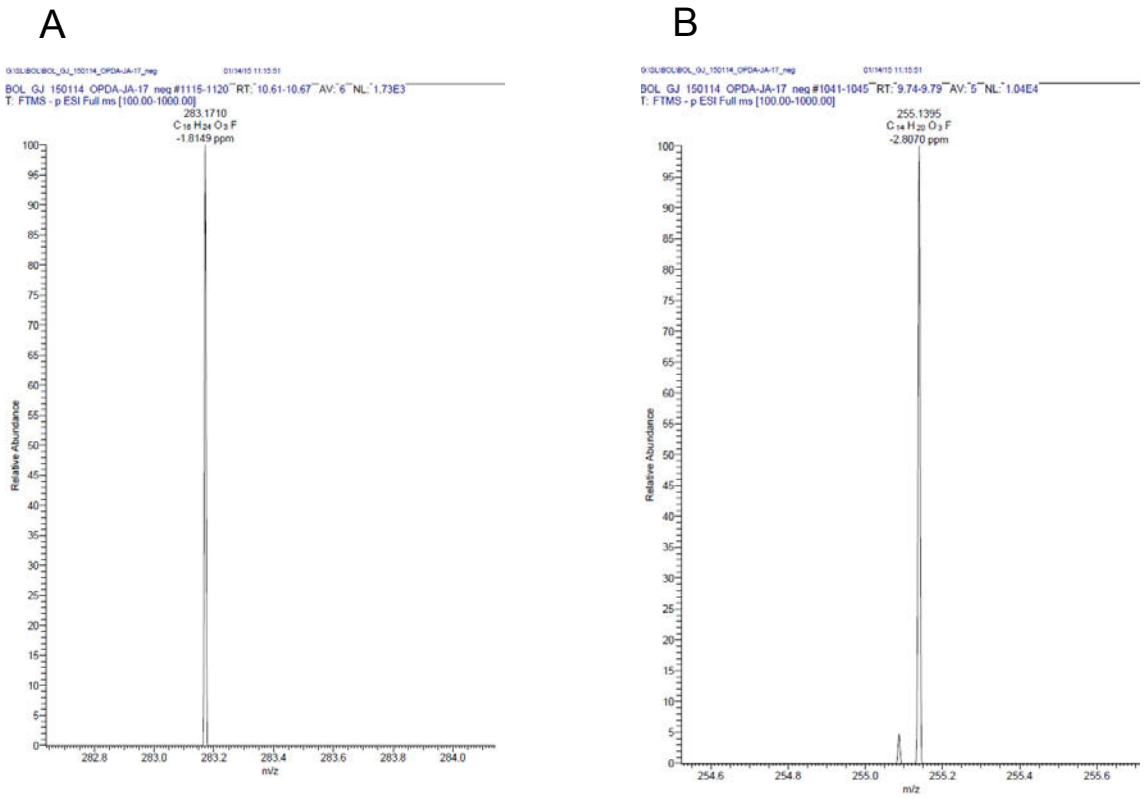


Fig. S4 HRMS spectra of compounds 5F-OPC-6:0 (**11**) (A) and 3F-OPC-4:0 (**12**) (B).

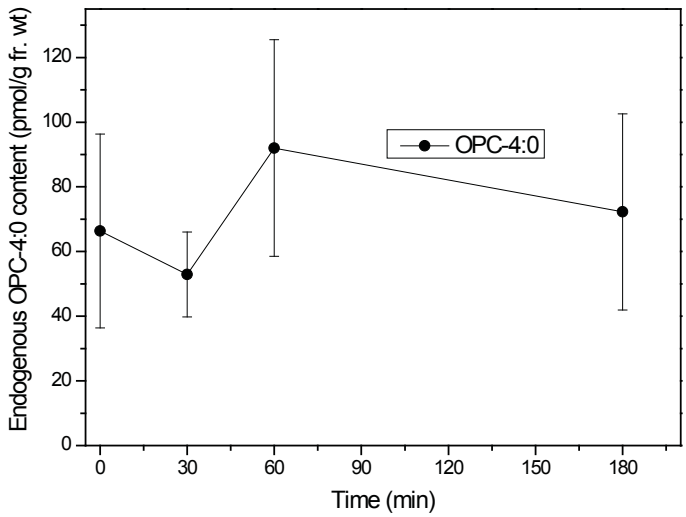
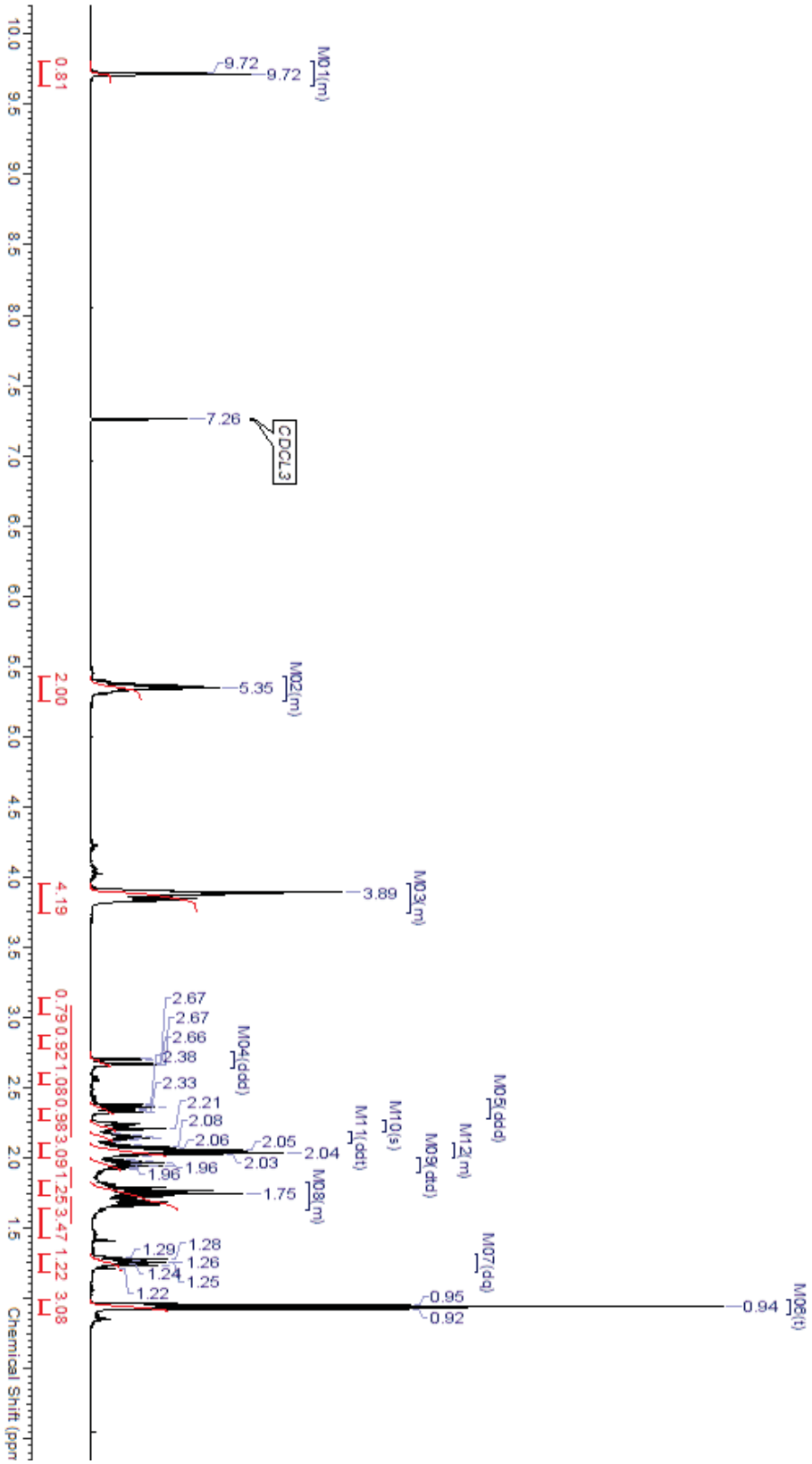


Fig. S5 Mean relative content (± s.e., n=5) of endogenous OPC-4:0 in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**). Measurements at 0 (control plants), 30, 60 and 180 min. Peak area was normalized to the IS $[^2H]_6$JA. No statistically significant differences were found between the time points that were analyzed by One-Way ANOVA.

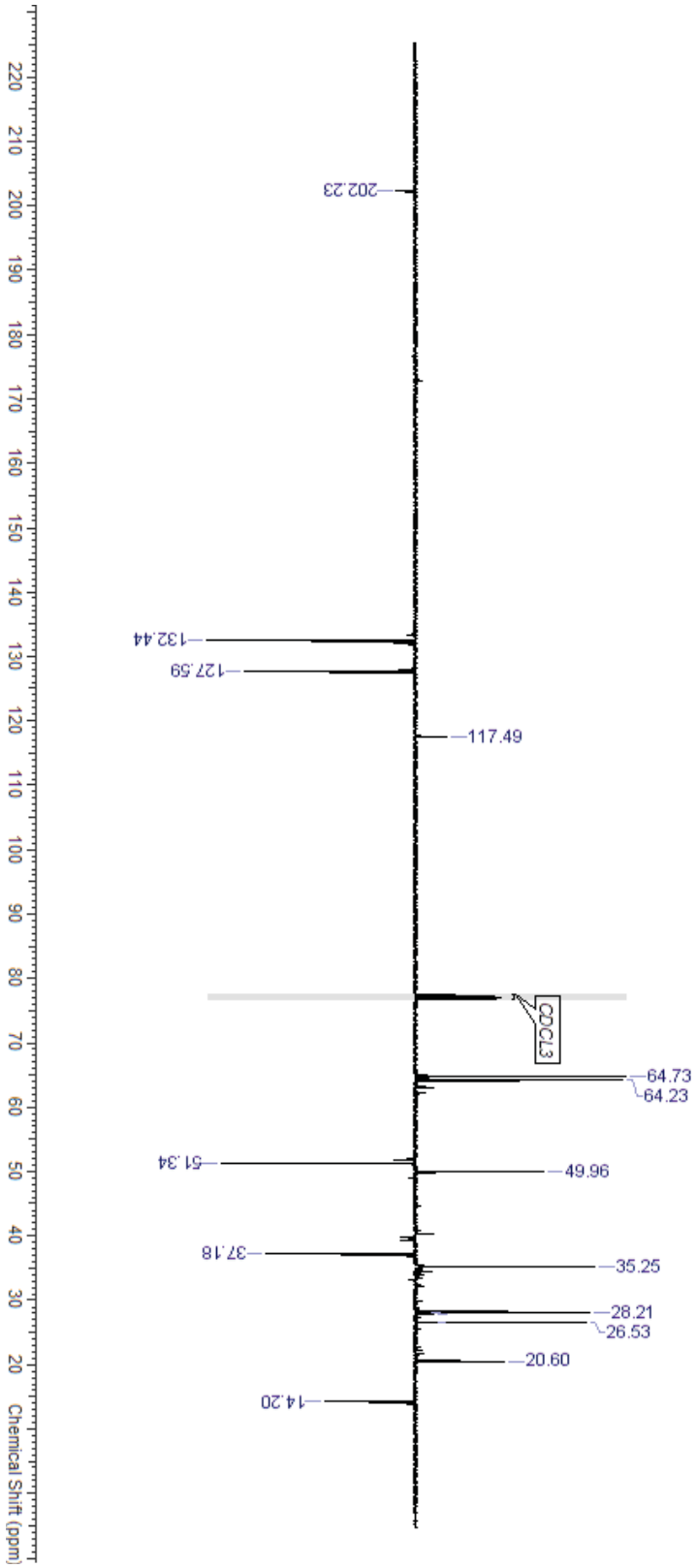
Table S1. Primers used for RT-PCR.

Target (Atg number)	Sequence
***RPS18B* (*At1g 34030*)**	5'- GTCTCCAATGCCCTTGACAT -3'
	5'- TCTTTCCTCTGCGACCAGTT -3'
OPR1 (At1g 76680)	5′- TGTGTCCTTGTTGTTGCAGGTTTTG -3′
	5′- TCCAACACGGTCTGGTCCGA -3′
OPR3 (At2g 06050)	5′- CCTTCTTCCAGATCGGCGGAGACAT -3'
	5′- GGCGCCAGAACCACTCGATGA -3'
GST1 (At1g 02930)	5'- GCCTTTCATCCTTCGCAACCCCT -3'
	5'- TCGCCATGTCCTTGCCAGTTGA -3'
VSP2 (At5g24770)	5'- ACGACTCCAAAACCGTGTGCAA -3'
	5'- CGGGTCGGTCTTCTCTGTTCCGT -3'
JAZ1 (AT1G19180)	5'- CGCGAGCAAAGGCACCGCTA -3'
	5'- TCCAAGAACCGGTGAAGTGAAGC -3'

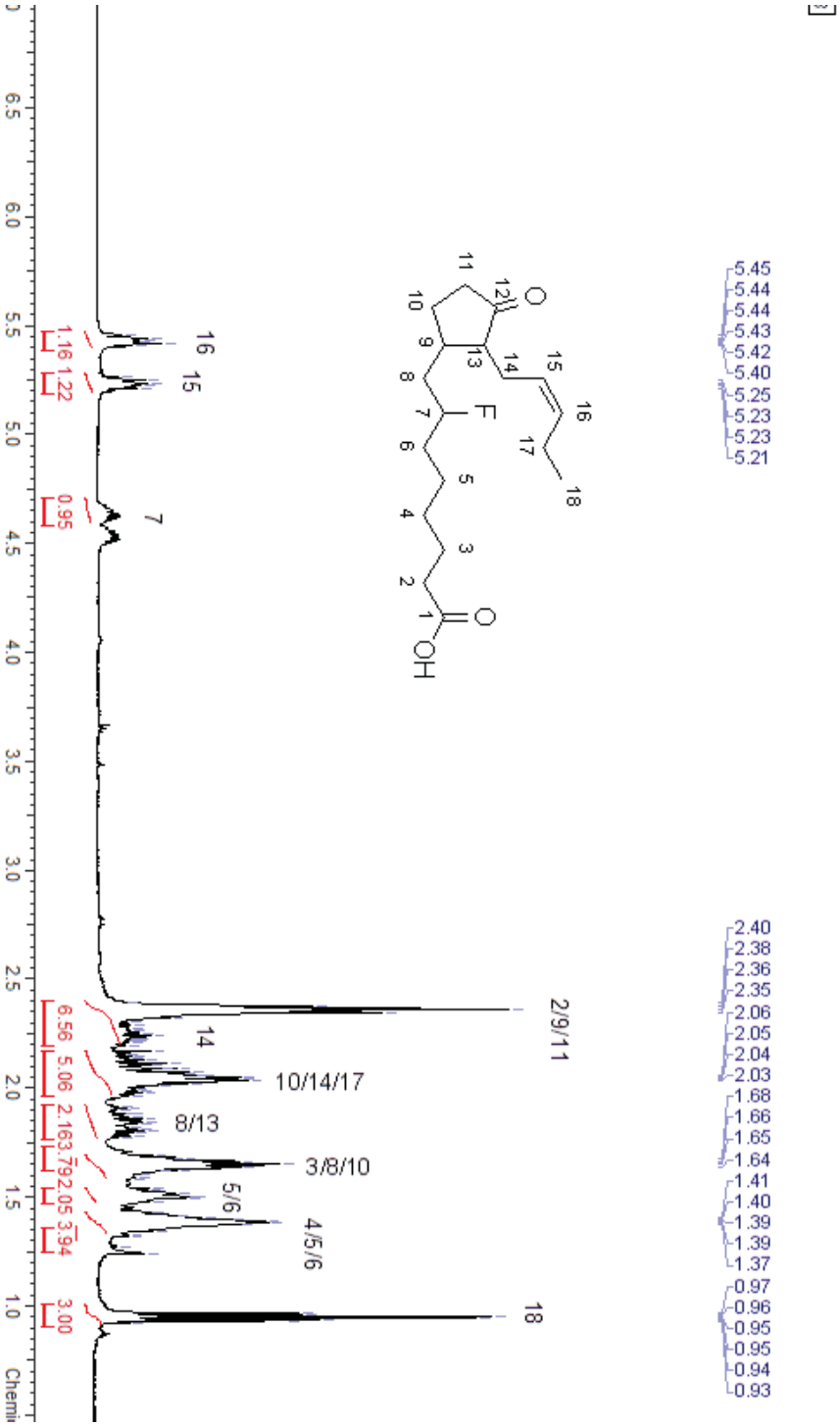
^{1}H-NMR Compound (**4**)



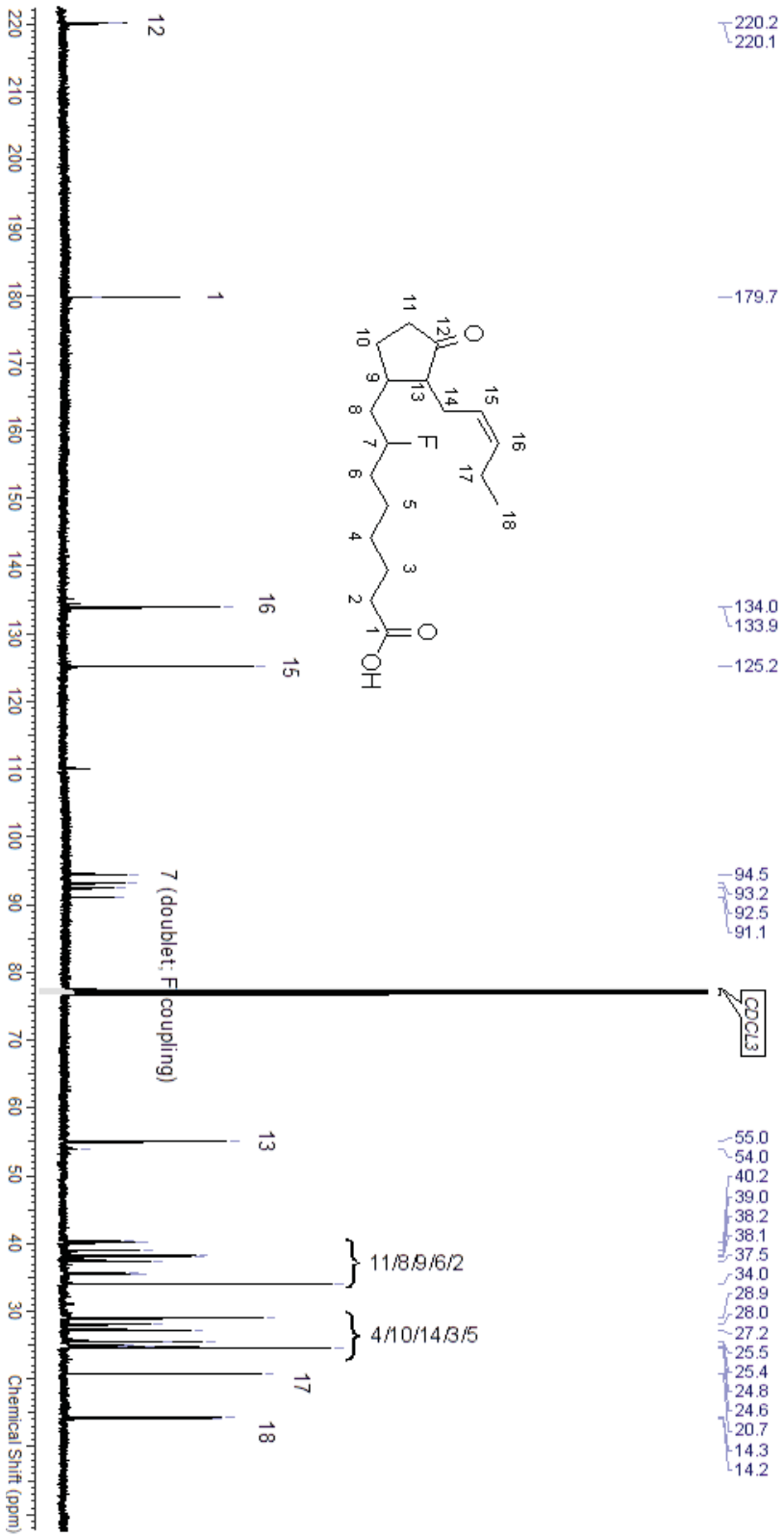
^{13}C- APT-NMR Compound (**4**)



^{1}H-NMR 7F-OPC-8:0 (**10**)



^{13}C NMR 7F-OPC-8:0 (**10**)



HRESIMS spectrum of 7F-OPC-8:0 (**10**)

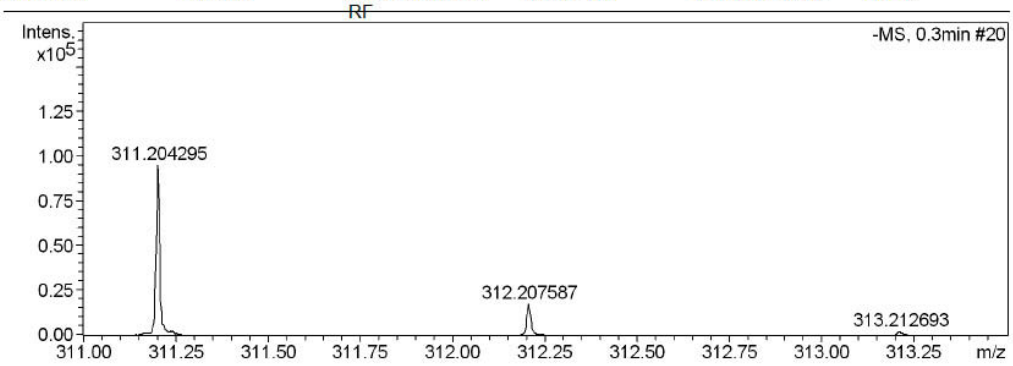
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\kepl2401\Guille_7fluoro8_cyclopentyloctanioicAcid_312neg_neuCalib.d	Acquisition Dat	12/5/2014 10:24:55 AM
Method	kp_tune_low_neg_250_JA.m	Operator	Admin
Sample Name		Instrument / Se	maXis 10078
Comment			

Acquisition Paramet

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	3000 V	Set Dry Heater	190 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	1000.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
311.204295	1	C 18 H 28 F O 3	100.00	311.202797	-1.498	-4.814	5.6	4.5	even	ok

Protein Interaction Data for CML37 (PAIR database)

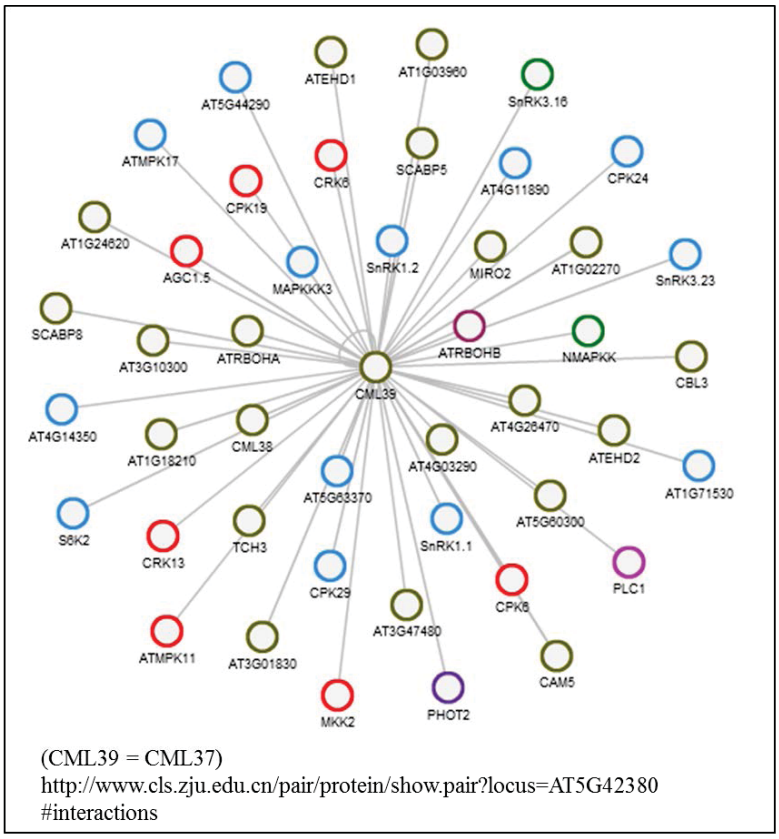


Figure S 3. Computational analysis of potential interaction partners of CML37 (= CML39).

Shown are the 46 possible interaction partners of AT5G42380 (CML37, CML39) based on computational calculation. Data were calculated by use of PAIR database (http://www.cls.zju.edu.cn/pair/home.pair).The ineracting partners are listed in Table S1.

Table S 1. Potential interaction partners of CML37 (= CML39) based on computational calculation. Calculation is based on the PAIR database entries (ID numbers). The score is an indicator of the prediction confidence, all scores greater than 1 is considered equally confident. In general, the higher the score, the more confident a prediction is (http://www.cls.zju.edu.cn/pair/home.pair).

ID	Partner A	Description A	Partner B	Description B	Score
21886	AT5G44290	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	protein kinase family protein	0.187245
23928	AT4G26470	calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0976034
26868	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	PLC1	PLC1 (PHOSPHOLIPASE C 1); phospholipase C	0.201623

26869	NMAPKK	MEK1 (MAP KINASE/ ERK KINASE 1); MAP kinase kinase/ kinase/ protein binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.249989
26870	ATMPK11	ATMPK11; MAP kinase/ kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0779014
26871	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	AT5G60300	lectin protein kinase family protein	0.116104
26872	SnRK3.23	CIPK23 (CBL-INTERACTING PROTEIN KINASE 23); kinase/ protein binding / protein serine/threonine kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0253147
26873	CML38	calcium-binding EF hand family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	1.37186
26874	CPK29	CPK29; ATP binding / calcium ion binding / calmodulin-dependent protein kinase/ kinase/ protein kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0386109
26875	ATRBOHA	ATRBOHA (respiratory burst oxidase homolog A); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.283213
26876	ATEHD2	ATEHD2 (EPS15 HOMOLOGY DOMAIN 2); GTP binding / GTPase/ calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.126341
26877	AT3G47480	calcium-binding EF hand family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0275765
26878	AT4G11890	protein kinase family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.342162
26879	CPK6	CPK6 (CALCIUM-DEPENDENT PROTEIN KINASE 6); ATP binding / calcium ion binding /	CML39	CML37 (CALMODULIN LIKE 37); calcium	0.1144

		calmodulin-dependent protein kinase/ kinase/ protein kinase/ protein serine/threonine kinase		ion binding	
26880	AT3G10300	calcium-binding EF hand family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0646465
26881	ATRBOHB	ATRBOHB (respiratory burst oxidase homolog B); FAD binding / calcium ion binding / electron carrier/ iron ion binding / oxidoreductase/ oxidoreductase, acting on NADH or NADPH, with oxygen as acceptor / peroxidase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	1.15933
26882	SnRK3.16	CIPK1 (CBL-INTERACTING PROTEIN KINASE 1); kinase/ protein binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.00428004
26883	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	PHOT2	PHOT2 (PHOTOTROPIN 2); FMN binding / blue light photoreceptor/ kinase/ protein serine/threonine kinase	0.130957
26884	MIRO2	MIRO2 (MIRO-RELATED GTP-ASE 2); GTPase/ calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0131524
26885	CRK6	CRK6 (CYSTEINE-RICH RLK 6); kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0106926
26886	AT1G24620	polcalcin, putative / calcium-binding pollen allergen, putative	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.117571
26887	CBL3	ATCBL3 (ARABIDOPSIS THALIANA CALCINEURIN B-LIKE 3); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.509189

26888	CAM5	CAM5 (CALMODULIN 5); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.281632
26889	AGC1.5	AGC1.5 (AGC KINASE 1.5); kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.00121386
26890	CPK19	CPK19; ATP binding / calcium ion binding / calmodulin-dependent protein kinase/ kinase/ protein kinase/ protein serine/threonine kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0355503
26891	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	AT5G63370	protein kinase family protein	0.111101
26892	MAPKKK3	MAP3KA; ATP binding / kinase/ protein kinase/ protein serine/threonine kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0652482
26893	SnRK1.1	AKIN10 (Arabidopsis SNF1 kinase homolog 10); protein binding / protein kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.488322
26894	ATMPK17	ATMPK17; MAP kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.00974092
26895	SCABP8	CBL10 (CALCINEURIN B-LIKE 10); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.508881
26896	AT1G02270	endonuclease/exonuclease/ phosphatase family protein / calcium-binding EF hand family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.288293
26897	CRK13	protein kinase family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.110216
26898	TCH3	TCH3 (TOUCH 3); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium	0.815963

				ion binding	
26899	AT1G71530	protein kinase family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0193371
26900	S6K2	S6K2 (ARABIDOPSIS THALIANA SERINE/THREONINE PROTEIN KINASE 2); kinase/ protein kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0906767
26901	MKK2	ATMKK2 (ARABIDOPSIS THALIANA MAP KINASE KINASE 2); MAP kinase kinase/ kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.355093
26902	SCABP5	CBL1 (CALCINEURIN B-LIKE PROTEIN 1); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.88678
26903	AT4G14350	protein kinase family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.166672
26904	AT3G01830	calmodulin-related protein, putative	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.198228
26905	AT4G03290	calcium-binding protein, putative	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.123432
26906	ATEHD1	ATEHD1 (EPS15 HOMOLOGY DOMAIN 1); GTP binding / GTPase/ calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.342258
26907	SnRK1.2	AKIN11 (Arabidopsis SNF1 kinase homolog 11); protein binding / protein kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.275558
26908	AT1G18210	calcium-binding protein, putative	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.159541

26909	CPK24	CPK24; ATP binding / calcium ion binding / calmodulin-dependent protein kinase/ kinase/ protein kinase/ protein serine/threonine kinase	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0639545
106052	AT1G03960	calcium-binding EF hand family protein	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.0621032
138502	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	CML39	CML37 (CALMODULIN LIKE 37); calcium ion binding	0.673265

8 Curriculum Vitae

Personal information

Name	Sandra Scholz
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Education and training

Since 07/2011	**PhD student** (Max Planck Institute for chemical Ecology, Jena)
	topic: Role of Calmodulin-like Proteins in Calcium-mediated Herbivore Defense Pathways in Arabidopsis.
	supervisors: PD Dr. Axel Mithöfer, Prof. Dr. R. Oelmüller, Dr. Jyothilakshmi Vadassery
11/2009 – 06/2011	**Diploma Theses** (Max Planck Institute for chemical Ecology, Jena)
	topic: Establishing of a flexible labeling strategy for soil bacteria with Fluorescent proteins.
	supervisors: Dr. Christian Kost, Prof. Wilhelm Boland, Prof. Erika Kothe
09/2005 – 11/2009	**Studies of Biology**
	Friedrich Schiller University, Jena

Graduate Academy

International Max Planck Research School Jena (IMPRS): Exploration of ecological interactions with molecular and chemical techniques.

Attended Courses

Feb 2012	Arabidopsis Protoplast Transfection, speaker: Dr. Jianqiang Wu
Nov 2012	MS-based Proteomics, speaker: Dr. A. Svatos, Dr. N. Wielsch, Y. Hupfer
Jan-Feb 2013	Introduction into the statistical program R, speaker: Dr. G. Kunert
Jun 2013	**Gene Safety Project Leader Certificate, speaker: JSMC**
Jul 2013	Fundamentals of Mass Spectrometry, speaker: Dr. A. Atthygalle, Dr. A. Svatos
Apr 2014	Professional Job Application for PhD Students, speaker: Barbara Hoffbauer, KEPOS
Jun 2014	Research Funding, speaker: Dr. Jan Kellmann
Jul 2014	JGA: Die schriftliche Bewerbung für den außeruniversitären Arbeitsmarkt, speaker: Evelyn Hohenheim
Jul 2014	Metabolomics principles and fundamental techniques, speaker: Prof. Georg Pohnert and co-workers
Okt 2014	**JGA: Introduction to the GxPs - with special focus on GMP and GLP, speaker: Prof. Dr. Michael Hildebrand**
May 2015	JGA: Projektmanagement, speaker: Peter Wagner
Mar 2015	NMR course, speaker: Dr. Bernd Schneider and co-workers
Jan 2015	Adobe Illustrator, speaker: Dr. Nico Überschaar
May 2015	JGA: Projektmanagement, speaker: Peter Wagner

Publications

2015

Jimenez-Aleman, G.H.*, Scholz, S.*, Heyer, M., Reichelt, M., Mithöfer, A., and Boland, W. (submitted). Synthesis, biological activity, metabolism and systemic transport of 7-fluoro-OPC-8:0, a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0. BBA Molecular and Cell Biology of Lipids, submitted 12.06.2015. (*Those authors contributed equally)

Scholz, S., Reichelt, M., Mekonnen, D., Ludewig, F., Mithöfer, A. (submitted). Insect herbivory-elicited GABA accumulation in plants is a wound-induced, direct, and jasmonate-independent defense response. Plant Cell & Environment, submitted 25.06.2015.

Scholz, S., Reichelt, M., Boland, W., Mithöfer, A. (submitted). Additional evidence against jasmonate-induced jasmonate induction hypothesis. Plant Science, resubmitted 16.06.2015.

Scholz, S., Reichelt, M., Vadassery, J., Mithöfer, A. (in press). Calmodulin-like protein CML37 is a positive regulator of ABA during drought stress in Arabidopsis. Plant Signaling & Behavior., 10(5), in press.

2014

Scholz, S. S., Vadassery, J., Heyer, M., Reichelt, M., Bender, K. W., Snedden, W. A., Boland, W., Mithöfer, A. (2014). Mutation of the Arabidopsis calmodulin-like protein CML37 deregulates the jasmonate pathway and enhances susceptibility to herbivory. Molecular Plant, 7, 1712-1726. doi:10.1093/mp/ssu102.

2012

Vadassery, J., Scholz, S. S., Mithöfer, A. (2012). Multiple calmodulin-like proteins in Arabidopsis are induced by insect-derived (Spodoptera littoralis) oral secretion. Plant Signaling & Behavior, 7(10), 1277-1280. doi:10.4161/psb.21664.

Oral Presentations

Scholz S. (2014). A calmodulin-like protein, CML37, acts as a positive regulator in jasmonate-mediated insect herbivory-induced plant defense in Arabidopsis. *Talk presented at 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE*

Scholz S. (2013). Calcium sensor CML37 – A player in herbivore induced plant defense. *Talk presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE*

Poster Presentations

Vadassery J., Scholz S., Heyer M., Reichelt M., Hause B., Boland W., Mithöfer A. (2011). Role of calcium sensors CML42 and CML37 in Arabidopsis response to insect herbivory. Poster presented at Botanikertagung 2011, German Botanical Society, Freie Universität Berlin, Berlin, DE

Scholz S., Vadassery J., Heyer M., Reichelt M., Hause B., Boland W., Mithöfer A. (2012). Role of calcium sensor CML37 in Arabidopsis response to insect herbivory. Poster presented at 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Scholz S., Vadassery J., Heyer M., Reichelt M., Hause B., Boland W., Mithöfer A. (2012). Role of calcium sensor CML37 in Arabidopsis response to insect herbivory. Poster presented at International Conference on Arabidopsis Research 2012, Vienna, AT

Scholz S., Vadassery J., Heyer M., Reichelt M., Hause B., Boland W., Mithöfer A. (2012). Role of calcium sensor CML37 in Arabidopsis response to insect herbivory. Poster presented at Black Forest Retreat 2012 on Molecular Plant Science, Feldberg, DE

Vadassery J., Scholz S., Heyer M., Reichelt M., Hause B., Boland W., Mithöfer A. (2012). Calcium signaling regulates herbivore-induced jasmonate pathway. Poster presented at SAB Meeting 2012, MPI for Chemical Ecology, Jena, DE

Scholz S., Vadassery J., Reichelt M., Hause B., Boland W., Mithöfer A. (2013). Calcium sensor CML37 - a player in herbivore induced plant defense. Poster presented at 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, DE

Scholz S., Vadassery J., Reichelt M., Hause B., Boland W., Mithöfer A. (2013). Calcium sensor CML37 - a player in herbivore induced plant defense. Poster presented at Botanikertagung 2013, Deutsche Botanische Gesellschaft, Tübingen, DE

Scholz S., Vadassery J., Reichelt M., Snedden W., Boland W., Mithöfer A. (2014). A calmodulin-like protein, CML37, acts as a positive regulator in jasmonate-mediated insect herbivory-induced plant defense in Arabidopsis. Poster presented at Plant Calcium Signaling Meeting 2014, Münster, DE

Scholz S., Vadassery J., Reichelt M., Hause B., Boland W., Mithöfer A. (2014). Calcium sensors CML37 and CML42 - two antagonistic players in herbivore defense. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE

Scholz S., Vadassery J., Reichelt M., Hause B., Boland W., Mithöfer A. (2014). Calcium sensors CML37 and CML42 - two antagonistic players in herbivore defense. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

9 Acknowledgement

First I want to say **THANK YOU** to our Jyothi. Yes, where should I start? Thanks for being my supervisor, for giving me the possibility to work on your project, for teaching me a lot of new things and techniques, for your help at any time (even from India). Thank you for a very nice and fruitful time together ☺.

Next I want to say Thank you to Christian Mazars, for being my external reviewer and for sharing information about CML9. Now I want to switch to German….

Es kommt mir noch wie gestern vor, als Moni und ich zeitgleich dabei waren unsere Diplom- bzw. Staatsexamensarbeit zu schreiben und wir uns gegenseitig Mut zusprachen und uns motivierten fertig zu werden ☺... Nun ist es wieder soweit und meine Dissertation ist nach 4 Jahren dabei „fertig" zu werden….

Dazu haben eine Vielzahl von Leuten beigetragen denen ich hiermit ein großes **DANKE** sagen möchte (ich hoffe ich vergesse niemanden...).

Zunächst erst einmal möchte ich mich bei Axel bedanken (ich schreibe jetzt nicht das P-Wort welches du so „gerne" hörst), der mir diese Position angeboten hat und mich immer in jeder Lage unterstützt und angespornt hat weiter zu machen und noch mehr zu erreichen. Das haben wir auf jeden Fall geschafft! Natürlich auch ein großes Danke dafür dass du meine Arbeit begutachten wirst.

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I’m done. ☺ ☺

10 Eigenstädigkeitserklärung

Ich erkläre hiermit, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist.

Ich habe die vorliegende Dissertation selbständig verfasst und alle Hilfsmittel und Quellen in der Arbeit angegeben.

Alle Personen, die an den Manuskripten durch Experimente, Auswertung und Schreiben mitgewirkt haben, sind im Kapitel „*Manuscript Overview*“ mit ihrem jeweiligen Anteil aufgelistet.

Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Arbeit stehen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit zu einer staatlichen oder anderen wissenschaftlichen Prüfung eingereicht. Ferner habe ich auch nicht versucht, die gleiche, eine in wesentlichen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Sandra Scholz