Linking early calcium signals to phytohormone responses: The role of different Calmodulin-like proteins in various stress reactions of *Arabidopsis thaliana*

Dissertation

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1 Introduction

1.1 Plants and environmental stress

Organisms live in a constantly changing environment. Abiotic factors like the temperature, humidity or light are fluctuating as well as the influence by interacting partners like symbionts, pathogens or predators (biotic factors). In order to deal with these environmental changes, organisms can either adapt to the new conditions or avoid unfavorable environments. Sessile organisms like plants are bound to their environment and cannot shortly escape if abiotic or biotic factors are changing. Thus, they needed to develop strategies to handle different abiotic and biotic stress situations. This dissertation focuses on three major environmental stressors of plants: Drought conditions, feeding of herbivores and infection by pathogens.

1.1.1 Plant defense against herbivores

Although plants are frequently attacked by herbivores throughout their lifetime, they are able to withstand these attacks by employing different defense strategies. These defenses can be either of mechanical or chemical nature. Mechanical barriers build the first layer of defense against herbivores (Scholz *et al.*, 2016). Hair-like structures on the leave surface called trichomes or epicuticular waxes are known to affect the feeding behavior of different herbivores (Eigenbrode and Espelie, 1995, Reymond *et al.*, 2004). Further, they prevent the oviposition of female herbivores and by this negatively influence the fitness of the herbivore (Eigenbrode and Espelie, 1995, Karley *et al.*, 2016, Silva *et al.*, 2017). However, the efficiency of this kind of physical barriers is highly dependent on the size of the herbivore (Hanley *et al.*, 2007). They might be more helpful against herbivores that are relatively small in size (Mitchell *et al.*, 2016). Further, the success of the physical barrier depends on the feeding mechanism of the herbivore: Leaf-mining insects that feed within the plant tissue are less affected by outer physical barriers than insects that chew on the plant tissue or suck on the cell content or the phloem (Hanley *et al.*, 2007, Mitchell *et al.*, 2016).
Thus, aside these physical barriers, plants produce a huge variety of secondary metabolites used as feeding deterrents and toxins (Mithöfer and Boland, 2012). In *Arabidopsis thaliana*, the model plant used in this study, the class of glucosinolates is of special interest as a defense against herbivores. Glucosinolates are *β*-

thioglucoside-N-hydroxysulfates with variable amino-acid derived side chains (Fahey *et al.*, 2001, Figure 1A). Depending on the biosynthetic origin of this side chain three groups of glucosinolates are distinguished: aliphatic (derived from methionine, leucine, isoleucine, alanine and valine), indole (derived from tryptophan) and aromatic glucosinolates (derived either from phenylalanine or tyrosine) (Halkier and Gershenzon, 2006). Glucosinolates themselves are not harmful to other organisms, but by cleaving off the glucose moiety they turn into toxins such as nitriles, thiocyanates, isothiocyanates, epithionitriles and oxazolidine-2-thiones (Halkier and Gershenzon, 2006, Figure 1B).

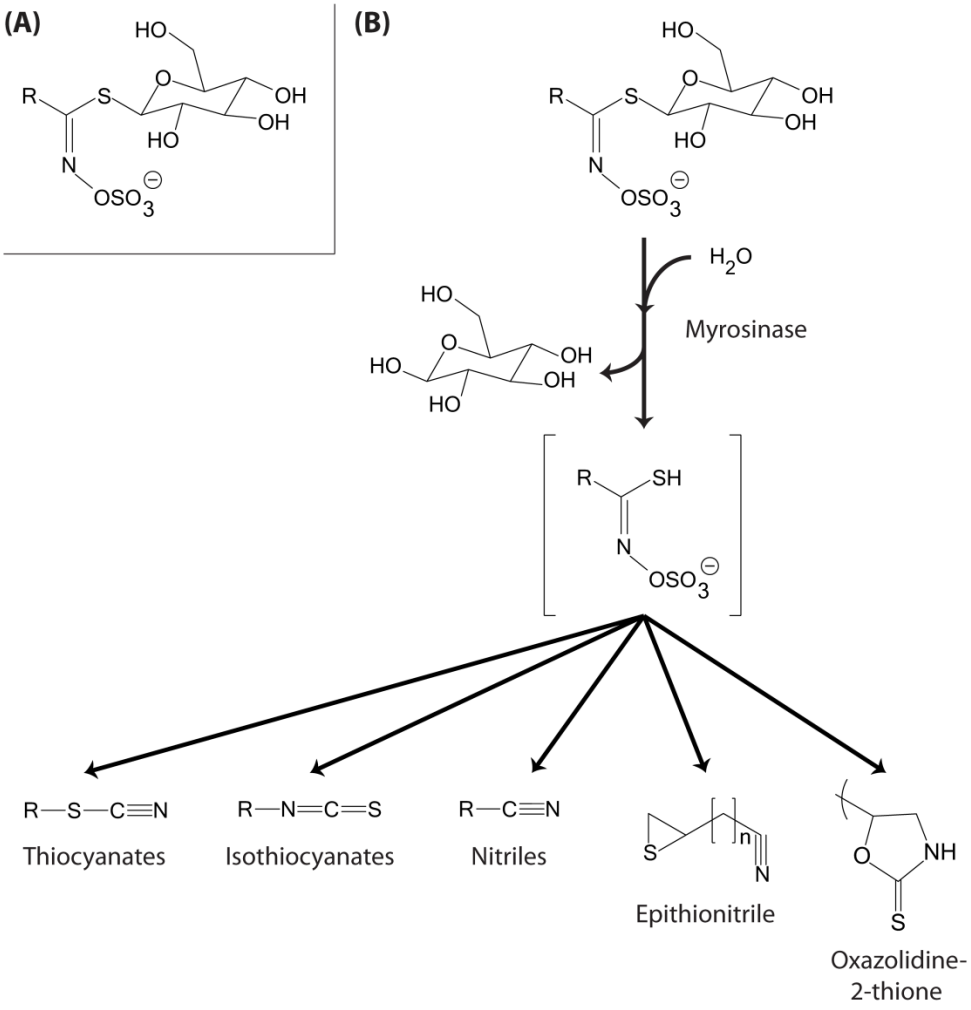

Figure 1. Glucosinolates and their breakdown products. (A) Basic chemical structure of a glucosinolate: The glucose is linked via a sulfur atom to the variable side chain R and the N-hydroximinosulfate ester (Halkier and Gershenzon, 2006). (B) Different hydrolysis products of Glucosinolates are shown. Both (A) and (B) are modified after Halkier and Gershenzon (2006).

The cleavage of glucosinolates into their toxic products is catalyzed by the enzyme myrosinase. Because of the high toxicity of the breakdown products for the plant itself, myrosinases are stored in separate cells, apart from the glucosinolates. However, upon tissue damage, e.g. by feeding of an insect, myrosinases and glucosinolates are mixed up and toxins are released (Mithöfer and Boland, 2012). Although it has been shown for different species that insects are able to circumvent the toxic effects of glucosinolate hydrolysis products by e.g. detoxification or excretion (Jeschke *et al.*, 2016), they have been proven to reduce feeding of many different insect species (e.g. Müller *et al.*, 2010, Jeschke *et al.*, 2017). However, to which extent secondary metabolites serve as feeding deterrents is highly dependent on the feeding strategy of the herbivore. Generalist herbivores, such as *Spodoptera littoralis*, that feed on a huge variety of host plants are more impaired than specialist insect that only feed on a few plants and thus are adapted to the feeding deterrents of the plant (Müller *et al.*, 2010, Schweizer *et al.*, 2013, Jeschke *et al.*, 2017). In some cases, specialists even use the specific secondary metabolites to distinguish their host plants from non-host plants, leading rather to an attracting effect of secondary metabolites than a deterring (Sun *et al.*, 2009).

Another form of chemical defense is the production of anti-herbivore proteins such as protease inhibitors that impair the digestion of the insects (Mithöfer and Boland, 2012). These proteins are taken up by the insects by feeding on the plant material and affect the use of nutrients as well as the destruction of plant-derived anti-herbivore proteins inside the insect gut (Mithöfer and Boland, 2012).

Besides secondary metabolites and proteins that directly act on the herbivore (thus called direct defense (Mithöfer and Boland, 2012)), plants produce also volatile compounds to attract predators or parasitoids of the feeding insect (e.g. Sabelis and Van De Baan, 1983, Whitman and Eller, 1990, Kessler and Baldwin, 2001). Since volatiles are actually not acting directly on the herbivore but are used as transmitters to employ other organisms for help, this kind of defense is called indirect defense (Mithöfer and Boland, 2012). Further, they also have been shown to act as systemic signals within plants, leading to priming of non-infested plant parts for an upcoming herbivore attack (Frost *et al.*, 2007, Heil and Silva Bueno, 2007).

Since the production of anti-herbivore compounds is quite costly to the plant, some defense metabolites are just produced upon actual feeding (Howe and Jander, 2008). They are classified as induced defenses (Mithöfer and Boland, 2012).

However, some of the defense mechanisms, especially the physical barriers, are also exhibited by non-attacked plants and thus are classified as constitutive defenses (Mithöfer and Boland, 2012). Nevertheless, most of the constitutive defense can also be induced upon insect attack, as e.g. glucosinolates, and thus can be classified as both (Halkier and Gershenzon, 2006).

1.1.2 Plant defense against pathogens

Similar to the defense against herbivores, plants developed physical and chemical barriers against the attack by pathogens. In order to successfully infect a plant, the pathogen needs to first enter the plant tissue. By installing different physical barriers, the plant tries to prevent the entry of the pathogen. Openings in the leaf surface, like stomata, facilitate the entrance into the inner leaf tissues. Thus, plants close their stomata upon pathogen attack (Melotto *et al.*, 2006). Further, they are known to strengthen their cell walls by e.g. lignification (Vance *et al.*, 1980) or callose deposition (Voigt, 2014). Also changing the composition of cuticular waxes or enhancing their quantity helps to prevent pathogen attacks (Reina-Pinto and Yephremov, 2009).

In addition, plants produce secondary metabolites that function as antimicrobial compounds. They are classified into phytoalexins and phytoanticipins. Phytoanticipins are compounds that are constitutively produced by plants without an actual pathogen infection (Vanetten *et al.*, 1994). Glucosinolates serve as phytoanticipins against some pathogens, although their function seems to be highly dependent on the attacking pathogen (Tierens *et al.*, 2001, Brader *et al.*, 2006, Bednarek *et al.*, 2009). On the other side, phytoalexins, are *de novo* produced upon pathogen infection (Vanetten *et al.*, 1994). The main phytoalexin in *Arabidopsis* is camalexin, which has been shown to be toxic to different fungal pathogens (Glawischnig, 2007).

Besides secondary metabolites, plants also use proteins as defense against pathogens. These pathogen-related proteins (PR-proteins) are produced upon pathogen attack and include e.g. proteinase inhibitors that block the lysis of the plant tissues by the pathogen, plant defensins (PDFs) that are known to display antifungal activities and chitinases or glucanases to destroy the cell walls of fungal pathogens (Loon *et al.*, 2006, Sels *et al.*, 2008).

Once the pathogen entered the plant tissue, plants try to inhibit the spreading of the pathogen amongst the tissue by programmed cell death around the infection side (Heath, 2000). However, this so-called hypersensitive response (HR, Heath, 2000) just helps to prevent growth of pathogens that feed on living plant tissue (biotrophs) (Mengiste, 2012). Necrotrophic pathogens that kill the plant tissue to get their nutrients from the plant, show even enhanced growth upon HR (Govrin and Levine, 2000, Mengiste, 2012). Thus, the success of defense against pathogens highly depends on the pathogens' lifestyle and the appropriate defense mechanisms.

1.1.3 Drought stress response

Water is essential for plants to survive. Thus, drought conditions cause severe stress in plants. In order to cope with drought conditions plants pursue two major strategies at once: minimize dehydration and maximize rehydration (Chaves *et al.*, 2003). In order to decrease water loss, one of the first reactions of plants is to close their stomata and by this limit the transpiration of water via the leaf surface (Daszkowska-Golec and Szarejko, 2013). However, the closure of stomata also limits the uptake of CO_2, used as carbon source for photosynthesis. Thus, a common response of plants to drought is a reduction of their photosynthetic activity (Chaves *et al.*, 2003). Besides closing stomata, the transpiration rate of the leaves can also be limited by an increase of the cuticular wax layer (Kosma *et al.*, 2009). Further, plants try to reduce transpiration by decreasing the leaf area by processes like leaf rolling (Kadioglu *et al.*, 2012) or by reduced growth of new leaves (Skirycz and Inzé, 2010).

On the other side, the uptake of water into the cells is maximized by the production of different osmotic compounds, like the amino acid proline, proteins such as the dehydrins or polyols like sorbitol or mannitol (Chaves *et al.*, 2003). They decrease the osmotic potential of the plant cell, allowing a water uptake even under low water conditions (Chaves *et al.*, 2003). Besides, they might also serve as protectant of the cell membrane stability or enzymes under drought conditions (Chaves *et al.*, 2003). Plants can also increase the water uptake by an enlargement of their root system, in order to get access to water in other soil layers (Chaves *et al.*, 2003, Werner *et al.*, 2010). However, tremendous growth changes might be rather a response to long-lasting drought conditions, than a short-term adaption (Chaves *et al.*, 2003).

1.2 Perception of stress in plants

The fact that defense mechanisms are inducible upon the occurrence of stress shows that plants can directly react to changes in their environment. A requirement for this is the perception of the stress conditions. In case of pathogens the recognition process is well studied. Plants perceive the pathogens via molecular patterns, either coming directly from the pathogen (pathogen-associated molecular patterns, PAMPs) or resulting from the infection (damage-associated molecular patterns, DAMPs) (Zipfel, 2014). Typical PAMPs are components existing in the surface of bacteria or fungi that are attacking plants, like chitin or peptidoglycans (Felix *et al.*, 1993, Gust *et al.*, 2007), or flagellin, the basic substance of the bacterial flagellum (Felix *et al.*, 1999). On the other side, DAMPs are plant-derived molecules emerging out of the damage caused by the pathogen (Choi and Klessig, 2016), like cell wall fragments (e.g. oligogalacturonides, Hahn *et al.*, 1981, Nothnagel *et al.*, 1983, Ferrari *et al.*, 2013), protein breakdown products (e.g. Peps in *Arabidopis*, Huffaker *et al.*, 2006), high mobility group box proteins (Choi *et al.*, 2016) or extracellular adenosine triphosphate (eATP, Chivasa *et al.*, 2009). PAMPs and DAMPs are perceived by the plant via pattern recognition receptors in the plasma membrane (Zipfel, 2014). Further, pathogens release so-called effectors upon infection, to impair the immune response of the plants (Jones and Dangl, 2006) that can be recognized by plants *via* intracellular receptors and lead to a secondary defense response (Zipfel, 2014).

Similar to the recognition of pathogens also herbivores are perceived by either elicitors originating their oral secretions (OS), so called herbivore-associated molecular patterns (HAMPs, Mithöfer and Boland, 2008) or by DAMPs released upon wounding caused by the herbivore feeding (Heil and Land, 2014). However, so far less is known about how HAMPs are perceived by the plants (Mithöfer *et al.*, 2005, Zipfel, 2014). Just for the fatty acid-amino acid conjugate volicitin, a HAMP in the OS of *Spodoptera exigua*, a putative receptor was revealed in maize plants (*Zea mays*, Truitt *et al.*, 2004). However, the protein was not further identified and HAMP receptors are still missing in other plant species. A limitation in the identification of HAMP receptors might be that also just a few HAMPs are identified (Basu *et al.*, 2017). Further, their role as recognition pattern seems to be highly dependent on the plant species, they are applied to (Acevedo *et al.*, 2015) e.g. Volicitin induced defense in maize, eggplant (*Solanum melongena*) and soybean (*Glycine max*)

(Alborn *et al.*, 1997, Schmelz *et al.*, 2009), but had no effect when applied to *A. thaliana* (Schmelz *et al.*, 2009). Besides HAMPs and DAMPs, the plant may recognize the insect feeding also by molecular patterns of the endosymbionts present in the gut or salivary glands of the herbivore (Acevedo *et al.*, 2015, Basu *et al.*, 2017).

In contrast to pathogens or herbivores the mechanisms underlying drought stress perception are not well understood. Although throughout the past years several receptors have been revealed to be important for the drought stress response, in most of the cases the ligands of the particular receptors are still unknown (Osakabe *et al.*, 2013). However, it seems that the recognition of drought stress is a multilayered sensing process, since it might involve perception of several markers at once like the turgor pressure, the osmotic potential of the plant cell or the integrity of the cellular membrane (Chaves *et al.*, 2003).

1.3 Signal transduction

Once the plant sensed a certain environmental change, by binding of the respective ligand to the receptors inside the cell or at the plasma membrane, this perception needs to be translated into the according stress responses. A complex network including different signaling molecules, like phytohormones, γ-aminobutyric acid (GABA), reactive oxygen species (ROS), phosphorylation events, pH, electrical signals or Calcium (Ca^{2+}), transfers the information about the environment from receptors into genetic and metabolic changes inside the cell, leading to the appropriate response (Mcdowell and Dangl, 2000, Chaves *et al.*, 2003, Maffei *et al.*, 2007, Plieth, 2016, Scholz *et al.*, 2016, Ramesh *et al.*, 2017). This thesis focuses mainly on the connection between two major signaling components: Phytohormones and Ca^{2+} signals.

1.3.1 Phytohormones mediate plant stress responses

1.3.1.1 Jasmonates

Jasmonates are phytohormones regulating a broad range of plant responses against environmental changes (Wasternack and Song, 2017). In particular, they are known for mediating plant defense against herbivores and as wound signaling

hormones (Howe and Jander, 2008, Koo and Howe, 2009). Further, it has been shown that they play a role in the signal transduction leading to defense against necrotrophic pathogens (Glazebrook, 2005) and to drought stress adaptations (Kazan, 2015).

Jasmonates are produced out of *α*-linolenic acid (Figure 2). Inside chloroplasts, *α*-linolenic acid is converted in three steps to 12-oxophytodienoic acid (OPDA), catalyzed by the enzymes lipoxygenase (LOX, Bell *et al.*, 1995, Bannenberg *et al.*, 2008), allene oxide synthase (AOS, Vick and Zimmerman, 1987, Song *et al.*, 1993, Laudert *et al.*, 1996) and allene oxide cyclase (AOC) (Hamberg, 1988, Ziegler *et al.*, 2000, Stenzel *et al.*, 2003). OPDA is then transported into the peroxisome were it is reduced by the oxophytodienoic acid reductase 3 (OPR3) to 8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (OPC-8:0, Vick and Zimmerman, 1986, Müssig *et al.*, 2000, Schaller *et al.*, 2000, Strassner *et al.*, 2002). Further OPC-8:0 undergoes three *β*-oxidations (Vick and Zimmerman, 1984). Each *β*-oxidation step shortens the carboxyl side chain by two carbons, leading to the production of 6-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)hexanoic acid (OPC-6:0), 4-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)butanoic acid (OPC-4:0) and finally to jasmonic acid (JA) (Miersch and Wasternack, 2000).

Recently Chini *et al.* (2018) showed, that JA can be also produced via an OPR3-independent pathway (Figure 2), where OPDA directly undergoes three *β*-oxidation cycles, leading to the production of first dinor-12-oxophytodienoic acid (dn-OPDA), then tretranor-12-oxophytodienoic acid (tn-OPDA) and finally 4,5-didehydro-jasmonic acid (4,5-ddh-JA). The 4,5-ddh-JA is then reduced by OPR2 to JA (Chini *et al.*, 2018).

Long time JA was thought to be the bioactive jasmonate. However, Staswick and Tiryaki (2004) and Fonseca *et al.* (2009) revealed that the isoleucine conjugate of jasmonic acid, jasmonoyl-isoleucine (JA-Ile), is the bioactive form. The production of JA-Ile in the cytoplasm is catalyzed by the jasmonic acid-amido synthetase JAR1 (JASMONATE RESISTANT 1, Staswick and Tiryaki, 2004, Figure 2).

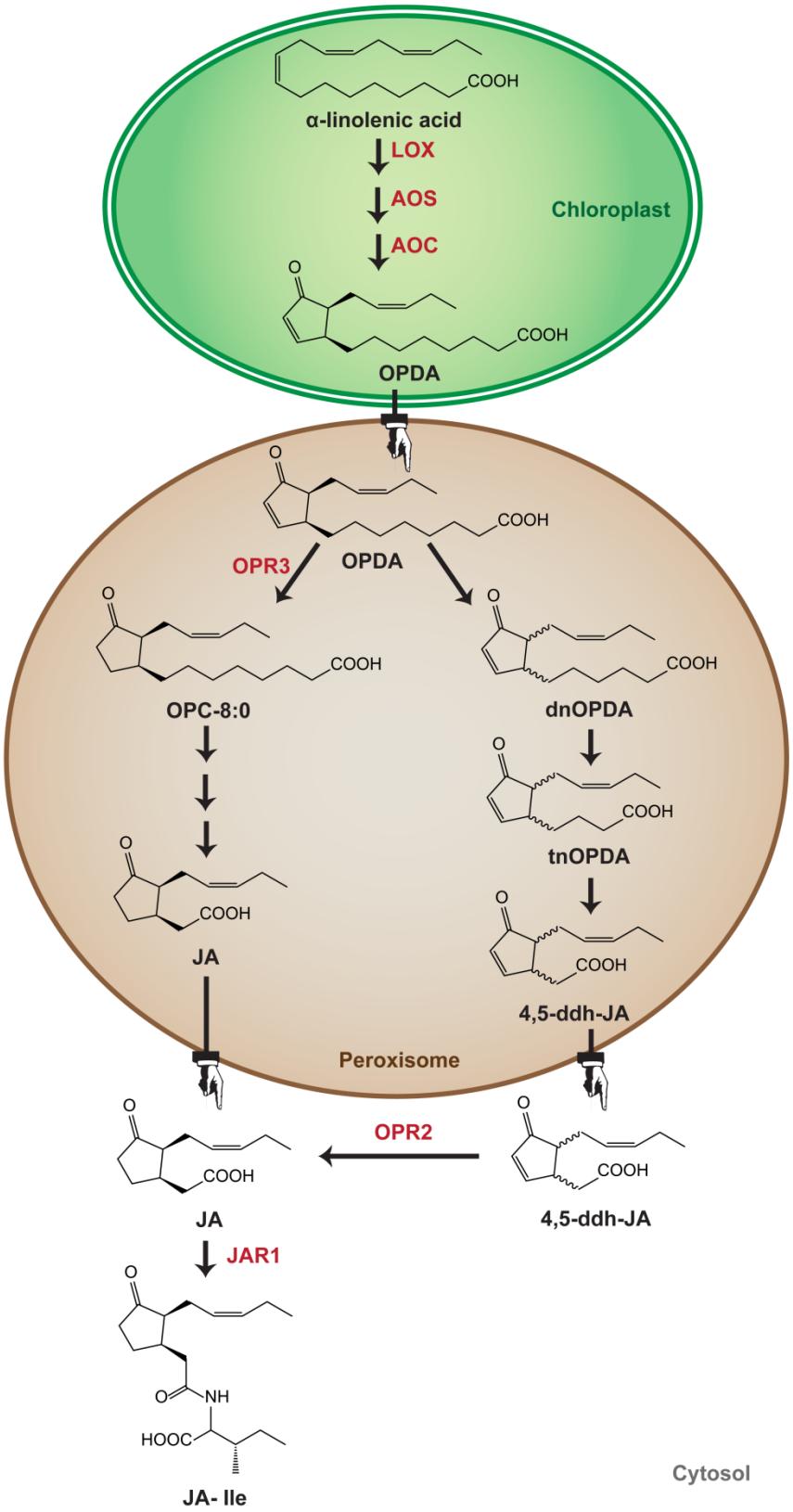

Figure 2. Jasmonate biosynthesis. Shown is a simplified scheme of the Jasmonate biosynthesis via the octadecanoid pathway modified after Wasternack and Song (2017), Chini *et al.* (2018). Enzymes indicated by the red color. Black arrows indicate chemical reactions. Hands indicate transport between cell compartments.

Once JA-Ile accumulates upon certain stimuli, it binds to its receptor CORONATINE INSENSITIVE 1 (COI1) (Fonseca *et al.*, 2009, Sheard *et al.*, 2010). COI1 is part of the SCF complex, an E3 ubiquitin ligase (Devoto *et al.*, 2002). Upon binding of JA-Ile, the SCF^{COI1} complex ubiquitinates the jasmonate-ZIM-domain (JAZ) proteins, leading to their degradation by the 26S proteasome (Chini *et al.*, 2007, Figure 3). JAZ proteins are transcription repressors, binding to MYC and MYB transcription factors under low JA-Ile levels (Chini *et al.*, 2007, Chini *et al.*, 2009, Fernandez-Calvo *et al.*, 2011, Song *et al.*, 2011). JA-Ile perception enables the degradation of JAZ proteins and thus the release of the transcription factors, leading to the expression of genes relevant for the plant defense (Chini *et al.*, 2007, Fernandez-Calvo *et al.*, 2011, Figure 3).

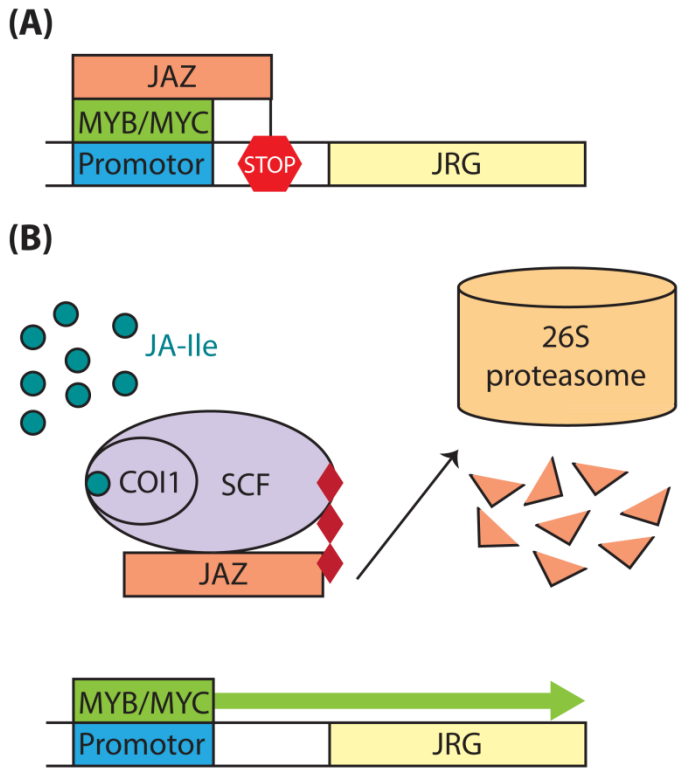

Figure 3. Perception of JA-Ile and induction of downstream defenses. Shown is a simplified scheme of the JA-Ile perception and downstream events modified after Wasternack and Song (2017). (A) The JAZ proteins repress MYC and MYB transcription factors in non-stressed plants (low JA-Ile) (B) Upon perception of JA-Ile by SCF^{COI1} complex, JAZ proteins are ubiquitinated and degraded in the 26S proteasome. MYC and MYB transcription factors are released and can induce expression of JA-responsive genes. Dark red diamonds indicate ubiquitin. The green arrow indicates gene expression

Besides JA-Ile it was also suggested that OPDA has a function as phytohormone on its own. It has been shown that OPDA can induce genes that are not inducible upon treatment with JA (Taki *et al.*, 2005). Further, OPDA can directly influence the development of insects (Dabrowska *et al.*, 2009). However, the mode of action of OPDA as independent signal is not clear.

1.3.1.2 Salicylic acid

Another important phytohormone in biotic stress signaling is salicylic acid (SA, Figure 4). SA is produced out of chorismate *via* two independent pathways with either isochorismate or phenylalanine as intermediate products (Dempsey *et al.*, 2011).

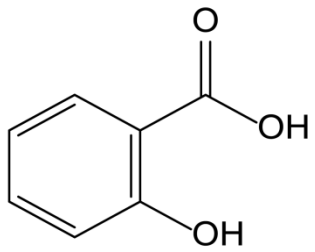

Figure 4. Chemical structure of the phyto-hormone SA.

SA is mainly known to mediate the defense against biotrophic pathogens (Glazebrook, 2005). Further, the defense against piercing-sucking herbivores relies on SA signaling. Feeding of aphids on the phloem of plants mainly triggers SA-dependent responses (Moran and Thompson, 2001, Li *et al.*, 2006). The defense against spider mites seems to be dependent on both JA and SA signaling (Kant *et al.*, 2004, Villarroel *et al.*, 2016).

However, in case of feeding of chewing herbivores or infection with necrotrophic pathogens SA is known to antagonize the defense responses induced by JA (Cipollini *et al.*, 2004, Spoel *et al.*, 2007, Bruessow *et al.*, 2010). It has been shown that SA can directly counteract JA-induced transcription by negatively influencing the accumulation of transcription factors necessary for expression of JA-responsive genes (Van Der Does *et al.*, 2013) or by inducing the expression of other transcription factors that suppress the expression of JA-responsive genes (Caarls *et al.*, 2015). On the other hand, some biotrophs produce coronatine, a chemical mimic of JA-Ile, to suppress SA-induced defense responses in plants, showing that the antagonism is bidirectional (Zheng *et al.*, 2012). It is hypothesized that the antagonism between the JA and SA pathway is used by the plant to regulate contrasting needs in case of the attack of several pathogens and herbivores at once (Pieterse *et al.*, 2012).

1.3.1.3 Abscisic acid

The responses to abiotic stress, like drought, are mainly mediated by the phytohormone abscisic acid (ABA, Figure 5) (Tuteja, 2007). ABA is a terpenoid, produced out of carotenoids (Nambara and Marion-Poll, 2005).

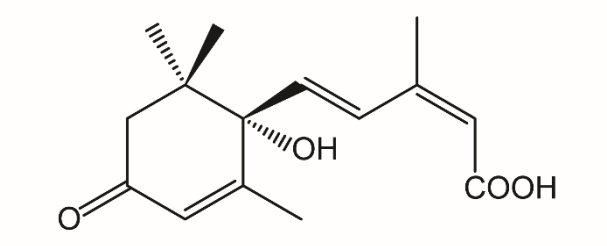

Figure 5. Chemical structure the phyto-hormone ABA.

The ABA pathway is closely interrelated with JA signaling. On one hand, ABA coregulates responses to drought together with JA, e.g. the closing of stomata in response to drought (Daszkowska-Golec and Szarejko, 2013). Further, it was shown that the ABA accumulation in drought stressed roots is dependent on JA-Ile (De Ollas *et al.*, 2015b). On the other side, it is known that ABA is also important for the defense against herbivores that is mainly mediated by jasmonates. ABA-signaling mutants are more susceptible to herbivory than wild type plants (Bodenhausen and Reymond, 2007). Further, MYC2, the transcription factor controlling expression of jasmonate responsive genes is coregulated by ABA (Kazan and Manners, 2013). In addition, ABA regulates enzymes releasing α-linolenic acid from the chloroplast membranes and thus stimulates jasmonate biosynthesis (Wang *et al.*, 2018). Thus, ABA seems to play a role in defining defense responses to biotic stress as well.

1.3.2 GABA: signaling molecule and defense compound

Besides phytohormones also other metabolites function as a signal transmitting information about the stimulus into a stress response. The non-proteinogenic amino acid GABA (Figure 6) is known for long time as neurotransmitter in animals and seems to function as signaling molecule in plants as well (Ramesh *et al.*, 2017).

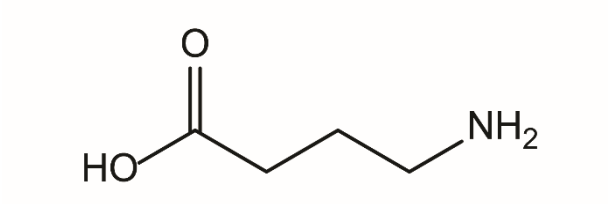

Figure 6. Chemical structure of the non-proteinogenic amino acid GABA.

GABA has been shown to modulate the activity of anion transporters in the plasma membrane of plant cells, leading to a change in the membrane potential and by this influencing downstream responses (Ramesh *et al.*, 2015). GABA is known to accumulate upon various abiotic and biotic stress treatments (Ramesh *et al.*, 2017). *Inter alia*, GABA is induced within a few minutes in wounded tissue (Ramputh and Bown, 1996) and it accumulates in response to herbivores as well (Bown *et al.*, 2002, Scholz *et al.*, 2015b). In both cases GABA signaling seems to act *via* a jasmonate independent pathway (Scholz *et al.*, 2015b). However, how GABA influences downstream defense responses as signaling molecule remains still unclear.

On the other hand, it was hypothesized that GABA might be the defense compound itself. Insects feeding on a GABA-rich diet showed reduced growth and delayed development (Ramputh and Bown, 1996, Bown *et al.*, 2006, Scholz *et al.*, 2017). Since GABA is a neurotransmitter in animals, activating chloride channels, high GABA levels in the diet may lead to negative effects on the nervous system of the larvae (Bown *et al.*, 2006, Scholz *et al.*, 2017). Thus GABA might function as signaling molecule in the defense and as defense itself.

1.3.3 Ca^{2+} as second messenger in stress responses

1.3.3.1 Ca^{2+} signals are fast and stimulus specific

Before the accumulation of signaling metabolites such as phytohormones, transient increases in the intracellular Ca^{2+} concentrations of plant cells are one of the earliest signals that can be measured in response to different environmental stimuli (Dodd *et al.*, 2010, see Figure 7 for time line of the signaling events).

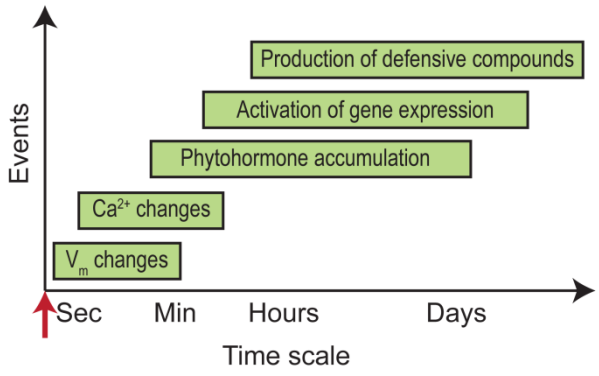

Figure 7. Time line of stress signaling in plants. Upon stress perception (indicated by the red arrow) changes in membrane potential (V_m) can be measured within seconds, followed by transient increases of the intracellular Ca^{2+} concentration. Phytohormone signaling starts within minutes after signal perception and can be long-lasting. Signaling events activate downstream the expression of stress responsive genes within minutes and hours which leads later to the production of defensive compounds. Figure is modified after Maffei *et al.* (2007).

In response to drought stress, *Arabidopsis* seedlings increase their intracellular Ca^{2+} concentration from approximate 100 nM at resting state up to 1.6 µM, within a few seconds (Knight *et al.*, 1997). Similarly, wounding or treatment with insect OS or PAMPs lead to a rapid increase in the cytosolic Ca^{2+} concentration of plants (Knight *et al.*, 1991, Mithöfer *et al.*, 1999, Blume *et al.*, 2000, Maffei *et al.*, 2004, Vadassery *et al.*, 2012a). Although Ca^{2+} signals can be measured in response to various biotic and abiotic stimuli, they differ depending on the particular stimulus (Mcainsh *et al.*, 1997). E.g. mannitol treatment to mimic drought induced the Ca^{2+} concentration to a higher extent than OS application (Knight *et al.*, 1997, Vadassery *et al.*, 2012a). However, Ca^{2+} increase was prolonged, lasting several minutes, when plants were treated with insect OS, whereas mannitol led only to an increase lasting less than 1 min (Knight *et al.*, 1997, Vadassery *et al.*, 2012a). Besides their duration and height, Ca^{2+} signals may also differ in their frequency and their intracellular or tissue-specific location (Mcainsh *et al.*, 1997, Dodd *et al.*, 2010). All these spatio-temporal characteristics of the calcium signals (calcium signature) encode for information about the stimulus occurring (Mcainsh *et al.*, 1997). Further, the interplay with other cellular messengers, such as reactive oxygen species, membrane potential changes or pH changes might transfer specific information about the environmental change into the plant cell (Mcainsh *et al.*, 1997, Plieth, 2016).

1.3.3.2 Decoding of Ca^{2+} signals by Ca^{2+} sensors

In order to use the information about the stimulus occurring that is mediated by the specific signature of the Ca^{2+} increase, the plant cell needs to decode these signals and transfer it into downstream responses like gene expression or production of metabolites. The sensing of Ca^{2+} ions by Ca^{2+} binding proteins is the first step of decoding.

In *Arabidopsis* a huge amount of approximately 250 Ca^{2+} sensing proteins have been identified (Day *et al.*, 2002). All of them carry at least one Ca^{2+} binding domain, the so called EF-Hand (Kretsinger and Nockolds, 1973, Day *et al.*, 2002). These domains are built up by two helices, called E- and F-helix respectively, that are connected *via* a loop binding the Ca^{2+} ion (Figure 8, Kretsinger and Nockolds, 1973).

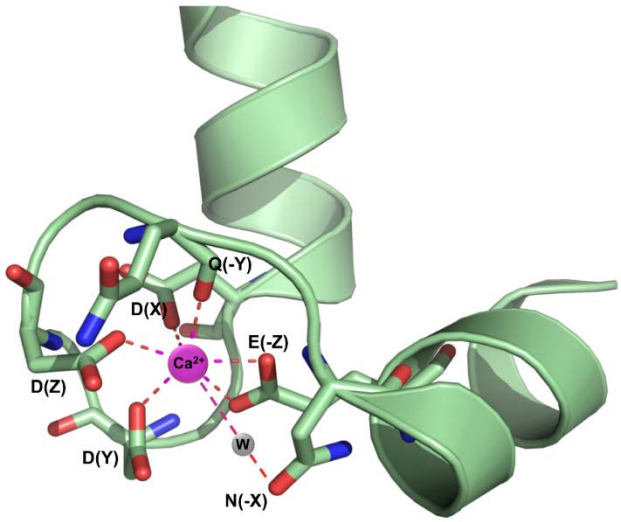

Figure 8. Structure of an EF-Hand. Shown is the 3D-structure of an EF-Hand of the Calmodulin CaM7 of *Arabidopsis thaliana*. The Ca^{2+} ion (pink) binds within the loop structure that is connecting the E- and the F-helix. W (grey) indicates a water molecule. The image is reused from La Verde *et al.* (2018).

Upon binding of Ca^{2+} these proteins undergo a conformational change, allowing them to bind to a downstream target, like kinases, phosphatases or other enzymes, ion pumps or channels, or transcription factors (Dodd *et al.*, 2010, La Verde *et al.*, 2018). This leads either directly to a change in gene expression and by this to a certain stress response or the Ca^{2+} signal is translated into another signal mode by interaction with the target, leading to further signal transduction events.

In addition to the EF-Hands some Ca^{2+} binding proteins possess other functional domains. They are classified as sensor responders (Sanders *et al.*, 2002). An example for sensor responders is the family of Ca^{2+}-dependent protein kinases

(CPKs) in *Arabidopsis* (Sanders et al., 2002). These proteins possess a kinase domain besides their EF-hands that is activated by the conformational change initiated by the Ca^{2+} binding (Sanders *et al.*, 2002). Thus they can directly transform the signal into other signals or gene expression changes.

Apart from sensor responders, most of the Ca^{2+} sensor families known in *Arabidopsis* just have EF-hands as functional domains and thus are only able to bind Ca^{2+}. They are called sensor relays (Sanders *et al.*, 2002). These proteins need to interact with other proteins to transform the Ca^{2+} signal into downstream signaling events or stress responses (Sanders *et al.*, 2002, Dodd *et al.*, 2010). Amongst the group of sensor relays are Ca^{2+} sensor families like the calcineurin B-like proteins (CBLs), calmodulins (CaMs) and calmodulin-like proteins (CMLs) (Sanders *et al.*, 2002).

Evolutionary studies revealed that amongst all the four families of Ca^{2+} sensing proteins only the CML family is unique to the plant kingdom (Edel *et al.*, 2017), suggesting that CMLs might have special roles in decoding plant specific Ca^{2+} signals (Zhu *et al.*, 2015). Indeed CMLs have been shown to be specifically regulated amongst different developmental stages as well as upon different stress treatments in *Arabidopsis* (Mccormack *et al.*, 2005). Further analysis of mutant lines of some CMLs revealed that they regulate plant stress responses. E.g. CML24 contributes to the HR and thus to the defense against the pathogen *Pseudomonas syringae* (Ma *et al.*, 2008). CML41 mediates the closure of stomata and callose deposition upon pathogen infection and thus enhances resistance against *P. syringae* (Xu *et al.*, 2017). Also CML9 (also known as CaM9) and CML8 have been shown to regulate the defense against *P. syringae* (Leba *et al.*, 2012, Zhu *et al.*, 2017). Besides its role in pathogen defense, CML9 is described to negatively regulate abiotic stress responses like the drought stress response (Magnan *et al.*, 2008). Further, transcripts of CML9 were induced upon mechanical damage and treatment with OS of *S. littoralis* larvae (Magnan *et al.*, 2008, Vadassery *et al.*, 2012b), suggesting that CML9 might regulate jasmonate dependent stress response as well. Thus CML9 might be a key player in regulating diverse stress responses. Similarly also CML42 and CML37 have been revealed as regulators of both abiotic and biotic stress responses. They mediate the drought stress response of *A. thaliana* as well as the defense against the herbivore *S. littoralis* (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c). Interestingly CML42 turns out to be

a negative regulator of both stress responses whereas CML37 is a positive one (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c), suggesting that both might act antagonistically to each other.

However, although for some CMLs their regulating functions in plant stress responses have been identified, less is known about how the Ca^{2+} sensing by CMLs is connected to downstream signaling events. Just for some CMLs the interacting partners are known (La Verde *et al.*, 2018). Recently it was shown that ACA8, an autoinhibited Ca^{2+} ATPase, is interacting with CML36 (Astegno *et al.*, 2017). Also CML42 and CML37 have been shown to interact with ACAs (Yilamujiang, 2012, Scholz, 2015), suggesting that ACAs might have an important function as downstream targets of CMLs.

1.3.4 Systemic signal transduction in *Arabidopsis* in response to herbivory

Unlike plants, herbivores are motile organisms that are able to move on the whole plant in order to feed. Thus it is important for plants to activate defense not only in the local infested leaf but also in the systemic parts of the plant that are not yet fed, to prepare them for upcoming attacks (Howe and Jander, 2008). In order to spread the information about the feeding on the local leaf amongst the whole plant, systemic signaling is necessary. Further, since insect movements are rather fast, the systemic signaling needs to be equally rapid to ensure a proper defense of systemic tissue. In animals, rapid systemic signaling is ensured by the nervous system. However, plants lack such a system and thus developed other ways of systemic signaling. In the past years it has been shown that signaling molecules can be rapidly propagated amongst the plant *via* the vascular system (Mousavi *et al.*, 2013, Salvador-Recatalà *et al.*, 2014, Gasperini *et al.*, 2015, Kiep *et al.*, 2015, Nguyen *et al.*, 2018). Hereby the plant uses the systemic connections between the vasculatures of the single leaves (Mousavi *et al.*, 2013, Salvador-Recatalà *et al.*, 2014, Gasperini *et al.*, 2015, Kiep *et al.*, 2015, Nguyen *et al.*, 2018).

In Arabidopsis, these connections result out of the development of the rosette (Dengler, 2006). The vasculature of the embryonic leaves, the cotyledons, and the first four juvenile leaves develops directly from the vascular cylinder of the hypocotyl (Figure 9, Busse and Evert, 1999, Kang *et al.*, 2003, Dengler, 2006). The vasculature of the following leaves 5 to 8 arises from branches of the vascular trace

of the first four leaves following either the rule n+3 or n+5, whereby n is the number of the antecedent leaf (Figure 9, Kang *et al.*, 2003, Dengler, 2006). Leaf 9 and following leaves are derived from branches following the rules n+8 and n+5 (Figure 9, Kang *et al.*, 2003, Dengler, 2006). However, they still share indirect connections to other leaves following the n±3 rule, so called contact parastichies (Figure 9, Dengler, 2006, Mousavi *et al.*, 2013). Thus all leaves amongst the *Arabidopsis* rosette are connected *via* their vasculature to other leaves following the rules n±3, n±5 and n±8.

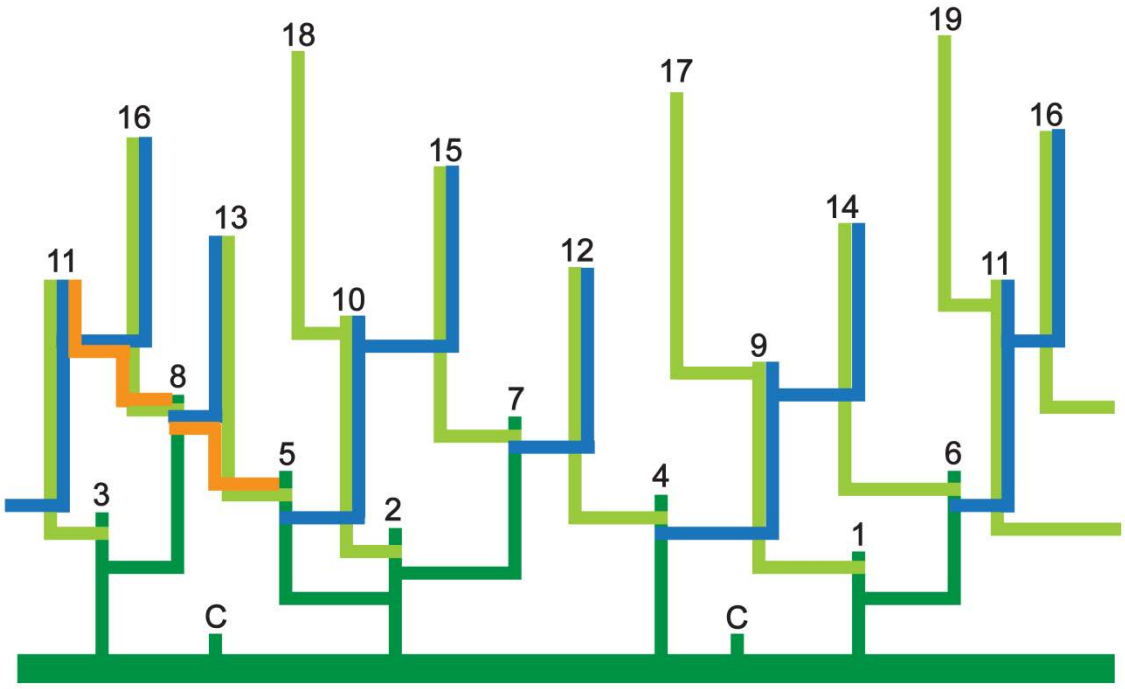

Figure 9. Vascular connections amongst leaves in an adult *Arabidopsis* rosette. Scheme represents vascular connections between the leaves of an *Arabidopsis* rosette. Leaves of the adult phase are directly connected following the rule n±8 (light green lines) and n±5 (blue lines), whereby n indicates a certain leaf number. Further indirect connections occur, where one leaf is connected via another connected leaf following the rule n±3. An example for such indirect connections is given for leaf 8, indicated by the orange lines. The vasculatures of leaves 1 to 4 develop directly from the vascular cylinder of the hypocotyl that is given by the dark green bar in the basis of the scheme. These juvenile leaves are connected to the older leaves *via* the n±3 or n±5 rule as indicated by the dark green lines. Numbers indicate the leaf number, ascending from oldest leaves to younger leaves. C indicates the cotyledons. Scheme is modified after Dengler (2006).

It is known from several signals in plants, that they follow these vascular connections, when they are systemically propagated. The second messenger Ca^{2+} has been shown to be only induced in systemic leaves that were connected to the

leaf locally fed by the herbivore *S. littoralis*, but not in other uninfested leaves of the *Arabidopsis* rosette (Kiep *et al.*, 2015). The same was true for electric signals that are required for the full systemic Ca^{2+} signal (Mousavi *et al.*, 2013, Nguyen *et al.*, 2018).

It has been shown that Ca^{2+} signaling can affect downstream phytohormone responses locally (Bonaventure *et al.*, 2007, Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Lenglet *et al.*, 2017), suggesting that also systemic phytohormone elevations might follow vascular connections. However, how phytohormones are systemically distributed is still not fully understood. Although some studies suggested that jasmonates might be induced in systemic connected leaves (Glauser *et al.*, 2009, Chauvin *et al.*, 2013), a full study amongst the whole *Arabidopsis* rosette is still missing. Further, less is known about the systemic role of other phytohormones like ABA and SA that interact with the jasmonate pathway. In addition, some studies point out that the phytohormones themselves get distributed among the rosette (Sato *et al.*, 2009, Sato *et al.*, 2011), whereas others show that they are *de novo* synthesized in the systemic leaves (Koo *et al.*, 2009). Still it is not clear how systemic phytohormone elevations develop. Thus, systemic phytohormone signaling still needs to be further investigated.

1.4 Aims of this study

As described in the chapters before, the signaling leading to a certain stress response is not fully understood. Especially the connection between early signaling events like the sensing of Ca^{2+} signatures and downstream signals like the elevation of phytohormones is still under investigation. CMLs might be an important link between Ca^{2+} and phytohormone signaling, since some have been shown to influence phytohormone pathways (Magnan *et al.*, 2008, Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c, Zhu *et al.*, 2017). Therefore, one aim of this study is to gain more knowledge about three CMLs that have been shown to regulate abiotic as well as biotic stress responses in *Arabidopsis thaliana* and, hence, might be important key players in the regulation of plant stresses in general: CML9, CML37 and CML42. Herein the following open questions concerning these three CMLs are addressed:

1. Does CML9 regulate JA-dependent stress responses like the defense against herbivores or the necrotrophic pathogen *Alternaria brassicicola*?
2. Are CML37 and CML42 antagonists in the regulation of herbivore defense and drought stress response?
3. Do CML37 and CML42 play a role in the defense against *A. brassicicola*?
4. How do CML37 and CML42 transfer their information sensed into downstream signaling events and what is the role of the possible interacting partner ACA4?

Besides studying local signal transduction after herbivory, this study aims to clarify also the systemic signaling events. Especially the systemic propagation of phytohormones needs further investigation. Here I address the following questions:

1. Can the jasmonate precursor OPC-8:0 be transported systemically throughout the plant?
2. Are jasmonates, ABA and SA systemically propagated after herbivore-related wounding along the vascular connections between the leaves?

Further, recently it was shown that also GABA is induced systemically in *Arabidopsis* plants (Scholz *et al.*, 2015b, Scholz *et al.*, 2017). However, in these studies just several leaves of the *Arabidopsis* rosette have been investigated. Here I studied systemic GABA elevations along with systemic phytohormone elevations in a holistic approach addressing the whole *Arabidopsis* rosette.

2 Manuscript overview

2.1 Manuscript 1

Herbivory-responsive calmodulin-like protein CML9 does not guide jasmonate-mediated defenses in *Arabidopsis thaliana*

Authors: Heyer, M., Scholz, S. S., Voigt, D., Reichelt, M., Aldon, D., Oelmüller, R., Boland, W., Mithöfer, A.

Status: published 2018, PLoS One, 13(5): e0197633. doi:10.1371/journal.pone.0197633.

Summary:

This study investigated the role of CML9 in different jasmonate-regulated defenses in *A. thaliana*. Examining the expression pattern of *CML9* revealed that it is upregulated by the oral secretion of the herbivore *S. littoralis* as well as mechanical damage and real insect feeding. However, the analysis of four independent knock-out and two overexpression lines in herbivore performance assays showed that CML9 is neither involved in the herbivore defense against *S. littoralis* nor against *T. urticae.* In addition, the response to the necrotrophic fungus *A. brassicicola* was investigated, since it is jasmonate-mediated as well. In line with the results of the herbivore treatment, *cml9* mutants displayed a wild type-like reaction to the fungus. These results suggest that CML9 is very likely not regulating jasmonate-mediated defense pathways. Furthermore the role of CML9 in the plant drought stress response was reexamined. In contrast to previous published studies, the drought stress reaction of *cml9* mutant lines was not altered, suggesting that CML9 is not a general regulator of this stress response.

Author contributions:

Conceptualization:	M. Heyer, D. Voigt, A. Mithöfer
Formal analysis:	M. Heyer, S. S. Scholz, M. Reichelt
Funding acquisition:	W. Boland
Investigation:	M. Heyer, S. S. Scholz, D. Voigt, M. Reichelt
Methodology:	D. Voigt, M. Reichelt
Project administration:	A. Mithöfer

Resources: D. Voigt, D. Aldon, R. Oelmüller, W. Boland

Supervision: R. Oelmüller, A. Mithöfer

Visualization: M. Heyer, S. S. Scholz

Writing – original draft: M. Heyer, S. S. Scholz, M. Reichelt, A. Mithöfer

Writing – review & editing: D. Voigt, D. Aldon, R. Oelmüller, W. Boland

Contribution of M. Heyer: 75%

M. Heyer

- planned all experiments (in consultation with A. Mithöfer and regarding Fig. 4 in consultation with D. Voigt)
- conducted the following experiments:
    - Fig. 1
    - Fig. 2
    - Fig. 3 (in collaboration with M. Reichelt, who performed the LC-MS/MS measurements and calculated the phytohormone content of single samples)
    - Fig. 6 (6b in collaboration with M. Reichelt, who performed the LC-MS/MS measurements and calculated the phytohormone content of single samples)
    - Fig. 7
    - S1 Fig
- did data processing and statistics of all experiments except Fig. 5
- visualized all data as the presented Figures, Fig. 5 was done in collaboration with S.S. Scholz
- wrote the original draft of the manuscript except of the methods part concerning the fungi treatment (S.S. Scholz), the methods part for the quantification of phytohormones was written in collaboration with M. Reichelt, the manuscript was revised by A. Mithöfer and the other authors

2.2 Manuscript 2

The Ca^{2+} sensor proteins CML37 and CML42 antagonistically regulate various plant stress responses by altering phytohormone responses

Authors: Heyer, M., Scholz, S. S., Chini, A., Reichelt, M., Kunert, G., Oelmüller, R., Mithöfer, A.

Status: submitted June 2019 to Plant, Cell & Environment

Summary:

The CMLs CML37 and CML42 have been shown before to regulate the defense against herbivores and the drought stress response in *Arabidopsis*, whereby CML37 turned out as positive and CML42 as negative regulator of both stress reactions. Here, we studied if these positive and negative effects of both CMLs are antagonistic to each other by employing a double knock out mutant line. Under drought as well as under herbivore pressure the double knock out mutants displayed wild type-like stress responses, suggesting that both CMLs are antagonistically to each other. In both cases CML37 and CML42 regulate the stress response by modulating the jasmonate or ABA signaling, respectively. We further investigated the interplay between both CMLs and the jasmonate pathway by studying possible interaction between the CMLs and the JAR1 enzyme and the JA-Ile receptor COI1. However, none of the CMLs interacted with JAR1 or COI1, suggesting that the CMLs might interact either with proteins upstream the phytohormone pathway or with transcription factors downstream the JA-Ile perception. We further show that CML37 and CML42 also antagonistically regulate the defense against the necrotrophic fungus *A. brassicicola*, suggesting a broad importance of the antagonism for jasmonate-mediated defense responses. The striking antagonism over all tested stress responses implies that CML37 and CML42 might have a balancing role in the coordination of different stress responses in the plant.

Author contributions:

Conceptualization:	M. Heyer, A. Mithöfer
Formal analysis:	M. Heyer, A. Chini, S. S. Scholz, M. Reichelt, G. Kunert
Investigation:	M. Heyer, A. Chini, S. S. Scholz, M. Reichelt
Methodology:	M. Reichelt

Project administration: A. Mithöfer
Resources: R. Oelmüller
Supervision: R. Oelmüller, A. Mithöfer
Visualization: M. Heyer, A. Chini, S. S. Scholz
Writing – original draft: M. Heyer, A. Chini, M. Reichelt, A. Mithöfer
Writing – review & editing: S.S. Scholz, G. Kunert, R. Oelmüller

Contribution of M. Heyer: 70%

M. Heyer

- planned all experiments (in consultation with A. Mithöfer and regarding Fig. 5 in consultation with A. Chini)
- conducted the following experiments:
    - Fig. 1
    - Fig. 2
    - Fig. 3 (in collaboration with M. Reichelt, who performed the LC-MS/MS measurements and calculated the phytohormone content of single samples)
    - Fig. 4 (in collaboration with M. Reichelt, who performed the HPLC/UV measurements and calculated the glucosinolate content of single samples)
    - Fig. 7 (7b in collaboration with M. Reichelt, who performed the LC-MS/MS measurements and calculated the phytohormone content of single samples)
- did data processing and statistics of all experiments, statistics for Fig. 4, 6 and 7 were done partially in collaboration with G. Kunert
- visualized all data as the presented Figures, Fig. 5 was done in collaboration with A. Chini and Fig. 6 was done in collaboration with S.S. Scholz
- wrote the original draft of the manuscript, the methods part for the quantification of phytohormones and glucosinolates was written in collaboration with M. Reichelt and the methods part for the yeast two-hybrid in collaboration with A. Chini, the manuscript was revised by A. Mithöfer and the other authors

2.3 Manuscript 3

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

Authors: Jimenez-Aleman, G.H.*, Scholz, S. S.*, Heyer, M., Reichelt, M., Mithöfer, A., Boland, W.

* These authors contributed equally to the work.

Status: published 2015, Biochimica et biophysica acta: BBA Molecular and cell biology of lipids, 1851(12), 1545-1553.
doi:10.1016/j.bbalip.2015.09.002

Summary:

In this study a fluorinated analog of the jasmonate precursor OPC-8:0 (7F-OPC-8:0) was synthesized in order to investigate its ability to be translocated systemically throughout the *Arabidopsis* rosette after wounding. It was shown that 7F-OPC-8:0 is a true mimic of the endogenous OPC-8:0, since it is metabolized *in vivo* similar to the natural compound. Due to its ability to be metabolized in the plant, treatment with 7F-OPC-8:0 lead to an increased jasmonate content and the upregulation of jasmonate responsive genes. After testing the biological activity, 7F-OPC-8:0 was used for translocation studies. It was applied to wounded local leaves and could be detected in vascular connected leaves after 1 h. This result suggests that OPC-8:0 can be transported systemically after wounding and by this contribute to systemic jasmonate signals. With these results, we show further that fluorinated jasmonates can be used as a tool to study the metabolism and transport of these compounds throughout the plant.

Author contributions:

Conceptualization:	G.H. Jimenez-Aleman, S.S. Scholz, M. Heyer, A. Mithöfer, W. Boland
Formal analysis:	G.H. Jimenez-Aleman, S. S. Scholz, M. Heyer, M. Reichelt
Funding acquisition:	W. Boland

Investigation:	G.H. Jimenez-Aleman, S. S. Scholz, M. Heyer, M. Reichelt
Methodology:	G.H. Jimenez-Aleman, M. Reichelt
Project administration:	W. Boland
Supervision:	W. Boland, A. Mithöfer
Visualization:	G.H. Jimenez-Aleman, S. S. Scholz, M. Heyer
Writing – original draft:	G.H. Jimenez-Aleman, S. S. Scholz, M. Heyer
Writing – review & editing:	A. Mithöfer, M. Reichelt, W. Boland

Contribution of M. Heyer: 15%

M. Heyer:

- planned experiment for Fig. 6 (in consultation with A. Mithöfer und G.H. Jimenez-Aleman)
- conducted the experiment for Fig. 6
- processed data and did statistics for Fig. 6
- visualized data as Fig. 6
- wrote the following parts of the original draft:
    - 2.5.
    - 3. in collaboration with G.H. Jimenez-Aleman und S.S. Scholz
    - 4.3.
    - 4.6. in collaboration with G.H. Jimenez-Aleman
- revised the whole manuscript

2.4 Manuscript 4

A holistic approach to analyze systemic jasmonate accumulation in individual leaves of Arabidopsis rosettes upon wounding

Authors: Heyer, M., Reichelt, M., Mithöfer, A.

Status: published 2018, Frontiers in Plant Science, 9(1569). doi:10.3389/fpls.2018.01569

Summary:

In this study we investigated the systemic pattern of different stress-associated phytohormones after herbivore-related wounding among the whole *Arabidopsis* rosette. By analyzing levels of jasmonates, SA and ABA, we showed that mainly JA and JA-Ile are induced systemically shortly after wounding. This jasmonate response is fast and takes less than 1.6 min to be fully pronounced. However, cutting the leaves, e.g. by sampling the leaves, is sufficient to induce similar systemic jasmonate levels as continuous wounding related to herbivores, causing false-negative results, if single leaves of the whole rosette are sampled at once. Thus we developed a sampling method, where rosette is frozen before cutting the leaves that allows studying the whole rosette at once in a holistic approach. Using this technique, we showed that the jasmonate distribution pattern follows the vascular connections between the leaves. As known from other systemic signals, JA and JA-Ile are distributed among vascular connected leaves of first and second order. Further, our data provide evidence, that the range of distribution can be even expanded to vascular connected leaves of third order. Besides JA and JA-Ile, we studied the systemic pattern of their precursor OPDA. However, OPDA was neither systemically induced by cutting nor by herbivore-related wounding, suggesting that the fast systemic JA and JA-Ile signal might be independent on systemic OPDA. Also SA and ABA did not play a role in rapid systemic signaling after wounding. In addition we reevaluated the systemic induction of GABA, showing that GABA induction follows the same vascular connections as jasmonate signals. However, simply cutting leaves was not able to induce systemic GABA elevations, suggesting that they are regulated via different time scale and mode than systemic phytohormone elevations.

Author contributions:

Conceptualization:	M. Heyer, A. Mithöfer
Formal analysis:	M. Heyer, M. Reichelt
Investigation:	M. Heyer, M. Reichelt
Methodology:	M. Heyer, M. Reichelt
Project administration:	A. Mithöfer
Supervision:	A. Mithöfer
Visualization:	M. Heyer
Writing:	M. Heyer, M. Reichelt, A. Mithöfer

Contribution of M. Heyer: 90%

M.Heyer:

- planned all experiments (in consultation with A. Mithöfer)
- conducted all experiments (in collaboration with M. Reichelt, who performed the LC-MS/MS measurements and calculated the phytohormone and GABA content of single samples)
- did data processing and statistics of all experiments
- visualized all data as the presented Figures
- wrote the original draft of the manuscript, the methods part for the quantification of phytohormones was written in collaboration with M. Reichelt, the abstract and the conclusions were written in collaboration with A. Mithöfer, the manuscript was revised by all authors

3 Manuscripts

3.1 Manuscript 1

PLOS ONE

RESEARCH ARTICLE

Herbivory-responsive calmodulin-like protein CML9 does not guide jasmonate-mediated defenses in *Arabidopsis thaliana*

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Competing interests: The authors have declared that no competing interests exist.

Abstract

Calcium is an important second messenger in plants that is released into the cytosol early after recognition of various environmental stimuli. Decoding of such calcium signals by calcium sensors is the key for the plant to react appropriately to each stimulus. Several members of Calmodulin-like proteins (CMLs) act as calcium sensors and some are known to mediate both abiotic and biotic stress responses. Here, we study the role of the *Arabidopsis thaliana* CML9 in different stress responses. CML9 was reported earlier as defense regulator against *Pseudomonas syringae*. In contrast to salicylic acid-mediated defense against biotrophic pathogens such as *P. syringae*, defenses against herbivores and necrotrophic fungi are mediated by jasmonates. We demonstrate that *CML9* is induced upon wounding and feeding of the insect herbivore *Spodoptera littoralis*. However, neither different *CML9* loss-of-function mutant lines nor overexpression lines were impaired upon insect feeding. No difference in herbivore-induced phytohormone elevation was detected in *cml9* lines. The defense against the spider mite *Tetranychus urticae* was also unaffected. In addition, *cml9* mutant lines showed a wild type-like reaction to the necrotrophic fungus *Alternaria brassicicola*. Thus, our data suggest that CML9 might be a regulator involved only in the defense against biotrophic pathogens, independent of jasmonates. In addition, our data challenge the involvement of CML9 in plant drought stress response. Taken together, we suggest that CML9 is a specialized rather than a general regulator of stress responses in *Arabidopsis*.

Introduction

The environment of organisms is continuously changing over their lifetime. Whereas most of the organisms are able to escape unfavorable conditions, plants are sessile and need to cope up with these changes. Thus, they developed a plenty of strategies to overcome biotic as well as abiotic stresses throughout evolution [1–3]. Since abiotic and biotic alterations of the

environment often occur simultaneously, a complex signaling network is coordinating all the different plant stress responses. Phytohormones play an essential role in these signaling pathways, such as abscisic acid (ABA) as key regulator of abiotic stress responses and salicylic acid (SA) and jasmonic acid (JA) as main mediators of biotic stress responses [3–5]. Resulting from the multiplicity of environmental changes, these stress-related phytohormone pathways overlap. For instance, defense reactions against the specialist herbivore *Pieris rapae* L. (Lepidoptera, Pieridae) and the generalist *Spodoptera littoralis* Boisd. (Lepidoptera, Noctuidae) are co-regulated by ABA and JA [6]. Plant-pathogen interactions are highly influenced by abiotic conditions and thus regulated by ABA as well [7]. Besides, the crosstalk between JA and SA has been largely investigated and several examples in herbivory and pathogen defense are known [3, 6, 8, 9].

Upstream of the phytohormone network, changes in the intracellular calcium (Ca^{2+}) level are one of the earliest signaling events after treatment with various environmental stimuli [10]. Depending on the stimulus different calcium signatures can be measured that vary in their location in the cell as well as in their dynamics [11]. To react appropriately to each stimulus, decoding of the particular calcium signature is necessary. The first step in translating the calcium code into a stress response is the recognition of the Ca^{2+} by sensor proteins. Calcium sensors are proteins that are able to bind Ca^{2+} via a helix-loop-helix structure, called EF-hand [12]. Two classes of calcium binding proteins are distinguished: sensor relays and sensor responders. Calcium sensor responders have an enzymatic function additional to their EF-hands that is activated upon binding calcium and by this initiating further signal transduction. In contrast, sensor relays have no other functional domain besides the EF-hands. By binding Ca^{2+} their conformation is changed, so that an interaction with the respective targets is possible [11]. In the model plant *Arabidopsis thaliana* (L.) Heynh., 250 calcium sensing proteins are identified, including sensor relays like calmodulins (CAMs), calmodulin-like proteins (CMLs) and calcineurin B-like proteins (CBLs) and sensor responders like Ca^{2+}-dependent protein kinases (CPKs) [13].

Among them, the group of CMLs is of particular importance for the plant calcium decoding, since they are unique for plants. Several studies revealed that they play a role in calcium perception in a wide range of plant stress responses. *CML24* is known to be regulated upon touch, extreme temperatures, darkness, ABA and H_2O_2 treatment and it is a regulator of salt stress response [14, 15]. *CML11*, *CML12*, *CML16*, *CML17* and *CML23* are induced by elicitors in insect oral secretions (OS), suggesting their possible role in defense against herbivores [16]. Furthermore, some CMLs are known to regulate abiotic as well as biotic stress responses and by this being of special interest in understanding the complex signaling network of the plant. Recently it was shown that CML37 and CML42 are regulators of plant defense against the herbivore *S. littoralis* and of drought stress. Whereas CML37 is a positive regulator of both stress responses, CML42 acts antagonistically to CML37 [17–19]. Besides, CML37 is induced upon infection with the (hemi)biotrophic pathogen *Pseudomonas syringae* (van Hall) as well [20]. Another CML coordinating different stress responses is CML9, also known as CAM9. It was suggested to be a negative regulator of the ABA pathway during seed germination and seedling growth. In addition, knock-out of *CML9* was leading to a higher salt and drought stress tolerance of the adult plants [21]. On the other hand, *CML9* was shown to be upregulated upon *P. syringae* infection and treatment with SA [22, 23]. Depending on the bacterial strain, CML9 was acting either as a positive or negative regulator of the plant immune reactions [23]. Additionally *CML9* is induced by mechanical stimuli and by *S. littoralis* OS [14, 16, 21], supposing that it might also play a role in herbivore defense.

In order to verify this hypothesis, we studied the functional relevance of CML9 in the defense against the insect herbivore *S. littoralis* and the spider mite *Tetranychus urticae* Koch (Trombidiformes, Tetranychidae). We show here that *CML9* is rapidly induced upon feeding

of the insect. The employment and analysis of *CML9* knock-out and overexpression mutants revealed that CML9 does not regulate the defense against these herbivores. To gain further insight into the putative role of CML9 in abiotic and biotic stress responses, we also investigated, if CML9 might regulate the defense against the necrotrophic pathogen *Alternaria brassicicola* (Schwein.) Wiltshire, and reexamined its function in drought stress reactions. Our data indicate that none of which is regulated by CML9. Thus, a role for CML9 as a coordinator of abiotic and biotic stress responses has to be reconsidered.

Material and methods

Plant growth

A. thaliana ecotype Col-0 and knock-out mutant lines *cml9-a* (SALK_006380C) and *cml9-b* (SALK_126787C; intronic T-DNA insertion lines, Col-0 background), obtained from Nottingham Arabidopsis Stock Center (NASC, Nottingham, United Kingdom), were used for the experiments. Additionally, feeding assays were repeated using the knock-out lines *cml9-1* (intronic T-DNA insertion, Col-8 background) and *cml9-2* (exonic T-DNA insertion, Ws-4 background) [21] and the overexpression lines OE-CC-2 and OE-CC-5 (Col-8 background) [23]. *A. thaliana* ecotypes Col-8 and Ws-4 were used as wild type, respectively. Most of the experiments were performed at MPI CE Jena. Seeds were sown in round pots with 10 cm diameter and kept at 4˚C for 2 d. After stratification, plants were grown under short day conditions (10 h : 14 h, light : dark) in a growth chamber at 21˚C and 50–60% humidity. FLUORA® bulbs (OSRAM, Garching, Germany) were used as light source and kept in 30 cm distance to the plants to achieve a light intensity of 100 μmol m^{-2} s^{-1}.

T. urticae assays were performed at the TU Dresden. Plants were grown under the described conditions at MPI CE Jena in 7 cm x 7 cm rectangular pots to allow monitoring of spider mite development. At the age of 4 weeks, plants were transferred to Dresden into a growth cabinet with slightly changed conditions. Humidity was 60–70%, temperature was around 19˚C, and a mixture of F32T8/TL741/Alto and F17T8/TL741/Alto bulbs (Philips, Hamburg, Germany) was used as light source with same light intensity as FLUORA bulbs.

Alternaria treatments were performed at the FSU Jena. Plants were cultivated as described in Johnson, Sherameti [24]. After 10 days, plants were transferred to soil and further incubated under the published short day conditions with 9 h photoperiod.

Insect and spider mite rearing

S. littoralis larvae were hatched from eggs (Bayer Cropsience, Monheim, Germany) and reared on an artificial diet consisting of 500 g ground beans, 1.2 L water, 9 g vitamin C, 9 g 4-ethylbenzoic acid, 9 g vitamin E Mazola oil mixture (7.1%), 4 mL formaldehyde, 1 g β-sitosterol, 1 g leucine, 10 g AIN-76 vitamin mixture, 200 mL agar solution (7.5%) (modified after Bergomaz and Boppré [25]). Insects were grown at 23–25˚C with a photoperiod of 14 h.

T. urticae females (Weixdorf population) were provided by D. Voigt, TU Dresden, Germany. They were kept on *Phaseolus vulgaris* (L.) 'Valja' and 'Saxa' plants (ISP-International Seeds Processing GmbH, Quedlinburg, Germany) at 22–24˚C, 50–65% humidity and a 16 h : 8 h light : dark cycle.

Growth and maintenance of fungi

A. brassicicola (FSU-218) was obtained from Jena Microbial Resource Centre, Jena, Germany. The fungus was grown on potato dextrose agar (PDA) medium (pH 5.6) at 22 ± 1˚C in a temperature-controlled chamber in the dark and 75% relative humidity for 2 weeks.

Plant treatments

5- to 6-week old plants were used for all herbivory-associated experiments. Insect biomass assays, short term feeding assays with *S. littoralis* larvae and OS treatment were performed according to Scholz, Vadassery [18]. MecWorm [26] was used for continuous mechanical wounding of the plant. Six different shaped areas (rectangular and circular), each lasting 30 min using a speed of 12 punches per minute, were designed to realize the given time points. Areas were arranged over the leaf lamina without wounding the midrib. Tissue samples for later extraction were immediately frozen in liquid nitrogen. For investigation of the possible interaction of ABA and wounding, plants were sprayed with a 100 μM ABA solution, containing 0.02% of ethanol. After 1 h, plants were treated with MecWorm for 30 min. Time points and ABA concentrations were chosen according to Magnan, Ranty [21] and prior results in this study.

For spider mite performance assay each plant was infested with one 3- to 5-day-old adult female. Mites were kept for 2 days on the plants to allow oviposition and were removed afterwards. Plants were monitored daily over development of one generation of spider mites. Number of eggs and immatures were counted and fitness parameters calculated.

For fungi treatment *A. brassicicola* spore suspension was prepared as follows: 5 ml of sterile-filtrated 0.01% Tween-20 were dropped on plates of 2-week old *A. brassicicola* cultures and plates were gently pivoted. Remaining spores were carefully removed with a spatula. The spore suspension was washed 3 times with 0.01% Tween-20 and filtrated through a 75 μm nylon membrane. The spore concentration was determined using a haemocytometer and was adjusted to 1 x 10^6 colony forming units (cfu) ml^{-1}. Mature leaves of 5- to 6-week-old *Arabidopsis* plants were used for inoculation assay. Single leaves were detached and placed in petri dishes containing sterilized filter paper soaked with 1.5 ml water. 2 μl of *A. brassicicola* spore suspension or solvent control was inoculated to each leaf. Plates were sealed to keep high humidity and incubated under continuous light as described above. To analyze the viability of treated leaves, chlorophyll fluorescence parameters were measured using a FluorCam FC 800-C (PSI, Brno, Czech Republic). Before measurement, the sealed plates were incubated in the dark for 20 min. Afterwards, plates were placed into the FluorCam and analyzed using following settings: Act 1: 50%, Act 2: 50%, Super: 100%. The QY_max (maximum PSII quantum yield) was recorded.

The drought stress assay was done with four week old plants as described in Scholz, Reichelt [19]. First drought period was extended to 11 days according to Magnan, Ranty [21]. After 11 days plants were watered till soil was fully soaked, followed by a second drought period of 1 week. To avoid competition between wild type and mutant plants, each was kept in single pots. Pots were placed randomly on the same tray, to minimize experimental variation.

Quantitative real time (qRT)-PCR

RNA from single leaves was isolated using TRIzol® Reagent (Invitrogen™, Darmstadt, Germany) according to the manufacturers' instructions with slight modifications. Leaf material was ground using 2010 Geno/Grinder® (SPEX®SamplePrep, Metuchen, USA) with a pre-cooled cryoblock. All centrifugation steps were performed at 4˚C and 16000 x g. After adding TRIzol®, samples were incubated for 20 min at room temperature. 300 μL chloroform was added, followed by incubation on ice for 20 min. The samples were centrifuged for 30 min. The aqueous phase was transferred into 600 μL isopropanol and samples precipitate overnight on -20˚C. To pellet the RNA, samples were centrifuged for 30 min. Pellet was washed with 80% ethanol and air dried. The dried pellet was dissolved in 80 μl preheated water. RNA was treated with TURBO DNase (TURBO DNA-*free*™ Kit, Invitrogen™, Darmstadt, Germany) to avoid DNA contamination. RNA concentration was measured with a photospectrometer and 1 μg of RNA was transcribed into cDNA using Omniscript® Reverse Transcription Kit

(Qiagen, Hilden, Germany) and Oligo(dT)$_{12-18}$ Primer (Invitrogen™, Darmstadt, Germany). *RPS18B* was used as housekeeping gene and primers published for *RPS18B* and *CML9* [16] were used for expression analysis of *CML9* after herbivore-associated stimuli. For quantification of exon1-exon3 fragment of *CML9*, primers (see S1 Table) producing a product of 168 bp, were designed in NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and cross checked in Vector NTI® *Express* 1.2.0 software (Thermo Fisher Scientific™, Schwerte, Germany). QRT-PCR was performed in 96 well plates in a CFX96 Touch™ Real-Time PCR System (Bio-Rad, München, Germany). Brilliant II QPCR SYBR green Mix (Agilent, Waldbronn, Germany) was used as master mix. The normalized fold expression was calculated with the ΔΔCP method [27]. Untreated plants or, in case of ABA spray, plants sprayed with 0.02% ethanol were used as controls and their expression level was defined as 1.

Phytohormone quantification

Phytohormone analysis was performed according to Jimenez-Aleman, Scholz [28] with modifications. Approximately 250 mg ground plant tissue was extracted with 1.5 mL methanol containing 60 ng D_6-abscisic acid (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), 60 ng of D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4-salicylic acid (Sigma-Aldrich) and 12 ng of jasmonic acid-$^{13}C_6$-isoleucine conjugate as internal standard. LC- MS/MS measurements were performed on an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) with a Turbo spray ion source in negative ionization mode. The analyte parent ion → product ion for multiple reaction monitoring (MRM) were the following: *m/z* 263.0 →153.2 (collision energy (CE) -22 V; declustering potential (DP) -35 V) for abscisic acid; *m/z* 269.0 →159.2 (CE -22 V; DP -35 V) for D_6-abscisic acid; *m/z* 209.1 →59.0 (CE -24 V; DP -35 V) for jasmonic acid; *m/z* 215.1 →59.0 (CE -24 V; DP -35 V) for D_6-jasmonic acid; *m/z* 136.9 →93.0 (CE -22 V; DP -35 V) for salicylic acid; *m/z* 140.9 →97.0 (CE -22 V; DP -35 V) for D_4-salicylic acid; *m/z* 290.9 → 165.1 (CE -24 V; DP -45 V) for *cis*-(+)-12-oxophytodienoic acid (*cis*-OPDA), *m/z* 322.2 →130.1 (CE -30 V; DP -50 V) for jasmonic acid-isoleucine conjugate; *m/z* 328.2 →136.1 (CE -30 V; DP -50 V) for jasmonic acid-$^{13}C_6$-isoleucine conjugate. For ABA quantification after drought stress the elution profile was modified as follows: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min 90–100% B; 4.02–4.5 min 100% B and 4.51–7.0 min 10% B keeping a flow rate of 1.1 mL/min.

Genotyping

For genotyping, DNA of single leaves of 3-week-old plants was isolated according to a modified protocol of Konieczny and Ausubel [29]. Samples were ground as described above. Extraction was performed using half of the given volumes of chemicals and buffer. All centrifugation steps were carried out at 16000 x g. After precipitation, sample was centrifuged for 20 min and pellet was directly washed in 70% ethanol. The dried pellet was dissolved in 30 μL water to achieve a higher concentration. Genotyping primers were designed with SALK T-DNA primer design tool (http://signal.salk.edu/tdnaprimers.2.html). Sequences of the primers are listed in S1 Table. Native Taq DNA polymerase and 10 mM dNTP Mix (both Invitrogen™, Darmstadt, Germany) were used for PCR. Mastermix was prepared according to the manufacturers' protocol and scaled down to a reaction volume of 10 μL, including 1.5 μL of template.

Semiquantitative reverse transcription (RT)-PCR

RNA isolation, DNase treatment and cDNA synthesis were carried out as described above. *ACTIN2* was used as housekeeping gene. The same primer sequences for *ACTIN2* were used as

described before [17]. *CML9* primers were designed with the same tools as qRT-PCR primers (see S1 Table). PCR was performed as described above.

Statistical analysis

Statistical significances were tested using t-test or Wilcoxon-test in RStudio 0.98.1103.0, or by ANOVA using SigmaPlot 12.2.0 (Systat Software GmbH, Erkrath, Germany). False discovery rate (FDR, [30]) was calculated if t-test or Wilcoxon-test was repeated more than 3 times in 1 data set. Statistical tests used for different experiments are indicated in the figure legends.

Results

Expression of *CML9* is induced upon herbivory

Several transcript studies in *Arabidopsis* already indicated that CML9 is induced by herbivore-associated stimuli [14, 17, 21]. Nevertheless, other data showed no change of the expression level after wounding or herbivory and even repression of the gene after application of methyl-JA [31]. In order to test these contradictory results, we treated the plants with various herbivore-associated stimuli and analyzed the *CML9* expression level using qRT-PCR. We found that *CML9* is significantly induced about 1.5-fold already after 30 min feeding of the chewing insect *S. littoralis* (Fig 1(*A*)). This induction was transient and reached the base level after 60 min, further reduction of the expression levels at 2 and 3 h was not significant. Since feeding of herbivores can be recognized by the mechanical wounding pattern as well as by elicitors in the OS of herbivores [32], we tested further if both stimuli can induce a change in *CML9* transcript level. Therefore, plants were mechanically wounded with a pattern wheel and either water or *S. littoralis*-derived OS was applied to the wounds. In both cases *CML9* was quickly and transiently upregulated (Fig 1(*B*)), as was found in the real insect treatment. After 30 min, wounding induced *CML9* one-fold, compared to the untreated controls. The application of insect OS increased *CML9* transcript level even two-fold compared to controls, suggesting that both stimuli cause the induction of *CML9* upon feeding of *S. littoralis* to a similar extend. Additionally, wounding treatment was repeated using MecWorm, a robotic larva mimicking the wounding pattern of a chewing insect [26], to confirm the results of the artificial wounding with a pattern wheel. MecWorm treatment caused the same expression pattern as all the treatments before (Fig 1(*C*)): *CML9* was induced about 1.5-fold shortly after wounding by MecWorm. This regulation was also transient and *CML9* transcript level decreased in later time points.

CML9 was shown to be induced by ABA as well [21]. Regarding to the given crosstalk between the ABA pathway and the defense against herbivores [6], we tested if there is an additive or synergistic effect of both treatments together on the *CML9* expression level. We compared the expression level of plants treated only with MecWorm or ABA with plants that were treated with both (Fig 1(*D*)). All three treatments induced *CML9* expression to the same extends. These data exclude an additive effect of ABA and wounding by an herbivore on the induction of *CML9*.

Herbivore performance is not affected by *CML9* knock-out or overexpression

Because of the fast induction of *CML9* after *S. littoralis* feeding, the functional relevance of CML9 to the plant defense against this herbivore was further investigated by studying *CML9* loss of function mutants. Two homozygous intronic T-DNA insertion lines (*cml9-a* and *cml9-b*) were used in a conventional one-week feeding assay. Feeding performance of the larvae was determined by measuring the larval weight (Fig 2). Larvae feeding on *cml9-a* gained

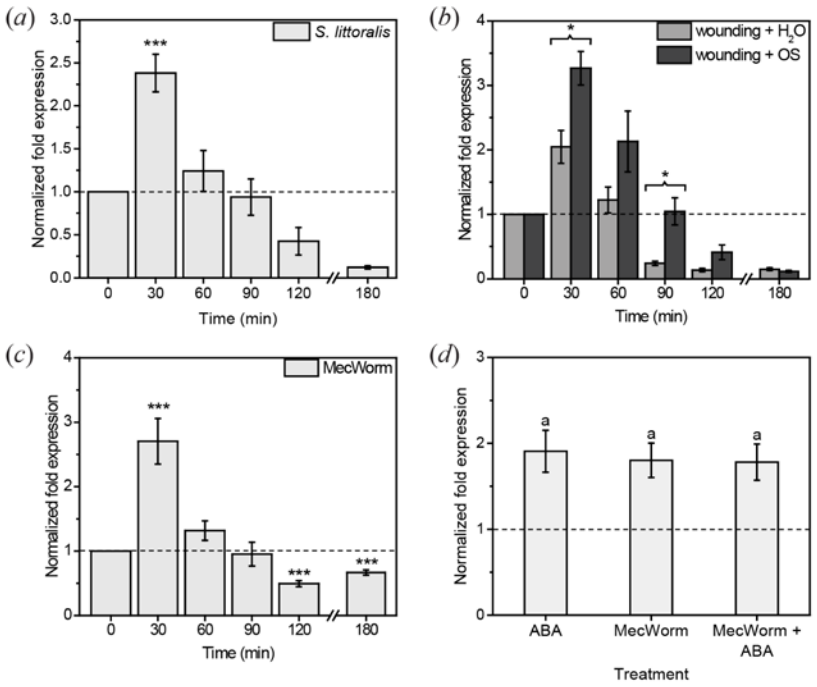

Fig 1. Expression of *CML9* in response to different herbivore-associated stimuli. Changes in *CML9* transcript level in *A. thaliana* wild type leaves (Col-0) upon *S. littoralis* feeding (*a*), mechanical wounding with a pattern wheel and application of either water or *S. littoralis* OS (*b*), mechanical damage by MecWorm (*c*) or ABA spray combined with MecWorm treatment (*d*) are plotted. Expression level in (*a*), (*b*) and (*c*) was determined after 30, 60, 90, 120 and 180 min of treatment. Untreated plants were used as controls. Fold expression in (*d*) was measured after 30 min of MecWorm treatment with a 60 min pre-incubation with 100 μM ABA solution or a 0.02% ethanol solution, or just incubation with ABA. Control plants were treated with 0.02% ethanol. The *CML9* fold expression was normalized with respect to the *RPS18B* transcript level and calculated relative to respective controls. Bars represent the means ± standard error (SE) ($n \geq 6$ (*a*), $n \geq 5$ (*b*), $n \geq 11$ (*c*), $n \geq 10$ (*d*)). Experiments were repeated at least two times independently. Statistically significant changes in expression levels were determined by one-sample Wilcoxon test (*a*) or one-sample t-test (*c*). Statistically significant differences between the different treatments were determined by unpaired two-sample Wilcoxon test at each time point separately (*b*) or by one way ANOVA (*d*). Asterisks indicate significances (* $P < 0.05$, *** $P < 0.001$). P-values for (*a*), (*b*) and (*c*) are FDR corrected. Letters in (d) indicate no statistic differences.

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little but significantly more weight than larvae feeding on corresponding wild type plants, although the measured effect was small. However, this result was not confirmed with the second knock-out line. Larvae feeding on *cml9-b* gained as much weight as on wild type plants (Col-0) (Fig 2(*A*)). Since the different response of these two loss-of-function mutants, two more knock-out mutant lines (*cml9-1* and *cml9-2* [21]) and two overexpression lines (OE-CC-2 and OE-CC-5[23]) were tested in feeding assays. All of the additional mutant lines display a different ecotype background: whereas *cml9-1* and both overexpression lines are in Col8 background, *cml9-2* is in Ws background. In general, *S. littoralis* larvae gained more weight on genotypes with the Ws ecotype than on those with the Col background (Fig 2(*B*)). However, the larvae gained as much weight on the knock-out lines as on the corresponding wild types, confirming the results of the *cml9-b* lines (Fig 2(*B*)). Moreover overexpression of CML9 did not influence the larval performance as well (Fig 2(*C*)).

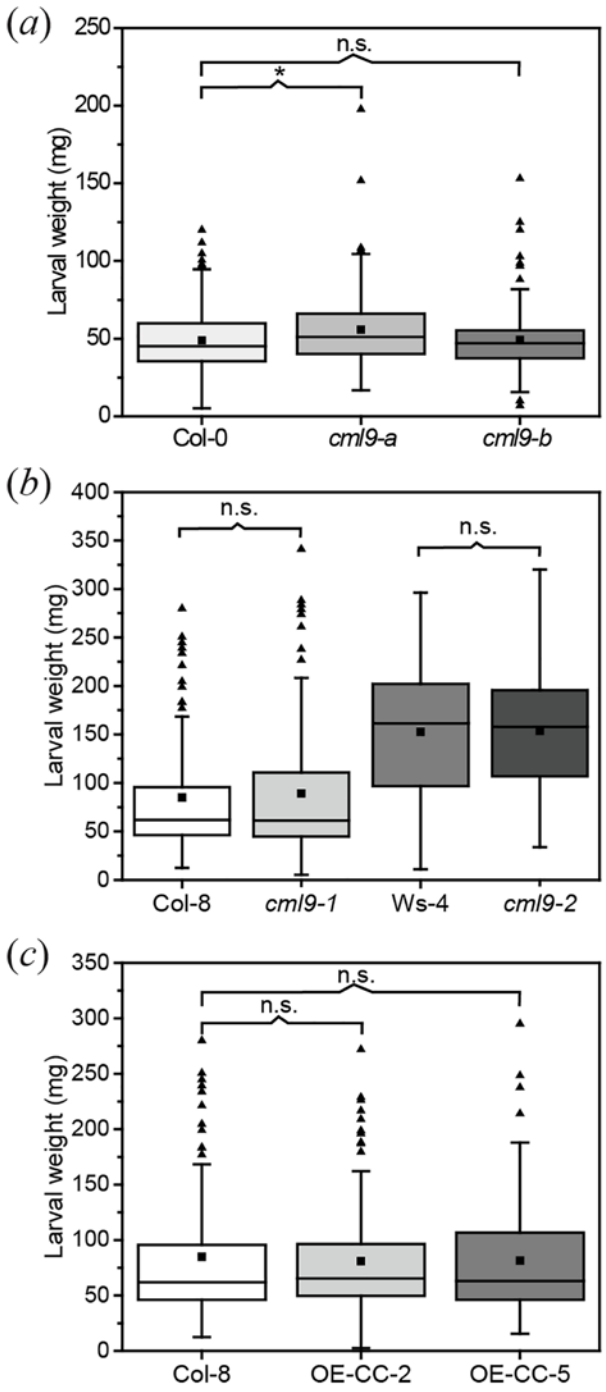
(a)
Larval weight (mg)
250
200
150
100
50
0
n.s.
*
Col-0
cml9-a
cml9-b
(b)
Larval weight (mg)
400
350
300
250
200
150
100
50
0
n.s.
n.s.
Col-8
cml9-1
Ws-4
cml9-2
(c)
Larval weight (mg)
350
300
250
200
150
100
50
0
n.s.
n.s.
Col-8
OE-CC-2
OE-CC-5

Fig 2. Susceptibility of different *Arabidopsis* mutant lines of *CML9* on *S. littoralis* feeding. Gain of larval weight was determined after feeding on Col-0 wild type plants and *cml9-a* and *cml9-b* knock-out lines (*a*), Col-8 and Ws-4 wild type plants and *cml9-1* and *cml9-2* knock-out lines (*b*) and Col-8 wild type plants and OE-CC-2 and OE-CC-5 overexpression lines (*c*) for one week. First instar larvae of *S. littoralis* were pre-weighed to reduce experimental variation. Three larvae were placed on each plant. After feeding period larval weight was determined. The boxplots show the distribution of the measured data. The box indicates the middle 50% of the data points. Black triangles represent outliers and the black squares the mean values. Whiskers are defined as 1.5 fold interquartile range (IQR). Experiments were repeated at least five times independently (n = 134 (Col-0), n = 133 (*cml9-a*), n = 139 (*cml9-b*), n = 98 (Col-8), n = 92 (*cml9-1*), n = 109 (Ws-4), n = 111 (*cml9-2*), n = 89 (OE-CC-2), n = 91 (OE-CC-5)). Statistically significant differences between larval weights of different genotypes were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (* $P < 0.05$), n.s. means not significant.

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To further investigate the different susceptibility of *cml9-a* and *cml9-b* to *S. littoralis* feeding, we measured the phytohormone content after feeding of the larvae. Especially the jasmonates are main regulators of this defense response [4]. Thus, we measured the content of JA, its precursor *cis*-12-oxophytodienoic acid (*cis*-OPDA) and the active jasmonate, jasmonic acid-isoleucine (JA-Ile) (Fig 3(*A*)–3(*C*)). All three jasmonates increased after feeding of *S. littoralis*, but to the same extend in both *cml9* lines as in Col-0 wild type plants. Besides, SA and ABA have been shown to modulate herbivore defense [6, 8, 9]. Hence, we analyzed the levels of these phytohormones additionally (Fig 3(*D*) and 3(*E*)). The ABA content was slightly increased upon insect feeding, whereas the SA level was nearly the same as in control plants. Nevertheless, there were no significant differences in the SA or ABA level obtained between the *cml9* lines and in comparison to the wild type. Both *cml9* lines show similar results for all tested phytohormones, thus slight differences observed in the feeding behavior of *S. littoralis* between the lines are not due to a change in phytohormone elevation.

Regarding the data of the *S. littoralis* feeding performance on the *cml9* lines, we tested the performance of a second herbivore with a different feeding strategy on *cml9-a* and *cml9-b* mutants in comparison to wild type plants. We used the piercing-sucking spider mite *T. urticae*. Different fitness parameters of *T. urticae* were monitored daily over development of one generation (Fig 4). Spider mites established successfully on all plants and dispersed over the leaves. Infested plants showed numerous chlorotic spots. All tested fitness parameters in the early stage of spider mite development that we obtained on the *cml9-a* and *cml9-b* were comparable to those on Col-0 wild type plants, such as fecundity, time of egg development and the egg mortality. Even the time of immature development and the sex ratio were not changed in *cml9-a* and *cml9-b* plants compared to the Col-0 wild type. Only the immature mortality of *T. urticae* reared on *cml9-a* plants was little higher than on wild type plants. Collectively, these results suggest that CML9 is not a regulator of plant defense against the tested herbivores.

Arabidopsis thaliana cml9 plants show a wild type-like response to *Alternaria brassicicola*

CML9 is described to modulate plant defense against different strains of the phytopathogenic bacteria *P. syringae* [23]. To further investigate if CML9 might act as a regulator in defense against other pathogens, we examined the reaction of *cml9-a* and *cml9-b* plants upon fungal infection with *A. brassicicola*. *A. thaliana* wild type Col-0 and *cml9-a* and *cml9-b* leaves were inoculated with a spore solution or mock treated and analyzed 3 and 4 days post-inoculation. Both *cml9* lines showed the same level of susceptibility than the wild type (Col-0) to the pathogen at tested time points (Fig 5(A)). There were no obvious differences in the formation and size of lesions at the macroscopic level observed. Additionally, we evaluated plant susceptibility by measuring chlorophyll fluorescence parameters of the inoculated leaves. In all plants the determined QY-max coefficient (0.75) indicated a decrease in fluorescence upon treatment

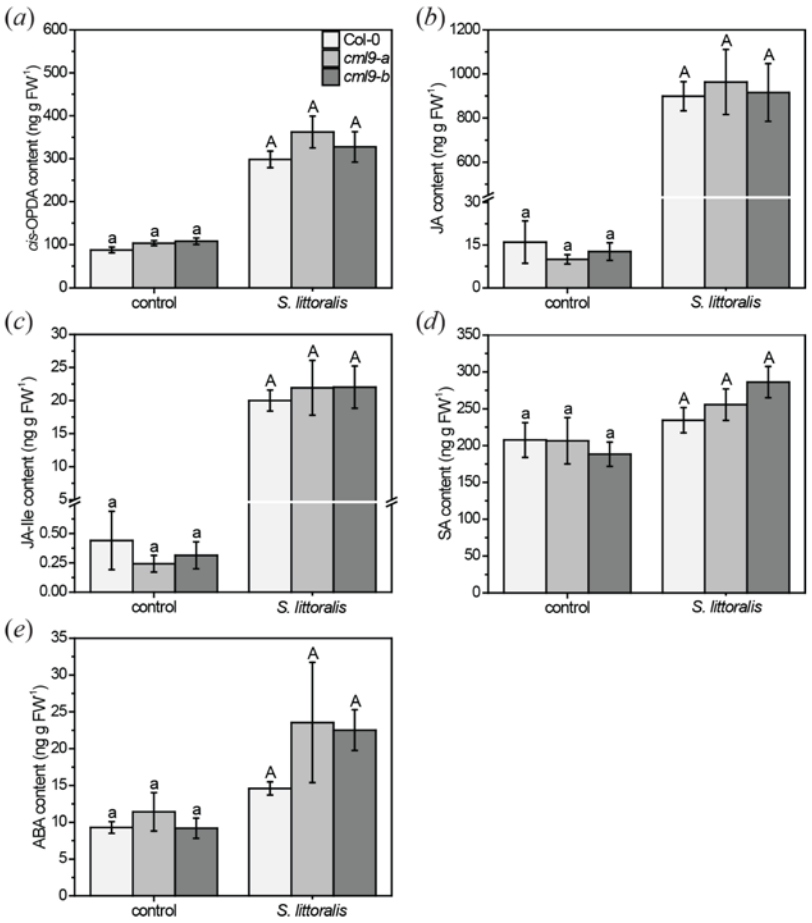

Fig 3. Phytohormone contents of *A. thaliana* wild type and *cml9* mutant plants after *S. littoralis* feeding. Levels of *cis*-OPDA (*a*), JA (*b*), JA-Ile (*c*), SA (*d*), ABA (*e*) after larval feeding for one week in ng g^{-1} fresh weight (FW). Phytohormones were extracted only from local fed leaves. Untreated plants were used as controls. Bars represent means ± SE. Experiment was repeated three times independently ($n \geq 13$). Statistically significant differences between phytohormone content of different genotypes among one treatment were determined by Kruskal-Wallis one-way ANOVA on ranks, using Dunn's method as post-hoc test. No significant differences were measured, as indicated by the letters. Legend for color code see (*a*).

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with *A. brassicicola* (Fig 5(B)). Nevertheless, chlorophyll fluorescence in the treated mutant lines was reduced to the same extent like in infected wild type (Col-0) plants, confirming the phenotypic results. Hence, CML9 does not seem to modulate plant defense against *A. brassicicola*.

Loss-of-function mutants are as susceptible to drought as wild type plants

In previous studies it was shown that CML9 is very likely a negative regulator of ABA-related stress responses, e.g. drought [21]. It was hypothesized that the higher drought tolerance of

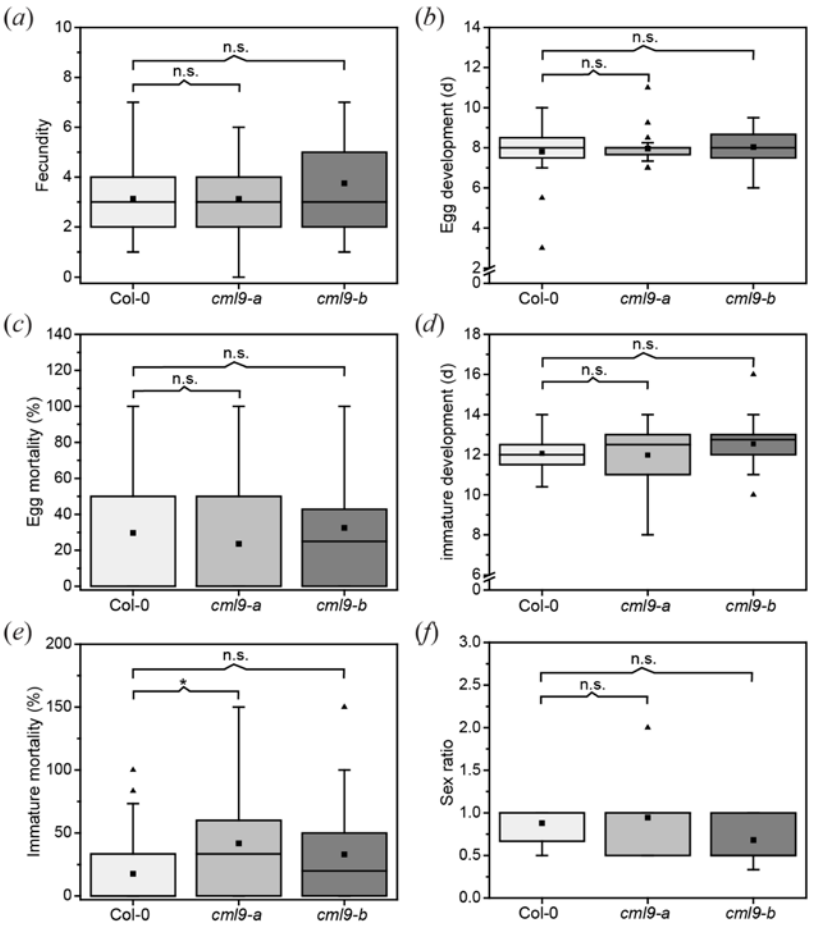

Fig 4. *T. urticae* performance on *cml9-a* and *cml9-b*. Different fitness parameter monitored over development of one generation of spider mites are shown in box plots: fecundity (number of eggs per female per 24 h) (*a*), egg development (means of all eggs on one plant) (*b*), egg morality (*c*), immature development (means of all immatures pooled together on one plant: larva, nymphochrysalis, protonymph, deutochrysalis, deutonymph, teleiochrysalis) (*d*), immature mortality (sum of mortality of all immature stages) (*e*), sex ratio (males : females) (*f*). The box indicates the middle 50% of the data points. At least n = 31 plants per genotype were used. Black triangles represent outliers and the black squares the means. Whiskers are defined as 1.5 fold IQR. Statistically significant differences between Col-0 wild type and mutant lines were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (* $P < 0.05$), n.s. means not significant.

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cml9 could be explained by hypersensitivity of the mutants to ABA, since both of the tested mutant lines showed a wild type-like ABA content upon drought [21]. Nevertheless, in that study ABA elevation was just analyzed after some hours of drought stress, but not in long term stress treatment like it was done for other CMLs by Scholz, Reichelt [19]. To elucidate whether a later increase in the ABA level could explain the higher resistance of *cml9* against drought, we kept the plants under drought conditions for 11 d or 18 d with watering once after 11 d. In contrast to previous published results, *cml9-a* and *cml9-b* mutants were as tolerant to drought

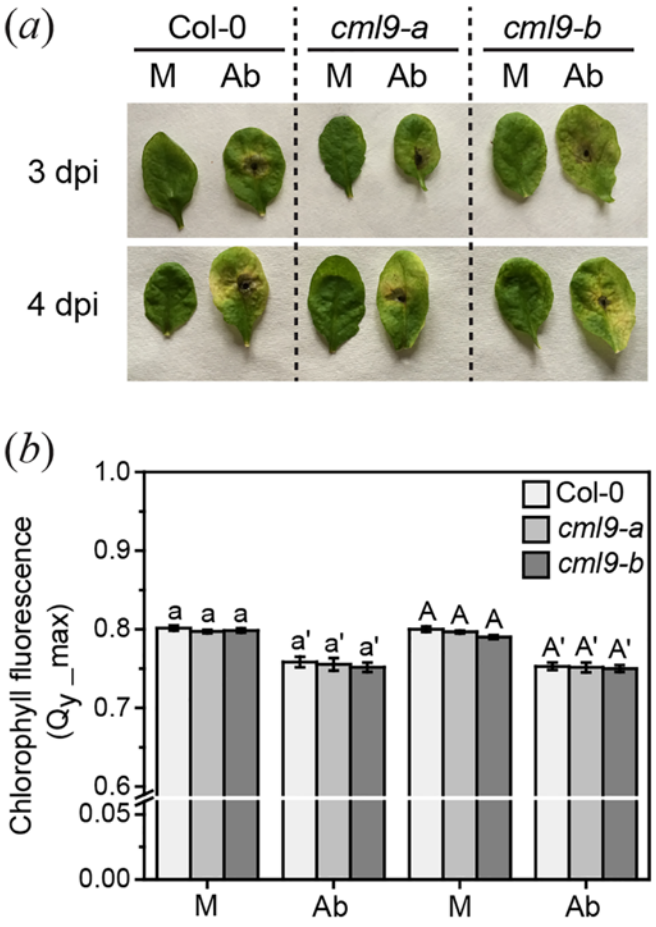

Fig 5. Response of *A. thaliana* wild type and *cml9* lines to *A. brassicicola* infection. Macroscopic observation of lesion formation (*a*) and measurement of chlorophyll fluorescence (*b*) of wild type and mutant leaves 3 and 4 day post-inoculation (dpi) with *A. brassicicola* spore suspension (Ab) or 0.01% Tween-20 solution as mock (M). Experiment was repeated three times independently. Plants shown are representative. Statistically significant differences in chlorophyll fluorescence of different genotypes among one treatment were determined by one-way ANOVA. No significant differences were measured, as indicated by the letters.

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as wild type plants (Fig 6(*A*)). Parallel to this stress treatment we quantified the ABA level in all genotypes. Without stress, all plants had similar endogenous ABA content (Fig 6(*B*)). As expected, a significant increase in this ABA content was observed in wild type (Col-0) 11 and 18 days after the beginning of the stress treatment (Fig 6(*B*)). Although we measured a slightly lower content of ABA at 11 d and 18 d in *cml9-a* and *cml9-b* control plants, the ABA elevation after drought showed the same profile and levels as the corresponding wild type line. Taken together, our data suggest that CML9 does not act as a key regulator of drought stress responses in plants.

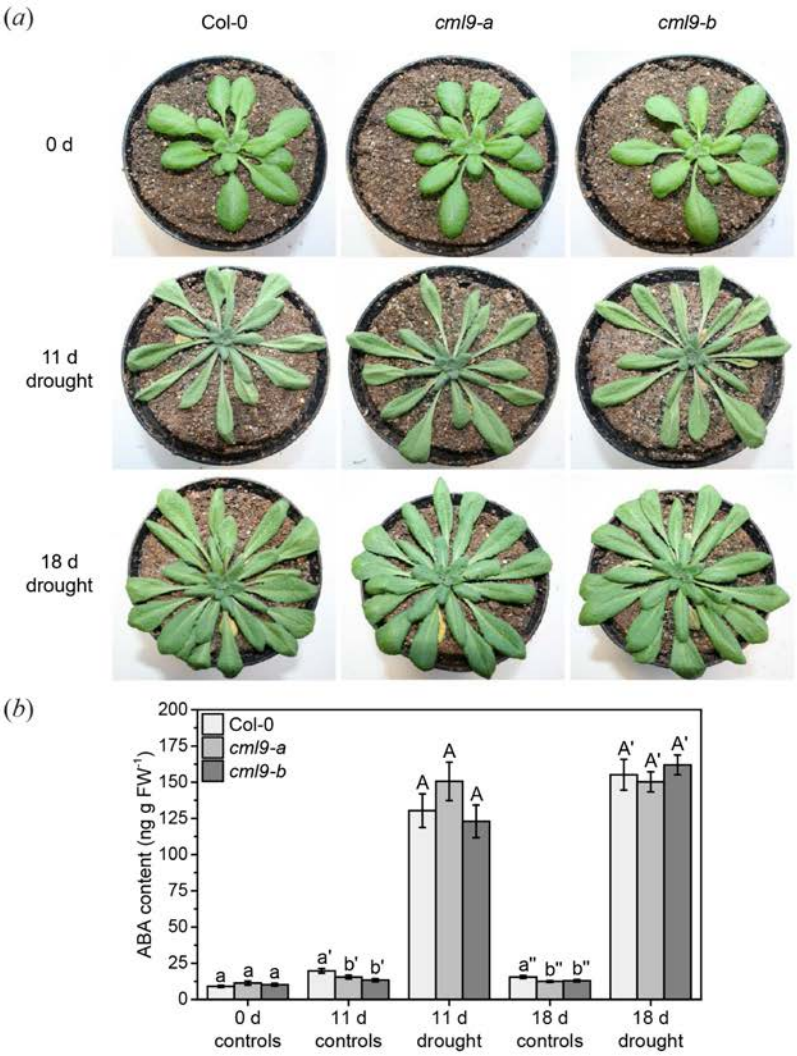

Fig 6. Comparison of drought stress response of wild type and *cml9* mutants. Representative pictures (*a*) and mean ABA level ± SE (*b*) of wild type, *cml9-a* and *cml9-b* before (0 d) and after drought (11 d and 18 d). Experiment was started with 4-week-old plants (0 d). Plants exposed to drought for 18 d were watered once after 11 d. All plants shown in (*a*) are independent from each other. Treatment was repeated 4 times independently (n = 20). Statistically significant differences between ABA content of different genotypes among one treatment were determined by one-way ANOVA, using Student-Newman-Keuls (SNK) method as post-hoc test. Different letters indicate significant changes ($P < 0.05$).

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cml9-a and *cml9-b* differ genetically

In this study two independent intronic T-DNA insertion lines were used as knock-out mutants, both with a T-DNA insertion in the third intron (Fig 7(*A*)). Prior to all experiments, the insertion of the T-DNA was confirmed by genotyping (Fig 7(*B*)). Two different primer

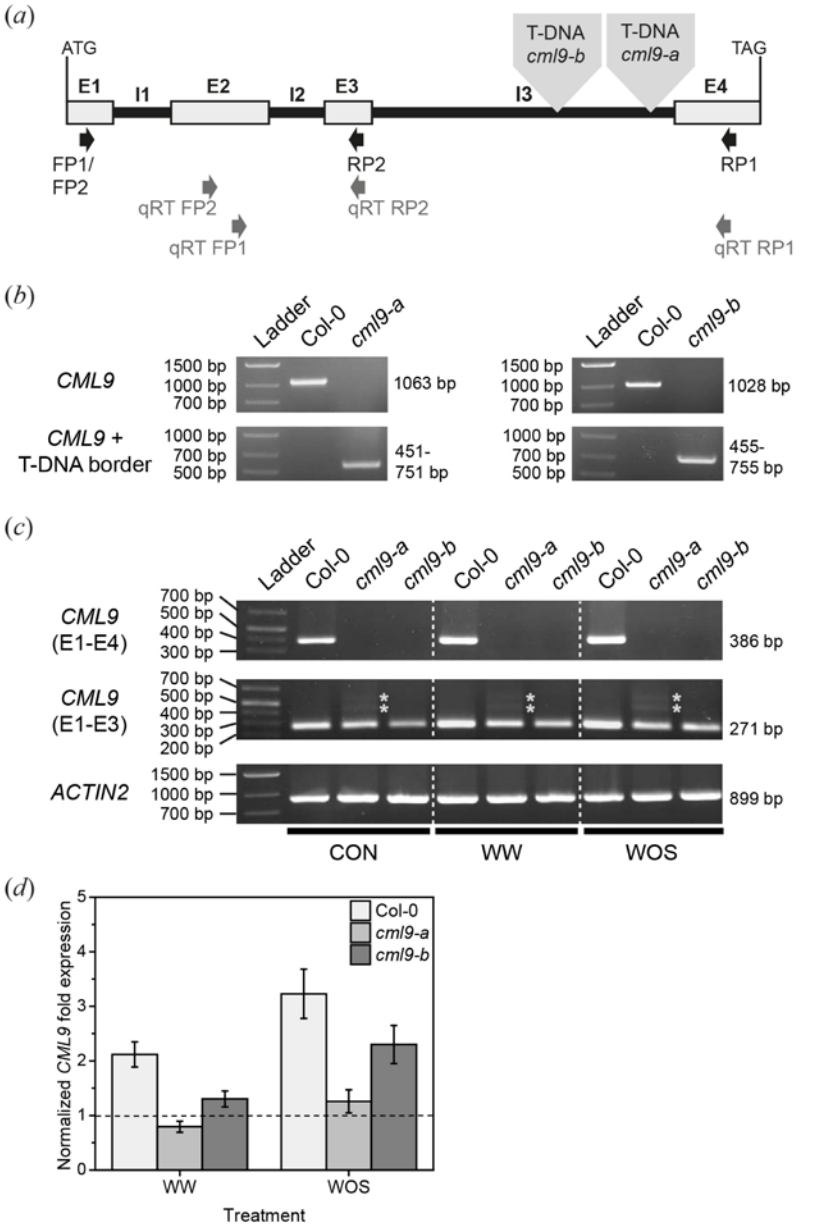

Fig 7. Genetic differences between *cml9-a* and *cml9-b*. (*a*) Schematic overview of *CML9* with T-DNA insertions and the used primers for RT- and qRT-PCR. Exons are indicated with E, introns with I. Light gray triangles indicate T-DNA insertions. RT primers used are indicated by black arrows, qRT primers by grey arrows (qRT FP1 and qRT RP1 are published as CML9 primers in Vadassery, Scholz [16]). Total length of *CML9* gDNA without insertions is 1137 bp. (*b*) Verification of T-DNA insertions in *CML9* by genotyping. The expected product length is indicated on the right sites of the respective pictures. (*c*) Semi quantitative RT-PCR analysis of *CML9* expression in wild type and knock-out mutants. Plants were treated with a pattern wheel and either water (WW) or *S. littoralis* oral secretion

(WOS) was applied for 30 min. Untreated plants were used as controls (CON). Besides full length expression, expression of the E1-E3 fragment, upstream of the intronic T-DNA insertions, is shown. Expression of *ACTIN* was used as quantitative control. The expected product length is written on the right sites of the respective pictures. Asterisks indicate unspecific bands in the *cml9-a* mutant. (*d*) Normalized fold expression of *CML9* E1-E3 fragment in wild type and *cml9-a* and *cml9-b* lines. Plants were treated as described in (*c*). Expression level was normalized with respect to the *RPS18B* transcript level. Bars represent the means ± SE ($n \geq 5$). Experiments were repeated two times independently. Statistically significant changes were determined by two-way ANOVA. Results of statistical analysis are shown in Table 1.

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pairs were used for PCR: one gene specific primer pair and a second one including the left border of the insertion. In both mutants there was no product for the intact *CML9* detectable (upper row, Fig 7(*B*)). Only a product consisting of truncated *CML9* and the T-DNA border was observed (lower row, Fig 7(*B*)). Both lines were verified as homozygous. The absence of the full length *CML9* transcript in MecWorm treated mutants was confirmed by semi quantitative RT-PCR (S1 Fig). Since these two *cml9* lines exhibited slightly different responses to herbivore treatments as described above, a more detailed genetic analysis for both lines was performed.

Although T-DNA insertion mutants are usually very stable, the use of intronic insertion lines can be problematic. It was shown that different environmental stimuli can cause alternative splicing of introns leading to a loss of the T-DNA insertion and, thus, to a wild type-like expression of the gene [33, 34]. In order to examine whether herbivory by *S. littoralis* is able to stimulate such an alternative splicing event in the two *cml9* lines, we analyzed the *cml9* gene expression by another semi quantitative RT-PCR. Since the first RT-PCR (S1 Fig) revealed that mechanical wounding by the larvae is not leading to any *CML9* transcript accumulation in the mutants, we investigated if treatment with the insect-derived OS may stimulate gene expression. The OS treatment was performed as described above. No *CML9* transcript was detectable in both mutants upon application of OS ((Fig 7(*C*)), upper row). Furthermore, the same was observed for the water treatment, confirming the result of the first RT-PCR. Thus, a loss of the entire T-DNA insertion by alternative splicing due to herbivory is unlikely for both *cml9* mutant lines.

The expression of fragments upstream or downstream of a T-DNA insertion can lead to the production of a truncated protein [35]. Just recently it has been shown, that two T-DNA alleles of a receptor kinase respond differently, due to the production of a truncated protein in one of these lines [36]. To exclude this possibility, the expression of the fragment upstream of the T-DNA insertion (from the first to the third exon) was investigated by RT-PCR. In all treatments and controls in the mutants, this fragment was expressed ((Fig 7(*C*)), middle row). In *cml9-a* plants, two additional unspecific bands were detectable. Sequencing of these fragments revealed that they are artefacts of an incorrect splicing event. The sequences or part of the sequences of the first and second intron are still included in the products. Thus, the T-DNA insertion in *cml9-a* seems to influence the splicing process of the *CML9* RNA, suggesting that it is unlikely that a truncated, but functional protein is produced. In *cml9-b* only the correctly spliced fragment was found. If a truncated protein was produced, two of the four EF-hands would miss, leading to a lower Ca^{2+} binding capacity and probably to reduced activities.

When comparing the expression of the expected *CML9* fragment with the house keeping gene expression ((Fig 7(*C*)), lower row), the transcript level of the fragment was lower in both mutants than in the wildtype. To refine this observation a qRT-PCR was performed (Fig 7(*D*)). Data were analyzed by two-way ANOVA, in order to test whether there is a difference among the treatments and the genotypes and if one of those could be explained by the other. The statistical analysis revealed that there is a significant increase in the fragment transcript

Table 1. Statistical analysis of qRT PCR of exon1-exon3 fragment of *CML9*. Data were log-transformed and analyzed by two-way ANOVA. SNK was used as post hoc test ($P < 0.05$). DF = Degrees of Freedom.

Tested Variables	Results two way ANOVA			Results SNK
	P	F	DF	
Treatment	<0.001	17.681	1	WW < WOS
Genotype	<0.001	26.179	2	*cml9-a* < *cml9-b* < wild type
Treatment x Genotype	0.846	0.168	2	-

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abundance upon OS treatment in all three genotypes (Table 1). Nevertheless, even when a very low threshold of two-fold was used, upon both treatments an induction of the fragment could only be observed in the wild type. In *cml9-b* the fragment is only induced upon OS treatment, whereas in *cml9-a* the fragment is not induced at all. Furthermore there is a significant difference between the genotypes. However, this statistical difference between the genotypes was not due to the treatments. This suggests that the two *cml9* lines vary genetically and this also might explain some varying results in herbivore treatments.

Discussion

In plants, the perception of environmental stimuli is followed by a fast calcium elevation inside the cells [10]. These calcium signals encode information about the stimulus that need to be translated into the appropriate response [11]. However, relatively little is known about the decoding of such calcium signals. CML proteins are important in sensing calcium signals after various external stimuli. Here we focused on the CML9. By using different CML9 mutant lines, we investigated the role of this calcium sensor in biotic and abiotic stress responses.

CML9 is not a regulator of plant herbivore defense

CML9 was described as a regulator of the plant defense against phytopathogenic bacteria [23]. The signaling cascade after recognition of a pathogen is also related to the signaling pathway after herbivory [32]. Thus, we examined if CML9 is also involved in the herbivore defense. First, we demonstrated that *CML9* is induced upon feeding by the insect herbivore *S. littoralis* (Fig 1(*A*)). This enhancement in the transcript level is caused by the mechanical wounding of the larvae as well as by the OS (Fig 1(*B*) and 1(*C*)). Unlike the known defense regulators *CML37* and *CML42* that are mainly regulated by one of the two stimuli [17, 18], *CML9* is equally induced by both. The result that *CML9* is a wound-inducible gene is of great interest, because the yet published literature was contradictory [21, 31]. Besides, our data indicate that the induction of the *CML9* transcript level after wounding is not synergistically regulated by ABA. Although *CML9* expression was upregulated by either of the treatments, ABA had no additional effect on the transcript level (Fig 1(*D*)). This suggests that the regulation of *CML9* mRNA levels after herbivory might be independent of ABA. Among all herbivore-associated treatments *CML9* displayed a characteristic expression dynamic. Like *CML42*, *CML9* expression was fast and transiently up-regulated and down-regulated at later time points. This result was quite surprising, since it was described that *CML9* expression occured late and remained high after OS treatment [16]. On the other hand, the fast and transient expression profile seems to be typical for *CML9*. The same dynamics were found after drought and pathogen-associated stress treatments [21, 23]. Compared to the stimulation of *CML37* and *CML42* mRNA levels after herbivory, the *CML9* transcript is only slightly induced [17, 18]. The same holds true for the *CML9* mRNA level after pathogen treatments [23]. Moreover, semi quantitative RT-PCR revealed that the basic level of the *CML9* transcript in untreated plants is already

quite high (Fig 7(C)), suggesting that *CML9* is rather constitutively expressed than strongly induced.

Despite the fast upregulation of *CML9* after herbivore treatment, the analysis of different *CML9* knock-out lines revealed that CML9 is not a key player in herbivore defense. In three independent lines (*cml9-b*, *cml9-1*, *cml9-2*) the performance of the chewing insect *S. littoralis* was unaffected by the loss of function of the gene (Fig 2*A and 2B*). Similar results were obtained for the performance of the piercing-sucking spider mite *T. urticae* on the knock-out line *cml9-b* (Fig 4). Only in *cml9-a* line, slight changes in the herbivore performance were observed. In detail, *T. urticae* displayed a higher immature mortality (Fig 4), which represents one out of six examined parameters. On the other hand, *S. littoralis* larvae performed better on the *cml9-a* line than on the Col-0 wild type (Fig 2(*A*)), suggesting a positive influence of CML9. However, compared to loss-of-function mutants of the positive defense regulator CML37 [18], *cml9-a* line was only slightly more susceptible to *S. littoralis* and more similar to the three additional knock-out lines tested (Fig 2(*A*) *and* 2(*B*)). As treatment with *T. urticae* showed similar results (Fig 4), all these data strongly suggest that the effects in *cml9-a* are not due to the loss-of-function of *CML9*.

Besides, throughout all feeding assays with *S. littoralis*, larvae gained more weight on plants with the Ws-4 ecotype background than on plants with Col-8 background (Fig 2(*B*)). This result is in agreement with previous studies showing that different insect species prefer Ws-0 to Col-0 ecotypes [37, 38] and could be correlated with lower glucosinolate content in the Ws-0 ecotype [37], a finding that also explains why the larvae fed better on Ws-4 lines (Fig 2(*B*)). Nevertheless, when using plants with different ecotype background for insect assays this difference between Col and Ws ecotypes should be taken into account.

Further analysis of *cml9-a* and *cml9-b* showed that both lines differ genetically (Fig 7(*C*) and 7(*D*)) that might explain the little variances in the herbivore treatment observed. Moreover, knock-out of *CML9* does not lead to a change in the phytohormone response to *S. littoralis* feeding (Fig 3) indicating once more that CML9 is not a key player in herbivore defense regulation.

In *Arabidopsis*, 50 CMLs are known and among them eight are regulated upon *S. littoralis* herbivory [16–18, 31]. Thus, it is conceivable that some of these proteins have redundant or overlapping functions. Inactivation of one of them does not necessarily have a great impact on a particular plant stress response. For example, the *cml24* but not *cml23* mutant shows under certain conditions a phenotype that differs from the wild type; however, in the *cml24*x*cml23* double mutant the *cml24* effect is modulated [39]. To exclude this scenario in case of CML9, *CML9* overexpression lines were examined. Both tested lines were as susceptible to *S. littoralis* feeding as their corresponding wild type, supporting the conclusion, that CML9 does not play any role in the defense against this herbivore.

Taken the results of gene expression data and the mutant analysis together, we suggest further to interprete gene regulations more cautiously. Even though *CML9* is significantly upregulated after herbivore treatment, it is not relevant for the defense response.

CML9 is not regulating defense against necrotrophs

In Arabidopsis 50% of the upregulated genes upon *A. brassicicola* infection are also induced upon *P. syringae* treatment, although the response to both microbes is mediated via different pathways [40]. Because CML9 is described as mediator of the defense against the biotrophic bacterial pathogen *P. syringae* [23], we also examined its role in the defense against the necrotrophic fungal pathogen *A. brassicicola*. We found that CML9 has no functional relevance in the plant immune response to *A. brassicicola*. Both mutant lines, *cml9-a* and *cml9-b*, were as

susceptible to the fungus as to Col-0 wild type plants (Fig 5). The defense against this fungus is mainly regulated by jasmonates like the defense against herbivores [3, 41], while the response to *P. syringae* is mediated mainly by SA [3]. Thus, our results are in one line with the results obtained in the herbivore assays and suggest that CML9 is also not regulating the defense against this necrotrophic pathogen. Hence, the different impact of CML9 on both pathogens might be explained by their different lifestyles that trigger different signaling pathways. Another explanation could be that CML9 is only coordinating the response to bacterial but not to fungal pathogens. The fact that CML9 has been shown to contribute to plant defense against bacteria mainly through a flagellin dependent pathway would favor this hypothesis [23]. Additional experiments with other pathogens with different lifestyle or virulence strategies will help to better position CML9 in plant defense pathways.

CML9 does not mediate drought stress tolerance in general

In contrast to previous studies, our data suggest that CML9 is not a common regulator of drought stress. It was reported earlier that CML9 negatively regulates the drought response [21], but under our experimental conditions both *cml9a* and *b* mutants displayed the same behavior as the wild type (Col-0) upon drought treatment (Fig 6(*A*)). Consistent with this observation, no significant difference in the kinetics and level of ABA elevation was observed between drought stress-exposed mutants and the wild type plants (Fig 6(*B*)). Thus, it is unlikely that CML9 plays a key role in the drought stress response. The divergent results of our and previous studies might be caused by different experimental setups: while our plants were grown in single pots during the drought stress, many plants were cultivated together in one pot in the previous study, a situation that can cause intra- and interspecific competition [21]. Furthermore we did our stress treatment under short day conditions while the other setup was done under long day conditions. It could well be that the role of CML9 in drought stress response is dependent on the length of the photoperiod. However, our data indicate that CML9 seems not to be a general regulator of the plant drought stress response.

Conclusion

Here, we investigated in more detail the role of CML9 in plant stress responses. CML9 was described to act as a calcium sensor at the crossroads of different pathways, like pathogen defense and abiotic stress responses [21, 23]. Our study specifies the known functions of CML9. Based on our data, CML9 does not regulate the plant drought stress response in general as otherwise suggested [21], but might have a role under certain conditions. It also does not regulate plant defense against herbivores, neither against chewing lepidopteran larvae nor against piercing-sucking spider mites. We further showed that knock-out of *CML9* does not affect the response of the plant to the fungal pathogen *A. brassicicola*. These results suggest that CML9 is not a general regulator of plant pathogen defense, but very likely specialized on defense against pathogenic bacteria or (hemi)biotrophic pathogens like *P. syringae* [23]. Moreover, the results of both the herbivore and pathogen assays further suggest that CML9 does not regulate jasmonate-mediated pathways at all. Therefore, we propose that CML9 should not be included in a group of CMLs that have a general role in plant stress regulation.

Supporting information

S1 Table. Primers used for different PCRs.
(DOCX)

S1 Fig. Verification of *cml9-a* and *cml9-b*. Semi quantitative RT-PCR analysis of *CML9* full transcript expression in wild type and knock-out mutants after 30 min of MecWorm treatment. Expression of *ACTIN2* was used as quantitative control. Water was used as negative control. The expected product length is written on the right sites of the respective pictures.
(DOCX)

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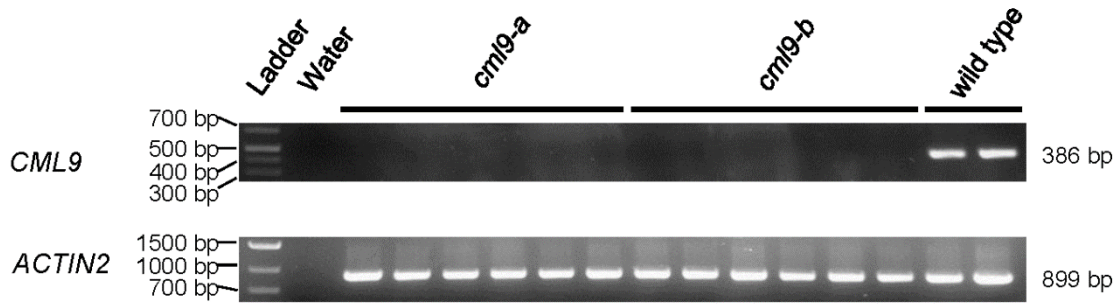
3.2 Supplementary Material Manuscript 1

S1 Table. Primers used for different PCRs.

Primer name	Sequence	PCR
LBb1.3	5′-ATTTTGCCGATTTCGGAAC-3′	PCR Genotyping
cml9-a LP	5′-TGAGCGATGTTGACATCTTTG-3′	PCR Genotyping
cml9-a RP	5′-TTTGGTTTGGTTCGAATTTTG-3′	PCR Genotyping
cml9-b LP	5′-GAATCGATCGGTTTGATGATG-3′	PCR Genotyping
cml9-b RP	5′-CATGGCATTTCACAAAATGC-3′	PCR Genotyping
FP1/FP2	5′-CAGATCCAAGAGTTTTACGAAGCC-3′	RT-PCR
RP1	5′-AGAAAACCATCGCCATCAAGG-3′	RT-PCR
RP2	5′-CATCTCCGTCTCTGTCGAAC-3′	RT-PCR
RPS18B F[a]	5′- GTCTCCAATGCCCTTGACAT -3′	qRT-PCR
RPS18B R[a]	5′- TCTTTCCTCTGCGACCAGTT -3′	qRT-PCR
qRT FP1[b]	5′- TTGGCAACGGTGGCATCACT -3′	qRT-PCR
qRT RP1[b]	5′- CCATCGCCATCAAGGTCGGCT -3′	qRT-PCR
qRT FP2	5′-CCAAAGGCGGAACAACTGCA-3′	qRT-PCR
qRT RP2	5′-ACCATCTCCGTCTCTGTCGAACAC-3′	qRT-PCR

[a] Ref [16]; [b] Ref [16, published as CML9]



S1 Fig.: Verification of *cml9-a* and *cml9-b*. Semi quantitative RT-PCR analysis of *CML9* full transcript expression in wild type and knock-out mutants after 30 min of MecWorm treatment. Expression of *ACTIN2* was used as quantitative control. Water was used as negative control. The expected product length is written on the right sites of the respective pictures.

3.3 Manuscript 2

Title:

The Ca^{2+} sensor proteins CML37 and CML42 antagonistically regulate plant stress responses by altering phytohormone signals

Running Title: The Antagonism of CML37 and CML42

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Abstract

A transient increase in the cytosolic calcium concentration is one of the first reactions that can be measured in plant cells upon abiotic as well as biotic stress treatments. These calcium signals are sensed by calcium binding proteins such as calmodulin-like proteins (CMLs), which transduce the sensed information into appropriate stress responses by interacting with downstream target proteins. In previous studies, CML37 has been shown to positively regulate the plants' defense against both the insect herbivore *Spodoptera littoralis* and the response to drought stress. In contrast, CML42 is known to negatively regulate those two stress responses. Here, we provide evidence that these two CMLs act antagonistically in the regulation of induced responses directed against both types of stress as well as in the defense against the necrotrophic pathogen *Alternaria brassicicola*. Both CMLs shape the plant reactions by altering the phytohormone signaling. Consequently, the phytohormone-regulated production of defensive compounds like glucosinolates is also antagonistically mediated by both CMLs. The finding that CML37 and CML42 have antagonistic roles in diverse stress-related responses suggests that these calcium sensor proteins represent important tools for the plant to balance and fine-tune the signaling and downstream reactions upon environmental stress.

Key words:

jasmonates, abscisic acid, drought, herbivory, necrotrophic pathogenes, defense, calcium, glucosinolates, *Spodoptera littoralis*, *Alternaria brassicicola*

1 Introduction

Plants are faced with a multiplicity of environmental changes at the same time. In order to adapt to their changing environment, each of these stimuli needs to be perceived and translated into an appropriate response via complex signaling networks. Calcium (Ca^{2+}) as a second messenger plays a central role in these signaling networks. In response to various abiotic and biotic stimuli, changes in the cytosolic Ca^{2+} concentration are reported, e.g. after drought and salinity stress (Knight, Trewavas & Knight, 1997), extreme temperature fluctuations (Knight, Campbell, Smith & Trewavas, 1991, Plieth, Hansen, Knight & Knight, 1999), light (Shacklock, Read & Trewavas, 1992), mechanical stimulation (Knight *et al.*, 1991) as well as after interaction with symbionts (Ehrhardt, Wais & Long, 1996, Vadassery *et al.*, 2009), pathogens (Knight *et al.*, 1991) or herbivores (Maffei, Bossi, Spiteller, Mithöfer & Boland, 2004). Each of those stimuli induces a Ca^{2+} oscillation in the cell, the so called Ca^{2+} signature, that can differ in location, duration, amplitude and frequency, reflecting the strength and the type of the stimulus (McAinsh, Brownlee & Hetherington, 1997). Together with other cellular messengers, such as reactive oxygen species, pH changes and membrane potential changes, these complex spatio-temporal Ca^{2+} signatures form a code, transferring specific information about the environment into the plant cell (McAinsh *et al.*, 1997, Plieth, 2016). In order to translate this code into the respective response of the plant, the cellular changes need to be sensed.

Ca^{2+} oscillations are sensed by Ca^{2+} binding proteins. These proteins possess Ca^{2+} binding motifs, consisting out of two helices, the E- and the F-helix, connected via a loop-structure and thus called EF-hand (Kretsinger & Nockolds, 1973). An EF-hand binds a single Ca^{2+} ion (Kretsinger & Nockolds, 1973), leading to a conformational change of the Ca^{2+} sensor protein

that allows subsequently the interaction with a certain target. Some sensors, so-called sensor responders, have additionally enzymatic domains, like the Ca^{2+}-dependent protein kinases (CDPKs) in plants that are activated upon binding of Ca^{2+}. However, most Ca^{2+} sensing proteins just possess EF-hands as functional domains and thus are dependent on an interacting protein to transduce the sensed signal into a response. In plants three major groups of these sensor relays are distinguished: calmodulins (CaMs), calmodulin-like proteins (CMLs) and calcineurin B-like proteins (CBLs) (Sanders, Pelloux, Brownlee & Harper, 2002). Amongst them, the family of CMLs is of special interest for decoding calcium signatures upon stress in plants, since they are unique to the plant kingdom (Bender & Snedden, 2013). Further, in comparison to the related CaMs, CMLs display a much more distinctive expression pattern upon various stress treatments in *Arabidopsis thaliana*, suggesting that they might play a role in decoding calcium signals upon stress in plants (McCormack, Tsai & Braam, 2005). For a few members of the CML family, there is also evidence that they mediate responses to various stresses in *A. thaliana*, although for most CMLs a functional characterization is still missing. Recently it was shown that CML41 reduces bacterial infection of *Pseudomonas syringae* by regulating the closure of plasmodesmata (Xu *et al.*, 2017). Also CML8, CML9 and CML24 are known to positively regulate the immune response to *P. syringae* (Leba *et al.*, 2012, Ma, Smigel, Tsai, Braam & Berkowitz, 2008, Zhu *et al.*, 2017). Besides, CML9 and CML24 have been shown to mediate the salt stress response in *A. thaliana* (Delk, Johnson, Chowdhury & Braam, 2005, Magnan *et al.*, 2008) and thus seem to play a role in abiotic as well as biotic stress regulation. Similarly, CML37 and CML42 are known to be important in regulating both, abiotic and biotic stress responses. They mediate the defense against the lepidopteran herbivore *Spodoptera littoralis* and the drought stress reaction of *A. thaliana* (Scholz, Reichelt, Vadassery & Mithöfer, 2015, Scholz *et al.*, 2014, Vadassery *et*

al., 2012). In both stress treatments it has been shown that CML37 and CML42 act via altering the phytohormone signaling in the plant. In response to *S. littoralis* feeding, loss-of-function mutants of CML37 accumulated less jasmonates, leading to a higher susceptibility of the plant to the herbivore (Scholz *et al.*, 2014). In contrast, knock out mutants of CML42 showed an upregulation of jasmonate-dependent defense responses, but a wild type-like jasmonate elevation, suggesting hypersensitivity to jasmonates and causing a higher resistance to the herbivore (Vadassery *et al.*, 2012). Similarly, *cml42* accumulated higher levels of abscisic acid (ABA) upon drought conditions and thus was more resistant to drought, whereas *cml37* displayed no ABA response at all and was highly more susceptible (Scholz *et al.*, 2015, Vadassery *et al.*, 2012).

Since CML42 turned out to be a negative regulator of both herbivore and drought stress response and CML37 a positive one, it was hypothesized that they might be antagonists in regulating these stress responses (Scholz *et al.*, 2015, Scholz *et al.*, 2014). In order to examine the interplay of CML42 and CML37, we constructed a double knock out mutant line and analyzed the response of this line upon drought and *S. littoralis* feeding. We show that effects of *cml37* abolish the effects of *cml42* in the double knock out mutant line and *vice versa*, verifying that CML37 and CML42 act antagonistically in regulating both stress responses. Further we demonstrate that CML37 and CML42 regulate the defense against the necrotrophic pathogen *Alternaria brassicicola* antagonistically, suggesting a general antagonistic role of CML37 and CML42 in the regulation of the jasmonate-dependent defense responses. To gain more insights into how both CMLs act on the phytohormone pathway, we tested possible interactions between both CMLs and downstream targets of the jasmonate pathway. By studying a double knock out

113 mutant of both CMLs we are now able to refine the roles of CML37 and CML42 in balancing
114 different stress responses.
115
116 **2 Materials and methods**
117 **2.1 Plant material**
118 *Arabidopsis* plants were grown under short day conditions at the MPI CE Jena in round pots
119 with 10 cm diameter as described in Heyer *et al.* (2018). The double knock out mutant line
120 *cml37 x cml42* was obtained by crossing *cml37-1* (SALK_011488C) (Scholz *et al.*, 2014) and
121 *cml42* (SALK_041400C) (Dobney, Chiasson, Lam, Smith & Snedden, 2009, Vadassery *et al.*,
122 2012). Plants were selected by genotyping and selection was proven by RT-PCR. Experiments
123 were performed with the F4 and F5 progeny of the crossed plants. *A. thaliana* ecotype Col-0 was
124 used as control.
125 Fungus treatments were performed at the FSU Jena and plants were grown on plates as described
126 in Johnson, Sherameti, Nongbri and Oelmüller (2013). After 21 d plants were transferred to soil
127 and further cultivated as described in Heyer *et al.* (2018).
128
129 **2.2 Insect rearing and oral secretion (OS) collection**
130 *Spodoptera littoralis* eggs were obtained from Syngenta Crop Protection AG (Stein,
131 Switzerland). Larvae were reared on an artificial diet based on ground beans modified after
132 Bergomaz and Boppré (1986) at 23 – 25 °C and with 14 h photoperiod. Modifications of the diet
133 composition are described in Heyer *et al.* (2018). For collection of OS, fourth instar larvae were
134 starved overnight and were allowed to feed on the respective plant genotype for one day. OS was

135 collected on ice and centrifuged at 13,000 rpm for 5 min after collection (Vadassery *et al.*, 2012).
136 It was stored at -80 °C until use.
137
138 **2.3 Fungal growth**
139 *Alternaria brassicicola* (FSU-218) was obtained from Jena Microbial Resource Center (Jena,
140 Germany). Fungus was grown according to Heyer *et al.* (2018).
141
142 **2.4 Plant treatments**
143 All herbivore-related experiments were done with 5 to 6 week old plants. One week feeding
144 assays were performed with first instar larvae as described in Vadassery *et al.* (2012) (see insect
145 biomass assay). For short term feeding assays fourth instar larvae were used. To ensure sufficient
146 feeding, larvae were starved 12 h prior the assays and three larvae were allowed to feed on one
147 plant (Scholz *et al.*, 2014, Vadassery *et al.*, 2012). OS treatments were done as described in
148 Vadassery *et al.* (2012).
149 Four week old plants were used for drought stress assays. Drought was applied for 1 or 2 weeks.
150 If applied for 2 weeks, plants were re-watered after first week of drought stress as described in
151 Scholz *et al.* (2015). Control and mutant plants were kept randomly distributed on the same tray
152 to minimize experimental variation.
153 Plant material collected for metabolite quantification or RT-PCR was immediately frozen in
154 liquid nitrogen and stored at -80 °C until extraction.
155 For pathogen treatments detached, fully expanded leaves of 5 to 6 week old plants were used.
156 *Alternaria* treatments were carried out as described in Heyer *et al.* (2018).
157

158 **2.5 Genotyping**

159 Single leaves of 3 week old plants were cut and immediately frozen in liquid nitrogen. Plant
160 material was ground using 2010 Geno/Grinder® (SPEX®SamplePrep, Metuchen USA). To avoid
161 defrosting of the samples, they were stored in precooled aluminum racks throughout grinding
162 process. DNA was extracted after a modified protocol of Konieczny and Ausubel (1993).
163 Modifications are described in Heyer *et al.* (2018). PCR was performed using native Taq DNA
164 polymerase and 10 mM dNTP Mix from Invitrogen™ by Thermo Fisher Scientific (Carlsbad,
165 USA). PCR mix was prepared according to manufacturers' protocol. The total reaction volume
166 was scaled down to 10 µl, including 1.5 µl of template. Primers published in Scholz *et al.* (2014)
167 and Vadassery *et al.* (2012) were used.

168

169 **2.6 Semi-quantitative reverse transcription (RT)-PCR**

170 Treated leaves were sampled and ground as described above. RNA was isolated using TRIzol®
171 Reagent (Invitrogen™ by Thermo Fisher Scientific, Carlsbad, USA). Extraction was perfomed
172 according to the manufacturers' protocol with modifications as described in Heyer *et al.* (2018).
173 To avoid DNA contamination, extracted RNA was treated with TURBO DNase (TURBO DNA-
174 *free*™ Kit, Invitrogen™ by Thermo Fisher Scientific, Vilnius, Lithuania). PCR was done as
175 described above. *ACTIN2* was used as housekeeping gene. Primers for *CML37* as published in
176 Scholz *et al.* (2014) and *CML42* and *ACTIN2* as described in Vadassery *et al.* (2012) were used.

177

178 **2.7 Phytohormone quantification**

179 Phytohormones were extracted from treated leaves using the protocol described in Jimenez-
180 Aleman *et al.* (2015) with slight modifications. Approximately 250 mg of ground leaf material

was extracted using 1.5 ml methanol containing 60 ng D_6-ABA (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), 60 ng of D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4-salicylic acid (Sigma-Aldrich, St. Louis, USA) and 12 ng of jasmonoyl-$^{13}C_6$-isoleucine (synthesized as described in Kramell, Schmidt, Schneider, Sembdner and Schreiber (1988)) as internal standard. Phytohormone analysis was performed according to the protocol of Vadassery *et al.* (2012). Protocol was modified as follows. For herbivore treated samples chromatography was performed on an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, USA), for drought stress samples chromatographic separation was done on an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, USA) using a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies, Santa Clara, USA) in both cases. Water containing 0.05% formic acid and acetonitrile were employed as mobile phases A and B, respectively. The elution profile for herbivore treated samples 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. In case of drought stress treated samples elution profile was: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min, 90–100% B; 4.02–4.5 min, 100% B and 4.51–7.0, min 10% B. Flow rate was kept at 1.1 ml/min and column temperature was maintained at 25°C. Mass spectrometry of herbivore treated samples was performed on an API 5000 tandem mass spectrometer (Applied Biosystems™, Darmstadt, Germany) and on an API 3200 tandem mass spectrometer (Applied Biosystems™, Darmstadt, Germany) in case of drought stressed samples. Both spectrometers were equipped with a Turbo spray ion source operated in negative ionization mode. The ion spray voltage was maintained at -4,500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi, and collision gas at 7 psi. The following analyte parent ion → product ion fragmentations were used for multiple reaction monitoring (MRM): mass-to-charge

204 ratio (*m/z*) 263.0 →153.2 (collision energy (CE) -22 V; declustering potential (DP) -35 V) for
205 ABA; *m/z* 269.0 →159.2 (CE -22 V; DP -35 V) for D_6-ABA; *m/z* 209.1 →59.0 (CE -24 V; DP -
206 35 V) for jasmonic acid (JA); *m/z* 215.1 →59.0 (CE -24 V; DP -35 V) for D_6-JA; *m/z* 136.9
207 →93.0 (CE -22 V; DP -35 V) for salicylic acid (SA); *m/z* 140.9 →97.0 (CE -22 V; DP -35 V) for
208 D_4-SA; *m/z* 290.9 → 165.1 (CE -24 V; DP -45 V) for *cis*-12-oxophytodienoic acid (OPDA), *m/z*
209 322.2 →130.1 (CE -30 V; DP -50 V) for jasmonoyl isoleucine (JA-Ile); *m/z* 328.2 →136.1 (CE -
210 30 V; DP -50 V) for JA-$^{13}C_6$-Ile. Both Q1 and Q3 quadrupoles were maintained at unit
211 resolution. Analyst 1.5 software (Applied Biosystems™, Darmstadt Germany) was used for data
212 acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution
213 series of standard mixtures. Phytohormones were quantified relative to the signal of their
214 corresponding internal standard. For quantification of OPDA the internal D_6-JA standard was
215 used applying experimental-determined response factors of 0.5 respectively. The response factor
216 was determined by analyzing a mixture of *cis*-OPDA (kindly provided by W. Boland, MPI for
217 Chemical Ecology, Jena, Germany; synthesized as described in Shabab, Khan, Vogel, Heckel
218 and Boland (2014)) and D_6-JA all at the same concentration. For JA-Ile quantification after
219 herbivory only the peak of the endogenous bioactive (+)-7-*iso*-jasmonoyl-L-isoleucine (Fonseca
220 *et al.*, 2009) was used.
221
222 **2.8 Quantification of glucosinolates**
223 Whole *Arabidopsis* rosettes where collected and freeze dried, to avoid fast degradation of
224 glucosinolates. Freeze-dried samples were ground to a fine powder in uncooled racks in 2010
225 Geno/Grinder® (SPEX®SamplePrep, Metuchen USA). Extraction was performed according to
226 Burow, Müller, Gershenzon and Wittstock (2006) with some modifications. For each sample,

227 approximately 25 mg per sample were extracted in 1 ml 80% methanol containing 50 µM 4-
228 hydroxybenzylglucosinolate (isolated from *Sinapis alba* seeds according to Thies (1988)) as
229 internal standard. Samples were mixed for 10 min at room temperature and pelleted by
230 centrifugation. 800 µl of the supernatant was transferred to columns containing 28 mg DEAE
231 Sephadex A25 (Sigma-Aldrich, St. Louis, USA) each. Columns were prewashed with 800 µl
232 water and 500 µl 80% methanol. After loading the samples, columns were washed with 500 µl
233 80% methanol and twice with 1 ml water. Afterwards they were rinsed with 500 µl 0.02 M MES
234 buffer (pH 5.2) and 30 µl of sulfatase (from *Helix pomatia*, Sigma Aldrich, St. Louis, USA) was
235 applied to the columns. Sulfatase was prepared according to Graser, Schneider, Oldham and
236 Gershenzon (2000). Columns were incubated for desulfation at room temperature overnight and
237 desulfoglucosinolates were eluted with 500 µl water. Desulfoglucosinolates were analyzed using
238 HPLC/UV and quantified as described in Vadassery *et al.* (2012).
239
240 **2.9 Yeast two-hybrid (Y2H) assays**
241 All vector constructs were produced using Gateway cloning technology (Invitrogen™ by Thermo
242 Fisher Scientific, Carlsbad, USA). The attB-flanked CML37 was amplified from c-DNA using
243 the following primers: CML37attB-F 5'-
244 GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACTCTCGCTAAGAACCAAA-3'
245 and CML37attB-R 5'-
246 GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAACGCATCATCAAAACAAAC-3'.
247 AttB-flanked amplicon was cloned into pDONR207 to generate the entry clone. Entry clones of
248 CML42 and COI1 where produced as described in Chini *et al.* (2007) and Vadassery *et al.*
249 (2012). Entry clone of JAR1 was kindly gifted by Sandra Fonseca. Entry clones were checked by

250 sequencing and further recombined with the pGADT7gateway (Gal4 Activation Domain; AD)
251 and pGBKT7gateway (Gal4 DNA-Binding Domain; BD) destination vectors in a LR reaction to
252 generate the Y2H expression vectors. To determine possible protein interactions, the respective
253 plasmids were co-transformed into *Saccharomyces cerevisiae* AH109 cells by heat shock
254 transformation (Schiestl & Gietz, 1989). Y2H assays were carried out as described in Chini,
255 Fonseca, Chico, Fernández-Calvo and Solano (2009). The empty vector pGADT7gateway was
256 also co-transformed with pGBKT7 constructs as negative control. To investigate the dependency
257 of the interaction on the availability of Ca^{2+}, the respective yeast synthetic dropout media was
258 complemented with 2 mM and 20 mM $CaCl_2$. Further, the dependency of the COI1 interaction
259 on the availability of its ligand JA-Ile was tested by supplementing the dropout media with the
260 JA-Ile mimic 10 µM Coronatine (COR).
261
262 **2.10 Chlorophyll fluorescence measurements**
263 Chlorophyll fluorescence parameters was measured in a FluorCam FC 800-C (Psi, Drasov,
264 Czech Republic) as described in Heyer *et al.* (2018).
265
266 **2.11 Statistical analysis**
267 Statistics were performed using R 3.5.1 (R Development Core Team, 2018) and SigmaPlot 11.0
268 (Systat Software, 2008). Differences in larval weight of *S. littoralis* were tested by Wilcoxon-
269 test. Differences in phytohormone concentration between two genotypes were determined with
270 Wilcoxon-test or t-test depending on the homogeneity and normal distribution of the data. In
271 order to test whether the glucosinolate content, chlorophyll fluorescence and ABA content
272 differed between different genotypes with different treatments, two-way ANOVAs was applied,

followed by Tukey tests as post-hoc test if necessary In case of variance inhomogeneity, generalized least square method (gls from the nlme library (Pinheiro, Bates, DebRoy, Sarkar & R Core Team, 2018)) with varIdent function was applied instead of atwo-way ANOVA. Whether the different variance of genotype, treatment, or the combination of both factors should be incorporated into the model was determined by comparing different variance structure models, selecting for the model with the lowest Akaike Information Criteria (AIC) (Zuur, Ieno, Walker, Saveliev & Smith, 2009). The influence of the different genotypes, treatments and the interaction of both was determined by likelihood ratio tests following the backward selection protocol of Zuur *et al.* (2009). To test for differences among the groups factor level reduction was used (Crawley, 2013). In order to obtain normal distribution of residuals, data were transformed before applying the statistic test, where it was necessary. The respective statistical test and transformation method is mentioned in the respective tables. Total number of replicates (n) is indicated in the figure legend. All experiments shown are repeated at least two times independently.

3 Results

3.1 Construction of the *cml37 x cml42* double knock out line

To investigate the possible antagonism of CML37 and CML42, a loss-of-function mutant of both proteins was generated by crossing the T-DNA insertion lines *cml37-1* (Scholz *et al.*, 2014) and *cml42* (Dobney *et al.*, 2009, Vadassery *et al.*, 2012). The homozygosity of the crossed line was confirmed by genotyping (Figure 1A). For both genes the product for the intact gene was not

295 detectable, whereas the product including the left border of the T-DNA was detectable in both
296 cases (Figure 1A), showing that *cml37 x cml42* is homozygous.
297 Further, the absence of *CML37* and *CML42* transcript was confirmed by RT-PCR. Since the
298 constitutive expression level of *CML37* in adult leaves is comparatively low (McCormack *et al.*,
299 2005), plants were wounded and treated with *S. littoralis* OS to stimulate the expression of the
300 CMLs. Whereas both CMLs are expressed in untreated and treated wild type plants, no product
301 was detected in case of the double knock out mutant (Figure 1B), confirming that it is a loss of
302 function of both CMLs.
303

304 **3.2 *S. littoralis* performance is not affected in *cml37 x cml42***

305 To test the hypothesis of the possible antagonism of CML37 and CML42 in the herbivore
306 defense, the *cml37 x cml42* mutant line was used for insect performance assays. First instar *S.*
307 *littoralis* larvae were allowed to feed on wild type and mutant plants for 1 week. The insect
308 performance was evaluated by the gain of weight. Results are presented in Figure 2. After 1
309 week, *S. littoralis* larvae gained as much weight on *cml37 x cml42* as on wild type plants,
310 suggesting the positive effect of *cml37* and the negative effect of *cml42* on the larval weight
311 gain (Scholz *et al.*, 2014, Vadassery *et al.*, 2012) compensate each other in the double knock out
312 mutant. Thus, both CMLs seem to be antagonistic in regulating the herbivore defense of *A.*
313 *thaliana*.
314

315 **3.3 *cml37 x cml42* displays a wild type-like phytohormone response after herbivory**

316 In former studies, the higher susceptibility of *cml37* to *S. littoralis* was shown to be caused by a
317 lower level of the jasmonate-precursor OPDA and the active jasmonate JA-Ile (Scholz *et al.*,

318 2014). However, in *cml42*, there was no difference in phytohormone concentration between the
319 wild type and the mutant (Vadassery *et al.*, 2012). Thus, we investigated the phytohormone
320 levels after *S. littoralis* feeding on *cml37 x cml42.* Similar to both single mutant lines, the levels
321 of SA and JA did not change in the double knock out mutant line compared to the wild type
322 (Figure 3A and C, Table 1). However, in contrast to the single mutant lines, *cml37 x cml42*
323 plants also displayed wild type-like OPDA and JA-Ile concentrations in non-treated controls and
324 after *S. littoralis* feeding (Figure 3B and D, Table 1). Even though CML42 was shown to have no
325 effect on phytohormone levels itself (Vadassery *et al.*, 2012), *cml42* is able to rescue the negative
326 effect of *cml37* on the OPDA and JA-Ile levels in *cml37 x cml42* after insect feeding.
327
328 **3.4 Induction of secondary metabolites is altered in *cml37 x cml42***
329 Vadassery *et al.* (2012) published that the higher resistance of *cml42* to insect feeding is, *inter*
330 *alia*, due to a higher constitutive level of defensive compounds, the glucosinolates. On the other
331 hand, loss-of-function mutants of CML37 displayed constitutive and induced glucosinolate
332 levels that were comparable to those of wild type plants (Scholz *et al.*, 2014). In order to
333 investigate if *cml37* is able to rescue the glucosinolate phenotype of *cml42*, we measured the
334 glucosinolate content of *cml37 x cml42* before and after *S. littoralis* feeding. There were no
335 differences in the total glucosinolate levels of untreated 5 and 6 week-old *cml37 x cml42* and
336 wild type plants (Figure 4A, Table 2). Since the higher total constitutive glucosinolate content in
337 *cml42* was due to an increased level of aliphatic glucosinolates (Vadassery *et al.*, 2012), we
338 further distinguished into aliphatic and indole glucosinolates. Untreated 5 and 6 week-old *cml37*
339 *x cml42* plants displayed a wild type-like constitutive level of aliphatic and indole glucosinolates,
340 showing that *cml37* rescues the effect on glucosinolates of *cml42* in the double knock out mutant

341 line (Figure 4B and C, Table 2). Further, no differences in the induced total, aliphatic or indole
342 glucosinolate content were measured after 1 day feeding between *cml37 x cml42* and wild type
343 plants (Figure 4, Table 2). However, after 1 week of feeding, the induction of glucosinolates in
344 *cml37 x cml42* was significantly lower than in wild type plants (Figure 4A, Table 2). This seems
345 to be due to both the lower level in aliphatic as well as in indole glucosinolates (Figure 4B and
346 C). Although the interaction of genotype and treatment was not significant in case of the
347 aliphatic glucosinolates there is a clear tendency (Table 2). This result seems to be a secondary
348 effect only detected in the double knock out mutant, since neither *cml37* nor *cml42* were
349 impaired in the induction of glucosinolates (Scholz *et al.*, 2014, Vadassery *et al.*, 2012).
350 However, the lower induction of glucosinolates after 7d does not cause better larval performance
351 after 1 week (Figure 2). Thus, it is unclear if this late reduction of the glucosinolates content in
352 *cml37 x cml42* is of functional relevance for the insect defense.
353
354 **3.5 CML42 and CML37 do not interact with JAR1 and COI1**
355 In a previous study, the lower amount of JA-Ile in *cml37* was explained by a lower activity of the
356 enzyme jasmonic acid-amido synthetase (JASMONATE RESISTANT 1, JAR1) (Scholz *et al.*,
357 2014) that is catalyzing the conjugation of JA and Ile (Staswick & Tiryaki, 2004). In order to test
358 if CML37 is able to directly influence the enzyme activity by binding to JAR1, we performed
359 Y2H assays to test the possible interaction. CML37 did not bind to JAR1 in yeast (Figure 5A).
360 However, since CML37 is changing its conformation upon Ca^{2+} binding (Scholz *et al.*, 2014), we
361 also examined the interaction of both proteins in the presence of Ca^{2+}. Two different $CaCl_2$
362 concentrations were tested, but CML37 still did not bind to JAR1 (Figure 5B and C). Since
363 CML42 was shown to counteract the effect of CML37 on the level of JA-Ile in the double knock

364 out mutant line, we next tested the possible interaction of CML42 and JAR1. CML42 also did
365 not interact with JAR1, under none of the tested conditions (Figure 5A-C).
366 Further it was hypothesized previously that CML42 might influence the binding affinity of JA-
367 Ile to its receptor CORONATINE INSENSITIVE 1 (COI1) (Fonseca *et al.*, 2009), since *cml42*
368 displayed a wild type-like jasmonate elevation, but enhanced jasmonate-dependent responses
369 (Vadassery *et al.*, 2012). Thus, we tested the possible interaction of COI1 and CML42. CML42
370 showed no interaction with COI1 under the tested conditions (Figure 5A-C). Also its antagonist
371 CML37 did not interact with COI1 (Figure 5A-C). On the other hand it has been shown that
372 interactions between proteins and COI1 can be dependent on the presence of its ligand JA-Ile
373 (Chini *et al.*, 2007). Thus, we further tested conceivable interactions between COI1 and both
374 CMLs in the presence of COR, a mimic of JA-Ile (Staswick & Tiryaki, 2004). However, none of
375 the CMLs interacted with COI1, neither when only COR was supplied nor when additionally
376 $CaCl_2$ was added (Figure 5D).
377 Next we wanted to examine if both CMLs can directly interact with each other and thus leading
378 to the antagonistic effects. However, in Y2H assay there was no interaction detected between
379 CML37 and CML42 (Figure 5A-C).
380

381 **3.6 *cml37 and cml42* display a different susceptibility to *A. brassicicola***

382 Besides mediating the defense against herbivores, jasmonates are also important signaling
383 components in the defense against necrotrophic pathogens (Glazebrook, 2005, Thomma *et al.*,
384 1998). Since both CML37 and CML42 are regulating the defense against the herbivore *S.*
385 *littoralis* by affecting the jasmonate pathway (Scholz *et al.*, 2014, Vadassery *et al.*, 2012), we
386 examined whether they are influencing the resistance to the necrotrophic fungus *A. brassicicola*

as well. In line with the results from the herbivore assays, both *cml* mutants displayed an antagonistic phenotype. Whereas *cml37* was much more susceptible to the fungus treatment, *cml42* seemed to be more resistant than the wild type (Figure 6A). After 3 d of treatment, necrotic lesions were slightly larger on *cml37* than on wild type leaves (Figure 6A). After 4 d, the necrotic area was even covering nearly the whole *cml37* leaf, whereas just a part of the wild type leaf was necrotic (Figure 6A). On the other hand, lesions on *cml42* leaves were smaller than on wild type plants at both time points (Figure 6A). Interestingly *cml37 x cml42* displayed an intermediate lesion forming phenotype that was comparable to wild type plants (Figure 6A), suggesting again that the negative effect of *cml37* and the positive effect of *cml42* are neutralizing each other in the double mutant.

In addition, the chlorophyll fluorescence of the leaves was measured to determine the differences in susceptibility. Corresponding to the phenotype, *cml37* leaves showed lower chlorophyll fluorescence than wild type plants after fungi treatment (Figure 6B, Table 3). Surprisingly, the chlorophyll fluorescence of *cml42* was similar to those of wild type leaves (Figure 6B, Table 3), although the necrotic area seemed to be smaller on mutant leaves (Figure 6A). However, in *cml37 x cml42* the chlorophyll fluorescence was comparable to wild type plants as well (Figure 6B, Table 3), implying that *cml42* can recover the strong negative impact of *cml37*. Thus, CML37 and CML42 seem to regulate the defense against *A. brassicicola* antagonistically as well.

3.7 Drought stress response is not altered in *cml37 x cml42*

An addition to their known function in jasmonate-mediated stress responses, CML37 and CML42 have been shown to regulate the drought stress response in *A. thaliana* (Scholz *et al.*,

410 2015, Vadassery *et al.*, 2012). It was observed that *cml37* plants are more sensitive to drought
411 stress than wild type plants, while *cml42* seemed to be less affected (Scholz *et al.*, 2015). The
412 drought stress phenotype of both mutants was reflected in altered ABA levels: *cml42* displayed
413 higher ABA levels after drought stress, whereas in *cml37* ABA was not induced at all upon
414 drought (Scholz *et al.*, 2015, Vadassery *et al.*, 2012). In order to test if CML37 and CML42 are
415 also antagonistically regulating the drought stress response, we investigated the response of
416 *cml37 x cml42* to drought. After 1 week without water, there was no difference in the drought
417 stress phenotype between *cml37 x cml42* and wild type plants (Figure 7A). Plants were than
418 watered and kept for a second week without water. Even after the second drought period, *cml37*
419 *x cml42* was as tolerant as the wild type plants (Figure 7A). Corresponding to that, *cml37 x*
420 *cml42* displayed wild type-like ABA levels among the whole treatment (Figure 7B, Table 4).
421 Again, the diverging effects of the single knock out mutants are balanced in the double knock out
422 mutant line, suggesting that CML37 and CML42 are antagonists in the regulation of the drought
423 stress response.
424
425 **4 Discussion**
426 The CMLs, a group of calcium sensors, has been shown to be responsible for sensing stress-
427 mediated Ca^{2+} signals and regulating downstream defense reactions of the plant (Delk *et al.*,
428 2005, Leba *et al.*, 2012, Ma *et al.*, 2008, Magnan *et al.*, 2008, Scholz *et al.*, 2015, Scholz *et al.*,
429 2014, Vadassery *et al.*, 2012, Xu *et al.*, 2017, Zhu *et al.*, 2017). However, not much is known
430 about the interplay between the different CMLs in mediating different stress responses. Here we
431 report about the antagonism of CML37 and CML42 in regulating the defense against herbivores
432 and pathogens, as well as the drought stress response of *A. thaliana.*

433

434 **4.1 CML37 and CML42 regulate the defense to *S. littoralis* antagonistically**

435 In previous studies CML37 was described as a positive regulator of defense against the insect
436 herbivore *S. littoralis* whereas CML42 was shown to negatively influence this defense response
437 (Scholz *et al.*, 2014, Vadassery *et al.*, 2012). Here we showed that if both CMLs are knocked
438 out, the positive effect of CML37 and the negative effect of CML42 neutralize each other,
439 suggesting that both CMLs act antagonistically. In feeding assays, *S. littoralis* larvae gained as
440 much weight on the double knock out mutant line as on wild type plants (Figure 2). In line with
441 this result, the double knock out mutant displayed a wild type-like phytohormone response upon
442 *S. littoralis* feeding (Figure 3). However in former studies with the corresponding single knock
443 out mutant lines, only *cml37* but not *cml42* had an effect on the level of phytohormones. In
444 detail, *cml37* elevated less OPDA and JA-Ile upon insect feeding than the wild type, whereas
445 *cml42* mutants accumulated the same amounts of jasmonates like the wild type (Scholz *et al.*,
446 2014, Vadassery *et al.*, 2012). Nevertheless, *cml42* is able to rescue the effect of *cml37* in the
447 double knock out mutant (Figure 3). This result suggests that *cml42* is able to positively
448 influence the jasmonate accumulation. This positive influence of *cml42* might not have been
449 displayed in the single mutant, since enhanced jasmonate levels are usually very costly to the
450 plants and negatively affect their fitness. Plants that exhibit higher levels of jasmonates are often
451 smaller and produce far less seeds (Baldwin, 1998, Cipollini, 2007). Thus the plant might control
452 via other regulators the level of jasmonates and limit them to a certain height. Nevertheless, it
453 might be possible that there are secondary effects that just appear in the double knock out
454 mutant, but not in the single mutants that explain the different effects of knocking out *CML42* in
455 single and double knock out mutant on the jasmonate elevation.

456 A similar rescue effect of one of the single mutants in the double knock out line could be shown
457 for the production of secondary metabolites. Here we investigated the levels of glucosinolates, a
458 class of secondary metabolites produced especially as defensive compounds against herbivores
459 in the Brassicaceae family (Halkier & Gershenzon, 2006). It was known that *cml42* produced
460 higher constitutive levels of mainly aliphatic glucosinolates (Vadassery *et al.*, 2012). In *cml37 x*
461 *cml42* the constitutive levels of both aliphatic and indole glucosinolates were comparable to
462 those of wild type plants (Figure 4B and C), suggesting that *cml37* is able to rescue the effect of
463 *cml42* on the constitutive levels of glucosinolates in *cml37 x cml42*. Thus *cml37* might
464 negatively influence the production of glucosinolates. However, such a negative effect on
465 glucosinolate production was not measured in the single knock out line of *CML37*. It displayed a
466 wild type-like glucosinolate pattern (Scholz *et al.*, 2014). On the other side it is known that
467 *cml37* alone negatively affected the biosynthesis of jasmonates (Scholz *et al.*, 2014) and that the
468 biosynthesis of glucosinolates is mainly regulated by the jasmonate pathway (Mewis *et al.*, 2006,
469 Schweizer *et al.*, 2013). Thus *cml37* might also negatively influence the glucosinolate
470 production. However, even *coi-1* mutants that are insensitive to jasmonates still possess a certain
471 level of glucosinolates (Mewis *et al.*, 2006, Schweizer *et al.*, 2013). Clearly *cml37* is impaired in
472 the jasmonate biosynthesis, but it displays a far less severe phenotype than *coi-1* (Scholz *et al.*,
473 2014). Thus it is conceivable that *cml37* has a negative impact on the production of
474 glucosinolates that is not pronounced in the single mutant, but strong enough to rescue *cml42* in
475 the double knock out plants. However, also here secondary effects that occur in the double knock
476 out mutant line cannot be fully excluded as a possible explanation of the rescue effect.
477 Since *cml37* is able to rescue effects of *cml42* and *vice versa*, we could clearly show that both
478 CMLs regulate the defense against the insect herbivore *S. littoralis* antagonistically.

4.2 CML37 and CML42 are not influencing the jasmonate pathway *via* direct interaction with COI1 or JAR1

CMLs belong to the class of Ca^{2+} sensor relays and thus just possess the functional domains to bind Ca^{2+} (Sanders *et al.*, 2002). Therefore, to transduce the information given in the Ca^{2+} signature into the appropriate defense response, they need to bind to a certain target protein. Both CML37 and CML42 have been shown to influence the jasmonate pathway by altering the biosynthesis of JA-Ile by the JAR1 enzyme as well as its recognition by the COI1 receptor (Scholz *et al.* (2014), Vadassery *et al.* (2012) and Figure 3). Thus JAR1 and COI1, respectively, might have been possible downstream targets for both CMLs. However, in Y2H assays, no interaction could be detected, neither between CMLs and JAR1 nor COI1 (Figure 5A-D).

Nevertheless, it cannot be excluded here that both CMLs might interact with transcription factors downstream of jasmonate perception *via* COI1 and, by this, influence jasmonate-dependent defense responses. For instance, it was shown that the soybean (*Glycine max*) GmCaM1 and GmCML1 (former GmCaM4) antagonistically regulate the activity of the transcription factor MYB2 in *Arabidopsis* (Yoo *et al.*, 2005) and, thus, the salt stress response. Similarly, CML37 and CML42 might influence transcription factors that are known to control the expression of jasmonate responsive genes. On the other hand, targets of the CMLs might be upstream of the jasmonate pathway and, hence, CML37 and CML42 might just indirectly influence JAR1 and COI1 *via* their interacting partners. Both theories need to be proven in further studies.

Besides possible interactions with COI1 and JAR1 we also examined the possibility that CML37 and CML42 might form heterodimers and by this leading to the antagonistic effects, like it is known from other antagonistic regulatory proteins (Epple, Mack, Morris & Dangl, 2003). However in case of CML37 and CML42 no interactions between the two calcium sensing

502 proteins were detected (Figure 5A-C), excluding the heterodimer hypothesis. Thus to understand
503 the antagonism between the two CMLs in detail, the downstream interacting proteins need to be
504 identified in future projects.
505
506 **4.3 The differential regulation of defense against necrotrophs by CML37 and CML42**
507 Besides their role in the herbivore defense, some CMLs have been shown to be involved in the
508 defense against pathogens (Leba *et al.*, 2012, Ma *et al.*, 2008, Xu *et al.*, 2017, Zhu *et al.*, 2017).
509 However, most of the research is focusing on the biotrophic bacterial pathogen *P. syringae*, but
510 less is known about the role of CMLs in the defense against necrotrophic pathogens. In gene
511 expression studies *CML37* and *CML42* were both upregulated after infection with the
512 necrotrophic fungus *Botrytis cinerea* (McCormack *et al.*, 2005). Here, we showed that CML37
513 and CML42 are also important for the regulation of the defense against the necrotrophic fungus
514 *A. brassicicola*. Whereas *cml37* was more susceptible to the fungus than the wild type plants,
515 *cml42* seemed to be more resistant (Figure 6), indicating that CML37 acts as a positive regulator
516 of the defense against the fungus and CML42 as a negative one. Further effects of *cml37* and
517 *cml42* neutralized each other in the *cml37 x cml42* double mutant (Figure 6), providing again
518 evidence that these two CMLs are antagonists in the regulation of this defense reaction.
519 The signaling pathways leading to defense against herbivores and those that are responsible for
520 the defense against necrotrophs are closely related as both of them rely on the jasmonate
521 pathway (Glazebrook, 2005, Howe & Jander, 2008, Thomma *et al.*, 1998). Since CML37 and
522 CML42 are influencing this phytohormone pathway (Scholz *et al.* (2014), Vadassery *et al.*
523 (2012) and Figure 3), it is not surprising that they have similar roles in the regulation of the
524 defense against the herbivore *S. littoralis* and the pathogen *A. brassicicola*.

525 **4.4 The antagonism of CML37 and CML42 in the drought stress response**

526 Besides biotic stress, plants have to cope with changing abiotic conditions. The availability of
527 water is one of the major abiotic factors. It is known that CMLs play important roles in
528 regulating the drought stress response. ShCML44, a CML isolated from wild tomato plants
529 (*Solanum habrochaites*), confers to drought tolerance when overexpressed in *Arabidopsis* (Munir
530 *et al.*, 2016). The same effect was shown for the rice (*Oryza sativa*) CML OsMSR2 (Xu *et al.*,
531 2011). Also CML37 and CML42 have been shown to mediate the drought stress response of
532 *Arabidopsis*, whereby CML37 was revealed as a positive regulator and CML42 as a negative
533 regulator (Scholz *et al.*, 2015, Vadassery *et al.*, 2012). By studying the drought stress response of
534 *cml37 x cml42*, we could show that CML37 and CML42 are also antagonists in the regulation of
535 the drought stress response. Similar to the response to biotic stress treatments, *cml37 x cml42*
536 displayed a wild type-like drought stress phenotype (Figure 7A), suggesting that the opposite
537 effects found in *cml37* and *cml42* neutralize each other in *cml37 x cml42*.
538 The response to abiotic stresses is mainly regulated by the phytohormone ABA (Vishwakarma *et*
539 *al.*, 2017). Both *cml37* and *cml42* displayed altered ABA levels upon drought stress. In *cml42*
540 levels of ABA were increased compared to wild type plants, whereas in *cml37* ABA was not
541 induced at all after drought treatments (Scholz *et al.*, 2015, Vadassery *et al.*, 2012). In line with
542 the drought stress phenotype of *cml37 x cml42*, plants elevated as much ABA upon drought as
543 wild type plants, confirming the thesis that CML37 and CML42 regulate the drought stress
544 response antagonistically.
545
546
547

548 **4.5 The function of the CML37/CML42 antagonism in plant stress regulation**

549 By investigating a *cml37 x cml42* double knock out line, we could show that the two Ca^{2+}
550 sensors CML37 and CML42 act antagonistically in both the jasmonate-mediated regulation of
551 biotic as well as ABA-mediated regulation of abiotic stress responses. Whereas CML37
552 performs in all cases as a positive regulator of the stress responses, CML42 is counteracting as a
553 negative regulator. Strikingly, in the *cml37 x cml42* plants both single effects are balanced out,
554 generating again a wild type-like phenotype (summarized in Figure 8). Similar effects have been
555 shown for two zinc finger proteins in *Arabidopsis*, LSD1 and LOL1, which antagonistically
556 regulate pathogen-induced cell death: A double knock out of both proteins led to a wild type-like
557 cell death response (Epple *et al.*, 2003). It was hypothesized by the authors that this antagonism
558 is used in the plant to control the cell death response: an imbalance towards the positive regulator
559 would activate the cell death response, whereas an imbalance towards the negative regulator
560 would counteract it (Epple *et al.*, 2003). Similarly, also CML37 and CML42 could fine tune
561 defense responses in the plant. This can be used by the plant also to coordinate competing
562 responses to stresses that occur at the same time. However, further studies dealing with parallel
563 stress treatments are needed to address this hypothesis.

564

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572

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736 **Tables**

737 **Table 1. Results of Wilcoxon and unpaired t-tests for analyzing differences in**

738 **phytohormone content after herbivory.**

Phythormone	Time point	Test	p-value
SA	0 h	t-test	0.307
	3 h	Wilcoxon	0.3245
	24 h	t-test	0.592
	48 h	Wilcoxon	0.5116
OPDA	0 h	t-test	0.7577
	3 h	Wilcoxon	0.5202
	24 h	t-test	0.5208
	48 h	Wilcoxon	0.9725
JA	0 h	Wilcoxon	0.1434
	3 h	Wilcoxon	0.4124
	24 h	t-test	0.4296
	48 h	Wilcoxon	0.9725
JA-Ile	0 h	Wilcoxon	0.5742
	3 h	Wilcoxon	0.6782
	24 h	t-test	0.271
	48 h	t-test	0.5196

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Table 2. Statistical values for the analysis of glucosinolates at different time points according to herbivory, plant genotype, and the interaction between herbivory and plant genotype. Either two-way ANOVA or generalized least square method was used to determine difference within one time point. In case of two-way ANOVA Tukey test was used as post-hoc test. Depending which statistical test was used F-values or Likelihood ratios (L-ratio) are given. L-ratios are given in italics. To account for the variance heterogeneity of the residuals data were either transformed before a two-way ANOVA or generalized linear models with the varIdent variance structure were used. Variance structures are given in italics.

Glucosinolate at time point	**Test**	**Transfor-mation/ Varaince structure**	**Factor**	**p-value**	**F-/L-ratio**	**Post-hoc/ factor level reduction ($p \leq 0.05$)**
Total glucosinolates 1d	Two-way ANOVA	$\log_{10}$ x	T[†]	< 0.001	80.687	control < feeding
			G[††]	0.967	0.002	not tested
			TxG[†††]	0.660	0.196	not tested
Total glucosinolates 7d	Two-way ANOVA	$\log_{10}$ x	T	< 0.001	826.717	control < feeding
			G	0.005	8.366	*cml37 x cml 42* < WT
			TxG	0.016	6.150	WT control = *cml37 x cml42* control < *cml37 x cml42* feeding < WT feeding
Aliphatic glucosinolates 1d	Two-way ANOVA	1/√x	T	< 0.001	71.162	control < feeding
			G	0.695	0.155	not tested
			TxG	0.738	0.113	not tested
Aliphatic glucosinolates 7d	Two-way ANOVA	$\log_{10}$ x	T	< 0.001	653.754	control < feeding
			G	0.033	4.740	*cml37 x cml 42* < WT
			TxG	0.053	3.874	not tested
Indole glucosinolates 1d	Generalized least squares	*different variances amongst T*	T	< 0.0001	*35.281*	control < feeding
			G	0.416	*0.661*	not tested
			TxG	0.561	*0.338*	not tested

Indole glucosinolates 7d	Generalized least squares	*different variances amongst T*	T	< 0.0001	*85.695*	control < feeding
			G	0.046	*3.996*	*cml37 x cml 42* < WT
			TxG	0.001	*10.478*	WT control = *cml37 x cml42* control < *cml37 x cml42* feeding < WT feeding

760 † T = Treatment

761 †† G = Genotype

762 ††† TxG = Interaction treatment and genotype

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779 **Table 4. Results of the generalized least square for analyzing the differences in the**
780 **chlorophyll fluorescence.** To define the variance structure, varIdent function was used, allowing
781 a different variance of all groups.

Time point	Factor	p-value	L-ratio
3 dpi	T[†]	<0.001	85.305
	G[††]	0.507	2.330
	TxG[†††]	0.002	14.440
4 dpi	T	<0.001	39.834
	G	0.406	2.911
	TxG	<0.001	27.571

782 [†] T = Treatment

783 [††] G = Genotype

784 [†††] TxG = Interaction treatment and genotype

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794 **Table 4. Results of the statistic tests for analyzing the differences in the ABA content.** Either
795 Wilcoxon-test, two-way ANOVA or generalized least square method was used to determine
796 difference within one time point. Depending which statistical test was used F-values or
797 Likelihood ratios (L-ratio) are given. L-ratios are given in italics. To account for the variance
798 heterogeneity of the residuals data were either transformed before a two-way ANOVA or
799 generalized linear models with the varIdent variance structure were used. Variance structures are
800 given in italics.

Time point	Test	Transformation/ Variance structure	Factor	p-value	F-/L-ratio
0 weeks	Wilcoxon	-	-	0.5203	-
1 week	Two-way ANOVA	$\log_{10} x$	T[†]	< 0.001	1653.535
			G[††]	0.235	1.438
			TxG[†††]	0.627	0.238
2 weeks	Generalized least square	*different variances amongst all groups*	T	<.0001	*104.059*
			G	0.3809	*0.768*
			TxG	0.7126	*0.136*

801 [†] T = Treatment

802 [††] G = Genotype

803 [†††] TxG = Interaction treatment and genotype

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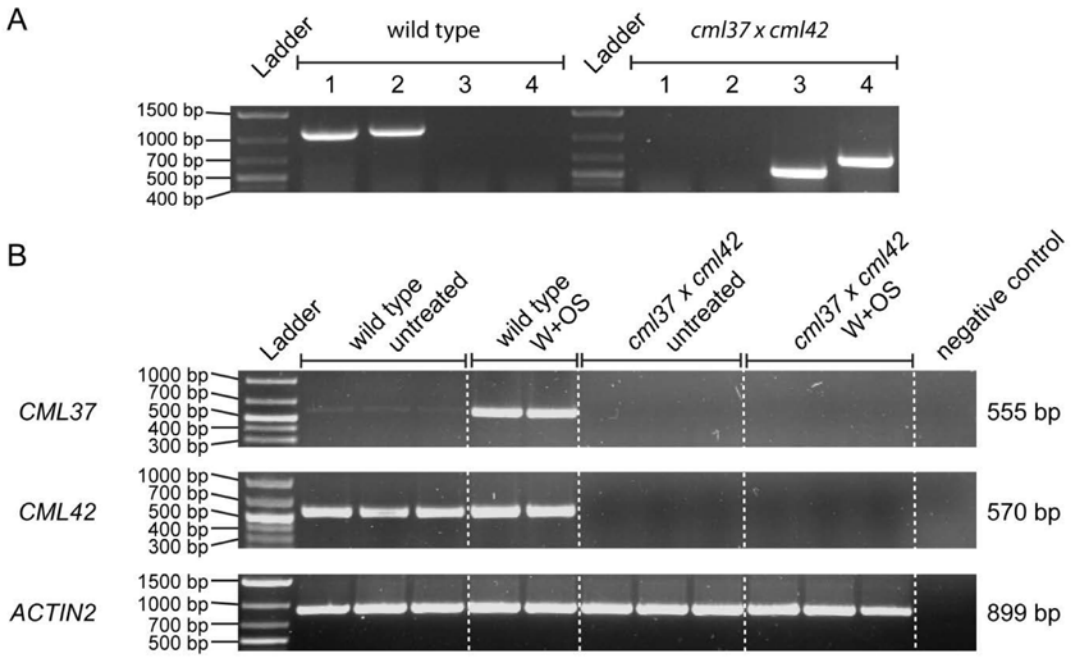
805 **Figures**



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807 **Figure 1. Genetic characterization of *cml37 x cml42*.** (A) Results of Genotyping *cml37 x*
808 *cml42*. Different numbers indicate different Primer-sets used: (1) *CML42* RP + *CML42* LP
809 (expected product size: 1120 bp) to verify the presence of the wild type *CML42* (2) *CML37* RP +
810 *CML37* LP (expected product size: 1158 bp) to verify the presence of the wild type *CML37* (3)
811 *CML42* RP + LBb1.3 (expected product size: 456-756 bp) to verify the presence of the T-DNA
812 insertion in *CML42* (4) *CML37* RP + LBb1.3 (expected product size: 599-899 bp) to verify the
813 presence of the T-DNA insertion in *CML37* (B) RT-PCR to confirm the absence of the *CML37*
814 and *CML42* transcripts in *cml37 x cml42*. Plants were wounded with a pattern wheel and treated
815 with oral secretion (W+OS) for 1 h or were used untreated. Water was used as negative control.
816 Expected product size is indicated to the right of the gel pictures. *ACTIN2* expression was used
817 as quantitative control.

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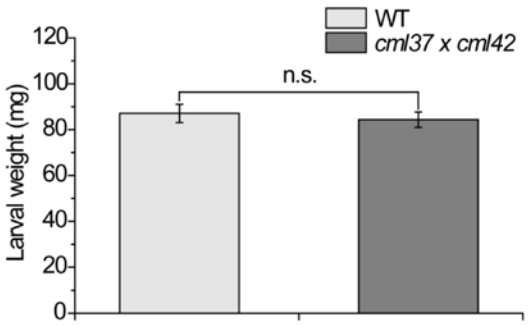
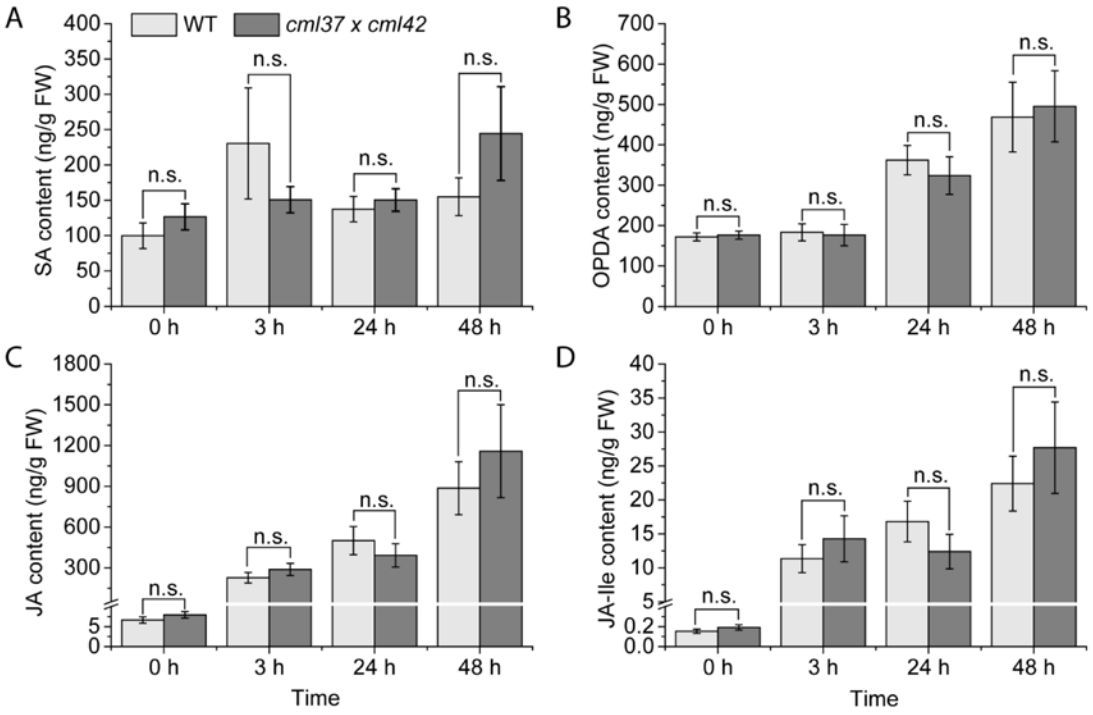


Figure 2. Feeding performance of *S. littoralis* larvae on *cml37 x cml42*. Larval weight gain in mg ± Standard error (SE) after 1 week of feeding on either *cml37 x cml42* or wild type (WT) plants. First instar *S. littoralis* larvae were pre-weighed to reduce experimental variation. Three larvae were placed on each plant. After 1 week of feeding, larval weight was determined. Experiment was repeated five times independently (n = 138 (Larvae on WT), n = 140 (Larvae on *cml37 x cml42*)). Larval weights of both genotypes were compared; n.s. means not significant (p = 0.9589).



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829 **Figure 3. Phytohormone contents of *cml37 x cml42* after feeding of *S. littoralis* larvae.**
830 Contents of (A) SA, (B) OPDA, (C) JA, and (D) JA-Ile in ng/g fresh weight (FW) ± SE in *cml37*
831 *x cml42* and WT plants after *S. littoralis* feeding. Larvae were allowed to feed for 3 h, 24 h and
832 48 h on the plants. Phytohormones were analyzed from local fed leaves. Untreated plants were
833 used as controls (0 h). Experiment was repeated four times independently (n ≥ 10).
834 Phytohormone contents of both genotypes within the time point were compared; n.s. means not
835 significant. The respective p-values are given in Table 1. Legend for color code see (A).

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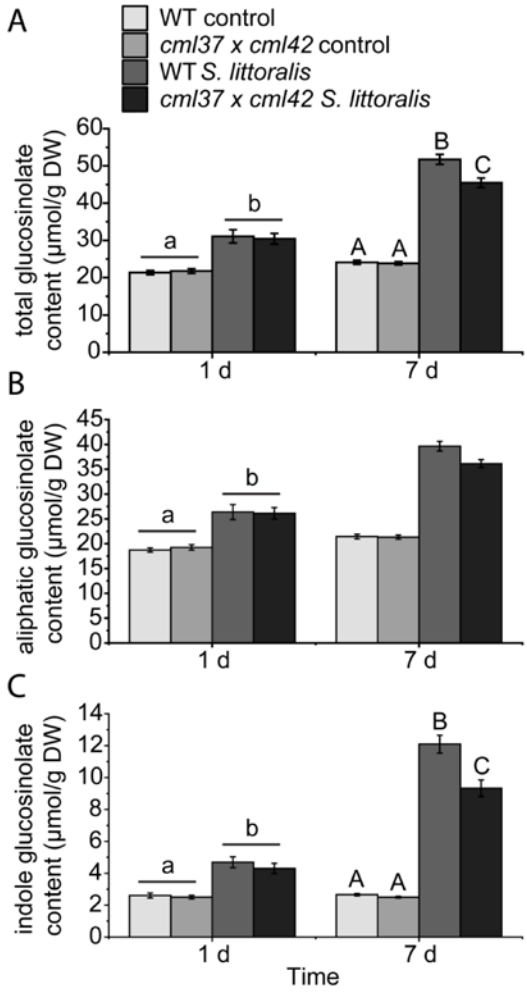

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839 **Figure 4. Glucosinolate content of *cml37 x cml42* after feeding of *S. littoralis* larvae.** (A)
840 Total glucosinolate, (B) aliphatic glucosinolate and (C) indole glucosinolate contents of WT and
841 *cml37 x cml42* plants in µmol/g dry weight (DW) ± SE after 1 d (5 week old plants) and 7 d (6
842 week old plants) of *S. littoralis* feeding. Untreated plants were used as controls. Glucosinolates
843 were extracted from whole *Arabidopsis* rosettes. Experiment was repeated at least four times
844 independently ($n \geq 14$). Differences between glucosinolate contents of controls, treated samples
845 and genotypes were determined within one time point by two-way ANOVA (A, B) and
846 generalized least square method (C). Differences between the groups are indicated by different

847 letters above the bars (p-value ≤ 0.05). Details about all the statistic tests including the respective
848 statistical values, are listed in Table 2. Legend for color code see (A).

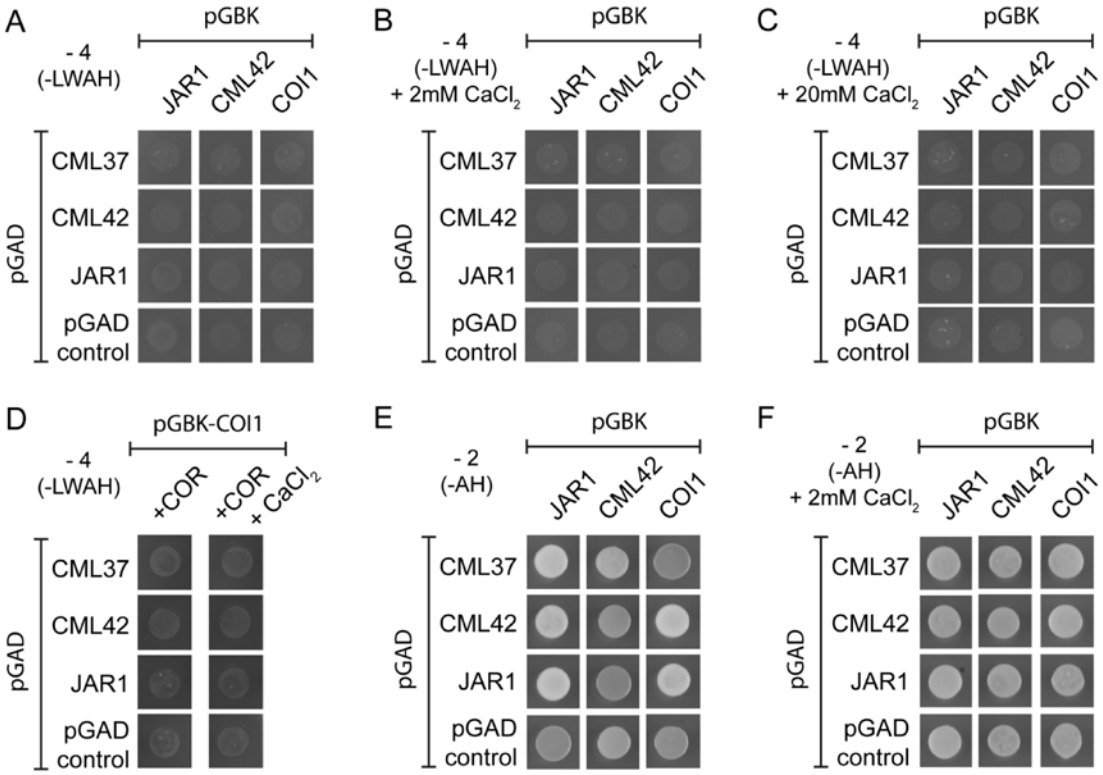


Figure 5. Interaction of CML42 and CML37 with different proteins of the jasmonate pathway. (A) Yeast cells co-transformed with pGAD (prey) and pGBK (bait) vectors were selected and subsequently grown on selective media lacking Ade, His, Leu and Trp (-4) to test protein interaction. (B) Testing the dependency of possible interactions on the availability of Ca^{2+}; the (-4) media was supplemented with 2 mM $CaCl_2$ (C) or a supplement of 20 mM $CaCl_2$. (D) Testing the dependency of possible interactions with COI1 on the presence of JA-Ile; the (-4) media was supplemented with 10 µM COR and 10 µM COR with 2 mM $CaCl_2$. (E and F) Yeast cells were grown on yeast synthetic drop-out lacking Leu and Trp (-2) (C) as transformation control either in the absence (E) or presence of Ca^{2+} (F). The empty vector pGAD was included as control to discard protein auto-activation. Experiments were repeated at least two times independently. Pictures shown are representative.

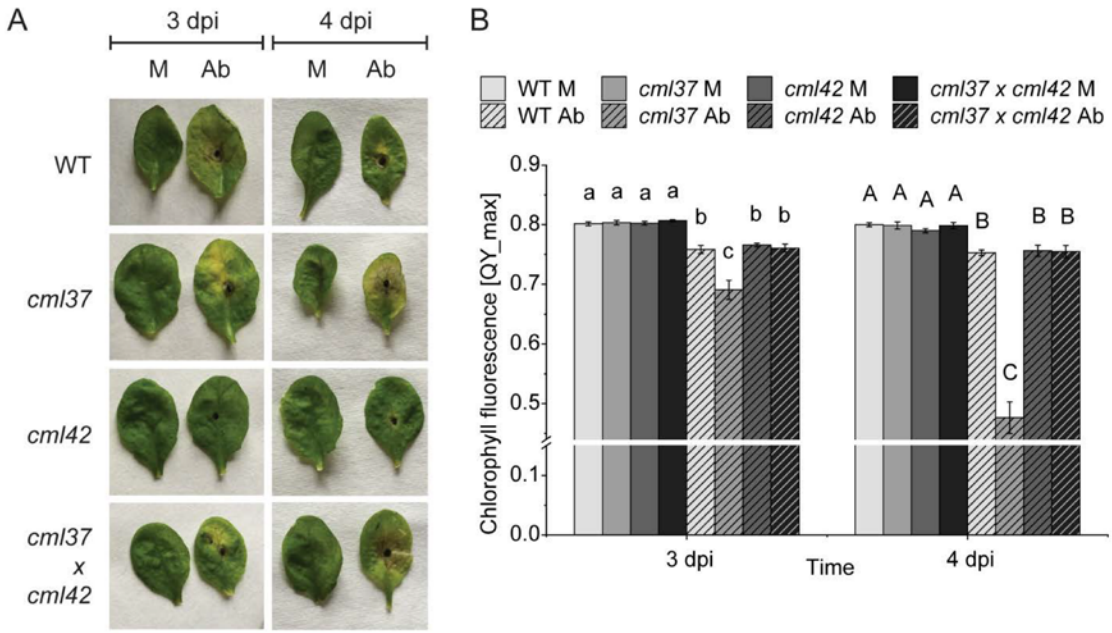
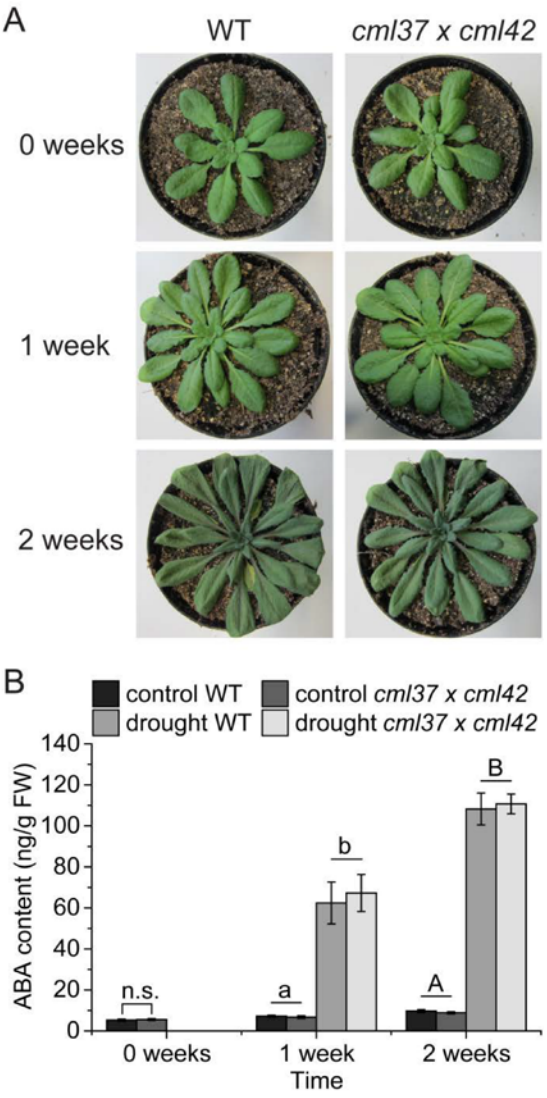


Figure 6. Different susceptibility of *cml37*, *cml42* and *cml37 x cml42* to *A. brassicicola*. (A) Necrotic lesion phenotype and (B) chlorophyll fluorescence (QY_max) ± SE of *cml37*, *cml42*, *cml37 x cml42* and WT 3 and 4 day post-inoculation (dpi) with *A. brassicicola* spore suspension (Ab) or 0.01% Tween as mock (M). Plants shown are representative. Experiment was repeated at least two times independently ($n \geq 12$ (3 dpi), $n \geq 6$ (4 dpi)). Differences in the chlorophyll fluorescence were tested by generalized least square method within the time point. Differences between the groups are indicated by different letters above the bars (p-value ≤ 0.05). Detailed information about the statistic tests and statistical values are listed in Table 3.



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872 **Figure 7. Drought stress responses of *cml37 x cml42*.** (A) Phenotypes and (B) ABA contents ±
873 SE of *cml37 x cml42* and WT plants after 1 or 2 weeks of drought stress. Plants exposed to
874 drought for 2 weeks were re-watered once after 1 week. Untreated plants were used as controls.
875 Plants shown are representative. Experiment was repeated four times independently ($n \geq 18$).
876 ABA levels of the genotypes at 0 weeks were compared by two-sample Wilcoxon test. The
877 influence of genotype and drought stress were tested with a two-way ANOVA (1 week) or a

878 generalized least square method (2 weeks). Differences between the groups are indicated by the
879 letters above the bars (p-value ≤ 0.05). For p-values see Table 4; n.s. means not significant.

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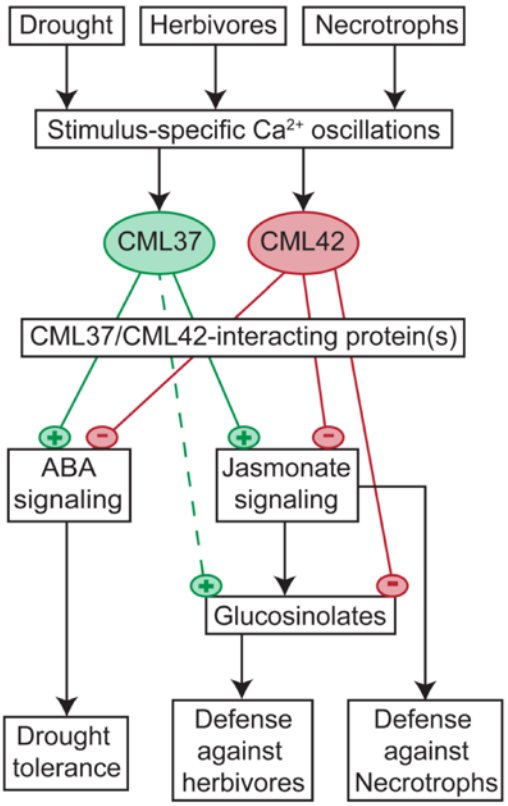

881

882 **Figure 8. Model for the antagonistic effects of CML37 and CML42 on different stress**
883 **responses in *Arabidopsis*.** CML37 and CML42 are Ca^{2+} sensors that act antagonistically on the
884 plant stress response to drought, herbivores and necrotrophs. After sensing Ca^{2+} elevations in the
885 cell, they bind to yet unknown targets (CML37/CML42 interacting protein(s)) and influence
886 downstream phytohormone pathways. CML37 positively influences the ABA elevation induced
887 by drought stress, enhancing drought tolerance. CML42 negatively affects the drought-induced
888 ABA elevation, and thus the drought tolerance. CML37 has a positive effect on the jasmonate
889 biosynthesis that is counteracted by CML42. CML42 negatively influences jasmonate-dependent
890 responses. Thus CML37 positively influences the defense against herbivores, whereas CML42
891 has a negative impact. Further CML37 and CML42 play the same roles in the defense against

892 necrotrophic fungi, which might be connected to their effects on the jasmonate signaling
893 pathway. In all shown defense reactions positive and negative effects of CML37 and CML42 are
894 balanced out. CML37 and CML42 might be used for fine tuning these stress responses. Symbols:
895 + means a positive influence, - a negative influence, dashed lines indicate that the influence was
896 proven indirectly by examining the double knock out line of *CML37* and *CML42*
897

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

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ABSTRACT

Jasmonates (JAs) 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, stable-isotope labeling of jasmonates does not allow the study of spatial and temporal distribution of these compounds in real time by positron emission tomography (PET). In this study, we explore whether a fluorinated jasmonate could mimic the action of the endogenous compound and therefore, be later employed as a tracer to study metabolic processes by PET. We describe the synthesis and the metabolism of (*Z*)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (7F-OPC-8:0), a fluorinated analog 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 its 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 analog 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.

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

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}, ubiquitin-ligase receptor complex; MRM, multiple reaction monitoring; HRMS, high resolution mass spectrometry.

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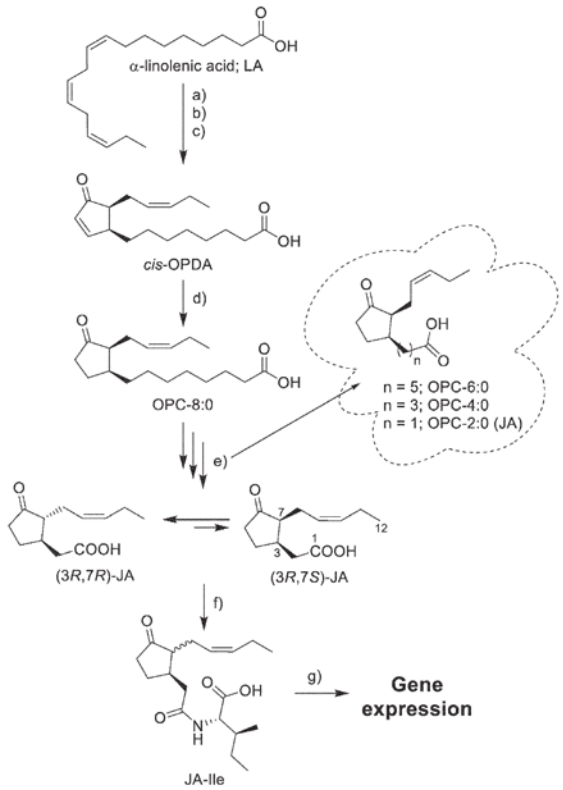

Fig. 1. Simplified scheme of the biosynthesis and signaling of jasmonates. In the plastids, LA is converted into *cis*-OPDA by the sequential action of LOX (a), AOS (b), and AOC (c). (3*R*,7*S*)-JA is formed in the peroxisomes by OPR3 (d) acting on *cis*-OPDA followed by three cycles of β-oxidation (e). (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*.

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.

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 analogs 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 ($\gamma = 40.05$ 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 describe the synthesis of 7F-OPC-8:0 (**10**), a fluorinated derivative of the JA precursor OPC-8:0. We study its ability to induce JRG expression and jasmonates accumulation in *Arabidopsis thaliana* plants. Additionally, the metabolic fate of **10** in the plant and the possibility of using this molecule as a probe to follow signal-trafficking *in planta* is explored. 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.

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 analog 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 capability of 7F-OPC-8:0 (**10**) to induce both the accumulation of jasmonates and gene expression of marker JRG was evaluated upon

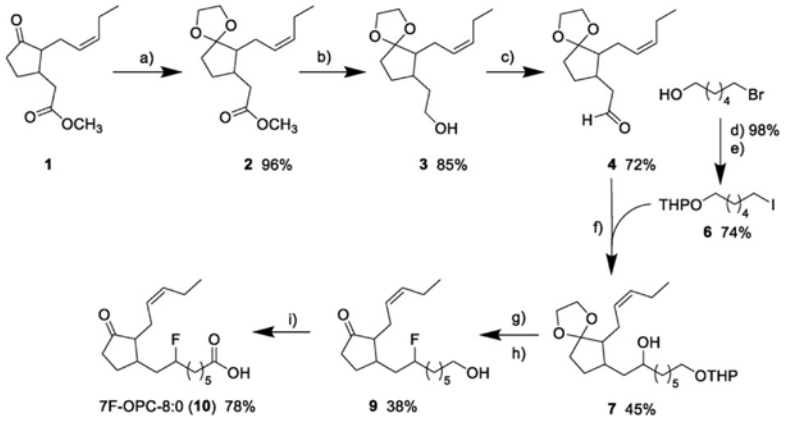

Fig. 2. Synthesis of 7F-OPC-8:0 (**10**). Reagents and conditions: a) 1,2-ethanediol/C_6H_6/*p*-TsOH, reflux; b) $LiAlH_4$/Et_2O; c) PCC/CH_2Cl_2/AcONa, 4 Å molecular sieves; d) NaI/Me_2CO; e) CH_2Cl_2/THP/*p*-TsOH, room temp.; f) *n*-pentane/Et_2O (3:2)/*t*-BuLi, −78 °C; g) DAST/CH_2Cl_2, −78 °C; h)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

application of the compound to *A. thaliana* plants. 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 treatment with several endogenous jasmonates or synthetic analogs [31,34,35]. To evaluate 7F-OPC-8:0 (**10**) capability of inducing the expression of marker JRG, *A. thaliana* plants were sprayed with this compound and the transcript level of the selected genes was monitored. For this purpose two genes, strongly induced by JA were chosen: *VSP2* and *OPR3* [36]. 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 [37].

On the other hand, some genes show a specific induction by the JA precursor *cis*-OPDA and are classified as *cis*-OPDA-responsive genes [36]. 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). Moreover, gene expression profiles observed upon 7F-OPC-8:0 (**10**) treatment are similar to the profiles observed for *cis*-OPDA (Fig. 1, reference [39]), which is directly metabolized in vivo to OPC-8:0 and finally JA, and thus, serves as a positive control for non-fluorinated jasmonates. Additionally, the gene coding for the transcriptional repressor of JA-signaling, JAZ1 [40], was induced after treatment with 7F-OPC-8:0 (**10**) (Fig. 2, reference [39]). All together those results suggest that 7F-OPC-8:0 (**10**) induces not only JA-responsive genes, but also genes responding to *cis*-OPDA, independent on the presence of the fluorine atom.

*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 jasmonates treatment. Fig. 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[2] 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. In order to examine the ability of **10** to initiate the accumulation of bioactive JA-Ile in comparison with *cis*-OPDA, both compounds were applied to *A. thaliana* leaves. Similar to the gene expression assays, the responses found for **10** again resembled the results found for *cis*-OPDA (Fig. 3, reference [39]).

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 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 1 h (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 [41]. Accumulation of arabidopsides has been reported during hypersensitive response and after wounding [42] 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 [43]. 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. 4, reference [39]). This supports that arabidopsides may represent the source for the increase in the *cis*-OPDA. Whether a similar phenomenon is observed with 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] The analytical method does not distinguish between 11-OH-JA and 12-OH-JA.

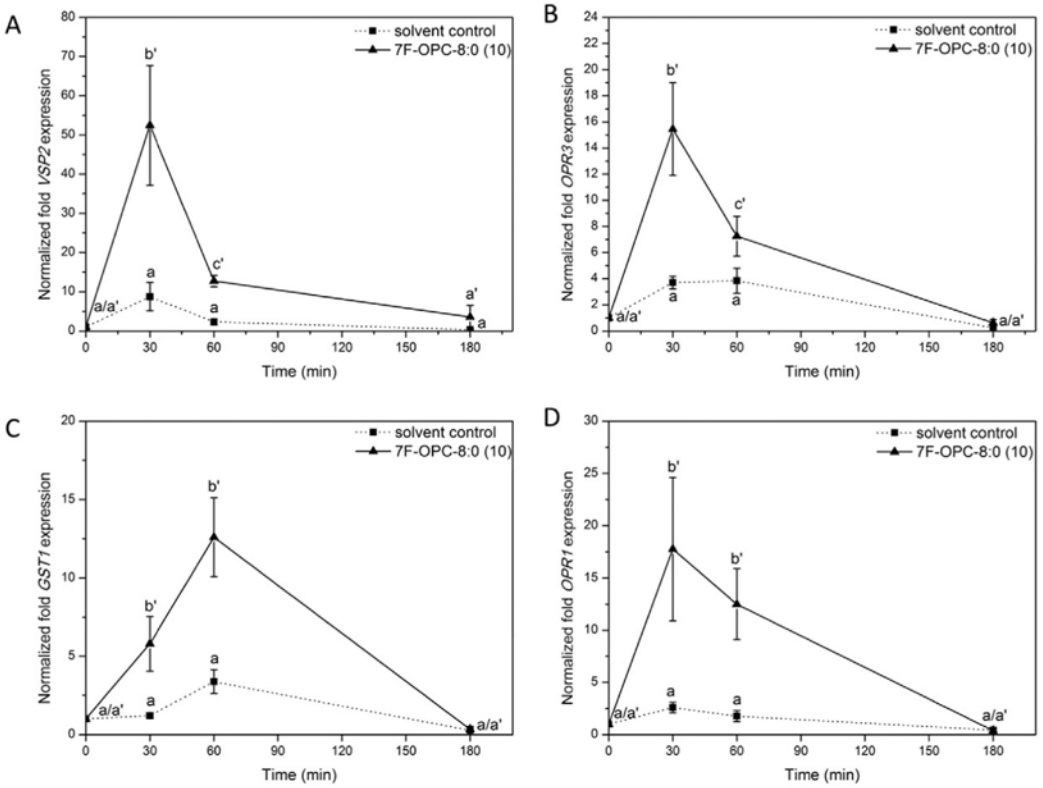

Fig. 3. Mean expression (±s.e., n = 5) of JA- (A, B) and *cis*-OPDA-responsive genes (C, D) in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Expression of *VSP2* (A), *OPR3* (B), *GST1* (C) and *OPR1* (D) was analyzed after 30, 60 and 180 min. All samples were normalized 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).

*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.

An 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. 5, reference [39]). 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. 6, reference [39]). The concentrations found for **11** and **12** showed a similar profile to that observed for other jasmonates in this study (Fig. 5).

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 (Fig. 5). The presence of the fluorine atom does not hamper the oxidative degradation, as shown by the two fluorinated metabolites **11** and **12.** A third round of β-oxidation seems reasonable, since the amount of **12** slowly decreases after reaching a maximum. A detailed analysis of this step would require an additional label to follow the molecule after removal of fluorine.

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 [44]. Following the nomenclature of Farmer et al. [45], 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).

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 levels of **11** and **12**were 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

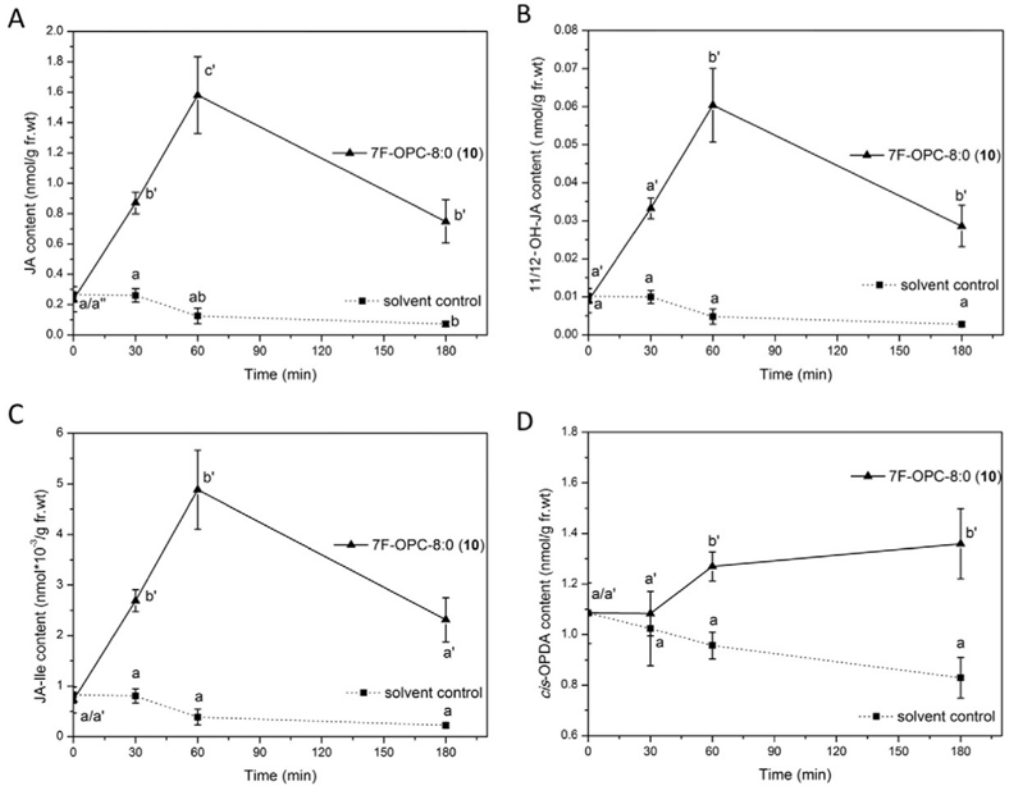

Fig. 4. Mean content (±s.e., n = 5) of jasmonate profiles in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. The content of JA (A), 11/12-OH-JA (B), JA-Ile (C) and *cis*-OPDA (D) was determined after 30, 60 and 180 min. Statistically significant differences were determined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

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 [44]. Leaves 5 and 11 were reported to react systemically when leaf 8 was wounded or feed by an insect, even though they do not share a direct vascular connection with leaf 8 [46,47]. 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 [46]. 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 these molecules as a systemic signal in a bidirectional way [44].

3. Conclusions

We developed a short synthesis of 7F-OPC-8:0 (**10**) – a fluorinated analog of the JA precursor OPC-8:0 – with good overall yield. This compound was shown to be 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 analog **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

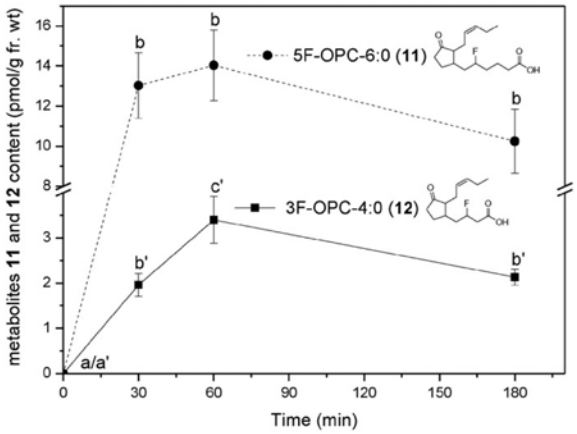

Fig. 5. Mean content (±s.e., n = 5) of the 7F-OPC-8:0 (**10**) derived metabolites 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) in *A. thaliana* Col-0 leaves after treatment with **10**. The content of 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) was determined after 30, 60 and 180 min. Statistically significant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

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-5 ms (30 m × 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 300 K 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 or the residual solvent peak. As compounds are mostly mixture of isomers, MS and NMR data are reported for the major isomer only. For copy of more important spectroscopic data see reference [39].

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

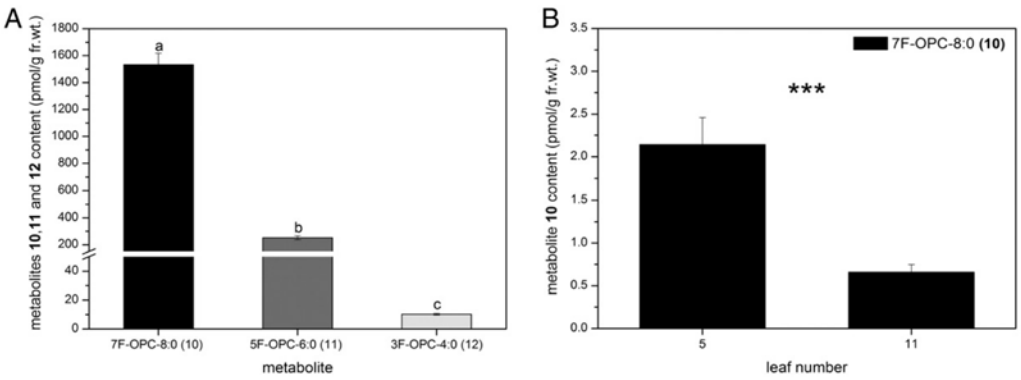

Fig. 6. Mean content (±s.e., n = 11) of 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) in *A. thaliana* Col-0 leaves after wounding and treatment with **10** for 60 min. (**A**) Contents of **10**, **11** and **12** in the treated local leaf 8. Statistically significant differences 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 ($p < 0.05$, *** $p < 0.001$).

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 (500 MHz, $CDCl_3$): δ 9.72 (s, 1 H), 5.30–5.40 (m, 2 H), 3.82–3.93 (m, 4 H), 2.54–2.71 (m, 1 H), 2.35 (ddd, J = 16.7, 9.5, 2.4 Hz, 1 H), 2.12–2.26 (m, 2 H), 2.01–2.10 (m, 3 H), 1.92–1.99 (m, 1 H), 1.61–1.83 (m, 3 H), 1.19–1.33 (m, 1 H), 0.94 ppm (t, J = 7.6 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 126 MHz): 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-2 H-pyran (**6**)

6-iodohexan-1-ol (**5**) was prepared as described in Ng and Fromherz [48]. 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-2 H-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). ^1H NMR (500 MHz, $CDCl_3$): δ5.30–5.43 (m, 1 H), 5.12–5.26 (m, 1 H), 3.63–3.71 (m, 1 H), 3.57 (t, J = 6.5 Hz, 2 H), 2.23–2.36 (m, 3 H), 2.06–2.23 (m, 2 H), 1.95–2.05 (m, 3 H), 1.73 (m, 2 H), 1.45–1.55 (m, 3 H), 1.22–1.44 (m, 10 H), 0.89 ppm (t, J = 7.5 Hz, 3 H); ^{13}C NMR (126 MHz, $CDCl_3$): 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) ^1H NMR (400 MHz, $CDCl_3$): δ5.36–5.54 (m, 1 H), 5.17–5.33 (m, 1 H), 4.44–4.74 (m, 1 H), 2.37 (m, 6 H), 1.95–2.19 (m, 5 H), 1.58–1.93 (m, 5 H), 1.30–1.56 (m, 6 H), 0.96 ppm (t, J = 7.5 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 101 MHz): 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

Plant treatments were carried out as described in reference. 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. [45]. 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. Leaves 5, 8 and 11 of each plant were harvested 60 min after treatment.

4.4. RNA-isolation, RT-PCR and primers

For all details concerning gene induction analyses see reference [39].

4.5. Quantification of phytohormones and Arabidopsides

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 [$^2H_6$]JA, and 12 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards. The homogenate was mixed for 30 min and centrifuged at 13,000 rpm for 20 min at 4 °C 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 °C 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 × 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 °C. An API 5000 tandem mass spectrometer (Applied Biosystems) equipped with a Turbo Spray 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 °C. 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 −30 V; DP −50 V) for JA-Ile; *m/z* 328.2 → 136.1

(CE −30 V; DP −50 V) 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. Arabidopside A and Arabidopside B were determined as described [39].

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

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**). 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 as described in [39].

Transparency document

The Transparency document associated with this article can be found, in online version.

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3.5 Supplementary Information Manuscript 3 (unpublished Reference [39])

Data article

Title: *Metabolism and chemical characterization of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0*

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Abstract

*Here we describe several physiological responses induced by a fluorinated mimic of the jasmonate precursor OPC-8:0 in Arabidopsis thaliana plants. In particular, we describe the induction of the jasmonate responsive genes (JRG) JAZ1, GST1 and OPR1 by the fluorinated compound 7F-OPC-8:0 (**10**). The (**10**)-dependent induced levels of JA-Ile, arabidopside A and arabidopside B are presented as well. Furthermore, we provide NMR and LC-MS characterization of compound **10** and some of its metabolic derivatives.*

Specifications Table

Subject area	*Chemistry and Biology*
More specific subject area	*Plant physiology*
Type of data	*Graph, figure, table*
How data was acquired	*RT-PCR: Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). NMR: Bruker DRX500; Bruker Avance 400. Mass spectroscopy: Bruker Daltonics - maXis Ultra High ResolutionTOF; API 5000 tandem mass (Applied Biosystems); LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany).*
Data format	*Analyzed*
Experimental factors	*RNA-isolation, phytohormone extraction,*
Experimental features	*RNA isolation: ca. 100 mg leaf material was homogenized and RNA extraction and cDNA synthesis as described in [1].*

	Phytohormone extraction: ca. 250 mg leaf material 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 as internal standards [2].
Data source location	*Jena, Germany.*
Data accessibility	*Data available with this article.*

Value of the data

- First physiological data for a fluorinated jasmonate compared to endogenous *cis*-OPDA.
- MS methods may be applicable for other fluorinated jasmonates
- Interesting profile for arabidopsides levels in fluorinated-jasmonate treated plants
- Useful NMR data of synthetic 7F-OPF-8:0

Data

Here we present how a fluorinated jasmonate precursor (7F-OPF-8:0) affects the expression of marker JRG *JAZ1*, *GST1* and *OPR1.* We characterize 7F-OPF-8:0 (**10**) and metabolic derivatives by LC-MS and quantify the levels of JA-Ile and arabidopsides A and B induced by **10**. Furthermore, we provide NMR spectra of this compound. In some cases data is directly compared to the endogenous jasmonate precursor *cis*-OPDA. Table S1 contains the list of primers employed for RT-PCR.

Experimental Design, Materials and Methods

Plant material and treatments: *Arabidopsis thaliana* ecotype Columbia was used for all experiments and plants were grown as previously described [2]. 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 *cis*-OPDA treatments, a solution of this compound (10 µM, containing 0.1% DMSO) was used. The solvent control was a 0.1% solution of DMSO. The *cis*-OPDA was synthesized according to the procedure previously described [3].

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 [1]. 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).

Quantification of JA-Ile, arabidopside A, arabidopside B, and MS data: Analysis of phytohormones followed previously described methods with some modifications [2]. 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 °C 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 °C. 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* 215.1 →56.0 (CE -24 V; DP -35 V) for [$^{2}H_{6}$]JA; *m/z* 322.2 →130.1 (CE -30V; DP -50V) for JA-Ile and *m/z* 328.2 →136.1 (CE -30V; DP -50V) for JA-[$^{13}C_{6}$]Ile conjugate. 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 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 described 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. 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.

HRMS (ESI$^-$) for compound **10** was performed on a Bruker Daltonics - maXis Ultra High ResolutionTOF instrument by direct injection of a pure sample. 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 °C.

NMR acquisition: 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 residual solvent peak $CDCl_3$. As compounds are mostly mixture of isomers, NMR data are reported for the major isomer only.

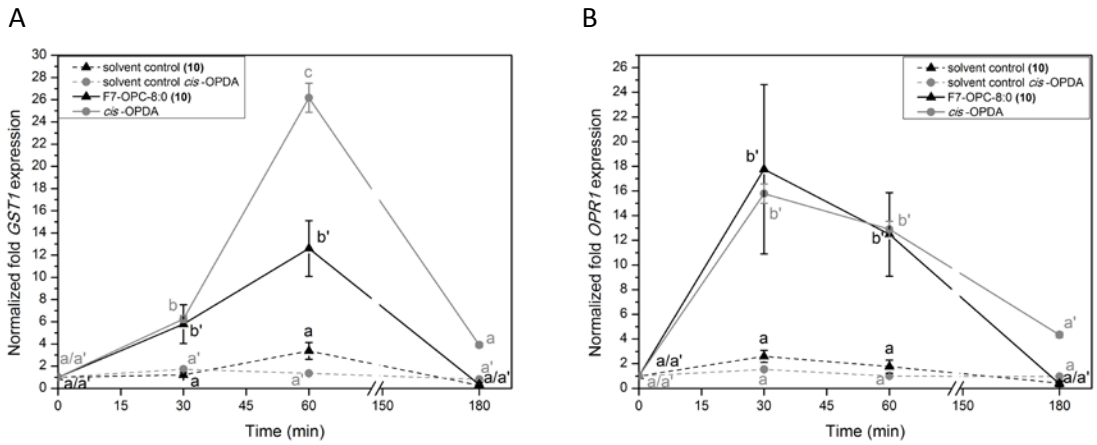


Fig. 1 Mean expression (± s.e., n=5) of *GST1* (A) and *OPR1* (B) in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**), *cis*-OPDA or the respective solvent control. Expression was analyzed after 30, 60 and 180 min. All samples were normalized to the *RPS18B* level and untreated plants were 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).

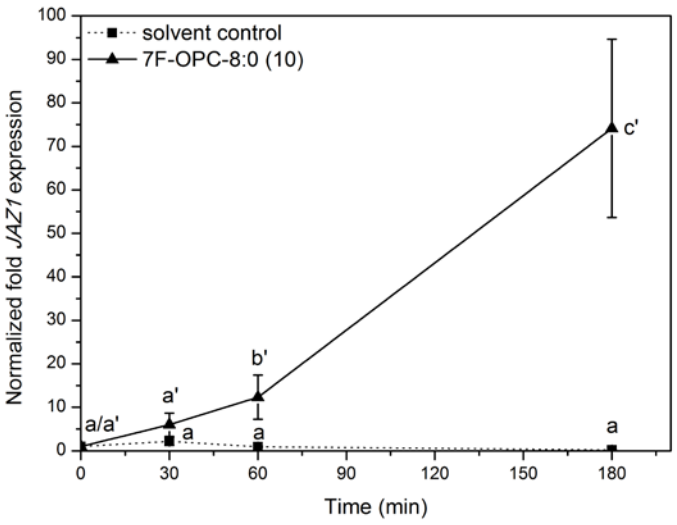


Fig. 2 Mean expression (± s.e., n=5) of *JAZ1* in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Expression was analyzed after 30, 60 and 180 min. All samples were normalized to the *RPS18B* level and untreated plants were used as control. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

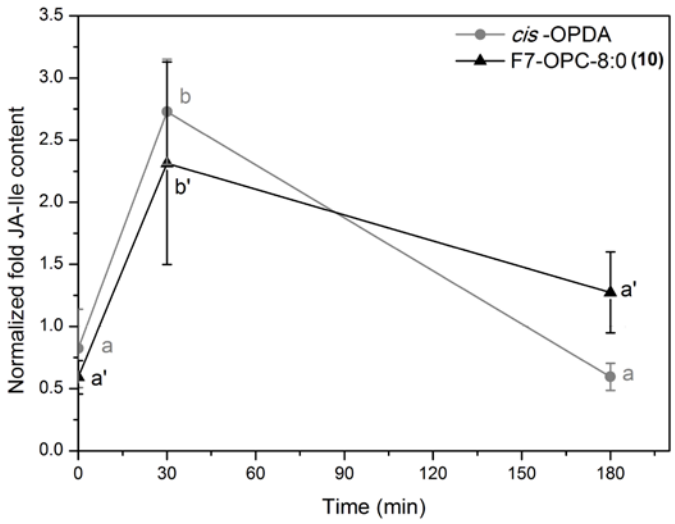


Fig. 3 Relative content (± s.e., n=5) of JA-Ile in *A. thaliana* Col-0 after treatment with *cis*-OPDA and 7F-OPC-8:0 (**10**). The content of JA-Ile was normalized to the respective solvent controls (level = 1). JA-Ile content was determined after 0 (untreated plants), 30, 180 min. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

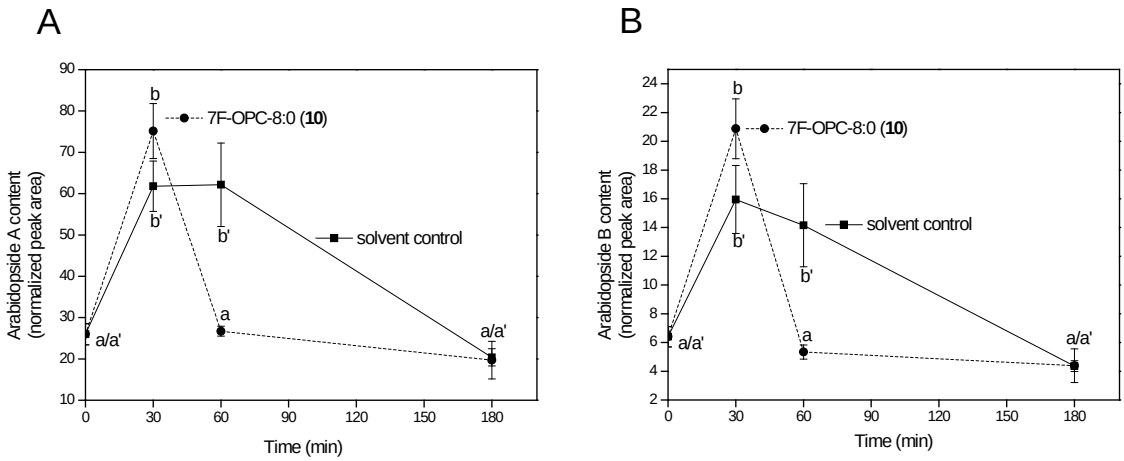


Fig. 4 Mean relative content (± s.e., n=5) of arabidopside A (A) and arabidopside B (B) in *Arabidopsis* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Measurements at 30, 60 and 180 min. Peak area was normalized to the IS $[^2H]_6$JA. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

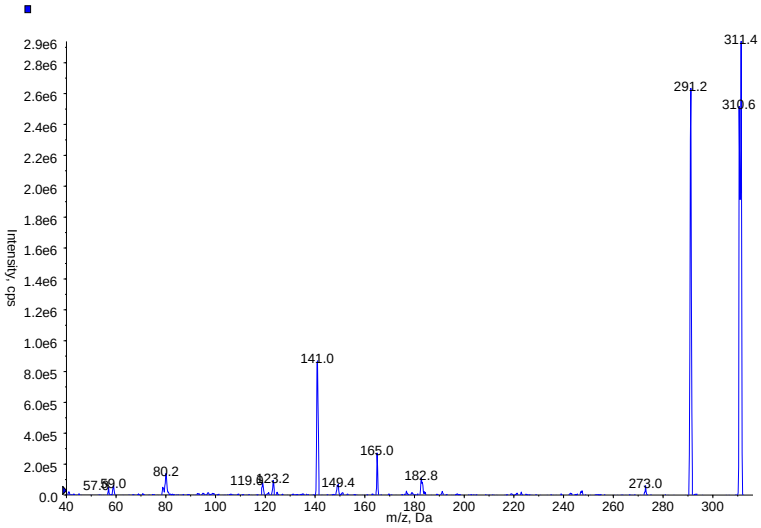


Fig. 5 MS2 spectrum of 7F-OPC-8:0 **(10)**. The fragmentation pattern of **10** reveals the molecular base peak $[M-H]^-$ (311.4 *m/z*) and a peak produced by the loss of HF ($[M-H-20]^-$, 291.2 *m/z*) as the most abundant fragments.

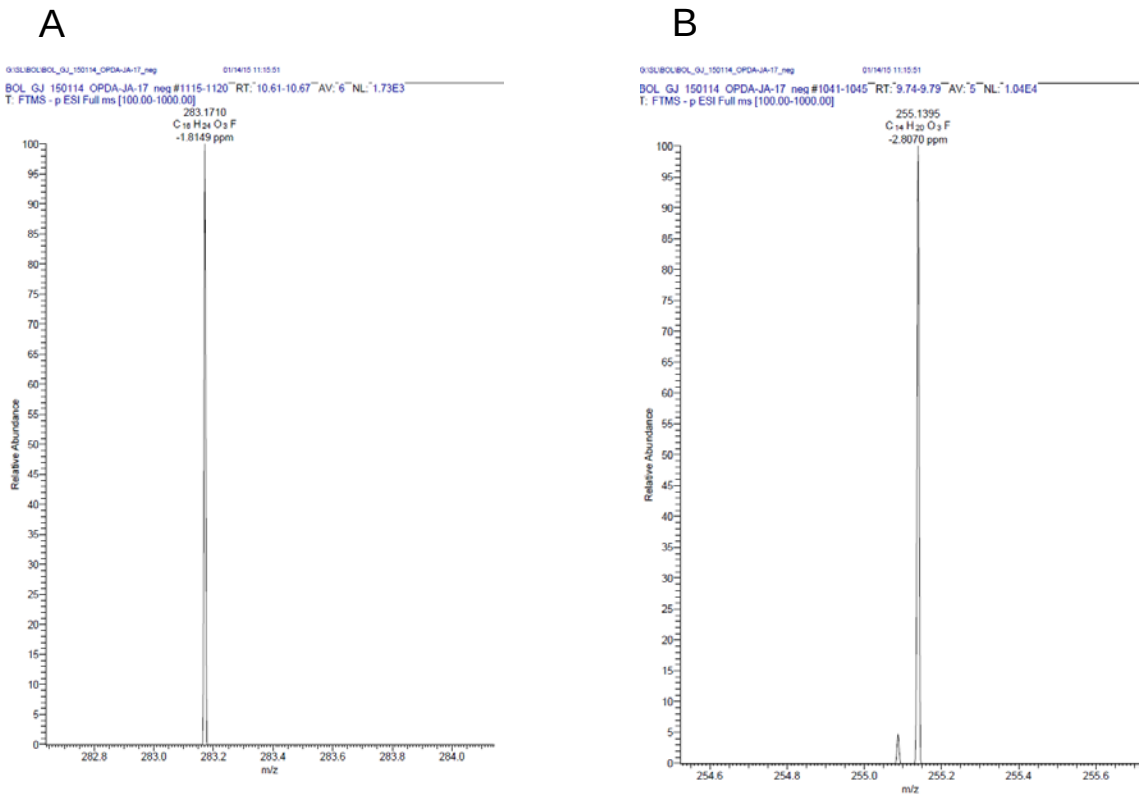
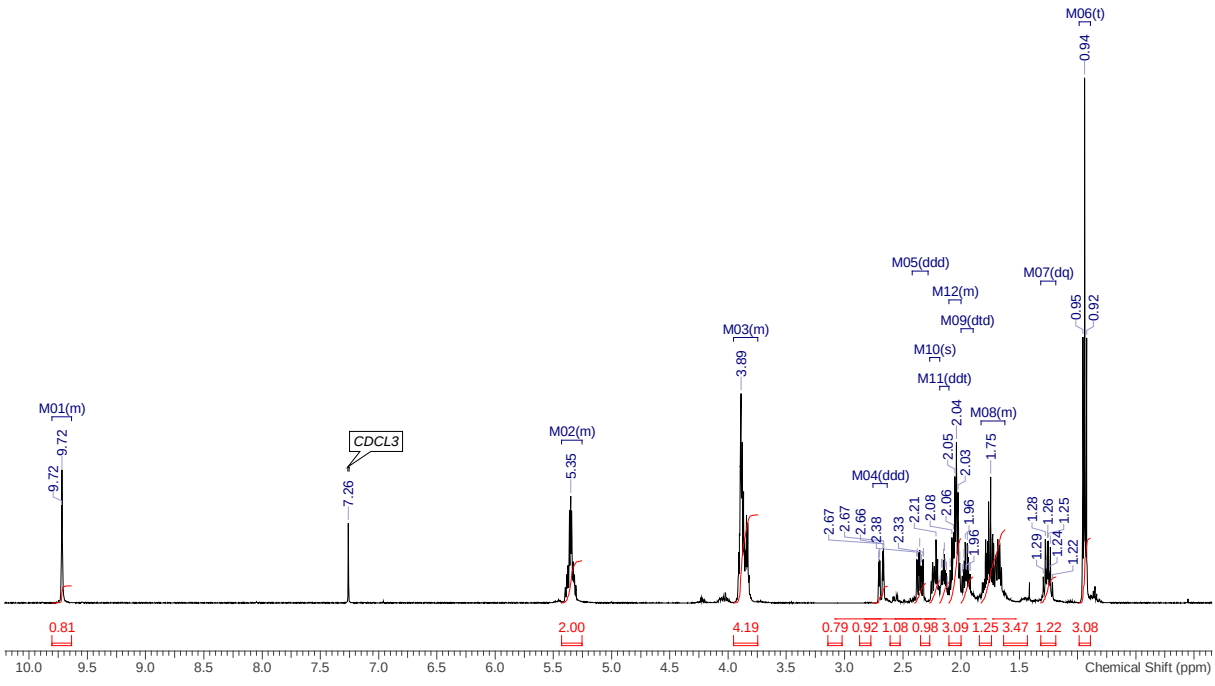


Fig. 6 HRMS spectra of compounds 5F-OPC-6:0 (**11**) (A) and 3F-OPC-4:0 (**12**) (B).

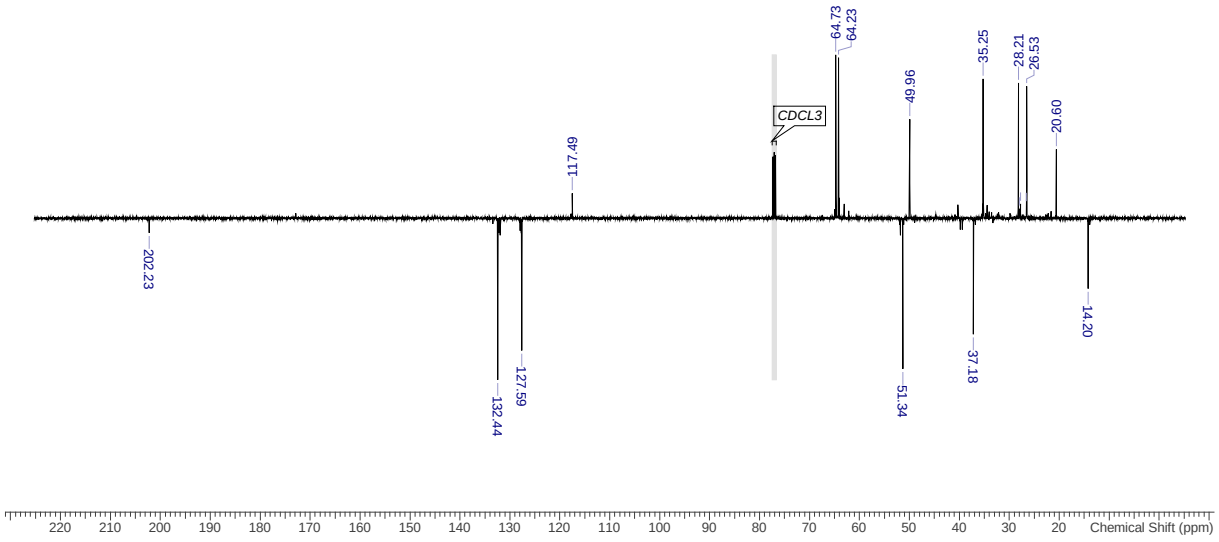
Table 1. Primers used for RT-PCR.

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'

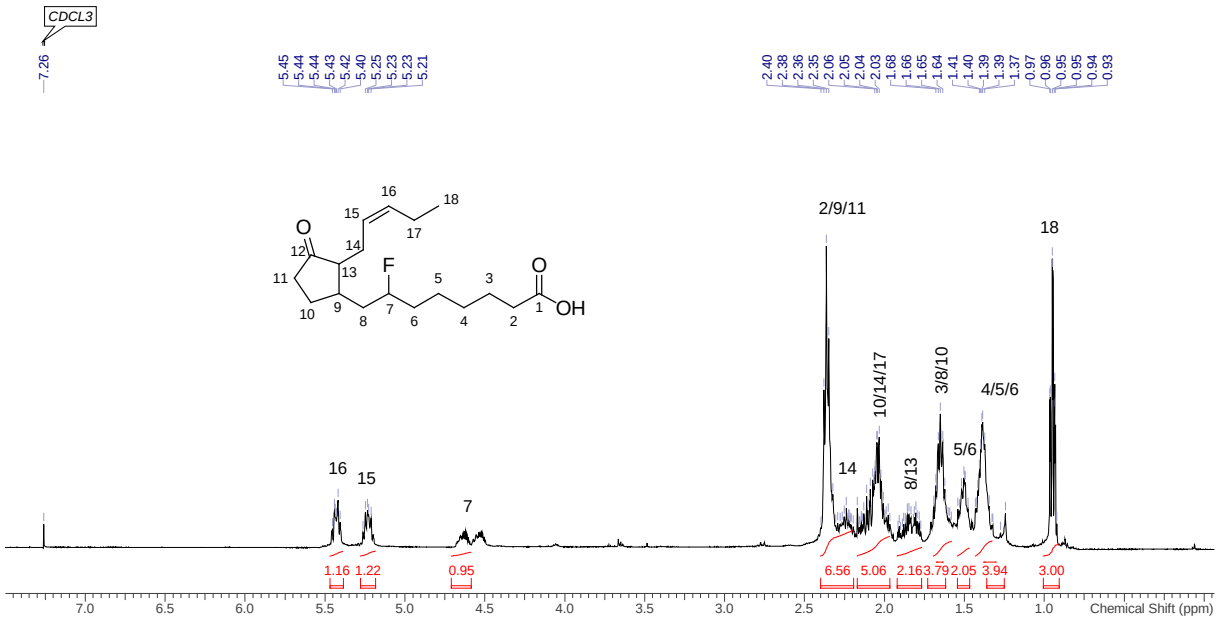
^{1}H-NMR Compound (**4**)



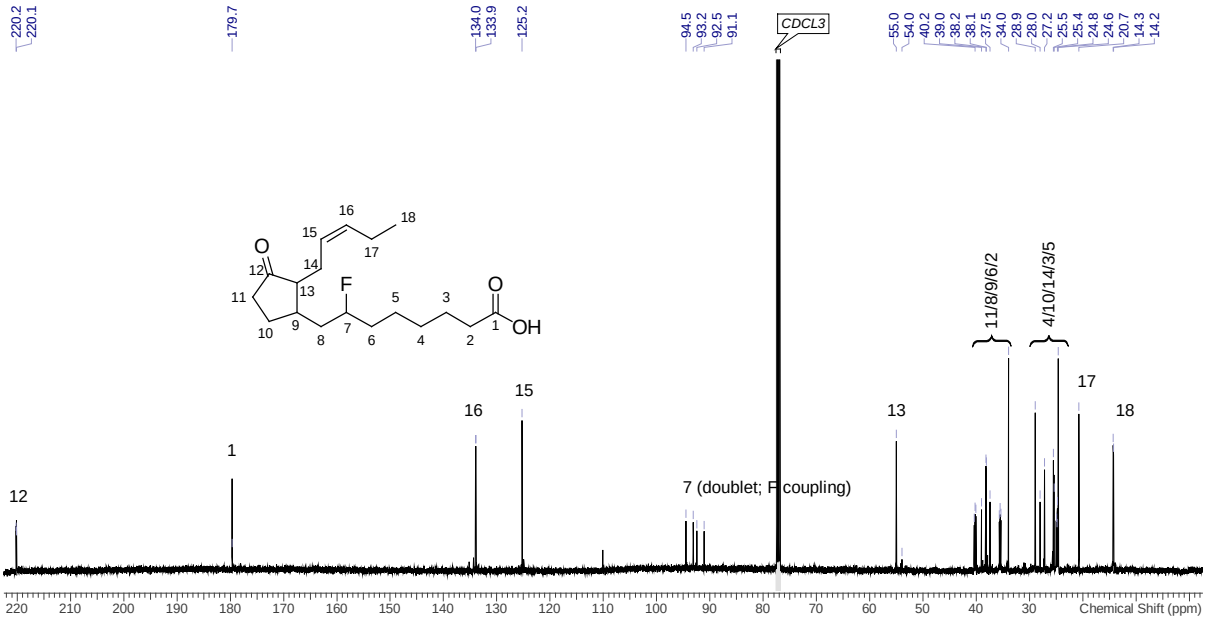
^{13}C- APT-NMR Compound (**4**)



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



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



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

Mass Spectrum SmartFormula Report

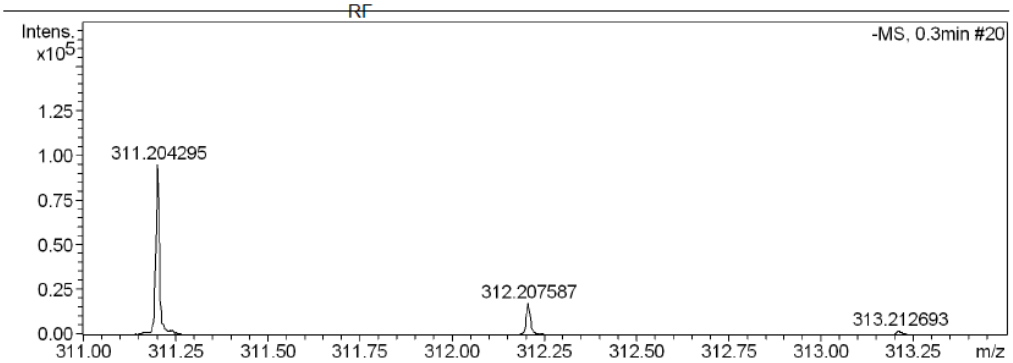
Analysis Info

Analysis Name D:\Data\kepl2401\Guille_7fluoro8_cyclopentyloctanioicAcid_312neg_AllCalib.d
Method kp_tune_low_neg_250_JA.m
Sample Name
Comment

Acquisition Dat 12/5/2014 10:24:55
Operator Admin
Instrument / Se maXis 10078

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

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3.6 Manuscript 4

frontiers in Plant Science

ORIGINAL RESEARCH
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A Holistic Approach to Analyze Systemic Jasmonate Accumulation in Individual Leaves of *Arabidopsis* Rosettes Upon Wounding

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Phytohormones, especially jasmonates, are known to be mediators of the plant responses to wounding and herbivore feeding. Their role in such stress responses has been largely studied locally in treated leaves. However, less is known about the induced systemic distribution of phytohormone signals upon these kinds of stresses. Here, a holistic approach was performed in order to investigate the systemic phytohormone pattern in the rosette of *Arabidopsis thaliana* after herbivore-related wounding. Levels of different stress-related phytohormones such as jasmonates, abscisic acid, and salicylic acid were analyzed in individual leaves. We demonstrate that the typically used sampling method, where leaves are first cut and immediately frozen, causes false-positive results since cutting already induces systemic jasmonate elevations within less than 1.6 min. Therefore, this approach is not suitable to study systemic phytohormone changes in the whole plant. By developing a new method where leaves are frozen first and subsequently cut, sampling-induced phytohormone elevations could be reduced. Using this new method, we show that jasmonic acid and its active isoleucine conjugate (jasmonoyl-isoleucine) are involved in the fast systemic wound response of *Arabidopsis*. A systemic induction of the jasmonates' precursor, 12-oxo-phytodienoic acid, was not observed throughout our treatments. The systemic phytohormone distribution pattern is strongly linked to the vascular connections between the leaves, providing further evidence that the vascular system is used for long distance-signaling in *Arabidopsis*. Besides already known vascular connections, we also demonstrate that the systemic distribution of jasmonate signals can be extended to distant leaves, which are systemically but indirectly connected *via* another vascularly connected leaf. This holistic approach covering almost the whole *Arabidopsis* rosette introduces a method to overcome false-positive results in systemic phytohormone determinations and demonstrates that wounding-induced long-distance signaling includes fast changes in jasmonate levels in systemic, non-treated leaves.

Keywords: systemic signaling, γ-aminobutyric acid, wounding, MecWorm, plant defense, phytohormones, jasmonates, *JASMONATE-ZIM DOMAIN (JAZ) 10*

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INTRODUCTION

In order to survive insect attacks, plants developed a variety of defense strategies that are locally induced by feeding of the herbivore (Mithöfer and Boland, 2012). Since the insect can easily move to other parts of the plant, it is essential for the plant to defend the non-fed systemic leaves as well as the local fed ones. Several studies have shown that defensive compounds accumulate both locally and systemically, like proteinase inhibitors in potatoes, tannins in aspen or γ-aminobutyric acid (GABA) in *Arabidopsis thaliana* (Pena-Cortes et al., 1988; Peters and Constabel, 2002; Scholz et al., 2015, 2017). To ensure a fast reaction to the insect feeding, these defensive compounds are most probably produced directly in the systemic parts rather than being transported from local to systemic leaves (Peters and Constabel, 2002; Scholz et al., 2017). This implies that there are signals traveling throughout the whole plant inducing systemic defenses. Several signals have been discussed to mediate systemic reactions to herbivores or herbivore-related stimuli, such as hydraulic changes (Farmer et al., 2014), electric signals (Mousavi et al., 2013; Salvador-Recatalà et al., 2014; Zimmermann et al., 2016), calcium waves (Kiep et al., 2015), reactive oxygen species (Miller et al., 2009), and phytohormones (Glauser et al., 2008a, 2009; Sato et al., 2009, 2011; Vos et al., 2013; Gasperini et al., 2015; Jimenez-Aleman et al., 2015).

Lacking a nervous system for rapid long distance signal transport, plants use their vascular system to transfer those systemic signals (Farmer et al., 2014; Salvador-Recatalà et al., 2014; Gasperini et al., 2015; Kiep et al., 2015; Nguyen et al., 2018). In *Arabidopsis,* leaves are connected *via* so called parastichies in a specific pattern according to their development: leaf n is directly connected to leaves n ± 5 and n ± 8 (connections of first order) and indirectly to leaves n ± 3 (connections of second order) (Dengler, 2006; Mousavi et al., 2013) (see example for leaf 8 parastichies **Supplementary Figure S1**). It was shown for several of the above mentioned systemic signals that their distribution among the *Arabidopsis* rosette corresponds to the vascular connections between the leaves, such as for leaf surface potential changes (Mousavi et al., 2013; Nguyen et al., 2018) or calcium signals (Kiep et al., 2015; Nguyen et al., 2018). Also for the phytohormone jasmonic acid (JA) and its active isoleucine conjugate (jasmonoyl-isoleucine, JA-Ile) that are known to be key regulators of the plant response to herbivory (Howe and Jander, 2008), there are hints that their systemic pattern corresponds to the parastichies between leaves. It was shown that the expression pattern of the JA responsive gene *JAMONATE ZIM-DOMAIN10* (*JAZ10*) among the whole *Arabidopsis* rosette follows the parastichious connections of the treated leaf (Mousavi et al., 2013). Furthermore Glauser et al. (2009) published that if two parastichious connected leaves are both wounded, a third non-treated parastichious connected leaf shows an increase in JA, but not an unconnected leaf. Similar results were obtained for JA-Ile (Chauvin et al., 2013) and a precursor of JA and JA-Ile, 8-oxopentenylcyclopentenyloctanoic acid (OPC-8:0) (Jimenez-Aleman et al., 2015).

Strikingly, a holistic study instead of picking selected leaves to investigate the phytohormone level among the whole *Arabidopsis* rosette and by this linking the phytohormone pattern to the known parastichies, is still missing. Even though a lot of work has been done on systemic jasmonates, less is known about other phytohormones. However, it has been long time speculated that salicylic acid (SA) as antagonist of JA might play a role in herbivore defense as well (Koornneef and Pieterse, 2008). Additionally ABA is known to regulate herbivore defense and to play a role in priming of systemic leaves (Bodenhausen and Reymond, 2007; Vos et al., 2013).

Here we present data, unraveling the systemic pattern of jasmonates as well as SA and ABA among the whole *Arabidopsis* rosette, by directly quantifying individual leaf phytohormone levels. We demonstrate that the sampling method is crucial for investigating the holistic jasmonate pattern. Furthermore, our data suggest that mainly JA and JA-Ile rather than their biosynthetic precursor 12-oxo-phytodienoic acid (OPDA) or SA and ABA mediate fast systemic herbivory-related defense responses.

MATERIALS AND METHODS

Plant Growth, Treatment and Sampling Procedures

Five week old *A. thaliana* Columbia-0 plants were used for all experiments. Plants were grown as described before (Scholz et al., 2017). Leaves were counted prior the treatment according to Farmer et al. (2013); the younger the higher the number. Plants were mechanically wounded at leaf 8 using MecWorm (Mithöfer et al., 2005) to ensure a continuous wounding. For 1 h treatment two rectangular shaped areas, each with a duration of 30 min, using a speed of 12 punches per minute, were designed to wound leaf 8 (Scholz et al., 2017). The midrib of the leaf was included in wounding. After treatment all leaves in the range of leaf 5 to leaf 13 were collected either from young to old or from old to young leaves. The sampling direction is mentioned in the respective figure legends. For experiments presented in **Figures 1**, **2**, **4**, **5**, **6**, **9** one leaf after another was cut and directly frozen following the sampling method of Glauser et al. (2008b) (further referred to as cut-and-freeze-method). For other experiments the freeze-and-cut-method was developed. The whole rosette was dipped in liquid nitrogen for 5–10 s. Leaves were cut of the frozen rosette starting from young leaves to old ones. Immediately after cutting each leaf was kept in liquid nitrogen. For all extraction procedures leaf material was ground in precooled cryoblocks using 2010 Geno/Grinder® (SPEX®SamplePrep, Metuchen, NJ, United States). Samples were stored in −80°C till the extraction procedure.

Extraction and Quantification of Phytohormones

Whole leaf was used for phytohormone extraction and weighed in frozen for quantification of the metabolites. Phytohormones were extracted following the single leaf extraction protocol described in Jimenez-Aleman et al. (2015) with some modifications. For phytohormone measurements shown in **Figures 1**, **5E,G**

samples were extracted using 1.0 ml methanol containing 40 ng D_6-ABA (Santa Cruz Biotechnology, Santa Cruz, CA, United States), 40 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany), 40 ng D_4-SA (Sigma-Aldrich) and 8 ng of JA-$^{13}C_6$-Ile conjugate as internal standard. All other extractions (**Figures 2**, **4**, **5A–D,F,H**, **7**, **8**) were performed using D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany), D_4-SA (Santa Cruz Biotechnology, Santa Cruz, CA, United States), D_6–ABA (Toronto Research Chemicals, Toronto, ON, Canada), D_6-JA-Ile (HPC Standards GmbH, Cunnersdorf, Germany) as internal standards, respectively. Chromatographic separation of phytohormones was performed on an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, United States) using a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm, Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B. The elution profile was: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min, 90–100% B; 4.02–4.50min, 100% B, 4.50–4.51 min 100–10% B and 4.51–7.00, 10% B. The mobile phase flow rate was 1.1 ml/min and column temperature was maintained at 25°C. Phytohormones were determined using an API 5000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, United States) with a turbospray ion source operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at −4500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, the heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion fragmentations as follows: m/z 136.9 →93.0 [collision energy (CE) −22 V; declustering potential (DP) −35 V) for SA; m/z 140.9 →97.0 (CE −22 V; DP −35 V) for D_4-SA; m/z 290.9 →165.1 (CE −24 V; DP −45 V) for OPDA; m/z 209.1 →59.0 (CE −24 V; DP −35 V) for JA; m/z 215.1 →59.0 (CE −24 V; DP −35 V) for D_6-JA; m/z 214.1 →59.0 (CE −24 V; DP −35 V) for D_5-JA; m/z 322.2 →130.1 (CE −30 V; DP −50 V) JA-Ile; *m/z* 328.2 →136.1 (CE −30 V; DP −50 V) for JA-$^{13}C_6$-Ile; m/z 328.2 →130.1 (CE −30 V; DP −50 V) for D_6-JA-Ile; m/z 327.2 →130.1 (CE −30 V; DP −50 V) for D_5-JA-Ile; m/z 263.0 →153.2 (CE −22 V; DP −35 V) for ABA; m/z 269.0 →159.2 (CE −22 V; DP −35 V)] for D_6-ABA. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.6 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. Since it was observed that both the D_6-labeled JA and D_6-labeled JA-Ile standards (HPC Standards GmbH, Cunnersdorf, Germany) contained 40% of the corresponding D_5-labeled compounds, the sum of the peak areas of D_5- and D_6-compound was used for quantification. For quantification of OPDA the internal D_5-/D_6-JA standard was used applying experimental-determined response factors of 0.5, respectively. The response factors were determined by analyzing a mixture of OPDA [kindly provided by W. Boland, MPI for Chemical Ecology, Jena, Germany; synthesized as described in Shabab et al. (2014)] and D_5-/D_6-JA all at the same concentration.

Extraction and Quantification of GABA

For GABA measurements amino acids were extracted with methanol following the single leaf extraction protocol for phytohormones (Jimenez-Aleman et al., 2015). The methanolic extract was diluted 1:10 (v/v) with water containing 10 µg/ml of ^{13}C, ^{15}N algal amino acid mix (Isotec, Miamisburg, OH, United States) as internal standard. GABA was determined using LC-MS/MS according to Scholz et al. (2015).

Quantitative Real Time (qRT)-PCR

RNA extraction, DNase treatment, reverse transcription and qRT-PCR were carried out as described in Heyer et al. (2018). The expression level of *JAZ10* was quantified with the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001; Ohlen et al., 2016) using *RPS18B* as housekeeping gene, were $2^{-\Delta CT} = 2^{CT}{}_{RPS18}/2^{CT}{}_{JAZ10}$. Untreated plants were used as controls. Primers used for *RPS18B* and *JAZ10* have been reported in Scholz et al. (2014).

Statistics

All statistical analysis was performed using RStudio Version 1.0.143. Statistic significant differences were tested using one sample or two sample Wilcoxon Test as indicated in the figure legends. False discovery rate (FDR) (Benjamini and Hochberg, 1995) was applied on all tests. For Phytohormone measurements a total number of biological replicates of at least $n = 11$ was used. In case of *JAZ10* expression studies the number of biological replicates was at least $n = 5$ and in case of GABA quantification it was at least $n = 6$. For detailed information of the number of biological replicates (n) used in each experiment, see the figure legends.

RESULTS

Sampling Induces Systemic Jasmonate Signaling

In order to investigate the systemic pattern of phytohormones in the *Arabidopsis* rosette, plants were wounded on leaf number 8 and all leaves from 5 to 13 were collected. The mechanical larva MecWorm (Mithöfer et al., 2005) was used for wounding treatment, since in comparison to previous studies (Glauser et al., 2008a, 2009; Chauvin et al., 2013) a constantly wounding of the leaf should be achieved to mimic the mechanical damage of real insect feeding. The midrib of the leaves was included into wounding to ensure a systemic wound response (Kiep et al., 2015). Phytohormones were measured in each leaf after 1 h of treatment. The time point was chosen according to previous investigations on systemic jasmonate related gene expression (Mousavi et al., 2013). Leaves were collected starting from oldest (leaf 5) to the youngest (leaf 13). Phytohormone contents of the individual leaves are displayed in **Figure 1**.

Jasmonates are known to be main mediators in wound signaling in plants (Koo and Howe, 2009) and there were hints that they accumulate in systemic leaves (Glauser et al., 2009; Koo et al., 2009; Chauvin et al., 2013; Jimenez-Aleman et al., 2015). As expected we saw a strong local response in case of OPDA,

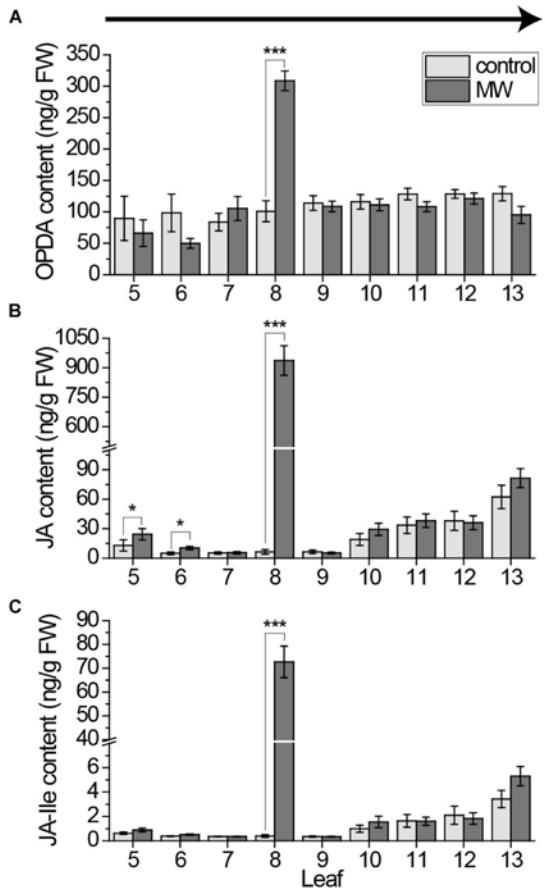

FIGURE 1 | Jasmonate contents in individual *Arabidopsis* leaves after wounding, sampled with the cut-and-freeze-method from old to young leaves. Mean levels of OPDA **(A)**, JA **(B)**, and JA-Ile **(C)** ±standard error (SE) in individual leaves after wounding leaf 8 with MecWorm for 1 h. Untreated plants were used as control. Legend for color code see **(A)**. Leaves were collected with the cut-and-freeze-method from leaf 5 (old) to leaf 13 (young) as indicated by the arrow in **(A)**. The experiment was repeated four times independently ($n \geq 15$). Statistically significant differences between phytohormone content of treated and untreated plants within the leaves were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (*$P < 0.05$, ***$P < 0.001$). *P*-values are False discovery rate (FDR) corrected.

JA and JA-Ile. All three jasmonates were significantly induced upon wounding in leaf 8 (**Figure 1**). However, compared to leaves of non-treated plants just for JA a small but significant increase in two systemic leaves of treated plants was measured, in the indirectly connected leaves 5 and 6 (**Figure 1B**). In contrast to previously published results, no difference could be shown in the directly connected leaf 13 (Glauser et al., 2009; Chauvin et al., 2013).

Furthermore, the jasmonate JA and JA-Ile content in younger leaves was detected to be much higher, even in untreated leaves, than in older leaves (**Figure 1**). In order to test if this rise in jasmonate content was age dependent or a matter of the sampling direction, we collected leaves from untreated plants from old to young leaves and the other way around. Independent of the sampling direction the level of OPDA in younger leaves was higher than in older leaves (**Figures 2A,B**). Strikingly, for JA as well as JA-Ile, the sampling direction was detected to cause differences in the phytohormone level between younger and older leaves. If leaves were collected from young to old, JA and JA-Ile levels increased toward the older leaves (**Figures 2D,F**); if collected from old to young they increased toward younger leaves (**Figures 2C,E**). This shows that cutting the leaves to sample plant material is already sufficient to induce systemic JA and JA-Ile accumulation.

To gain further insight into the speed of the cutting-induced systemic JA and JA-Ile elevation, we measured the time that is necessary to harvest all nine leaves of interest. The average sampling time was 2.36 min (**Figure 3**). Since sampling-induced JA and JA-Ile elevation started after cutting 3 to 5 leaves, it took less than 1.57 min to induce these phytohormones systemically.

To investigate whether these sampling-induced signals are as high as the wounding treatment-induced signals and thus causing false negative results, we repeated the 1 h MecWorm treatment with collecting leaves from young to old and measured the jasmonate content. In line with the data from the untreated plants (**Figures 2A,B**), OPDA was only locally but not systemically induced, providing evidence that OPDA is not fast induced (**Figure 4A**). In contrast, JA and JA-Ile levels strongly increased in the wounded leaf 8 as well as in the directly connected leaf 13 and, in case of JA, in the indirectly connected leaf 11 (**Figures 4B,C**). However, the significant induction in leaves 5 and 6 found after harvesting leaves from 5 to 13 (**Figure 1**) was not visible anymore. Thus, the systemic JA und JA-Ile elevation might follow the vascular connections of the leaves, but sampling-induced signals attenuate the treatment effects and impede a reliable analysis of the whole rosette pattern at once.

Because of the known crosstalk between Jasmonates and SA as well as ABA (Koornneef and Pieterse, 2008; Vos et al., 2013), we next investigated the systemic pattern of these hormones. To proof if the sampling direction has any effect on the levels of these two phytohormones, we first measured the SA and ABA level in untreated plants, when sampled from old to young and from young to old. Both SA and ABA were not influenced by the sampling direction (**Figures 5A–D**). The SA level is decreasing with the increasing leaf age, while ABA is increasing slightly independent on the sampling direction. We further investigated if these hormones play a role in systemic signaling after wounding. Neither SA nor ABA showed a clear systemic pattern after 1 h MecWorm treatment, independent on the sampling direction (**Figures 5E–H**). Just sporadically few leaves showed a significant increase or decrease of either SA or ABA, which might be rather artifacts than real systemic signals.

JAZ10 Expression Is Unaffected by Sampling

Previous studies showed that the jasmonate-regulated gene *JAZ10* is upregulated in systemic leaves that are vascularly

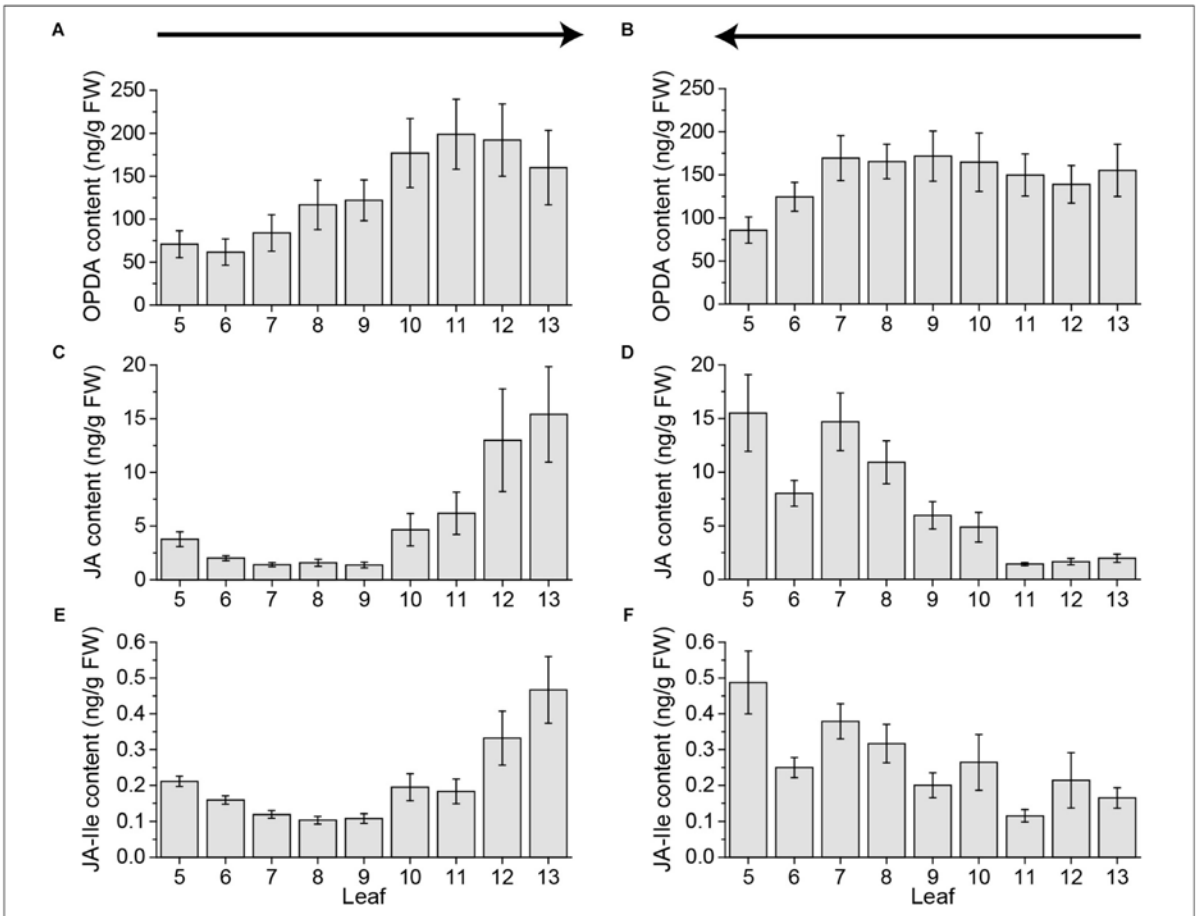

FIGURE 2 | Jasmonate contents of untreated *Arabidopsis* leaves. Mean levels of OPDA **(A,B)**, JA **(C,D)**, and JA-Ile **(E,F)** ± SE in individual leaves of untreated *Arabidopsis* plants. Single leaves were collected with the cut-and-freeze-method from leaf 5 (old) to leaf 13 (young) **(A,C,E)** or from young to old **(B,D,F)**. Sampling directions are indicated by arrows. The experiment was repeated two times independently ($n \geq 11$).

connected to the wounded leaf, but not in non-connected leaves (Glauser et al., 2009; Mousavi et al., 2013). Because we showed that sampling induces JA and JA-Ile to the same extend as 1 h wounding by MecWorm, we examined if sampling with the cut-and-freeze-method can induce jasmonate-regulated gene expression as well. Therefore, plants were 1 h MecWorm-treated and *JAZ10* expression was determined using qRT-PCR, sampling once from young to old and from old to young leaves. The sampling direction had no effect on the systemic *JAZ10* expression. The expression levels in leaves of untreated plants were comparable between the two sampling directions (**Figures 6A,B**). However, after wounding leaf 8, the *JAZ10* expression increased in the local leaf as well as in the directly connected leaf 13 and the indirectly connected leaves 5 and 11 (**Figures 6C,D**) in both sampling directions. Leaf 6 showed an increase in *JAZ10* expression over both treatments as well, even though this result was just significant when sampling from young to old leaves (**Figures 6C,D**). However, this leaf is known as a variable leaf (Mousavi et al., 2013). Surprisingly, the unconnected leaf 9 displayed a small but significant difference between control and treatment when sampling from young to old (**Figure 6D**). Nevertheless, this increase in *JAZ10* expression was quite low compared to the increase in other systemically connected leaves and thus might be not of biological relevance. Taken these results together, in contrast to the jasmonate levels the expression of the jasmonate regulated gene *JAZ10* was not induced by sampling.

Freeze-and-Cut-Method Overcomes Sampling Induced Signaling

Because of the sampling time needed for a whole *Arabidopsis* rosette, the cut-and-freeze-method was no longer appropriate as shown above. To overcome the problem of sampling-induced phytohormone elevations, a new sampling method was developed. It was shown before that rapid freezing of the plant material can decrease the amount of sampling induced JA and JA-Ile (Glauser et al., 2008b). Thus, we tested if freezing the

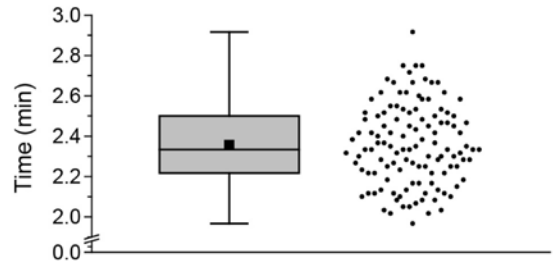

FIGURE 3 | Sampling time for one *Arabidopsis* rosette. Boxplot (left) and scatter plot (right) of times needed for sampling 9 leaves. Both plots show the distribution of the measured data. The scatter plot shows each time measured. The box indicates the middle 50% of the data points. The black square indicates the mean value and the line within the box the median. Whiskers are defined as 1.5 fold interquartile range. In total, time was measured for 119 sampling events.

sample before cutting (freeze-and-cut-method) might solve the problem of sampling-induced phytohormone elevations. We froze untreated plants by dipping the whole rosette in liquid nitrogen and cut the frozen leaves from young to old ones, to avoid destruction of the leaves. The new freeze-and-cut-method drastically decreased the levels of OPDA, JA, and JA-Ile in all leaves (**Figures 7A–C**). Thereby the sampling-induced increase of JA and JA-Ile in older leaves was reduced as well; the amount of JA could be lowered up to 18 times and the amount of JA-Ile up to 9 times. (**Figures 7B,C**). In line with the results above, SA and ABA levels were not influenced by the sampling method. The amount of SA and ABA in single leaves of the freeze-and-cut-method was comparable to those of the cut-and-freeze-method (**Figures 7D,E**). Thus the freeze-and-cut-method can be used to investigate systemic phytohormone patterns by sampling the whole rosette of *Arabidopsis*.

JA and JA-Ile Are the Key Systemic Phytohormones Mediating Early Wound Responses

Using the new method, we re-investigated the systemic phytohormone pattern after 1 h continuous wounding of leaf 8 with the MecWorm. The results are shown in **Figure 8**. In line with the results shown above, SA was neither upregulated in the local leaf nor in the systemic leaves (**Figure 8A**); thus, SA can be excluded as phytohormone mediating early wound responses both locally and systemically. Also ABA seems not to be a key phytohormone in the early systemic wound response. Although a systemic reduction of ABA after wounding leaf 8 in the systemically connected leaf 13 could be measured, none of the other leaves displayed any change in the ABA level after treatment (**Figure 8B**). As in all measurements done before, OPDA was just upregulated locally but not systemically (**Figure 8C**), as reported before (Glauser et al., 2009). Only JA and JA-Ile increased locally and systemically (**Figures 8D,E**). Both phytohormones were upregulated in the connected leaves 13, 11, and 5. Interestingly,

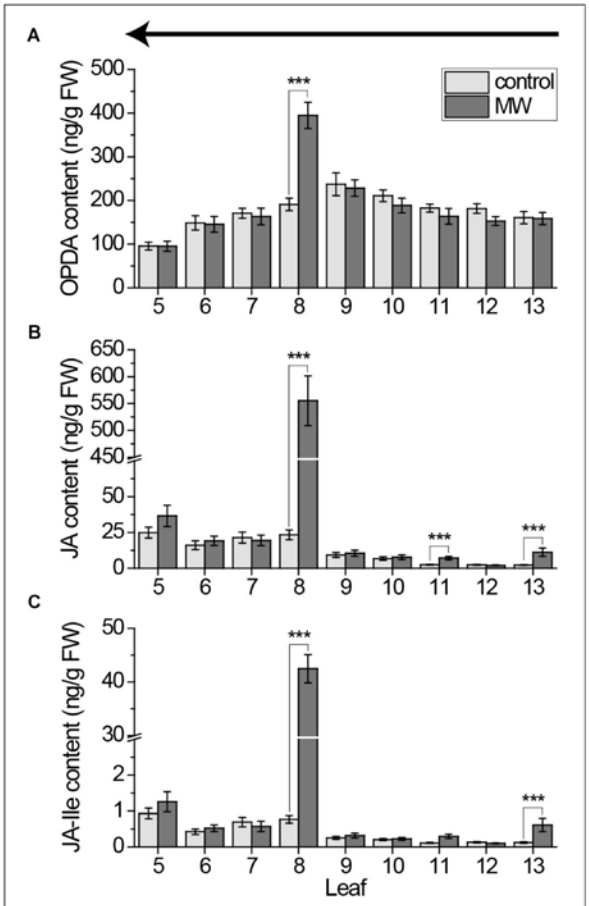

FIGURE 4 | Jasmonate content in individual *Arabidopsis* leaves after wounding, sampled with the cut-and-freeze-method from young to old leaves. Mean levels of OPDA **(A)**, JA **(B)**, and JA-Ile **(C)** ±SE in individual leaves after wounding leaf 8 with MecWorm for 1 h. Untreated plants were used as control. Legend for color code see **(A)**. Leaves were collected with the cut-and-freeze-method from leaf 13 (young) to leaf 5 (old) as indicated by the arrow in **(A)**. The experiment was repeated three times independently ($n \geq 13$). Statistically significant differences between phytohormone content of treated and untreated plants within the leaves were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (***$P < 0.001$). *P*-values are FDR corrected.

leaf 10 that is directly connected to leaf 5 and indirectly to leaf 13 displayed JA and JA-Ile elevation as well, suggesting a vascular connections of third order.

Systemic GABA Elevations Are Not Influenced by the Sampling Method

In previous studies it was shown that the non-proteinogenic amino acid GABA is systemically induced in vascularly connected leaves upon MecWorm treatment (Scholz et al., 2015, 2017). Furthermore it was known that the local GABA level can be already upregulated within several minutes by touch

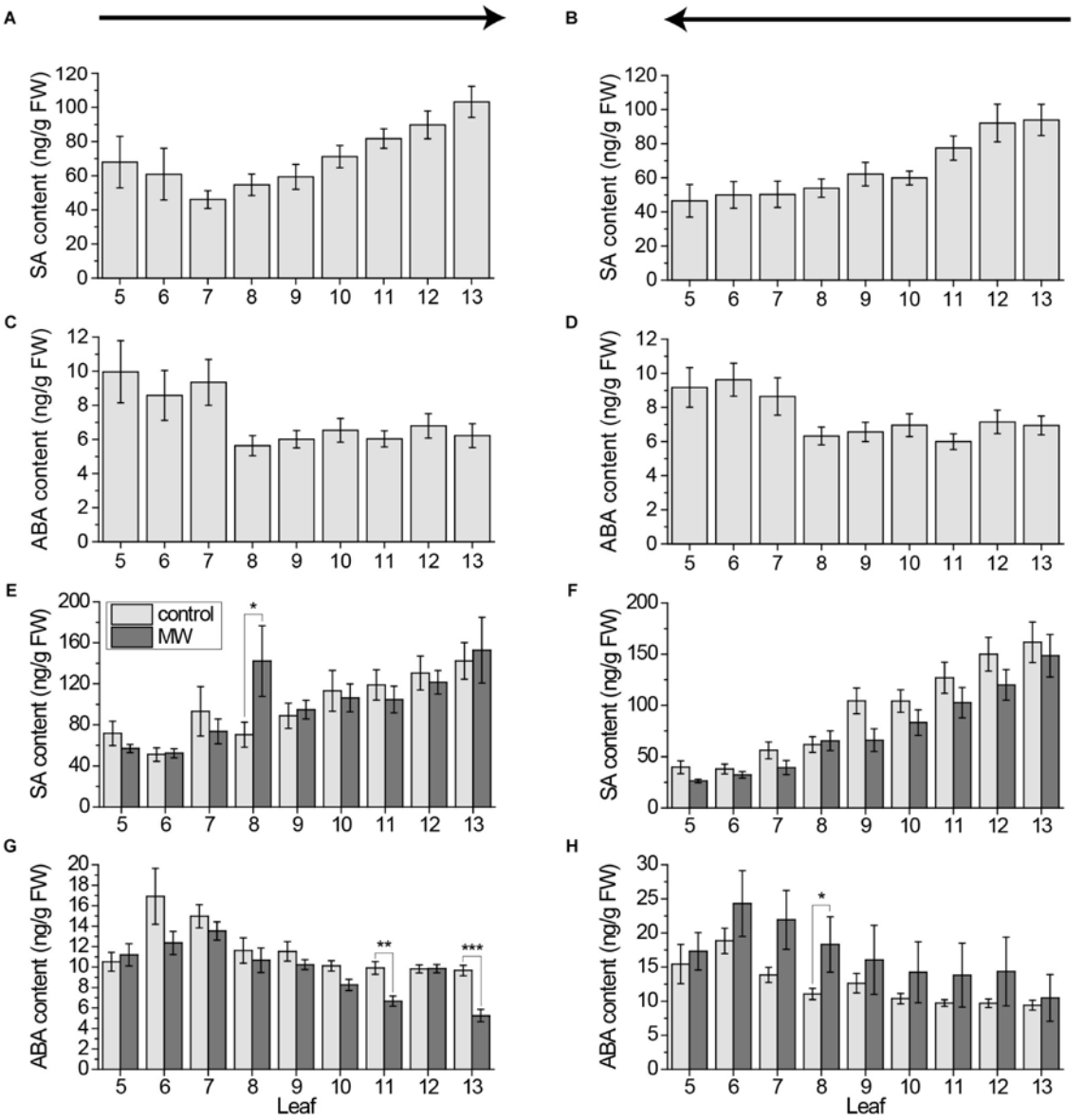

FIGURE 5 | Systemic pattern of SA and ABA in individual leaves of the *Arabidopsis* rosette. Mean levels of SA **(A,B,E,F)** and ABA **(C,D,G,H)** ±SE in individual leaves of *Arabidopsis* plants. In **(A–D)** untreated plants were used. Experiment was repeated two times independently ($n \geq 11$). In **(E–H)** plants were wounded for 1 h with MecWorm and phytohormone content of the leaves was compared to unwounded plants. See **(E)** for color coding legend. Experiment was repeated at least three times independently ($n \geq 13$). All samples over all experiments were collected using the cut-and-freeze-method. Sampling direction is indicated by arrows above the figures. In **(A,C,E)** and **(G)** rosette was collected from old (5) to young leaves (13) and in **(B,D,F,H)** from young to old leaves. Statistically significant differences between phytohormone content of treated and untreated plants within the leaves in experiments **(E–H)** were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). *P*-values are FDR corrected.

(Ramputh and Bown, 1996; Bown et al., 2002) and is thus a highly sensitive wound response in plants. Therefore, we investigated if systemic GABA elevations are rapidly inducible by cutting and subsequent sampling similar to jasmonates. In order to test this, we cut the leaves of untreated plants from old to young and from young to old. Independent on the sampling direction the GABA level increased slightly toward the older leaves (**Figures 9A,B**). Thus GABA seems to be not induced by sampling.

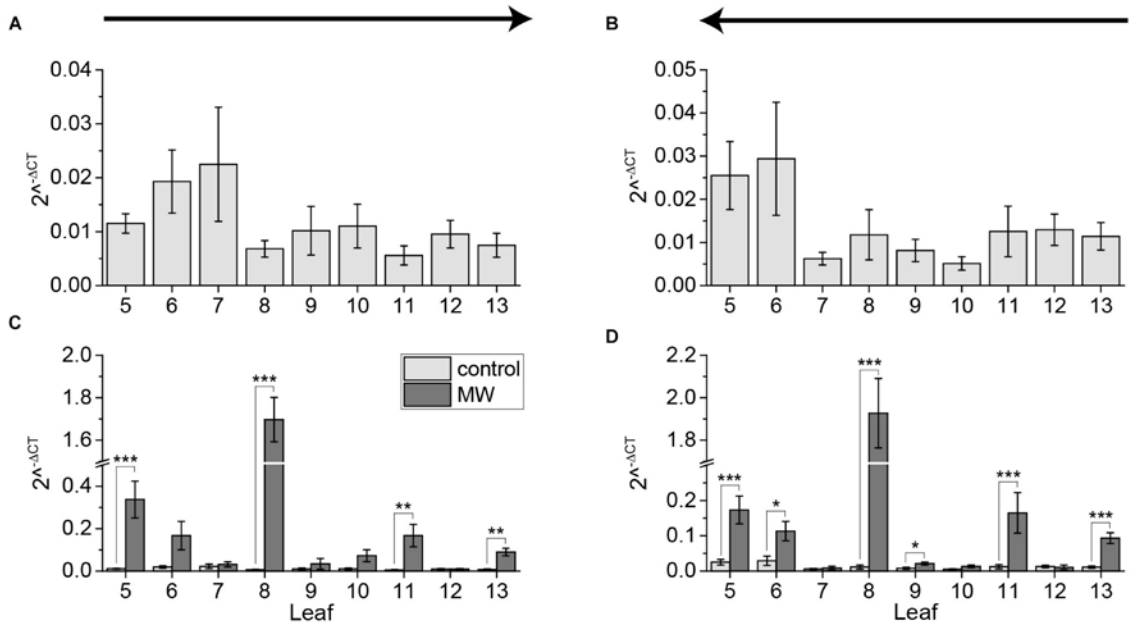

FIGURE 6 | Systemic pattern of *JAZ10* gene expression. Mean ± SE expression of *JAZ10* gene in untreated control plants **(A,B)** and in comparison to plants treated for 1 h with MecWorm at leaf 8 **(C,D)**. For color coding see legend in **(C)**. Individual leaves were collected using the cut-and-freeze-method. Sampling direction is indicated by arrows above the figures. In **(A)** and **(C)** rosette was collected from old (5) to young leaves (13) and in **(B)** and **(D)** from young to old leaves. Statistically significant differences between *JAZ10* expression levels of treated and untreated plants within the leaves in experiments **(C)** and **(D)** were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). *P*-values are FDR corrected. Whole experiment was repeated at least two times independently ($n \geq 5$).

Furthermore we measured the GABA levels in control and MecWorm-treated plants, when sampled with the freeze-and-cut-method. Similar to previous reported results (Scholz et al., 2017), the GABA level was drastically induced in the treated leaf 8 and upregulated in the directly connected leaf 13 (**Figure 9C**). Additionally, the indirectly connected leaf 11 and leaf 10, which likely is connected to leaf 8 *via* third order, showed a significant increase in the GABA level (**Figure 9C**). Nevertheless, we measured no differences in GABA concentration between control plants and treated plants in the indirectly connected leaf 5. Since induced systemic GABA levels are quite low, this might be that due to the higher variability of the GABA levels in leaf 5 (**Figure 9C**).

DISCUSSION

Phytohormones are known to play an important role as systemic signaling components after herbivory (Howe and Jander, 2008; Vos et al., 2013). For other systemic signals it is known that their distribution among the *Arabidopsis* rosette is dependent on the vascular connections between the single leaves. Up to now, most of the systemic phytohormone measurements done, did not take these connections into account. Systemic leaves are chosen randomly (Glauser et al., 2008a; Koo et al., 2009) or just few leaves that share a connection to the treated leaf were investigated (Glauser et al., 2009; Chauvin et al., 2013; Jimenez-Aleman et al., 2015). A holistic approach to measure phytohormones in the whole rosette to get insights into their distribution pattern, as done for systemic electric signals or calcium waves (Mousavi et al., 2013; Kiep et al., 2015), was missing. Furthermore, most of the studies performed, focused only on the distribution of JA and its active conjugate JA-Ile and very often jasmonate reporters are used to determine the systemic pattern of these jasmonates instead of measuring directly the phytohormone content of individual leaves (Glauser et al., 2008a, 2009; Chauvin et al., 2013; Mousavi et al., 2013; Salvador-Recatalà et al., 2014). Thus, in this study the systemic distribution of OPDA, JA, JA-Ile, SA, and ABA by measuring single leaf phytohormone contents were investigated and linked to the vascular connections between the leaves in the *Arabidopsis* rosette.

Sampling Matters – The Cut-and-Freeze-Method Is Not Appropriate for Holistic Approaches

Glauser et al. (2008a) showed that cutting a single leaf can induce a local JA elevation within an average time of 1.2 min. To avoid such rapid local sampling-induced signals, they developed the cut-and-freeze-method (Glauser et al., 2008a,b). Nevertheless,

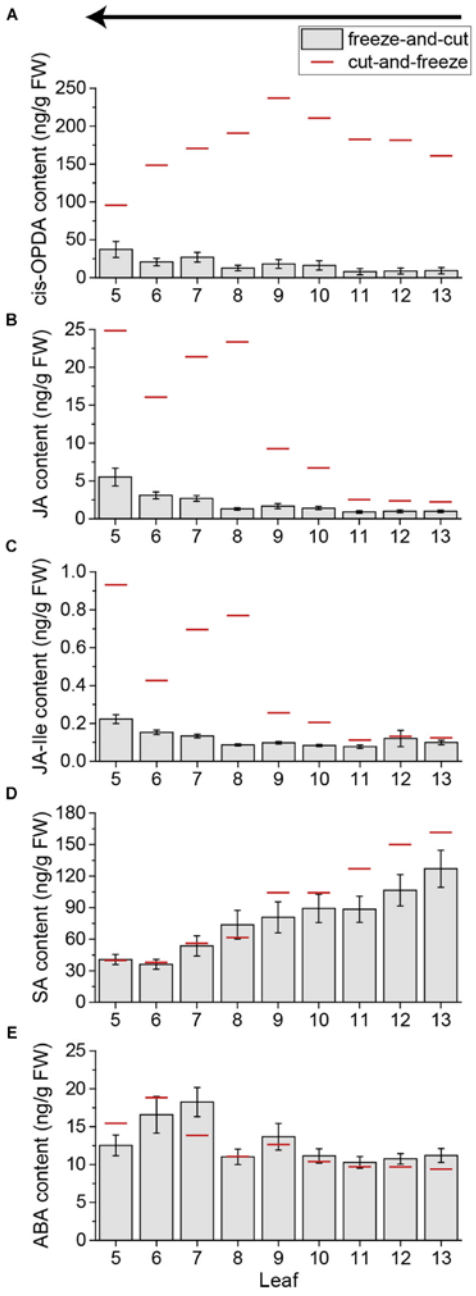

FIGURE 7 | Phytohormone content of untreated *Arabidopsis* leaves using the freeze-and-cut-method. Bars represent mean levels of OPDA **(A)**, JA **(B)**, JA-Ile **(C)**, SA **(D)**, and ABA **(E)** ± SE in individual leaves of untreated *Arabidopsis* plants. Individual leaves were collected with the freeze-and-cut-method from leaf 13 (young) to leaf 5 (old). Sampling direction is indicated by an arrow above the figures. The experiment was repeated six times independently ($n \geq 19$). Red lines indicate the mean values of untreated plants collected with the cut-and-freeze-method in the same sampling direction (see **Figures 4, 5F,H**).

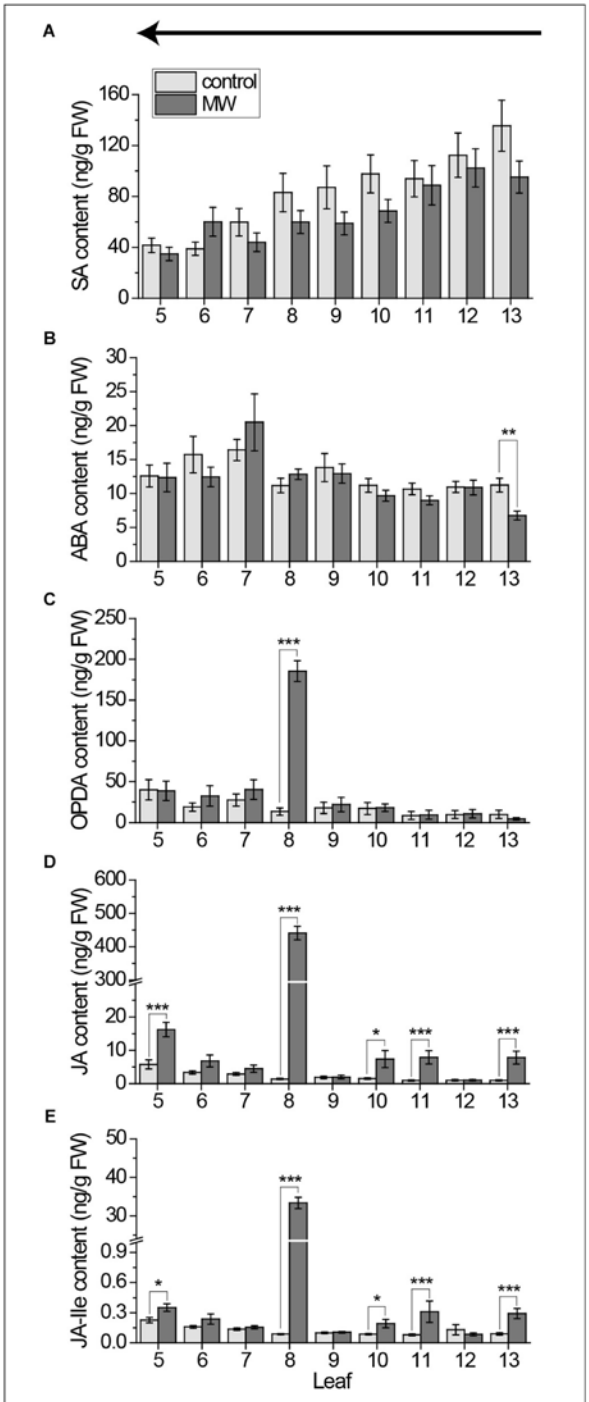

FIGURE 8 | Systemic pattern of phytohormones in individual leaves of the *Arabidopsis* rosette after wounding using the freeze-and-cut-method for sampling. Mean levels of SA **(A)**, ABA **(B)**, OPDA **(C)**, JA **(D)**, and JA-Ile **(E)** ± SE in individual leaves after wounding leaf 8 with MecWorm for 1 h.

(Continued)

FIGURE 8 | Continued
Untreated plants were used as control. Legend for color code see **(A)**. Leaves were collected with the freeze-and-cut-method from leaf 13 (young) to leaf 5 (old) as indicated by the arrow above the figures. The experiment was repeated five times independently ($n \geq 16$). Statistically significant differences between phytohormone content of treated and untreated plants within the leaves were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). *P*-values are FDR corrected.

when rosettes (leaves 5 to 13) were systematically sampled for phytohormone measurements using the cut-and-freeze-method (**Figures 1–4**) sampling-induced systemic JA and JA-Ile elevations were detected. Depending on the sampling direction, the amount of jasmonates increased toward the younger leaves (**Figures 1B,C**, **2C,E**) or the older ones (**Figures 2D,F**, **4B,C**). The systemic JA and JA-Ile increase occurred after sampling one third to one half of the rosette. Since sampling of a whole rosette takes 2.36 min (**Figure 3**), systemic signals induced by cutting took less than 1.57 min. These findings are supported by Chauvin et al. (2013) who reported a time of 1.5 min for the systemic JA signals to be measured in the connected leaf 13 after wounding up to 50% of leaf 8. For JA-Ile we could show that its systemic induction in leaf 13 seems to be even faster than the reported 3 min after wounding (Chauvin et al., 2013).

Interestingly, simply cutting the leaves increased the amount of systemic JA and JA-Ile to the same extend as the continuous wounding for 1 h on the local leaf 8, (**Figures 1B,C**, **4B,C**), causing false-negative results if the cut-and-freeze-method is used for sampling the whole rosette. These data suggest that the systemic JA and JA-Ile signals are an on-off-system and independent on time of wounding at the local leaf. Once the systemic leaf is activated through its vascular connection to the local leaf, the systemic JA and JA-Ile signals are switched on. Thus, cutting one leaf after the other using the cut-and-freeze-method causes a systemic activation in the leaves that are still attached to the rosette and connected *via* the vascular system with the cut leafs. This gains high, unrealistic background levels of JA and JA-Ile already in the controls. Subsequently, if compared to these controls, the interpretation of treatment effects are affected because existing differences can be diminished. Consequently, previous results on systemic phytohormones achieved by the cut-and-freeze-method should be interpreted carefully, especially, if more than 3–5 leaves are collected consecutively.

Fast sampling-induced phytohormone elevations were detected for JA and JA-Ile only, whereas their precursor OPDA, as well as SA and ABA were not influenced by cutting the leaves (**Figures 1A**, **2A,B**, **4A**, **5**). In addition, the cut-and-freeze-method seems to be just problematic, when real phytohormone levels are measured. If the *JAZ10* gene was employed for measuring systemic jasmonate responses, the controls did not display any sampling dependent increase in *JAZ10* expression (**Figures 6A,B**). The systemic pattern of *JAZ10* expression after wounding was independent on the sampling direction as well. The directly connected leaf 13

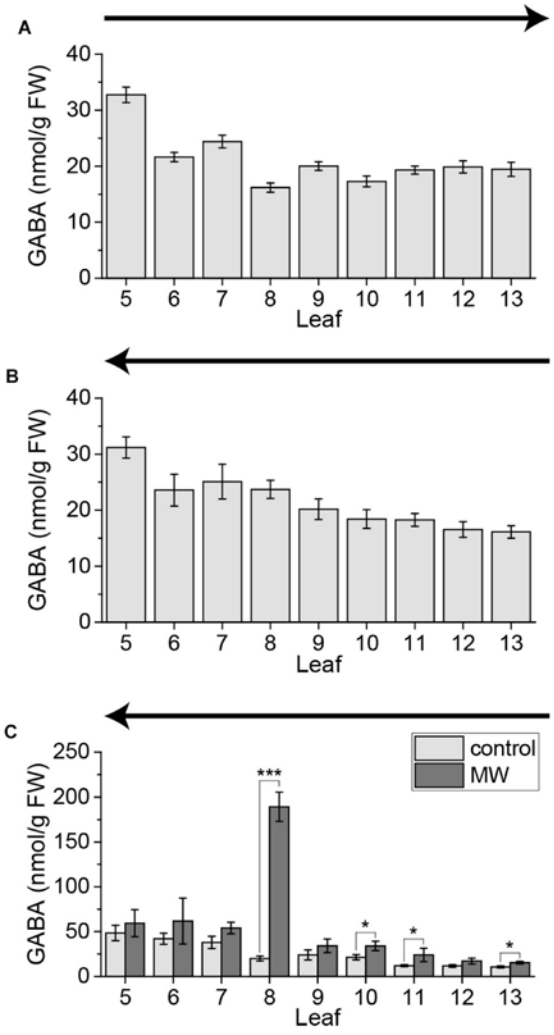

FIGURE 9 | GABA content of untreated and wounded *Arabidopsis* leaves. GABA levels of individual leaves of untreated *Arabidopsis* rosettes sampled with the cut-and-freeze-method **(A,B)**. **(C)** GABA levels of individual leaves of untreated control plants and plants that were treated for 1 h with MecWorm sampled with the freeze-and-cut-method. Sampling direction is indicated in each figure by an arrow. The experiment was repeated two times independently ($n \geq 6$) for **(A,B)** and five times independently ($n \geq 15$) for **(C)**. Statistically significant differences in **(C)** between GABA content of treated and untreated plants within the leaves were determined by unpaired two-sample Wilcoxon test. Asterisk indicates significance (*$P < 0.05$, ***$P < 0.001$). *P*-values are FDR corrected.

and the indirectly connected leaves 5 and 11 and sometimes leaf 6 showed a clear increase in *JAZ10* expression upon wounding leaf 8 (**Figures 6C,D**) as reported before (Mousavi et al., 2013). The differences in the outcome of the marker gene study and real jasmonate measurements using the same sampling method might be due to the time delay between

phytohormone burst and the gene expression following the burst. *JAZ10* has been shown to accumulate earliest 1 h after wounding treatment (Chung et al., 2008). Sampling-induced JA and JA-Ile signals in the control plants just lasted less than 2 min before freezing the leaves. This short time period is not sufficient to transduce the signal into *JAZ10* gene expression. Hence, the fast cutting-induced JA and JA-Ile signals in the control plants are not visible, when the marker gene is taken as readout.

JA and JA-Ile Are Key Mediators of the Early Systemic Wound Response

To avoid sampling-induced jasmonate signals, the freeze-and-cut-method was developed in this study. We showed that using this new method, the amount of jasmonates in control plants is much lower compared to those sampled with the cut-and-freeze-method (**Figures 7A–C**), allowing the investigation of the real systemic pattern of the phytohormones.

In line with our *JAZ10* expression data (**Figure 6**) and those published before (Mousavi et al., 2013), we could show that the directly connected leaf 13, and the indirectly connected leaves 5 and 11 display a significant increase in JA and JA-Ile level after treatment of leaf 8 (**Figures 8D,E**). In addition, a significant JA and JA-Ile increase was measured in leaf 10. It is connected to leaf 8 in third order *via* connected leaves of second and first order. Recently, Salvador-Recatalà et al. (2014) reported that if larvae cut the midvein of leaf 8 while feeding, leaf 10 is depolarized and the expression of the marker gene *JAZ10* is upregulated in leaf 10. Thus it might be possible, due to the continuous wounding along the midvein of leaf 8 in our experiment, that the systemic signal gets extended to leaves of third order. By this the radius of defended leaves gets increased (**Supplementary Figure S1**). However in our experiments, as well as in the experiments done by Mousavi et al. (2013), *JAZ10* expression was not increased in leaf 10 (**Figures 6C,D**), even though the midvein was wounded in both. It might be that such signals of third order occur with delay after those of first and second orders. Such time delay would explain why third order responses can be missed; or in case of leaf 6 are variable in *JAZ10* marker studies. However, this hypothesis needs to be proven in further studies.

Besides JA and JA-Ile, we investigated the systemic pattern of their precursor OPDA. The systemic role of this phytohormone is highly controversial. Koo et al. (2009) reported a rapid dropdown in OPDA levels 5 min after wounding in systemic leaves, used as a proof that OPDA is used as a store for fast synthesis of JA for rapid wound responses Glauser et al. (2009). measured an increase in OPDA in leaves distal to the wounded leaf about 3 min after treatment, suggesting that it is *de novo* synthesized fast after wounding. On the other hand a systemic role of OPDA after herbivore treatment was excluded by Vos et al. (2013). Here we could show, that systemic OPDA levels are not changing after continuous wounding of leaf 8 (**Figure 8C**) for 1 h. Furthermore, systemic OPDA levels were neither fast induced nor reduced when leaves were harvested using the cut-and-freeze-method (**Figures 1A**, **2A,B**, **4A**). Over all treatments, OPDA was just increased in local leaves (**Figures 1A**, **4A**, **8C**), like reported before after herbivory (Vos et al., 2013). This might be a hint that systemic JA and JA-Ile elevations are not due to *de novo* synthesis, but rather due to transport between the leaves as shown in *Nicotiana* plants (Sato et al., 2009, 2011). Nevertheless, it might be possible as well that they are synthesized out of the bound OPDA in the Arabidopside pool or other sources like OPCs as suggested by Jimenez-Aleman et al. (2015) and Gasperini et al. (2015). In any case, our data exclude free OPDA as rapid systemic wound signal. The same holds true for SA and ABA. None of which showed a constant local or systemic pattern over all treatments (**Figures 5E–H**, **8A,B**). Nevertheless, SA, ABA, and OPDA might still play a role at later time points or after priming as shown for ABA (Vos et al., 2013). Taken together our results suggest that mainly JA and JA-Ile mediate the rapid systemic wound response following the vascular connections between the local and systemic leaves.

Systemic GABA Elevations Are Not Rapid Induced by Sampling

Bown et al. (2002) reported that GABA can be locally induced by insects crawling on leaf surfaces of different plant species. First significant GABA inductions were reported after 5 min of insect crawling, but a slight trend was already seen after 2 min in tobacco leaves (Bown et al., 2002). In a previous work they reported local GABA elevations in soybeans after mechanical stimulation already after 1 min (Ramputh and Bown, 1996). We showed recently that GABA can be induced systemically after continuous wounding with MecWorm in parastichious connected leaves (Scholz et al., 2015, 2017). Here we show that simple cutting was not sufficient to trigger systemic GABA elevations (**Figures 9A,B**) in contrast to JA and JA-Ile elevations. It is known that systemic GABA production is independent on jasmonate signaling (Scholz et al., 2015, 2017). Therefore, it might be that systemic GABA elevations are regulated in a different time scale compared with JA and JA-Ile. Furthermore, if local GABA responses take place at first after 1–5 min (Ramputh and Bown, 1996; Bown et al., 2002), it is conceivable that sampling the whole rosette is faster than the induction of systemic synthesis of GABA. On the other hand GABA is known as a defense compound against insect herbivores (Ramputh and Bown, 1996; Scholz et al., 2015). So, it might be necessary to wound the local leaf severe and continuously like done with MecWorm, to trigger systemic GABA synthesis. Thus, we measured GABA in the whole rosette using the freeze-and-cut-method after MecWorm treatment. As reported before, the connected leaves 13 and 11 displayed a significant increase in GABA, after treatment of leaf 8 (**Figure 9C**), confirming that the signal inducing GABA synthesis follows the parastichious connections between the leaves (Scholz et al., 2017). Interestingly, again we could measure a systemic response in leaf 10, as already shown for JA and JA-Ile (**Figures 8D,E**), supporting once more the hypothesis that systemic signals might be extended to third level connections.

CONCLUSION

In this work a holistic approach for systemic phytohormone elevations in the *Arabidopsis* rosette after herbivore-related wounding was performed. Detailed investigation of jasmonates in individual leaves revealed intrinsic problems of methods used previously to study systemic phytohormone changes. A modified method was developed that overcomes these problems and avoids false-negative results. Employing the new method in combination with targeted analysis of different phytohormones it became clear that very likely JA and JA-Ile are the key phytohormones involved in a rapid systemic response. The systemic jasmonate response seems to be independent on changes in free OPDA levels. It was also demonstrated that these systemic signals strictly follow the vascular connections of first, second, and third orders between the leaves. The distance that can be bridged between a locally treated and systemically activated leaves might depend on the kind of wounding treatment and the nature of the yet non-identified traveling signal(s). This needs to be studied in future.

AUTHOR CONTRIBUTIONS

MH and AM conceived and designed the research and interpreted the data. MH and MR performed the experiments and analyzed the data. MH, AM, and MR wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01569/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.7 Supplementary Material Manuscript 4

Supplementary Material

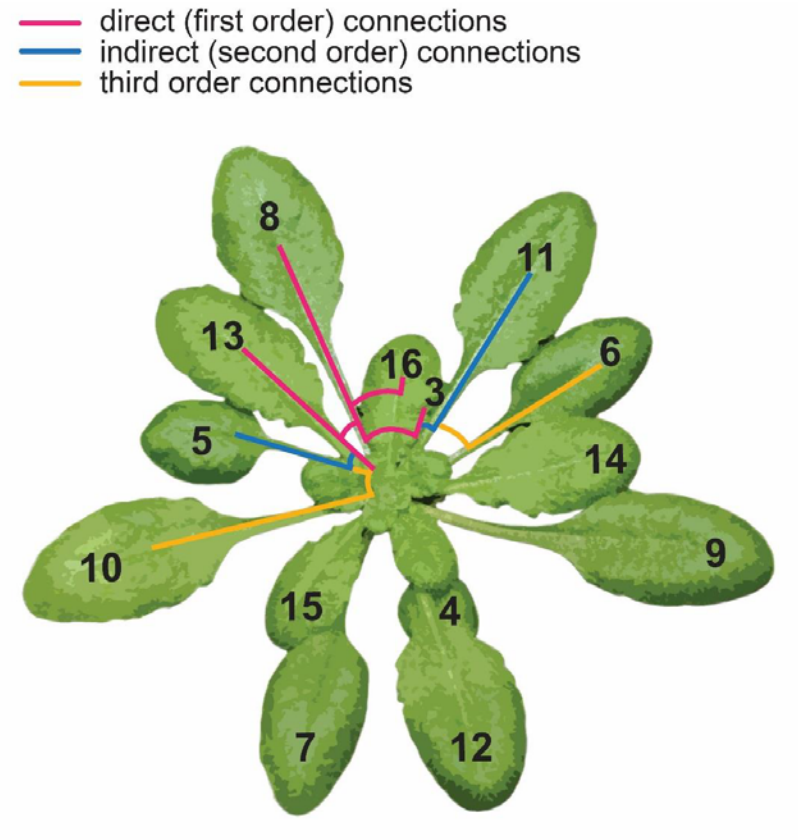

Figure S1. Vascular connections of leaf 8 in a 5-week-old *Arabidopsis thaliana*. Shown are the vascular connections of leaf 8 that was used as local leaf for all treatments. Red lines indicate first order connections (8±5 and 8+8). Those leaves share a direct connection to leaf 8. Blue lines indicate second order connections (8±3). Leaves are indirectly connected to leaf 8. Yellow lines indicate possible third order connections to leaf 8 via indirectly or directly connected leaves of leaf 8.

4 Unpublished results

4.1 *cml9-1* and *cml9-2* display a wild type-like drought stress response

4.1.1 Material and Methods

4.1.1.1 Plant growth

A. thaliana T-DNA insertion lines *cml9-1* (T-DNA insertion in an intron, Col-8 background) and *cml9-2* (T-DNA insertion in an exon, Ws-4 background) described in Magnan *et al.* (2008) were used for drought stress treatments. *A. thaliana* ecotype Col-8 and Ws-4 were used as wild type controls respectively. Plants were grown under short day conditions as described for Max Planck Institute for chemical Ecology (MPI CE) Jena in Heyer *et al.* (2018b, Manuscript 1).

4.1.1.2 Drought stress treatment

Drought stress treatment was performed as described in Heyer *et al.* (2018b, Manuscript 1). Pictures were taken at 0 d, 11 d and 18 d of drought treatment to document the drought stress reaction of the plants. Experiment was repeated three times independently (n = 17). Pictures shown are representative individual plants from one independent repeat.

4.1.2 Results

Magnan *et al.* (2008) reported that CML9 is a negative regulator of the drought stress response in *A. thaliana*. Both tested knock out mutant lines *cml9-1* and *cml9-2* displayed a higher survival rate upon drought stress treatment (Magnan *et al.*, 2008). However, in Heyer *et al.* (2018b, Manuscript 1), the drought stress response of two other knock out mutant lines (*cml9-a* and *cml9-b*) of *CML9* was investigated and both of them displayed a wild type-like drought stress phenotype. To exclude that the different response is due to a secondary effect of the mutant lines used in both studies, I re-investigated the drought stress reaction of *cml9-1* and *cml9-2* under the drought stress conditions published in Heyer *et al.* (2018b, Manuscript 1). As reported for *cml9-a* and *cml9-b*, *cml9-1* and *cml9-2* were as drought stress

tolerant as the corresponding wild type plants. There was no difference neither after 11 d nor 18 d of drought in the phenotype of wild type and mutant plants (Figure 10).

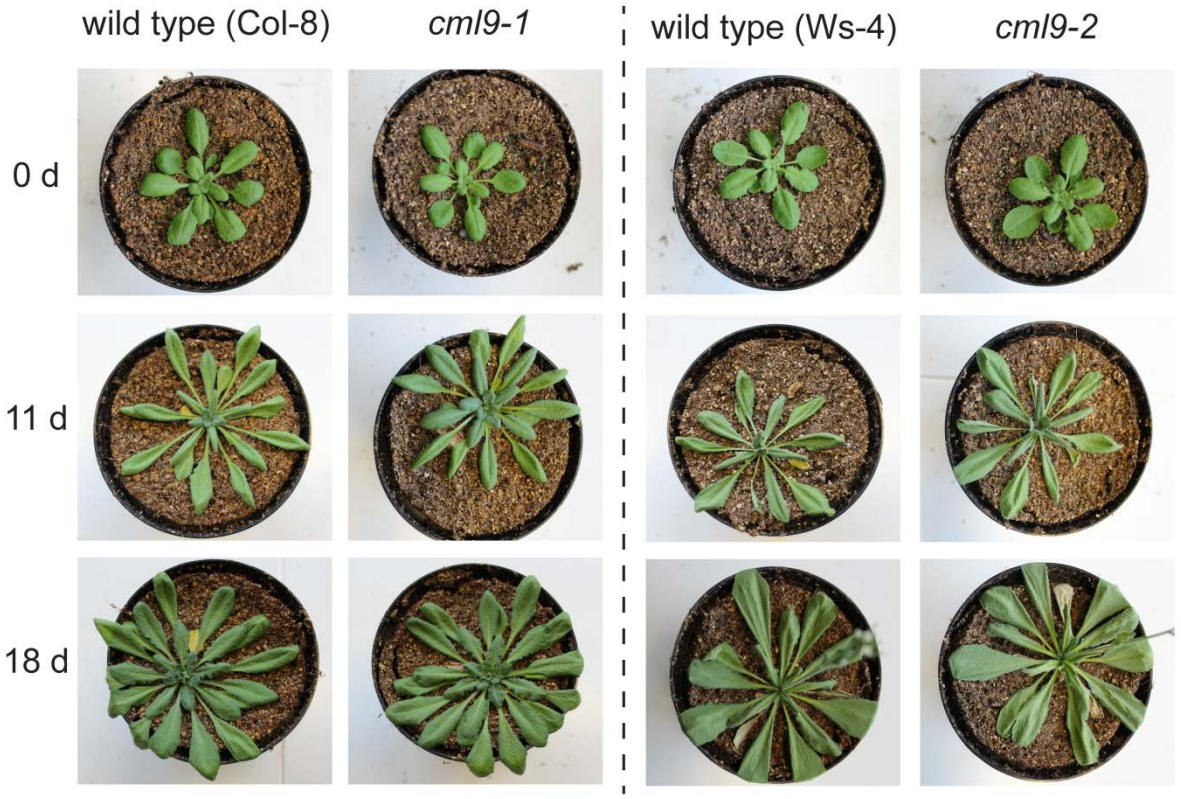

Figure 10: Drought stress phenotype of *cml9-1* and *cml9-2.* *cml9-1*, *cml9-2* and *A. thaliana* ecotype Col-8 and Ws-4 plants after 11 d and 18 d without watering. Untreated plants were used as controls (0 d). Plants were re-watered once after 11 d, when drought stress was applied for 18 d.

4.2 *aca4* is more susceptible to *S. littoralis* feeding

4.2.1 Material and Methods

4.2.1.1 Plant growth and insect rearing

Mutant lines *aca4-1* and *aca4-3* were kindly provided by J. Harper (Boursiac *et al.*, 2010). SALK_108323C was ordered at the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, United Kingdom). *Arabidopsis* ecotype Col-0 was used as control in the larval performance assay. Plants were grown under short day conditions as described for MPI CE Jena in Heyer *et al.* (2018b, Manuscript 1).
S. littoralis larvae were reared from eggs provided by Syngenta Crop Protection AG (Stein, Switzerland) as described in Heyer *et al.* (2018b, Manuscript 1).

4.2.1.2 Semi-quantitative Reverse Transcription (RT)-PCR

RNA isolation, DNA digestion and cDNA synthesis were performed as described in Heyer *et al.* (2018b, Manuscript 1). Primers for *ACA4* were designed using NCBI Primer-BLAST (Ye *et al.*, 2012) and Vector NTI1 Express 1.2.0 software (Thermo Fisher Scientific™, Schwerte, Germany). The following Primers were used: ACA4F 5' - ATGTCGAACTTGCTAAGGGATTTCG - 3' and ACA4R 5' - GGCAGAGTTGGAAGAAGAAGGAC - 3'. *ACTIN2* was used as housekeeping gene. Primers used for *ACTIN2* are published in Vadassery *et al.* (2012a). Phusion® High-Fidelity DNA Polymerase (New England BioLabs Inc., Ipswich, USA) was used, to allow amplification of the whole *ACA4* product. PCR was performed according to the manufacturer's protocol, using 10 mM dNTP Mix (Invitrogen™, Darmstadt, Germany). Since residuals of cDNA synthesis are known to block the Phusion Polymerase, only 12.5 ng cDNA were added to the PCR reaction.

4.2.1.3 Larval performance assays

Larval performance assays were performed as described as insect biomass assay in Scholz *et al.* (2014).

4.2.1.4 Statistics

Larval performance assay was repeated five times independently. For *aca4-3* n = 100 replicates were used and for wild type n = 93. Difference in the gain of weight of larvae feeding on either *aca4-3* or wild type plants were determined by unpaired two-sample Wilcoxon test using R 3.5.1 (R Development Core Team, 2018).

4.2.2 Results

ACA4 is a vacuolar Ca^{2+} pump in *Arabidopsis* that has been shown to be wound-inducible and plays a role in the defense against pathogens (Geisler *et al.*, 2000, Boursiac *et al.*, 2010, Gfeller *et al.*, 2011). Further, it displays a CaM binding domain at its N-terminus. The N-terminus of ACA4 is an auto-inhibitory region, preventing ACA4 to pump Ca^{2+} under normal conditions. Upon a Ca^{2+}-signal, CaMs binds to the N-terminus and activates the ACA function (Geisler *et al.*, 2000, Heven Sze *et*

al., 2000). Previous works have shown that CML37 as well as CML42 interact with ACA4 (Yilamujiang, 2012, Scholz, 2015). To further unravel how ACA4 might interplay with CML37 and CML42 in the regulation of the defense against herbivores, I examined the functional relevance of ACA4 for the defense response.

The knock-out mutant *aca4-3* (Boursiac *et al.*, 2010) was used for larval performance assays. To confirm the full knock out, the absence of the full length *ACA4* transcript was proven by semi-quantitative RT-PCR. There was no full-length *ACA4* transcript in *aca4-3* mutants, whereas the wild type expressed *ACA4* clearly, suggesting that *aca4-3* is a full knock out (Figure 11A). Nevertheless, in *aca4-3* there was a fragment of about 1100 bp detected (Figure 11A). Sequencing revealed that this fragment consists out of exon E1, E4, E5, E6 and E7 of *ACA4*. *ACA4* consists out of 7 exons in total. The T-DNA in *aca4-3* is inserted in the biggest exon, E3 (Figure 11B). Since the fragment consists out of exons before and after the T-DNA insertion, it might be that there was an alternative splicing event, resulting in the deletion of E2 and E3 in the mRNA.

However, although this fragment is expressed, it is highly unlikely that a functional Ca^{2+} pump is still produced in the mutant, if it is processed into a protein. ACAs consist out of 10 transmembrane domains (TMD) in total. TMD1 to TMD6 build the actual Ca^{2+} transport domain, whereas TMD7 to TMD10 are domains to stabilize the structure of the pump (Geisler *et al.*, 2000, Demidchik *et al.*, 2018 and Figure 12C). E3 encodes for the first 4 TMD and partially for the cytoplasmic domains that contain the ATP binding site (nucleotide binding site, N) as well as the domain that is phosphorylated (P) and the phosphatase that dephosphorylates the P-domain again for the next catalytic cycle (actuator domain, A) (The uniprot consortium, 2016, Demidchik *et al.*, 2018 and Figure 11C). Thus the expression of the fragment cannot lead to a functional ACA. Further, *aca4-3* was already successfully used as knock out mutant in other studies and confirmed by other lines (Boursiac *et al.*, 2010). Thus *aca4-3* was used for larval performance assays.

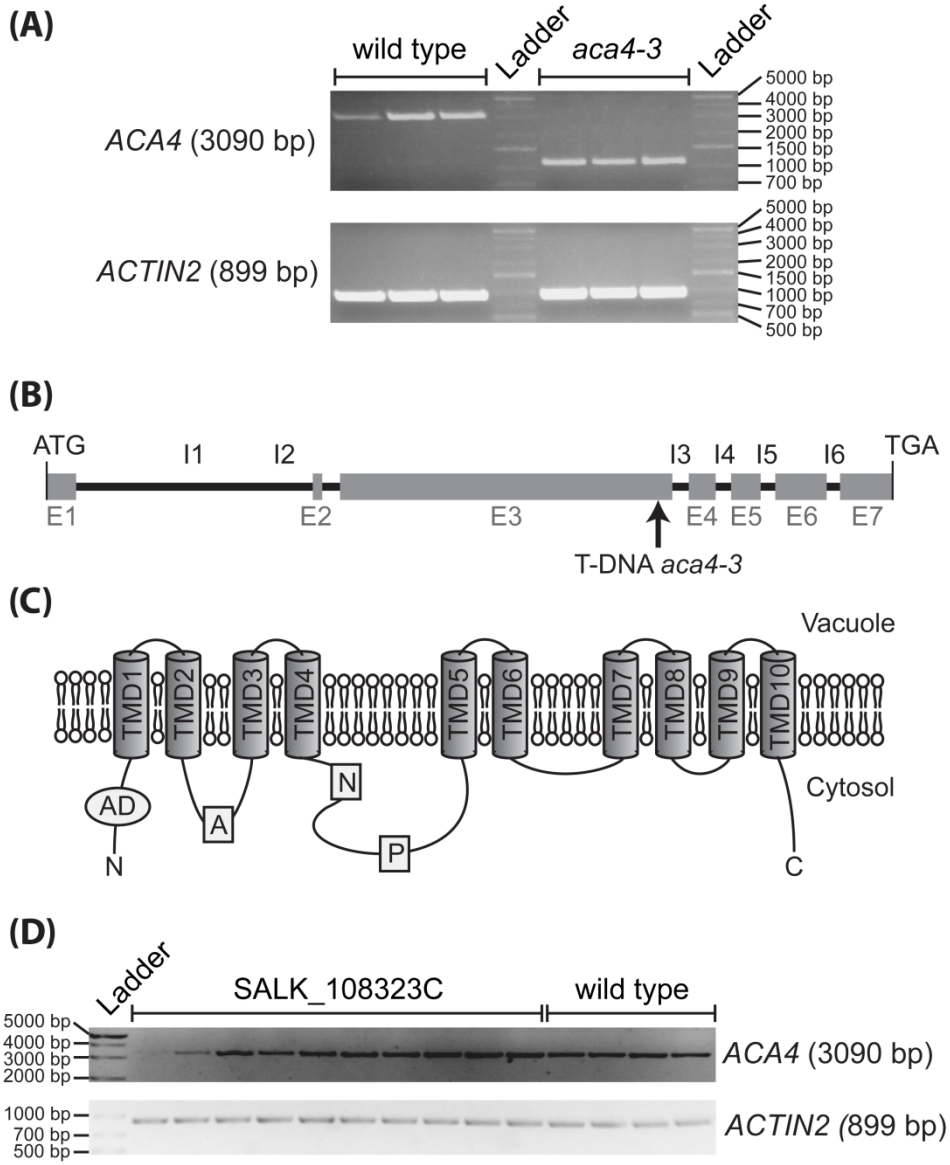

Figure 11. Characterization of the *aca4-3* mutant line. (A) Semi-quantitative RT-PCR amplifying *ACA4* of *aca4-3* and wild type plants using *ACTIN2* as housekeeping gene. Expected product sizes are mentioned behind the product name in brackets. Unexpected fragment is labeled with an asterisk. (B) Schematic overview of the genetic structure of *ACA4*. Exons are marked with E (thick grey bar) and intron with I (thin black bar). T-DNA insertion of *aca4-3* is indicated by the arrow. Scheme was modified after Boursiac *et al.* (2010). (C) Simplified scheme of the ACA4 protein in the membrane modified after Demidchik *et al.* (2018). Transmembrane domains (TMDs) are indicated by grey cylinders. N and C mark the N- and C-terminus respectively. At the N-terminus there is the auto-inhibitory domain including the CaM-binding site (AD), indicated by a circle. The actuator domain (A), the nucleotide-binding domain (N) and the phosphorylation domain (P) are indicated by squares. (D) Semi-quantitative RT-PCR amplifying *ACA4* of SALK_108323C and wild type plants using *ACTIN2* as housekeeping gene. Expected product sizes are mentioned on the left side of the pictures. Color was inverted to enhance visibility of the bands.

After feeding, *S. littoralis* larvae gained significantly more weight on *aca4-3* than on wild type plants (Figure 12). The knock out of *ACA4* positively influenced the larval performance, suggesting that ACA4 is a positive regulator of the defense response against *S. littoralis*.

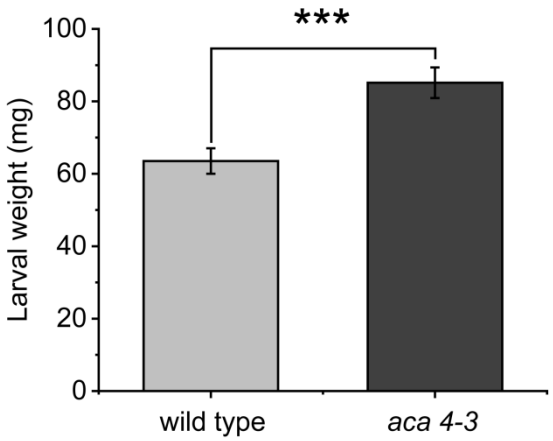

Figure 12. Larval performance on *aca4-3* compared to wild type plants. Bars represent the gain of weight of *S. littoralis* larvae ± standard error after feeding for one week on either *aca4-3* or wild type (*A. thaliana* Col-0) plants. Experiment was started with first instar larvae that were preweighed to reduce variation. Three larvae were placed on each plant. Gain of weight was determined by weighing larvae after one week again. Difference in the gain of weight were determined by unpaired two-sample Wilcoxon test ($p = 0.00001508$) and is indicated by the three asterisks.

In order to proof the results of the *aca4-3* line, two more mutant lines were investigated: the *aca4-1* line (Boursiac *et al.*, 2010) and SALK_108323C (NASC, Nottingham, United Kingdom). However, the SALK_108323C line expressed full length transcript of *ACA4* and thus was not a knock out mutant (Figure 12D). Further, the seed stock of *aca4-1* seems to be to old und thus failed to germinate and could not be used for experiments. In future studies other lines should be employed and further investigated. However, the first results with the *aca4-3* line already indicate that ACA4 might positively influence the defense against *S. littoralis*.

5 Discussion

In order to react appropriately to abiotic and biotic stresses, plants need to perceive and to transduce environmental changes into the respective responses to survive the stress situations. Ca^{2+} and phytohormones are important signals linking the perception of environmental stresses to the appropriate stress response (Chaves *et al.*, 2003, Maffei *et al.*, 2007, Dodd *et al.*, 2010, Mengiste, 2012, Verma *et al.*, 2016). The aim of this study was to gain more mechanistically insights into the connection between these two signaling elements and how they influence downstream stress responses. Thereby, special emphasis was placed on jasmonate dependent stress responses such as the defense against herbivores and necrotrophs, and the ABA mediated drought stress response.

5.1 CMLs – missing links between Ca^{2+} and phytohormone signaling?

The family of CMLs is of great importance for sensing stress-mediated Ca^{2+} signals, as described above. Further, some of them are known to influence downstream phytohormone biosynthesis as well as phytohormone perception (Magnan *et al.*, 2008, Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c, Zhu *et al.*, 2017) and thus might be important linkers between early Ca^{2+} signals and phytohormone responses in the signal transduction after stress. In order to proof this hypothesis, I studied the role of three different CMLs in the response to herbivory, drought and necrotrophic fungi.

5.1.1 CML9 is not a key regulator of jasmonate dependent responses

One of the CMLs investigated here is CML9. CML9 was chosen as a candidate, since it was suggested to modulate SA as well as ABA dependent stress responses (Magnan *et al.*, 2008, Leba *et al.*, 2012). It mediates the response to abiotic stress by influencing the ABA perception (Magnan *et al.*, 2008) and contributes to SA dependent responses in the defense against the biotrophic pathogen *P. syringae* (Leba *et al.*, 2012). In addition, expression of CML9 is induced by both ABA and SA (Magnan *et al.*, 2008, Leba *et al.*, 2012, Heyer *et al.*, 2018b, Manuscript 1 (Figure 1)). However, less was known about the role of CML9 in jasmonate dependent stress responses.

It was shown for two other CMLs, CML37 and CML42, that they are induced upon herbivory and that they also play important roles in regulating the defense against herbivores (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014). Both of them mediate defense responses by altering the jasmonate signaling following herbivore feeding (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014), and thus demonstrate an important link between Ca^{2+} and phytohormone signals. Also CML9 was known to be induced upon treatment with OS of the herbivore *S. littoralis* (Vadassery *et al.*, 2012b). Further, CML9 is inducible by herbivore-related wounding and feeding of the herbivore itself (Heyer *et al.*, 2018b, Manuscript 1 (Figure 1)), suggesting that it might be an important regulator of the defense against herbivores as well.

Thus, in Manuscript 1 (Heyer *et al.*, 2018b) the effect of CML9 on the herbivore defense of *A. thaliana* was intensively studied, employing loss-of-function as well as overexpression mutants. However, *CML9* mutants displayed mostly a wild type-like phenotype to feeding of the herbivores *S. littoralis* and *T. urticae* (Heyer *et al.*, 2018b, Manuscript 1 (Figure 2 and 4)). Also herbivore-induced phytohormones did not differ between *cml9* and wild type lines (Heyer *et al.*, 2018b, Manuscript 1 (Figure 3)), suggesting that CML9 is not a linker between Ca^{2+} and phytohormone signals in herbivore defense.

To further investigate the role of CML9 in other jasmonate mediated stress responses, the functional relevance of CML9 for the defense against the necrotrophic fungus *A. brassicicola* was examined. The *cml9* mutants showed a wild type-like reaction to the fungus treatment (Heyer *et al.*, 2018b, Manuscript 1 (Figure 5)), suggesting that CML9 is not relevant for the defense against the necrotroph. Together the results of the herbivore and the pathogen assays rather exclude a fundamental role of CML9 in jasmonate mediated defense responses. It seems that CML9 has only a very specific role in defense reactions that are more connected to the SA signaling pathway, like the defense against *P. syringae* as shown by Leba *et al.* (2012). However, this influence on SA dependent stress responses does not seem to interfere with jasmonate mediated defenses.

5.1.2 CML9 is not a general regulator of ABA signaling

Magnan *et al.* (2008) described CML9 as an essential modulator of ABA mediated stress responses. Loss-of-function mutants of *CML9* were more tolerant to drought

stress than wild type plants, indicating that CML9 negatively regulates the drought stress response (Magnan *et al.*, 2008). Surprisingly, these results were not confirmed when reexamining the drought stress response of two other *cml9* lines in Manuscript 1 (Heyer *et al.*, 2018b). Both lines investigated, *cml9-a* and *cml9-b*, performed as good under drought stress conditions as the wild type plants and induced similar levels of ABA as the wild type upon drought (Heyer *et al.*, 2018b, Manuscript 1 (Figure 6)), suggesting rather that CML9 is not essential for this stress reaction.

However, in Manuscript 1 (Heyer *et al.*, 2018b) *cml9* mutants with a different ecotype background were used for the drought stress treatments than in Magnan *et al.* (2008). The ecotype background can have an impact on the outcome of a stress performance assay, since there is a natural variation among different ecotypes of *Arabidopsis* concerning their drought tolerance (Bouchabke *et al.*, 2008). To exclude such secondary effects, the drought stress experiment was repeated using the mutant lines published in Magnan *et al.* (2008) and their respective ecotype wild type plants. Nevertheless, there was no difference in the drought stress reaction between the *cml9* mutants and the wild type plants (Figure 10), pointing out once more that CML9 is not relevant for the drought stress reaction in general as before proposed by Magnan *et al.* (2008).

On the other side, it cannot be fully excluded, that CML9 might have regulating functions under certain conditions. Magnan *et al.* (2008) showed, that *cml9* seeds are hypersensitive to ABA, suggesting that CML9 is interfering with the ABA pathway in early developmental stages of the plant. The plants used in the drought assays of Magnan *et al.* (2008) were only three weeks old and thus slightly younger than in the experiments performed here. If CML9 plays a role in ABA signaling only in younger plants it might be one reason for the contradictory results of the two drought assays. In addition also different growth conditions and experimental setup might have led to a different outcome, as discussed in Manuscript 1 (Heyer *et al.*, 2018b). It might be as well that CML9 has other functions that under certain conditions can influence the drought stress tolerance secondarily. However, the additional drought assays performed here indicate that CML9 is not necessarily a regulator of the drought stress response and ABA signaling.

5.1.3 The two antagonists CML37 and CML42 act at the crossroads of JA and ABA signaling

Besides CML9, CML37 and CML42 are Ca^{2+} sensors that were known before to play a role in abiotic stress responses depending on ABA signaling as well as in biotic stress responses depending on jasmonate signaling (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c). In particular they have been shown to modulate the defense against the insect herbivore *S. littoralis* (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014). There, CML37 acts as a positive regulator and CML42 as a negative regulator of the defense and both are able to influence the defense directly *via* modulating the JA signaling (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014). Further, CML42 was known to directly affect defense strategies of the plants against herbivores negatively, like the constitutive content of aliphatic glucosinolates (Vadassery *et al.*, 2012a). Because of the opposite function of both CMLs, one research question of this thesis was, if they both antagonize each other.

To tackle this question, in Manuscript 2 (Heyer *et al.*, 2019) the double knock out mutant line *cml37 x cml42*, generated by crossing the single mutants used in previous studies (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014), was further investigated. The *cml37 x cml42* plants were as susceptible as wild type plants to *S. littoralis* herbivore feeding and exhibited similar levels of jasmonates and constitutive glucosinolates as the wild type (Heyer *et al.*, 2019, Manuscript 2 (Figure 2 - 4)). The wild type-like phenotype of *cml37 x cml42* clearly indicates that the positive effect of *cml42* and the negative impact of *cml37* are balanced out in the double knock out mutant, implying that CML37 and CML42 are actually antagonistic regulators of the defense against this insect. Similarly, the antagonism of two zinc finger proteins was also verified by observing a wild type-like phenotype of the respective double knock out line (Epple *et al.*, 2003).

Moreover, studying the double knock out mutant lines of both CMLs revealed new insights into the interplay of CML37 and CML42 in the defense against *S. littoralis*. Previous studies with the respective single knock out lines proposed a model, where CML42 and CML37 both act on the JA signaling pathway at different positions: Whereas CML37 was shown to directly act on the biosynthesis of OPDA and JA-Ile, CML42 was thought to influence only the JA-Ile perception but not its biosynthesis (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014). However, the induced and constitutive jasmonate levels in *cml37 x cml42* were comparable to those of wild

type plants (Heyer *et al.*, 2019, Manuscript 2 (Figure 3)), indicating that *cml42* can compensate the effects of *cml37* in the double knock out line and thus has an impact on the jasmonate biosynthesis, even though it might be only indirect. The same holds true for the regulation of the constitutive glucosinolate levels. After studying the single knock out lines, only CML42 seemed to have an impact on the constitutive levels of aliphatic glucosinolates, whereas CML37 did not (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014). Nevertheless, *cml37* can compensate this effect of *cml42* in the double knock out line (Heyer *et al.*, 2019, Manuscript 2 (Figure 4)) and thus has an influence on the glucosinolate content. Therefore, a new model of the defense regulation by CML37 and CML42 is predicted here (Heyer *et al.*, 2019 (Figure 8)), where positive effects of CML37 and negative effects of CML42 on the jasmonate and glucosinolate production are balancing each other out and by this fine tune the defense reactions against the herbivore.

Corresponding to their role in the herbivore defense of *A. thaliana*, CML37 and CML42 have similar functions in the defense against the necrotrophic fungus *A. brassicicola* (Heyer *et al.*, 2019, Manuscript 2). Whereas *cml37* is more susceptible to the fungus, *cml42* seems less affected by the pathogen (Heyer *et al.*, 2019, Manuscript 2 (Figure 6)), suggesting that CML37 is a positive and CML42 a negative regulator of the defense against the necrotroph. Further, CML37 and CML42 are acting as antagonists again, since the *cml37 x cml42* displays a wild type-like phenotype upon the pathogen treatment (Heyer *et al.*, 2019, Manuscript 2 (Figure 6)). Because of their known influence on the jasmonate pathway in the herbivore defense and the fact that the defense against necrotrophs is regulated *via* the same phytohormones (Glazebrook, 2005), it is convincing that both CMLs might act on the defense reaction *via* jasmonates here as well. This suggests that these CMLs might play a role in stress reactions that are mediated by jasmonates in general.

On the other side, CML37 and CML42 are also known as regulators of the drought stress response *via* influencing the ABA pathway (Vadassery *et al.*, 2012a, Scholz *et al.*, 2015c). CML37 is positively regulating the ABA elevation upon drought and thus the response to this stress, whereas CML42 is a negative regulator (Vadassery *et al.*, 2012a, Scholz *et al.*, 2015c). Similar to biotic stress responses, CML37 and CML42 act antagonistically in the drought stress regulation as well, as *cml37 x cml42* again displays a wild type-like phenotype and ABA elevation upon drought (Heyer *et al.*, 2019, Manuscript 2 (Figure 7)). Their role in ABA mediated stress

responses might be connected with their function in jasmonate signaling and vice versa. De Ollas *et al.* (2015b) provided evidence that JA-Ile is necessary for the drought stress induced ABA elevation in roots. Since CML37 and CML42 regulate the jasmonate elevation upon herbivory it might be interesting to investigate if this is also the case for drought stress to test whether there is a connection to the regulation of the ABA levels. However, such a dependency between JA-Ile and ABA induction has been only shown for roots and has been excluded for the aerial parts of the plants (Savchenko *et al.*, 2014, De Ollas *et al.*, 2015a). Unfortunately, our knowledge about the function of CML37 and CML42 is restricted to the shoots. Studying their role in the roots might help us to gain more knowledge about the possible dependencies between ABA and jasmonate signaling in drought stress.

On the other side, ABA is also known to be essential for the full defense response against herbivores (Bodenhausen and Reymond, 2007) and has been shown to regulate jasmonate biosynthesis (Wang *et al.*, 2018) and to coregulate jasmonate dependent gene expression (Kazan and Manners, 2013). Thus it might be possible that the effect of CML37 and CML42 on jasmonate signaling upon herbivory is connected to their effect on the ABA pathway. However, previous studies about these CMLs did not record ABA upon herbivory in the single mutants. Collecting those additional data will help us to understand in detail how CML37 and CML42 are influencing both pathways and if they are connected to each other in a signaling network. The fact that CML37 and CML42 have similar functions in both pathways suggests that there might be such connections.

Furthermore since CML37 and CML42 act at the crossroads of these two signaling pathways leading to various stress responses, they might be also important in balancing different needs of the plants if multiple stressors occur.

5.1.4 Interacting partners of CML42 and CML37

Although previous studies and the results obtained here strongly indicate that CML42 and CML37 link Ca^{2+} signaling to the jasmonate and ABA pathway (Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c, Heyer *et al.*, 2019, Manuscript 2), it is still unclear how they are linking Ca^{2+} and phytohormone signals in detail. To answer this question a further investigation of possible CML37

and CML42 interacting proteins was needed. Here, special attention was drawn on their possible targets in the jasmonate pathway.

In previous studies it was shown that CML37 regulates the JA-Ile levels by positively influencing the expression and activity of the JA-Ile biosynthesis enzyme JAR1 (Scholz *et al.*, 2014). On the other hand, it was speculated that CML42 might affect the JA-Ile perception *via* its receptor COI1 (Vadassery *et al.*, 2012a). However, here it was shown that none of the two CMLs interacted directly with JAR1 or COI1 (Heyer *et al.*, 2019, Manuscript 2 (Figure 5)). Nevertheless, preliminary data of yeast two-hybrid experiments indicate that CML42 might influence the jasmonate dependent gene expression by interacting directly with the transcription factor MYC2 (A. Chini, unpublished results/personal communication). Although this interaction has been only shown for CML42 so far, CML37 might interact with MYC2 or other transcription factors controlling jasmonate dependent gene expression as well. Similar to soybean (*Glycine max*) GmCaM1 and GmCML1 (previously named GmCAM4) that are antagonistically regulating the MYB2 transcription factor and by this the salt stress response of the plant (Yoo *et al.*, 2005), CML37 and CML42 might interact both with MYC2 and by this fine tune jasmonate dependent defense gene expression. As MYC2 is in addition controlling the expression of glucosinolate biosynthesis genes (Schweizer *et al.*, 2013) and also ABA dependent gene expression (Abe *et al.*, 2003), an interaction between these CMLs and MYC2 might also explain the effects of these CMLs on the level of glucosinolates and the drought stress response. However, the experiments are still ongoing and these hypotheses needs to be further investigated.

On the other hand, MYC2 cannot be the only interacting protein of the CMLs, since they are also influencing the jasmonate biosynthesis (Scholz *et al.*, 2014, Heyer *et al.*, 2019, Manuscript 2). However, a positive feedback loop, where jasmonates itself control their biosynthesis has been excluded recently (Scholz *et al.*, 2015a). Thus, the effects of both CMLs on the jasmonate biosynthesis cannot be explained by the possible interaction with MYC2 that is downstream of the JA-Ile perception, because that would imply such a positive feedback mechanism. Therefore, CML37 and CML42 might interact with other proteins upstream of JAR1 and by this control the JA-Ile biosynthesis indirectly.

In previous studies it was shown that some CMLs interact with different ACAs (Yilamujiang, 2012, Scholz, 2015, Astegno *et al.*, 2017). Interestingly CML37 as well

as CML42 interact both with ACA4 (Yilamujiang, 2012, Scholz, 2015), a Ca^{2+} pump that controls the uptake of Ca^{2+} ions into the vacuole (Geisler *et al.*, 2000). It is thought that ACA4 plays mainly a role in Ca^{2+} homeostasis after Ca^{2+} increase upon a certain stimulus in regaining the basal Ca^{2+} level in the cell and thus helps to define the Ca^{2+} signal itself (Geisler *et al.*, 2000). Further, ACA4 is known to be wound-inducible and to negatively regulate together with ACA11 the programmed cell death upon pathogen attack (Boursiac *et al.*, 2010, Gfeller *et al.*, 2011). However, nothing was known so far about the roles of ACAs in herbivore defense. Here, it could be shown that ACA4 might play an important role in the defense against the herbivore *S. littoralis*. Similar to the results gained on knock-out plants of its interacting partner CML37, *S. littoralis* larvae performed better on the *aca4-3* mutant than on wild type plants (Figure 12), indicating that the activity of ACA4 is necessary for the full defense response against that herbivore. Since it is known from the *aca4/11* double knock out mutants that their phenotypes concerning the defense against *P. syringae* is dependent on the presence of the phytohormone SA (Boursiac *et al.*, 2010), it might be that there is a connection between ACA4 and jasmonates in the defense against herbivores. Thus, ACAs might link CMLs and phytohormones. Investigation of the phytohormone levels after *S. littoralis* feeding in *aca4-3* will help to gain more insights into that possible connection.

The fact that ACA4 interacts with CML37 as well as with CML42, implies that ACA4 might be an important control point for the antagonism of both CMLs. However, binding of one of the CMLs to the CAM-binding domain would always lead to an activation of the Ca^{2+} pump activity (Tidow *et al.*, 2012, Demidchik *et al.*, 2018). Since a functioning ACA4 is influencing the herbivore defense positively, this raises the question how the two CMLs can antagonistically regulate ACA4. Tidow *et al.* (2012) showed that ACAs have two levels of activation since they possess two CAM-binding domains: In the basal activated state only one of the CAM-binding sites is filled, leading to only low Ca^{2+} transport activity (Tidow *et al.*, 2012). In order to achieve full activity, the second domain needs to be filled by another CAM or CML, allowing a higher transport rate of Ca^{2+} (fully activated state, Tidow *et al.*, 2012). Thus, the activity of the ACA4 might be fine-tuned by CML37 and CML42 by differential binding to the two CAM-binding domains. Different kinetics in binding or also differences in the affinity of both CMLs to the binding site might further

contribute to this fine tuning. Investigating these interactions further will help to understand the interplay of ACA4, CML37 and CML42 in detail.

5.2 Systemic signaling

5.2.1 Phytohormones are systemic signals following the vascular connections between the leaves

Besides local signaling events after herbivore feeding as described above, plants also propagate signals systemically in order to prepare the unfed parts for a possible upcoming attack (Howe and Jander, 2008). Ca^{2+} has been shown to play an important role as such a systemic signal upon wounding and herbivory that is propagated among the *Arabidopsis* rosette *via* the vascular connections between the single leaves (Kiep *et al.*, 2015, Nguyen *et al.*, 2018). As described in previous chapters, there is a strong connection between Ca^{2+} and phytohormones in local stress signaling in plants (e.g. Vadassery *et al.*, 2012a, Scholz *et al.*, 2014, Scholz *et al.*, 2015c, Zhu *et al.*, 2017, Heyer *et al.*, 2019, Manuscript 2). However, the propagation of systemic phytohormone signals after herbivore feeding or wounding was far less understood than in case of Ca^{2+} signals.

Several studies showed before that especially jasmonates accumulate in distal leaves of the plant upon wounding or herbivory (Glauser *et al.*, 2009, Koo *et al.*, 2009, Chauvin *et al.*, 2013, Vos *et al.*, 2013). However, there is still an ongoing debate if these systemic jasmonate accumulations are due to a transport of the phytohormones or by induced *de novo* synthesis in the distal leaves (Koo and Howe, 2009). Throughout the literature there is evidence provided for both theories: Whereas studies in *Nicotiana tabacum* and *Solanum lycopersicum* with labeled compounds show that JA and JA-Ile can be transported (Sato *et al.*, 2009, Sato *et al.*, 2011), a study performed in *Arabidopsis* rather suggest that they are *de novo* synthesized in systemic leaves (Koo *et al.*, 2009). Here, in Manuscript 3 (Jimenez-Aleman *et al.*, 2015) it was shown with the help of a fluorinated compound, that the jasmonate precursor OPC-8:0 is transported among the *Arabidopsis* rosette. The 7F-OPC-8:0 could be found in the locally treated leaf 8, as well as in the distal leaves 5 and 11 that are vascularly connected to leaf 8 (Jimenez-Aleman *et al.*, 2015 (Figure6)). Having verified that 7F-OPC-8:0 behaves similar to the endogenous compound (Jimenez-Aleman *et al.*, 2015 (Figure 3 and 4)), the

translocation study with 7F-OPC-8:0 indicates that the endogenous compound might be transported in a similar way upon wounding. This supports the theory that jasmonates can be transported within the plant and thus that systemic phytohormone elevations are not only due to *de novo* biosynthesis.

The fact that the 7F-OPC-8:0 was translocated to leaves that were vascularly connected to the local leaves raised the question if jasmonates are propagated among the vascular connections similar to Ca^{2+} signals (Kiep *et al.*, 2015). Evidence for such a transport along the vascular connections was also provided by the work of Glauser *et al.* (2009), showing that wounding of leaf 5 and 8 led to a rapid JA increase in leaf 13 but not in the unconnected leaves 12 and 9. Similar results were also shown for JA-Ile (Chauvin *et al.*, 2013). However, in these studies only a few leaves of the *Arabidopsis* rosette were measured, but a study on the whole rosette level similar to those performed for Ca^{2+} signals or electric signals (Mousavi *et al.*, 2013, Kiep *et al.*, 2015) was still missing for jasmonates. Others retraced systemic jasmonate levels only based on reporter genes, without measuring the real phytohormone levels (Mousavi *et al.*, 2013). Furthermore such systemic studies did not exist for other phytohormones like SA or ABA that are interconnected with the jasmonate pathway (Cipollini *et al.*, 2004, Bodenhausen and Reymond, 2007, Vos *et al.*, 2013). Thus, in Manuscript 4 (Heyer *et al.*, 2018a) a holistic study of jasmonates, SA and ABA after herbivore-mimicking wounding was performed.

However, applying the traditional sampling method on a whole rosette scale experiment revealed that already cutting the leaves for sampling purposes induces systemic JA and JA- Ile levels (Heyer *et al.*, 2018a, Manuscript 4 (Figure 1 and 2)). Additional measurements of the time for sampling per rosette (Heyer *et al.*, 2018a, Manuscript 4 (Figure 3)) indicated that these signals were induced within less than 1.6 min, showing that these systemic jasmonate elevations are extremely fast. Similar fast systemic JA and JA-Ile elevations have been reported before by Chauvin *et al.* (2013). However, there leaves were severely wounded and not just cut as in case of sampling. Here, cutting the leaves induced similar systemic JA and JA-Ile signals as severe, continuous wounding (Heyer *et al.*, 2018a, Manuscript 4 (Figure 1 and 4)). This suggests that the systemic JA and JA-Ile signals are not enhanced by further wounding once they are turned on in the single leaves (Heyer *et al.*, 2018a, Manuscript 4, reffered as on-off-system)).

Furthermore, fast sampling induced systemic signals were only recorded on the level of phytohormone measurements and not when employing *JAZ10* as a reporter gene (Heyer *et al.*, 2018a, Manuscript 4 (Figure 6)), indicating the need of a phytohormone analysis based method to investigate systemic phytohormone signaling in detail.

In order to record systemic phytohormone signals without any distortion by sampling induced signals, the freeze-and-cut method was successfully developed in Manuscript 4 (Heyer *et al.*, 2018a). By freezing the whole rosette before sampling, cutting induced systemic signals could be drastically reduced (Heyer *et al.*, 2018a, Manuscript 4 (Figure 7)), allowing the monitoring of systemic phytohormone signals. Using this new method, it was shown that JA and JA-Ile are only induced in leaves that are vascularly connected to the local leaves similar to Ca^{2+} signals (Kiep *et al.*, 2015, Heyer *et al.*, 2018a, Manuscript 4 (Fgure 8)), proving that systemic signaling in plants is carried out *via* the vasculature of the plants as suggested before (Mousavi *et al.*, 2013, Salvador-Recatalà *et al.*, 2014, Gasperini *et al.*, 2015, Kiep *et al.*, 2015, Nguyen *et al.*, 2018). Furthermore, besides the known vascular connections, the results here suggest that the plant also uses additional indirect connections amongst the leaves as those predicted before from the vascular architecture of the rosette (Dengler, 2006, Heyer *et al.*, 2018a, Manuscript 4 (Supplementary Figure S1), referred as third order connections). For instance, leaf 10 was reacting to wounding of leaf 8 (Heyer *et al.*, 2018a, Manuscript 4 (Figure 8)), although following the rules of Dengler (2006), it is not vascularly connected to leaf 8 (see also Fig. 9). Similar results for leaf 10 have been also obtained by Salvador-Recatalà *et al.* (2014). However, leaf 10 shares a connection with leaf 5 and 13 that are vascularly connected to leaf 8. These third order connections might be used by the plant in case of severe wounding, as cutting the midvein, to increase the radius of the systemic reacting leaves and by this enhance the chance to defend them against herbivore feeding as discussed in Manuscript 4 (Heyer *et al.*, 2018a). Also in case of Ca^{2+} signals, the way of wounding had a huge impact on the appearance of systemic signals: Only if the midvein was wounded, there were Ca^{2+} signals in the distal leaves (Kiep *et al.*, 2015). Thus it seems that the plant is indeed reacting with different systemic signal patterns according to the severeness of wounding. Furthermore in case of herbivory also the elicitors in the OS of the herbivore might shape the systemic answer of the plant (Kiep *et al.*, 2015). Although this might be

true for Ca^{2+} signals, this needs to be investigated in further studies for phytohormones. Preliminary trials of such a holistic approach investigating systemic phytohormones upon *S. littoralis* feeding where not successful (data not shown), since the insect needs to be fixed on the local leaf with a cage that in control plants already injured the leaf enough to induce systemic JA and JA-Ile signals on its own. However, there might be the possibility to investigate the influence of OS on the systemic phytohormone patterns by using the so called SpitWorm. The SpitWorm combines mechanical wounding by MecWorm that was used here, with the application of insect oral secretion (Li *et al.*, 2018) and thus allows to discriminate between effects of wounding and effects of OS. Using such a system would help to investigate the impact of OS on systemic phytohormone elevations in future.

In contrast to the clear systemic induction of JA and JA-Ile, neither SA nor ABA nor the jasmonate precursor OPDA was systemically induced upon continuous herbivore-related wounding (Heyer *et al.*, 2018a, Manuscript 4 (Figure 8)). In line with that, they also did not show any sampling-induced systemic effects in the previous experiments with the old sampling method (Heyer *et al.*, 2018a, Manuscript 4 (Figure 1, 2 and 5)), indicating that they do not function as rapid systemic signal upon wounding. Furthermore, since OPDA seems to play only a role in the local wound response (Heyer *et al.*, 2018a, Manuscript 4 (Figure 8)), the rapid systemic JA and JA-Ile signals are independent of free OPDA and thus not due to a full *de novo* biosynthesis as suggested by Koo *et al.* (2009). This strengthens again the theory that jasmonates might be transported directly from the local to the distal leaves. However, as shown in the study of Sato *et al.* (2011) and also in manuscript 3 (Jimenez-Aleman *et al.*, 2015) jasmonates are only transported to a low extent, indicating that the systemic signals are not totally explained by the transport of these compounds. Aside the transport, JA and JA-Ile might be also fast produced from stored precursor molecules like arabidopsides that contain OPDA or dnOPDA (Hisamatsu *et al.*, 2003). Thus, both theories of the origin of systemic jasmonates signals might work together: Partially the phytohormones are transported *via* the vascular connections of the leaves (Heyer *et al.*, 2018a, Manuscript 4), sometimes even as precursor molecules (Jimenez-Aleman *et al.*, 2015, Manuscript 3), but partially they might be also synthesized, although not *de novo*.

5.2.2 GABA as systemic defense

In addition to Ca^{2+} and jasmonates, it was also known that GABA accumulates in leaves that are vascularly connected to the wounded leaf (Scholz *et al.*, 2015b, Scholz *et al.*, 2017). However, similar to phytohormones a holistic approach taking the whole rosette into account was missing for GABA as well. Further, GABA has been shown to accumulate in several plant species rapidly, within minutes after mechanical stimulation in the local leaves (Ramputh and Bown, 1996, Bown *et al.*, 2002), raising the question if GABA might be similar to JA and JA-Ile signals stimulated by sampling already and thus question previous results obtained with the old sampling method (Scholz *et al.*, 2015b, Scholz *et al.*, 2017). However, as shown here in Figure 9 (Manuscript 4, Heyer *et al.*, 2018a) GABA is not induced systemically by leaf cutting within the rosette sampling time of 2.36 min. This result is also in line with previous experiments by Scholz *et al.* (2017), where wounding with a pattern wheel did neither induce local nor systemic GABA elevations within the first hours after wounding. Thus, against the results obtained in other plant species (Ramputh and Bown, 1996, Bown *et al.*, 2002), GABA accumulation in *A. thaliana* seems to require a severe wounding, like obtained here by treatment with the robotic larvae MecWorm.

In accordance with the fact that sampling cannot induce GABA on its own, previous results obtained by Scholz *et al.* (2017) could be verified using the freeze-and-cut method, showing that GABA is accumulating in most of the vascular connected leaves, whereas it does not accumulate in unconnected leaves (Heyer *et al.*, 2018a, Manuscript 4 (Figure 9)). Similar as already shown for the phytohormone experiments, the additional leaf 10 was showing an induced GABA content after MecWorm wounding, supporting the theory of third order connections. Taken together with the results obtained for systemic jasmonate signals, this promotes the theory that systemic signaling in plants happens along the vasculature, as shown before by other studies (Mousavi *et al.*, 2013, Salvador-Recatalà *et al.*, 2014, Gasperini *et al.*, 2015, Kiep *et al.*, 2015, Nguyen *et al.*, 2018).

However, although GABA displays a similar induction pattern after severe wounding as Ca^{2+} and jasmonates signals (Kiep *et al.*, 2015, Heyer *et al.*, 2018a, Manuscript 4), both of them were excluded as trigger for systemic GABA elevations (Scholz *et al.*, 2015b, Scholz *et al.*, 2017). Also the data obtained here with simply cutting leaves implies that systemic GABA and jasmonate signals are differentially

regulated, since GABA is showing a distinct pattern here (Heyer *et al.*, 2018a, Manuscript 4 (Figure9)). Further, also GABA itself has been excluded as systemic signal (Scholz *et al.*, 2017), implying that there might be another jasmonate and Ca^{2+} independent signaling cascade that is able to induce defense responses, such as the accumulation of GABA, in the plant. Since local GABA application can trigger systemic GABA production (Scholz *et al.*, 2017) and GABA can influence anion transporters and thus shape the membrane potential (Ramesh *et al.*, 2015), Scholz *et al.* (2017) suggested that electric signals might be the systemic signal inducing GABA production in distal leaves. On the other side, electric signals were also shown, to precede systemic Ca^{2+} as well as jasmonate signals (Mousavi *et al.*, 2013, Nguyen *et al.*, 2018). Thus, electric signals might be initial for many systemic effects, leading to several independent signaling cascades in distal leaves.

5.3 Outlook

As described above, Ca^{2+} and phytohormones are central signaling compounds in several stress reactions of the plant. In the present thesis it is shown that CMLs as Ca^{2+} sensors act as linkers between Ca^{2+} and phytohormone signals in different stress responses. Hereby especially CML37 and CML42 are important players in the investigated stress treatments. The fact that CML37 as well as CML42 act at the crossroads of ABA as well as jasmonate signaling in similar roles suggest that they might be also important in balancing these different stress responses. In contrast to the greenhouse treatments performed here, plants have to deal with several environmental stimuli at once in nature. A tight balancing of different responses is thus needed to react appropriate and finally to survive. Thus, future studies should include experiments were multiple stressors are applied at once, to untangle how CML37 and CML42 interact with the other signaling components in the signal network behind these stress responses. Also in this case a further investigation of interacting proteins would help to understand the interplay of CML37 and CML42 on the signaling network.

Although this thesis provides strong evidence that CML37 and CML42 are key players in the regulation of jasmonate and ABA dependent stress responses, the wild type-like phenotype of *cml37 x cml42* double knock out mutant also suggests, that there might be other Ca^{2+} sensors that are able to overtake the functions of

CML37 and CML42 in the double knock out. One possible candidate was CML9. However, it could be excluded here that it would overtake the same functions as CML37 or CML42. Other promising candidates might be CML11, CML12, CML16, CML17 and CML23 as they have been shown to be upregulated upon *S. littoralis* OS treatment as well (Vadassery *et al.*, 2012b) or CML24, a touch and ABA regulated gene, known to mediate the salt stress response (Delk *et al.*, 2005, Lee *et al.*, 2005). Further, also other Ca^{2+} sensor families might step in, like CPKs that are known to regulate the herbivore defense or drought stress as well (Kanchiswamy *et al.*, 2010, Xu *et al.*, 2010, Yang *et al.*, 2012, Zou *et al.*, 2015). Future studies might address how different Ca^{2+} sensors interact in the regulation of the plant stress responses.

Further, by developing the freeze-and-cut sampling method a tool to investigate systemic phytohormone signaling is provided here. In contrast to the local signaling pretty less is known about the interplay of different signaling components in the systemic signal transduction. CMLs might play also an important role in linking Ca^{2+} and phytohormone signals in systemic leaves. A further investigation of the systemic phytohormone patterns of *cml* mutants, will unravel the possible systemic role of those Ca^{2+} sensors.

6 Summary

Throughout evolution plants developed a huge variety of protective mechanisms in order to cope with their constantly changing environment. Aside constitutive defenses most of those protective mechanisms are inducible upon occurrence of stress. The inducibility of these defenses implies that plants perceive environmental stress and transduce this perception *via* signal transduction cascades into the appropriate response. Calcium (Ca^{2+}) as second messenger plays an important role in the early signal transduction processes in reaction to many environmental stresses. Furthermore, downstream of Ca^{2+} signals, phytohormones such as jasmonates, abscisic acid (ABA) and salicylic acid (SA) are known to mediate plant stress responses. However, less is known about how these two parts of the signal transduction cascade are interconnected. Ca^{2+} binding proteins, such asCalmodulin-like proteins (CMLs), might be important linkers between these two parts of the signaling cascade, since they are able to sense the Ca^{2+} signals and transfer it into downstream signaling events.

Previous studies already indicated that CML9, CML37 and CML42 are able to regulate abiotic as well as biotic stress reactions. However, the data presented here exclude CML9 as a master regulator of the tested stress responses. It is shown, that CML9 is neither essential for the defense against the herbivores *Spodoptera littoralis* and *Tetranychus urticae* nor the defense against the necrotroph pathogen *Alternaria brassicicola*, that are all mediated by jasmonates. Furthermore, it is demonstrated that CML9 is rather not a regulator of the drought stress response mediated by ABA, as suggested before by another study. Thus, CML9 is neither a key regulator of jasmonate mediated nor of ABA mediated stress responses.

On the other side, this thesis provides insights into the interplay of CML37 and CML42 in controlling stress response. It is demonstrated that both are antagonists in the regulation of the defense against herbivore *S. littoralis* and necrotroph *A. brassicicola* and in the drought stress response. Thereby, CML37 is the positive regulator and CML42 the negative one. Together they balance the herbivore stimulated jasmonate elevation as well as the drought stress induced ABA elevation and thus demonstrate important links to the phytohormone signaling upon these stresses. By fine tuning the phytohormone signaling they are also able to influence downstream defense mechanisms, like the content of glucosinolates that act as feeding deterrents against herbivores.

Further, it was investigated how both CMLs are able to affect jasmonate elevations by interacting with downstream targets. It is excluded that they directly affect the biosynthesis and the perception of the bioactive jasmonate jasmonoyl-isoleucine (JA-Ile), since they neither interact with the enzyme promoting the JA-Ile synthesis (JAR1), nor with its receptor COI1. This suggests that CML37 and CML42 might influence the jasmonate pathway either indirectly by interacting with targets upstream the jasmonate production or directly by influencing other components of the pathway. The autoinhibited Ca^{2+}-ATPase ACA4 might be such an upstream target, since it was shown to interact with both CMLs before. Here evidence is provided that ACA4 is necessary for the herbivore defense and thus might be a target for CMLs that links to downstream signaling events.

Taken together, all these results show clearly that CML37 and CML42 are important Ca^{2+} sensing proteins that are the first step in the signal transduction cascade after Ca^{2+} signals leading to phytohormone signaling and by this to the final defense response. Because of their striking antagonism in different pathways they might be playing a role in the fine tuning of different stress responses.

Besides these local signaling events, also systemic leaves of a plant are reacting to e.g. feeding of an herbivore or wounding. However, these systemic signaling processes are far less understood than the local ones - especially in case of phytohormones. Here it is shown that a jasmonate precursor 8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (OPC-8:0) can be translocated from wounded to distal unwounded leaves, by using a fluorinated compound 7F-OPC-8:0. Furthermore a holistic approach to monitor systemic phytohormone elevations amongst the whole *Arabidopsis* rosette was performed. It is demonstrated that jasmonates are the key phytohormones in rapid systemic signaling upon wounding, whereas ABA and SA do not seem to play a role. This systemic jasmonate signals were extremely fast, taking less than 1.6 min and could be already induced by simply cutting the leaves leading to distorted results using old sampling methods. Thus, a new sampling method where leaves are frozen before sampling was developed. Using this method it could be shown that jasmonic acid (JA) and JA-Ile were only induced in leaves that are directly or indirectly connected *via* their vasculature to the treated leaf, a systemic pattern that has been reported before for Ca^{2+} signals as well. These results support the hypothesis that plants use their vascular system to propagate systemic signals. Further, this systemic jasmonate induction is independent of the

jasmonate precursor 12-oxophytodienoic acid (OPDA), suggesting that systemic JA and JA-Ile signals are not due to *de novo* synthesis. In combination with the results obtained in the translocation study with 7F-OPC-8:0, this suggests that they might be partially transported or synthesized from other precursor forms.

Furthermore, using the new sampling method it was verified that the defense compound γ-aminobutyric acid (GABA) is only induced in systemic leaves with vascular connections to the local leaf. However, in difference to systemic jasmonate signals GABA is not induced by sampling, showing that results of previous studies using the old sampling method are reliable. Furthermore the different induction patterns of jasmonates and GABA provide additional evidence that GABA induction is independent of jasmonate signaling. Nevertheless, GABA is a systemic defense following a systemic signal propagated among the vasculature of the plant, pointing once more out that systemic signaling happens along the vascular system.

Thus, this work provides new insights into the local and systemic stress signaling in plants.

7 Zusammenfassung

Während der Evolution bildeten Pflanzen verschiedene Schutzmechanismen aus, um sich an ihre ständig ändernde Umwelt anzupassen. Neben konstitutivem Schutz verfügen sie auch über Abwehrstrategien, die nur induziert werden, wenn der jeweilige Stress auftritt. Die Induzierbarkeit dieser Mechanismen setzt jedoch voraus, dass die Pflanze die Umweltveränderung wahrnehmen kann und diese Wahrnehmung mittels Signaltransduktionskaskaden in die entsprechende Abwehr umsetzen kann. Der Botenstoff Calcium (Ca^{2+}) spielt in der frühen Signaltransduktion eine wichtige Rolle und wird durch viele Umweltreize induziert. Neben Ca^{2+}-Signalen werden Stressreaktionen außerdem durch Phytohormone wie Jasmonate, Abscisinsäure (ABA) und Salizylsäure (SA) vermittelt. Allerdings ist wenig darüber bekannt wie Ca^{2+}- und Phytohormonsignale miteinander in der Signaltransduktionskette verbunden sind. Ca^{2+}-Bindeproteine, wie z.B. Calmodulin-like Proteine (CMLs), könnten ein wichtiges Bindeglied zwischen diesen zwei Teilen der Signaltransduktion sein, da sie Ca^{2+}-Signale wahrnehmen und dadurch die folgenden Signalprozesse beeinflussen können.

Vorherige Studien zeigten bereits, dass CML9, CML37 und CML42 Reaktionen auf abiotischen sowie biotischen Stress regulieren können. Allerdings schließen die hier gezeigten Ergebnissen CML9 als Hauptregulator der getesteten Stressreaktionen aus. CML9 ist weder für die Abwehr gegen die Herbivoren *Spodoptera littoralis* und *Tetranychus urticae* notwendig noch in der Abwehr gegen den nekrotrophen pathogenen Pilz *Alternaria brassicicola*. All diese Abwehrmechanismen werden durch Jasmonate vermittelt. Des Weiteren wird hier im Gegensatz zu vorherigen Studien gezeigt, dass CML9 kein essentieller Regulator der Trockenstressreaktion ist, die durch ABA gesteuert wird. Daher ist CML9 weder ein zentraler Regulator von Jasmonat- noch von ABA-vermittelten Stressreaktionen.

Andererseits liefert die hier vorliegende Arbeit neue Einblicke in das Zusammenspiel von CML37 und CML42 bei der Steuerung verschiedener Stressreaktionen. Es wird gezeigt, dass beide sowohl die Abwehr gegen den Herbivoren *S. littoralis* und das nekrotrophe Pathogen *A. brassicicola* antagonistisch steuern sowie die Trockenstressreaktion. Dabei ist CML37 immer der positive Regulator und CML42 der negative. Zusammen steuern sie sowohl die durch Herbivorenfraß induzierte Jasmonatausschüttung der Pflanze als auch die durch Trockenstress vermittelte ABA-Erhöhung und sind somit wichtige Verbindungsglieder zu den

Phytohormonsignalen dieser Stressreaktionen. Durch die Feinabstimmung der Phytohormonsignale beeinflussen sie außerdem die entsprechenden Abwehrmechanismen, wie z.B. den Gehalt an Glukosinolaten, welche als Fraßabwehrstoffe gegen Herbivore produziert werden.

Des Weiteren wurden die Interaktionspartner beider CMLs untersucht, um herauszufinden, wie sie die Jasmonatausschüttung kontrollieren können. Da CML37 und CML42 weder physisch mit dem Enzym JAR1, welches die Synthese des bioaktiven Jasmonates Jasmonoyl-Isoleucin (JA-Ile) vermittelt, noch mit dem JA-Ile-Rezeptor COI1 interagieren, kann ausgeschlossen werden, dass sie direkt die Biosynthese oder Wahrnehmung dieses Moleküls beeinflussen. Daher scheinen sie entweder mit Proteinen zu interagieren, welche selbst den Jasmonatsignalweg beeinflussen können, und haben somit nur einen indirekten Effekt auf Jasmonate oder sie beeinflussen andere Komponenten des Jasmonatsignalweges direkt. Die autoinhibitorische Ca^{2+}-ATPase könnte ein möglicher Interaktionspartner sein, der die CMLs mit weiteren Teilen der Signaltransduktionskette verbindet, da zuvor gezeigt wurde, dass beide CMLs mit ACA4 interagieren. In dieser Arbeit wird außerdem gezeigt, dass ACA4 für die Abwehr gegen Herbivore notwendig ist. ACA4 könnte somit ein wichtiges Bindeglied zwischen den CMLs und folgenden Signalen in der Signaltransduktion sein.

Insgesamt deuten all diese Ergebnisse darauf hin, dass CML37 und CML42 wichtige Ca^{2+}-Sensoren sind, die den Anfang in der Signaltransduktionskaskade ausgehend von Ca^{2+}-Signalen zu Phytohormonsignalen bilden und somit die davon abhängigen Abwehrmechanismen steuern. Durch ihren ausgeprägten Antagonismus in verschiedenen Signalwegen könnten sie außerdem eine Rolle in der Feinabstimmung unterschiedlicher Abwehrreaktionen spielen.

Neben der lokalen Signaltransduktion regieren auch systemische Blätter einer Pflanze auf z.B. Herbivorenfraß oder Verwundung. Jedoch sind diese systemischen Signalprozesse weniger gut untersucht als die lokalen, was vor allem auf systemische Phytohormone zutrifft. In der hier vorliegenden Arbeit konnte mit Hilfe einer fluorinierten 8-(3-Oxo-2-(pent-2-en-1-yl)cyclopentyl)-Oktansäure (7F-OPC-8:0) gezeigt werden, dass die Jasmonatvorstufe OPC-8:0 von verwundeten Blättern in unverwundete, distale Blätter transportiert werden kann. Außerdem wurden systemische Phytohormonerhöhungen in einem holistischen Ansatz, bei welchem die gesamte *Arabdiopsis*-Rosette untersucht wurde, erforscht. Dabei konnte gezeigt

werden, dass Jasmonate die zentralen systemischen Phytohormone nach Verwundung sind, wohingegen ABA und SA in der schnellen, systemischen Verwundungsreaktion keine Rolle spielen. Die systemischen Jasmonatsignale sind enorm schnell. Sie kamen bereits nach weniger als 1.6 min auf und konnten bereits durch einfaches Abschneiden der Blätter induziert werden. Dies führte jedoch zu verfälschten Aussagen über die systemische Verteilung der Jasmonate, wenn die gesamte Rosette durch einzelnes Abschneiden der Blätter eingesammelt wurde. Daher wurde eine neue Methode entwickelt, bei der die Rosette vor dem Ernten gefroren wurde. Mit Hilfe dieser Methode konnte gezeigt werden, dass Jasmonsäure (JA) und JA-Ile nur in systemischen Blättern akkumulieren, die indirekt oder direkt über das Vaskularsystem mit dem verwundeten Blatt verbunden sind. Ein ähnliches systemisches Verteilungsmuster wurde bereits für Ca^{2+} beschrieben. Die hier erzeugten Ergebnisse für Phytohormone unterstützen daher die Hypothese, dass Pflanzen ihr Vaskularsystem für die Verbreitung systemischer Signale nutzen. Außerdem ist diese systemische Jasmonaterhöhung unabhängig von dem Jasmonatvorläufer 12-Oxophytodiensäure (OPDA). Daraus folgt, dass die systemischen JA- und JA-Ile-Signale nicht durch *de novo* Synthese entstehen. Zusammen mit den Ergebnissen der Transportstudie mit 7F-OPC-8:0 deutet dieses Ergebnis darauf hin, dass Jasmonate zum Teil transportiert werden oder aus Vorläufermolekülen im systemischen Blatt freigesetzt werden.

Des Weiteren konnte mit Hilfe der neuen Erntemethode verifiziert werden, dass γ-Aminobuttersäure (GABA) nur in Blättern induziert wird, welche vaskulär mit dem verwundeten Blatt verbunden sind. Außerdem wird GABA, anders als JA und JA-Ile, nicht bereits durch das Abschneiden von Blättern induziert, wodurch sichergestellt werden konnte, dass die Ergebnisse vorheriger Studien, die die alte Erntemethode nutzten, ebenfalls verlässlich sind. Diese Unterschiede in der Induzierbarkeit zwischen Jasmonaten und GABA unterstützt außerdem die Theorie, dass die GABA-Induktion nicht durch Jasmonate gesteuert wird. Trotzdem wird GABA durch ein systemisches Signal induziert, welches entlang der vaskulären Verbindungen der Blätter verbreitet wird. Dies unterstützt ein weiteres Mal die Theorie, dass Pflanzen ihr Vaskularsystem zur Signalverbreitung nutzen.

Somit stellt diese Arbeit sowohl neue Einblicke in lokale sowie systemische Signalprozesse zur Verfügung.

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9 Eigenständigkeitserklärung

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

Monika Heyer

10 Curriculum vitae

Monika Heyer

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Work experience

since 10/2014 PhD student in the Research Group Plant Defense Physiology at the Max Planck Institute for Chemical Ecology in Jena

Topic: Linking early calcium signals to phytohormone responses: The role of different Calmodulin-like proteins in various stress reactions of *Arabidopsis thaliana*

08/2012 – 07/2014 Teacher training at Gymnasium Dresden Plauen

11/2010 – 06/2011 State examination thesis project in the Research Group Plant Defense Physiology at the Max Planck Institute for Chemical Ecology in Jena

Topic: Untersuchungen zur Rolle von CML37 in der Herbivorenabwehr von *Arabidopsis thaliana*

09/2008 – 01/2011 Student assistant in the Research Group Plant Defense Physiology at the Max Planck Institute for Chemical Ecology in Jena

04/2008 – 07/2008 Tutor at the Institute for General Botany, Friedrich Schiller University in Jena

Education

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07/2014 Second state examination for teaching profession (Gymnasium) of the Free State of Saxony

06/2012 First state examination for teaching profession (Gymnasium) of the Ministry for Education, Sciences and Culture of Thuringia

10/2006 – 07/2012 Studies of Biology and Philosophy

Publications

06/2019 Heyer, M., Scholz, S. S., Chini, A., Reichelt, M., Kunert, G., Oelmüller, R., Mithöfer, A. (submitted): The Ca^{2+} sensor proteins CML37 and CML42 antagonistically regulate various plant stress responses by altering phytohormone responses, Plant, Cell and Environment

10/2018 **Heyer, M.**, Reichelt, M., Mithöfer, A.: A holistic approach to analyze systemic jasmonate accumulation in individual leaves of *Arabidopsis* rosettes upon wounding. Frontiers in Plant Science, 9(1569).

05/2018 **Heyer, M.**, Scholz, S. S., Voigt, D., Reichelt, M., Aldon, D., Oelmüller, R., Boland, W., Mithöfer, A. (2018). Herbivory-responsive calmodulin-like protein CML9 does not guide jasmonate-mediated defenses in *Arabidopsis thaliana*. PLoS One, 13(5): e0197633.

03/2017 Scholz, S., Malabarba, J., Reichelt, **M., Heyer**, M., Ludewig, F., Mithöfer, A. (2017). Evidence for GABA-induced systemic GABA accumulation in *Arabidopsis* upon wounding. Frontiers in Plant Science, 8(388).

10/2016 Scholz, S., **Heyer, M.**, Vadassery, J., Mithöfer, A. (2016). A role for calmodulin-like proteins in herbivore defense path-ways in plants. Endocytobiosis and Cell Research: journal of the International Society of Endocytobiology, 27(1), 1-12.

12/2015 Jimenez-Aleman, G. H., Scholz, S. S., **Heyer, M.**, Reichelt, M., Mithöfer, A., Boland, W. (2015). Synthesis, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0. Biochimica et Biophysica Acta: BBA Molecular and cell biology of lipids, 1851(12), 1545-1553.

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Conference presentations

Oral presentations

12/2018 *The antagonism of two Calmodulin-like proteins in plant herbivore defense* XXVI Workshop advances in molecular biology by young researchers abroad, Centre for Biotechnolgy, Madrid, ES

09/2017 *Keeping the balance - How two antagonistic calmodulin-like proteins mediate various plant stress responses*
Metabolic Biology seminar, John Innes Centre, Norwich, UK

03/2017 *About keeping the balance – How to antagonistic calmodulin-like proteins mediate various plant stress responses*
16th IMPRS Symposium, Max Planck Institute for Chemical Ecology, Dornburg, DE

10/2016 *Kommunikation in grün*
Annual meeting of Anbaugemeinschaft Bio-Zierpflanzen, ÖKOmene and LVG Heidelberg, Topic: Bio-Zierpflanzen & -Kräuter, Nachhaltiger Gartenbau, Heidelberg, DE

09/2016 *Small herbivore, great performance: Signalling and its counteraction after feeding of* Tetranychus urticae *on* Arabidopsis *plants*
ICE Symposium, Max Planck Institute for Chemical Ecology, Jena, DE

12/2015 *Taking the CML forward: New stories and results about CML9*
Kick-off meeting for the Max-Planck-Partner-Group, National Institute of Plant Genome Research NIPGR, Delhi, IN

Poster presentations

09/2017 **Heyer M.**, Scholz S., Reichelt M., Vadassery J., Oelmüller R., Boland W., Mithöfer A. *About keeping the balance - How two antagonistic calmodulin-like proteins mediate various plant stress responses*. Plant Calcium Signalling Conference 2017, John Innes Centre, Norwich, UK

12/2016 **Heyer M.**, Scholz S., Reichelt M., Vadassery J., Oelmüller R., Boland W., Mithöfer A. *Two antagonistic calmodulin-like proteins modulate various plant stress responses*. SAB Meeting 2016, Max Planck Institute for Chemical Ecology, Jena, DE

06/2016 **Heyer M.**, Scholz S., Reichelt M., Vadassery J., Boland W., Mithöfer A. *Two antagonistic calmodulin like proteins modulate plant defense against insect herbivores*. 4th International Symposium on Plant Signaling & Behavior 2016, St. Petersburg, RU

02/2016 **Heyer M.**, Reichelt M., Boland W., Mithöfer A. *The calcium sensor protein CML9 – A defense regulator in plants?*. 15th IMPRS Symposium, Max Planck Institute for Chemical Ecology, Dornburg, DE

09/2015 **Heyer M.**, Scholz S., Vadassery J., Reichelt M., Boland W., Mithöfer A. *Differentially expressed calmodulin-like proteins regulating defense against insect herbivores*. ICE Symposium, Max Planck Institute for Chemical Ecology, Jena, DE

02/2015 **Heyer M.**, Vadassery J., Boland W., Mithöfer A. (2015). *CML9 - A calcium sensor protein involved in plant defense?*. 14th IMPRS Symposium, Max Planck Institute for Chemical Ecology, Dornburg, DE

Advanced Training and Workshops

01/2019	Plant Morphometry, IMPRS Max Planck Institute for Chemical Ecology, Jena
10/2018	Project Management, Graduate Academy Friedrich Schiller University Jena
02/2018	The basics of light and fluorescence microscopy, IMPRS Max Planck Institute for Chemical Ecology, Jena
06/2017	Fundamentals of Mass Spectrometry, IMPRS Max Planck Institute for Chemical Ecology, Jena
04/2017	Adobe Illustrator, IMPRS Max Planck Institute for Chemical Ecology, Jena
08/2016 – 09/2016	Introduction to Basic Statistics and R, IMPRS Max Planck Institute for Chemical Ecology, Jena
05/2016	Scientific Writing and Publishing for Natural Scientists - the Basics, Graduate Academy Friedrich Schiller University Jena
03/2016	NMR course, IMPRS Max Planck Institute for Chemical Ecology, Jena
03/2016	Grant Proposal Writing, IMPRS Max Planck Institute for Biogeochemistry, Jena
02/2016	Leadership Skills in Academia and Industry, Graduate Academy Friedrich Schiller University Jena
03/2015	R course - The Basics, IMPRS Max Planck Institute for Biogeochemistry, Jena
03/2015	Gene Safety Project Leader Certificate, JSMC Jena
03/2015	Rules for good scientific practice, IMPRS Max Planck Institute for Biogeochemistry, Jena

Collaboration visits

12/2016 – 01/2017	Max-Planck-Partner-Group, National Institute of Plant Genome Research NIPGR, Delhi, IN

Committee work

10/2016 – 05/2019	PhD representative of the Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena

Teaching experience

09/2017	Supervision internship of Zoe Heckhausen (Bachelor student), Max Planck Institute for Chemical Ecology, Jena
May 2016	Training for Gymnasium teachers: Pflanzen sind langweilig? - Ein großer Irrtum!, Max Planck Institute for Chemical Ecology, Jena
11/2015 – 12/2015	Supervision internship of Thomas E. Burks (Master student), Max Planck Institute for Chemical Ecology, Jena
05/2015 – 06/2015	Supervision internship of Anja Meents (Master student), Max Planck Institute for Chemical Ecology, Jena
08/2012 – 07/2014	Teacher training at Gymnasium Dresden Plauen
10/2010	Teaching internship at Goethe Gymnasium, Weimar
04/2008 – 07/2008	Tutor at the Institute for General Botany, Friedrich Schiller University in Jena
03/2008 – 04/2008	Teaching internship at Ernst-Abbe-Gymnasium, Jena

Skills

IT

MS-Office	■■■■□
Endnote	■■■■□
R	■■■□□
Sigmaplot	■■■□□
Origin	■■■■□
Adobe Illustrator	■■■□□

Languages

German	■■■■■
Englisch	■■■■□

11 Acknowledgement

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