



A role for calmodulin-like proteins in herbivore defense pathways in plants

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During their lifetime, plants need to adapt to various stimuli originating from the abiotic and biotic environment, some of which represent stress factors. One major biotic stress factor is the attack of herbivorous insects feeding on the plant. But plants are not defenseless; they are equipped with an arsenal of different defense layers. Additionally to mechanical barriers, which are a first line of defense, the plant can produce a variety of chemicals like toxic secondary metabolites, anti-herbivory proteins or compounds involved in indirect defense. The production of many of these defensive compounds is triggered by HAMPs, elicitors in insects' oral secretions (OS), which come in contact with the wounded plant tissues while insects are feeding. The early events upon perception of these stimuli are still poorly understood. Elevations in cytosolic calcium are one of these early events, which activate the downstream defense signaling network, including certain phytohormones. To reach this, a proper decoding of calcium signals by different calcium sensor proteins is important. In *Arabidopsis thaliana*, several members of the calmodulin-like proteins (CMLs), one group of calcium sensor proteins, is induced upon treatment with OS of the generalist herbivore *Spodoptera littoralis*. Some of these CMLs are involved in herbivore defense by modulating the jasmonate pathway.

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Plant defense strategies against insect herbivory

During their lifespan, plants have to deal with a multitude of stress factors originating from the abiotic as well as the biotic environment. Main abiotic environmental cues influencing the plants performance and fitness include drought and

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

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

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

Mechanical defenses

The plant's mechanical defenses are the first layer of defense that a herbivorous insect encounters while feeding on them.

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

Chemical defenses

A. thaliana processes a huge arsenal of inducible chemical herbivore defense mechanisms which contribute to direct and indirect defense by influencing the insect's feeding behavior and fitness. One well studied indirect defense of *Arabidopsis* plants is the emission of VOCs after herbivore attack (Van Poecke 2007). Main components of VOCs are the fatty acid derivative (*Z*)-3-hexenyl acetate (Hex-Ac), the phenolic compound methyl-salicylate (MeSA) and the monoterpene linalool (Dicke et al. 1990). The blend of volatiles differs in *Pieris rapae* infested and undamaged plants and functions as attractant for parasitic wasps like *Cotesia rubecula*, which are specifically predated on *P. rapae* caterpillars (Van Poecke et al. 2001).

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

Plants of the family Brassicaceae (like *Arabidopsis*) mainly store glucosinolates which are nitrogen- and sulfur-containing compounds (Halkier and Gershenzon 2006), (Figure 1A). These can be classified upon their biosyntheses into aliphatic (mainly produced from methionine) and aromatic (produced from tryptophan, phenylalanine and tyrosine) glucosinolates (Glawischnig et al. 2003; Halkier and Gershenzon 2006; Bidart-Bouzat and Kliebenstein 2008). The glucosinolates are not toxic per se, but upon hydrolysis by a myrosinase, toxic products like nitriles, thiocyanates and isothiocyanates are formed. In undamaged *Arabidopsis* leaves, glucosinolates and myrosinase are stored spatially separated. When an herbivorous insect is chewing on a leaf,

both components are mixed together; the toxic degrading products are formed and can act as feeding deterrent (Burrow et al. 2006; Wittstock and Burrow 2010; Schramm et al. 2012). Glucosinolates are not uniformly distributed in all leaves of an *Arabidopsis* plant (Shroff et al. 2008; Shroff et al. 2015) and are induced upon herbivore feeding (Textor and Gershenzon 2009). Insects that feed on *Arabidopsis* plants developed different detoxification mechanisms to deactivate the glucosinolate breakdown products. *S. littoralis* larvae detoxify isothiocyanates by forming conjugates with amino acids or glutathione which are further hydrolyzed (Schramm et al. 2012).

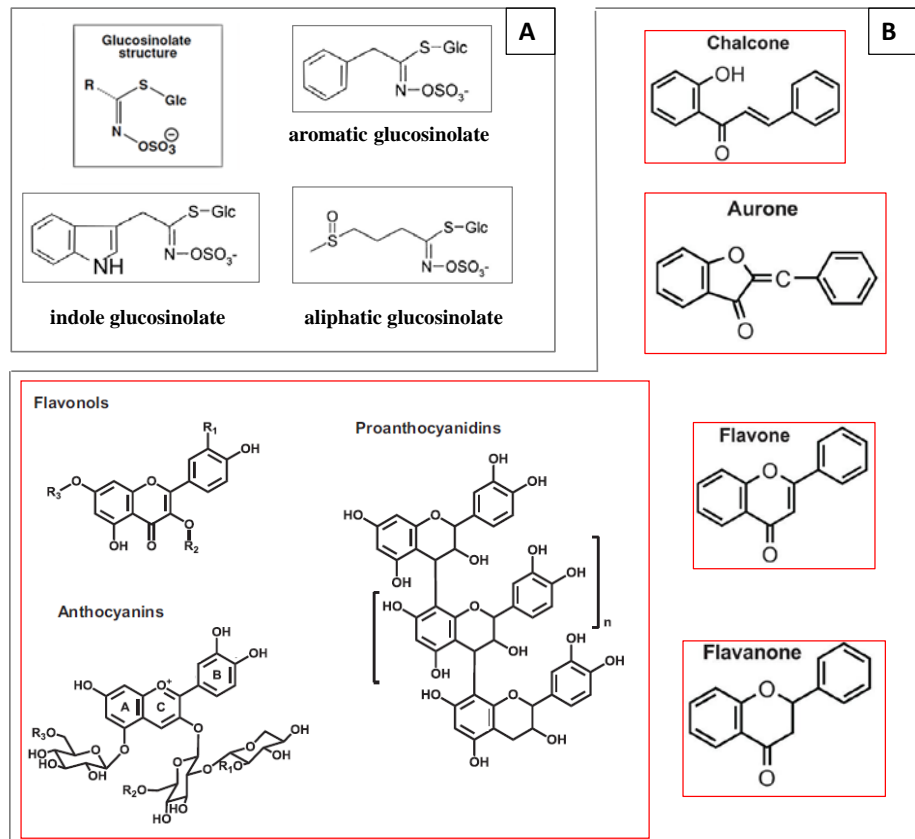


Figure 1: General structures of secondary metabolites in *A. thaliana*. Shown are the structures of glucosinolates (A) and flavonoids (B) occurring in *A. thaliana*. (A) Shown are the basic core structure of glucosinolates (encircled) and the major groups of glucosinolates detected in *A. thaliana* (adapted from Halkier and Gershenzon 2006; Van Poecke 2007). (B) Shown are the groups of flavonoids detected in *A. thaliana* (adapted from Falcone Ferreyra et al. 2012; Saito et al. 2013).

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

Anti-herbivore proteins

Many defense compounds like anti-insect proteins produced by the plant act directly on the metabolism or development of feeding insects. So are by the plant produced protease inhibitors (Molina-Rueda et al. 2015) able to disturb the digestion of ingested food material (Green and Ryan 1972) and, as a consequence of this, to slow down the development of the insect (Ryan 1990). Well studied defensive proteins produced by Arabidopsis are the translated products encoded of JA-responsive genes *VSP2*, *THI2.1* and *PDF1.2*. In previous studies it was demonstrated that *VSP2* (VEGETATIVE STORAGE PROTEIN2) is induced by wounding, methyl jasmonate, insect feeding, and phosphate deprivation. The *VSP2* protein shows phosphatase activity in acid pH range corresponding to the pH of insect gut lumen. Here, *VSP2* could significantly delay development of the insects and increase their mortality (Berger et al. 1995; Liu et al. 2005). The expression of *VSP2* could also be inhibited by neomycin application in Arabidopsis (Vadassery et al. 2014). Another JA-responsive gene induced by wounding of plant tissue and methyl jasmonate is *THI2.1*, encoding the antimicrobial protein thionin which might also contribute to herbivore defense (Epple et al. 1997; Bohlmann et al. 1998; Vignutelli et al. 1998). *PDF1.2*, encoding another defensin in Arabidopsis is also activated upon methyl jasmonate and Spodoptera feeding (Manners et al. 1998; De Coninck et al. 2010; Kan-chiswamy et al. 2010).

GABA as possible player in plant-herbivore defense

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

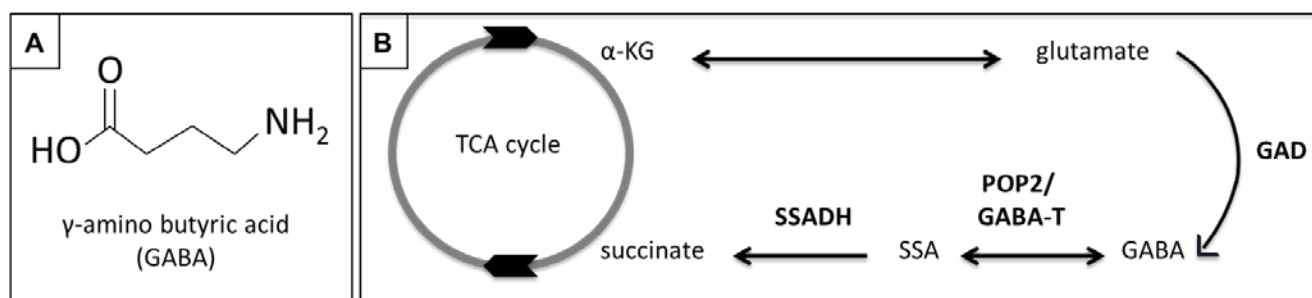
than is exported from mitochondria or oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH) (Breitkreuz et al. 2003; Ludewig et al. 2008; Michaeli et al. 2011).

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

Plant-herbivore interaction

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

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



GABA is mainly produced by decarboxylation of L-glutamate catalyzed by glutamate decarboxylases (GADs) in the cytosol (Turano and Fang 1998; Zik et al. 1998). The catabolism of GABA into alanine and succinic semialdehyde (Figure 2B) is localized in the mitochondrial matrix, where a GABA transaminase (GABA-T) removes the amino group of GABA and transfers it onto pyruvate. The succinic semialdehyde

Figure 2: Structure, syntheses and metabolism of γ -amino butyric acid (GABA, adapted from Ludewig et al. 2008). Shown is the molecular structure of the non-protein amino acid GABA (A) and the metabolism and catabolism of GABA (B). GAD glutamate decarboxylase, GABA-T GABA transaminase, SSA succinic semialdehyde, SSADH succinic semialdehyde dehydrogenase.

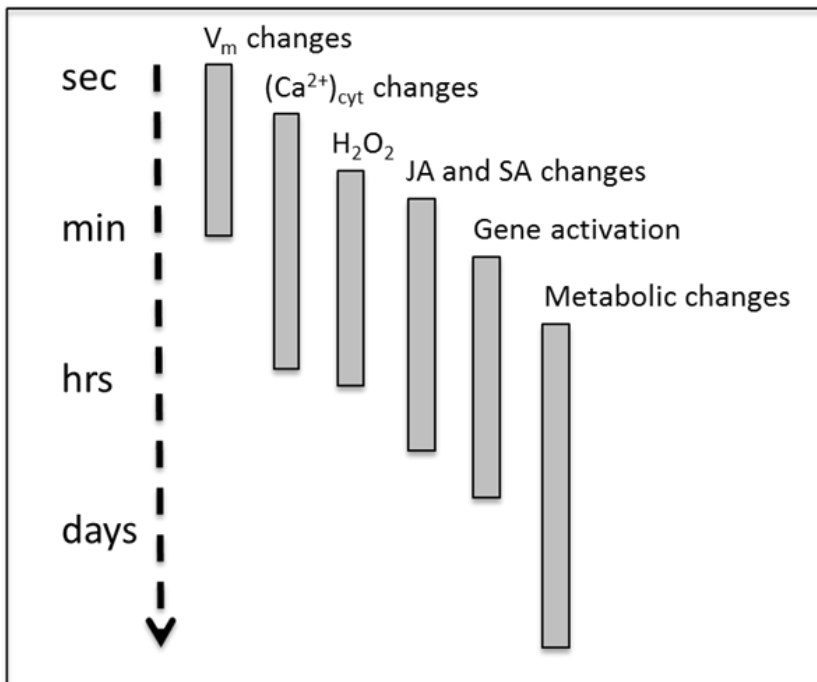


Figure 3: Early events in plant-herbivore interaction (adapted from Maffei et al. 2007a). Shown are the first steps of plant herbivore perception, which occurs in the first minutes and hours after attack. After the stimulus perception, the membrane depolarizes and initiates a spike in the cytosolic Ca^{2+} level. This activates a signaling cascade which leads among others to ROS production and the accumulation of phytohormones. These induce the expression of responsive genes and transcripts which in turn can modulate the plants metabolism.

Herbivore-associated molecular patterns (HAMPs) and receptors

The plant recognizes attacks by perception of different molecular patterns (MPs), which are associated with the outer surface or released components of the aggressor (Taylor et al. 2004; Ausubel 2005; Mithöfer and Boland 2008). It is known that the conserved microbe-specific molecules, referred to as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs), are recognized by pattern recognition receptors (PRRs). MAMPs like flagellin (Flg22), elongation factor Tu (EF-Tu), peptidoglycan (PGN), lipopolysaccharides (LPS), Ax21 (Activator of Xa 21-mediated immunity in rice), fungal chitin, and β -glucans from oomycetes are recognized by plant surface localized PRRs (Jones and Dangl 2006; Newman et al. 2013; Ranf et al. 2015). Herbivore-associated molecular patterns (HAMPs) are postulated to be present in insect oral secretions and are of two kinds: (i) chemical elicitors derived from insect oral secretions and oviposition fluids; and (ii) plant-derived self-recognition factors, DAMPs (damage-associated molecular patterns) occurring due to a specific pattern of wounding (Mithöfer and Boland 2008; Heil and Land 2014). Insect OS contain elicitors, notable examples are inceptins, which are peptides formed as proteolytic products of plant chloroplastic ATP synthase formed in caterpillar midgut, and fatty acid-amino acid conjugates (FACs) such as volicitin in maize (Alborn et al. 1997).

Upon herbivore attack, the plants encounter two main components of herbivore feeding: the wounding of plant tissue and recognition of elicitors in OS. HAMPs include the oral secretion of the larvae composed of saliva and regurgitant,

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

Membrane depolarization

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

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

The calcium ion (Ca^{2+}) plays an important role as a second messenger in varied signaling networks of plant cells (Dodd et al. 2010). Plant cells maintain a level of 100-200 nM free cytosolic calcium $[\text{Ca}^{2+}]_{\text{cyt}}$, the so called Ca^{2+} homeostasis. This incident is due to the fact that high concentrations of cytosolic Ca^{2+} have a cytotoxic effect on phosphate-containing components, including proteins and nucleic acids. To maintain this low level of Ca^{2+} in the cytosol, several active transporters like Ca^{2+} -ATPases (ACAs) located in organelle- and cell membranes pump the Ca^{2+} into the stores (Sze et al. 2000). The Ca^{2+} is stored in high concentrations (105 times higher than cytosolic concentration) in different intra- and extracellular stores. While the apoplast serves as external calcium store, different organelles like the vacuole or chloroplasts store Ca^{2+} inside the cell (Knight et al. 1996; Peiter 2011; Stael et al. 2011). This high gradient of Ca^{2+} concentrations is the basis for a fast response to stress stimuli. Here an

influx of Ca^{2+} from the stores into the cytosol induces a calcium signature, whose specific shape, amplitude and duration encode the information perceived (Lecourieux et al. 2006; McAinsh and Pittman 2009; Dodd et al. 2010). To achieve a specific decoding of Ca^{2+} signals both in the nucleus and the cytosol, the plant processes an arsenal of different calcium sensor proteins (DeFalco et al. 2010).

Calcium sensors

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

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

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

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

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

Calmodulin-like proteins

CMLs are one class of calcium sensor proteins, which act as sensor relays where they are propagating the Ca^{2+} signal. To

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

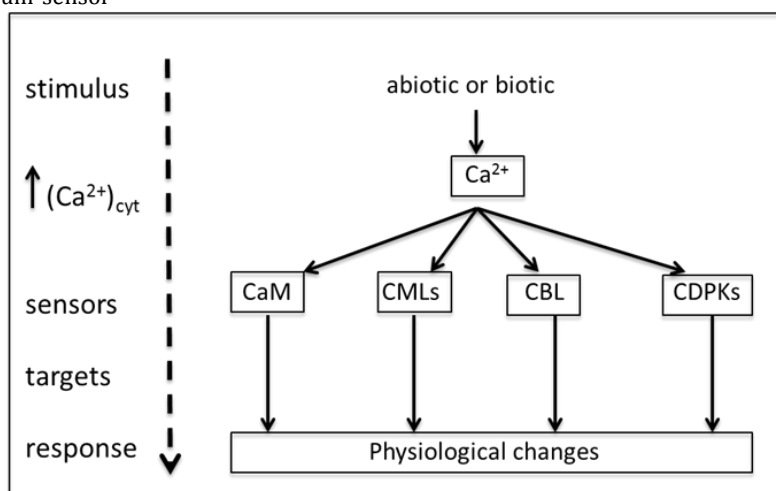


Figure 4. Different classes of calcium sensor proteins activated upon abiotic and biotic stimuli (adapted from DeFalco et al. 2010). Shown are the classes of Ca^{2+} sensor used to decode cytosolic Ca^{2+} spikes induced by diverse stimuli. Here, calcium sensor proteins function as signal relays (CaM/CMLs and CBLs) or primary responders (CDPKs).

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

It was demonstrated that the expression of CMLs is induced by diverse abiotic as well as biotic stimuli. So is *CML8* induced by SA and salt stress (Park et al. 2010). *CML9* is also induced by SA as well as by infection with *P. syringae* and can alter plant responses to ABA and abiotic stress (Magnan et al. 2008; Leba et al. 2012). *CML24* modulates ABA level during ion stress, regulates pollen tube growth and can induce changes in flowering time (Delk et al. 2005; Hubbard et al. 2008; Yang et al. 2014). Additionally it was shown that expression of *CML37*, *CML38* and *CML39* are regulated by salt- and drought stress, phytohormones and *P. syringae* infection

(Vanderbeld and Snedden 2007) and CML42 is involved in trichome branching (Dobney et al. 2009). Recently, it was shown that one member of the CML-family, CML42, is involved in *A. thaliana* defense against *S. littoralis* herbivory.

CML42 acts as a negative regulator of plant defense against herbivory and affects JA perception of the plant. CML42 gene expression is herbivore elicitor-specific and is not activated upon mechanical wounding (Vadassery et al. 2012a). It was additionally observed that the gene expression of eight CMLs is induced by insect OS (Figure 5, arrows, Vadassery et al. 2012b; Scholz et al. 2014). In contrast,

CML37 was identified as positive regulator of plant defense against herbivory. This CML is strongly induced by mechanical wounding and was shown to downregulate the most bioactive form of jasmonates, the isoleucine-jasmonic acid conjugate JA-Ile (Scholz et al. 2014). The exact position of CMLs in the signaling cascade and the further processing of the signal by target proteins are still unknown.

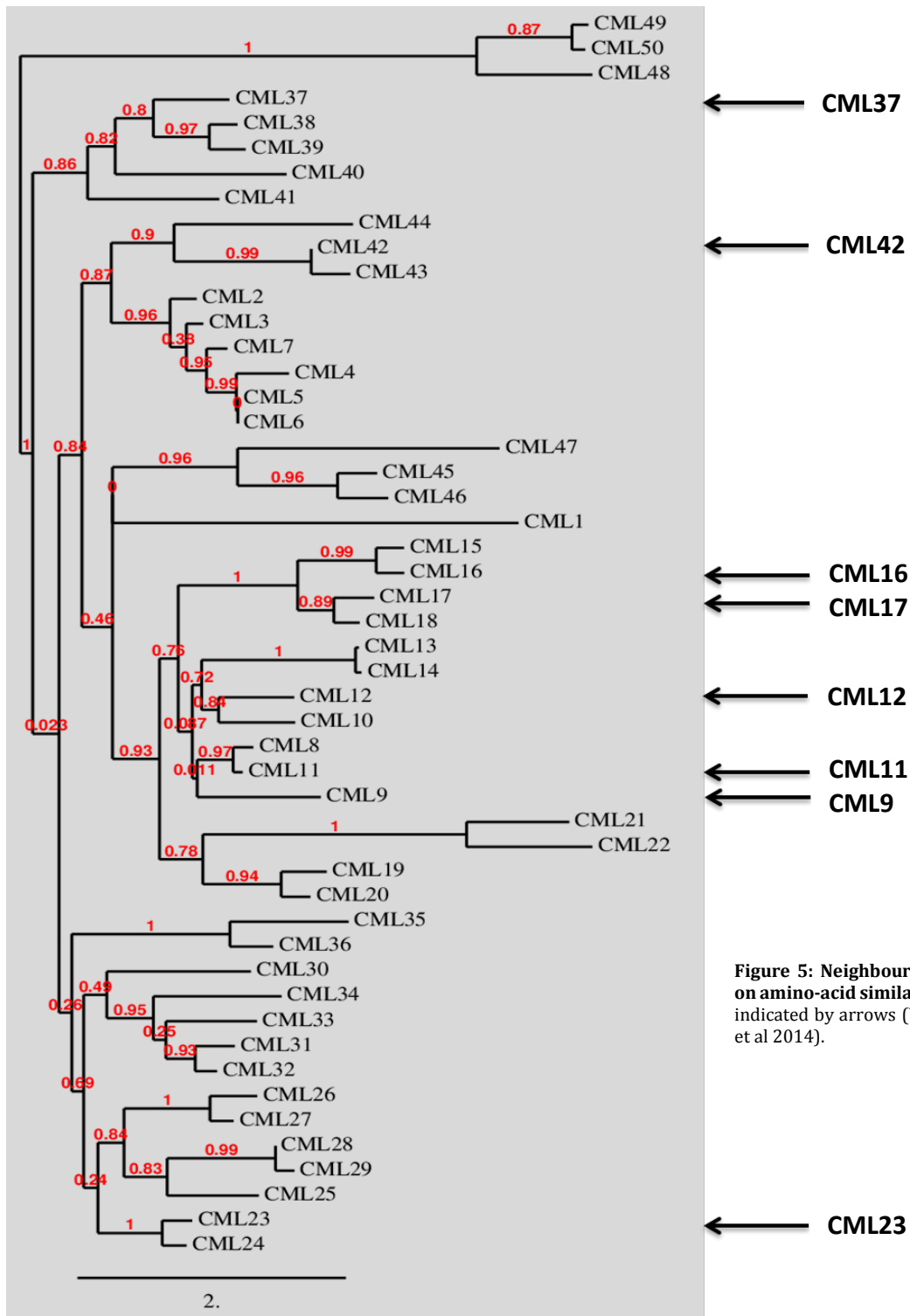


Figure 5: Neighbour-joining tree of CMLs based on amino-acid similarities. CMLs induced by OS are indicated by arrows (Vadassery et al. 2012b; Scholz et al 2014).

Downstream signaling

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

The production of ROS, which include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), and hydroxyl radical ($\cdot OH$), is well studied as a part of plant response to pathogens (Lamb and Dixon 1997). In recent studies it became clear that ROS production is also involved in herbivore defense. *Medicago truncatula* plants accumulated ROS only after herbivory while wounding did not induce ROS production (Leitner et al. 2005). In lima bean plants (*Phaseolus lunatus*) it was similarly shown that the production of ROS after herbivory was much higher than that after mechanical wounding alone (Maffei et al. 2006). So showed soybean plants challenged with *Helicoverpa zea* an elevated lipid peroxidation and OH radical formation (Bi and Felton 1995).

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

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

In plant defense against herbivory, the most important and most studied class of phytohormones is the one of JAs (Wasternack 2007). JAs are lipid-derived molecules originating from plastid membrane-bound α -linolenic acid. The JA biosynthetic pathway is well understood and the enzymes participating in it are well characterized (Vick and Zimmerman 1984; Schaller and Stintzi 2009). In the chloroplast, the released α -linolenic acid is metabolized in several steps to form OPC-8:0 followed by *cis*-OPDA, which is catalyzed by

lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). After a translocation to the peroxisome, the *cis*-OPDA is further processed to form JA (Schaller and Stintzi 2009). The active phytohormone JA-Ile is formed by a conjugation of JA and the amino acid isoleucine catalyzed by JASMONATE RESISTANT1, JAR1 (Staswick and Tiryaki 2004). Activation of the receptor complex SCF-CO11 by JA-Ile (Figure 7) triggers the degradation of JAZ proteins, the transcriptional repressors of JA responsive genes. This removal of repression leads to activation of the transcription factor MYC2 and the expression of anti-insect JA-responsive genes including *PDF1.2*, *Thi2.1* and *VSP2* (Wasternack and Kombrink 2010). Mutants of receptor CO11 like *coi1-1* and *coi1-16*, *jar1* and *jaz1* show higher susceptibility to herbivore feeding (Feys et al. 1994; Chung et al. 2008; Westphal et al. 2008; Chung et al. 2009; Abe et al. 2013).

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

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

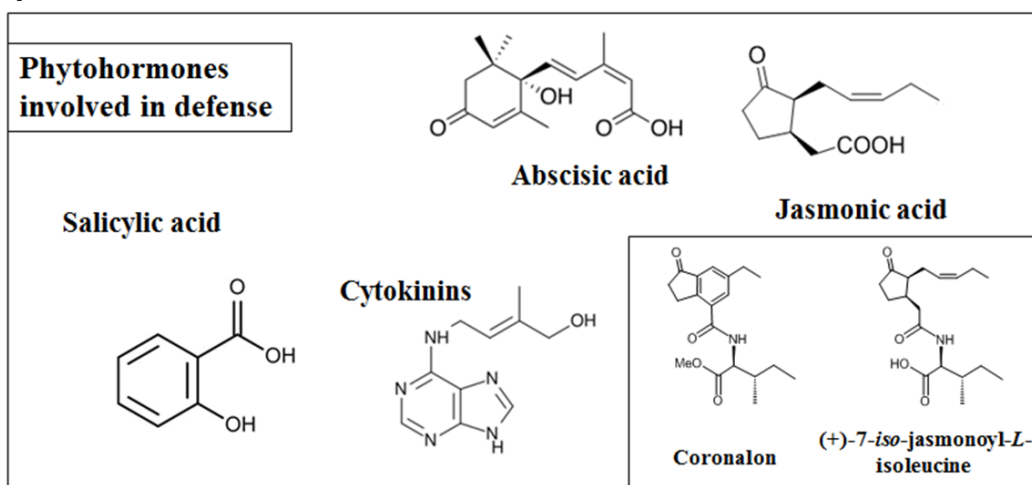


Figure 6: Structure of different phytohormone classes. Shown are the basic structures of phytohormone classes and the structural JA-Ile mimic coronalon in comparison to JA-Ile itself (inlet).

A major player in adaptation to abiotic stress stimuli is the sesquiterpenoid abscisic acid (ABA), which is mediating resistance to salt, drought and cold stress by regulation of stomata closure (Zhu 2002). ABA is also involved in embryo maturation, seed dormancy, germination, cell division and elongation (Finkelstein 2013). Interestingly it was shown, that ABA is also involved in plant defense signaling. Here the

complex interplay between ABA and JA-ethylene signaling pathways can regulate plant defense (Anderson et al. 2004).

All these examples demonstrate that herbivore-defense in *Arabidopsis thaliana* is provided by different pathways interacting in a complex signaling network. The complexity of plant defense ensures survival of the plant and hampers rapid adaptation of feeding herbivores.

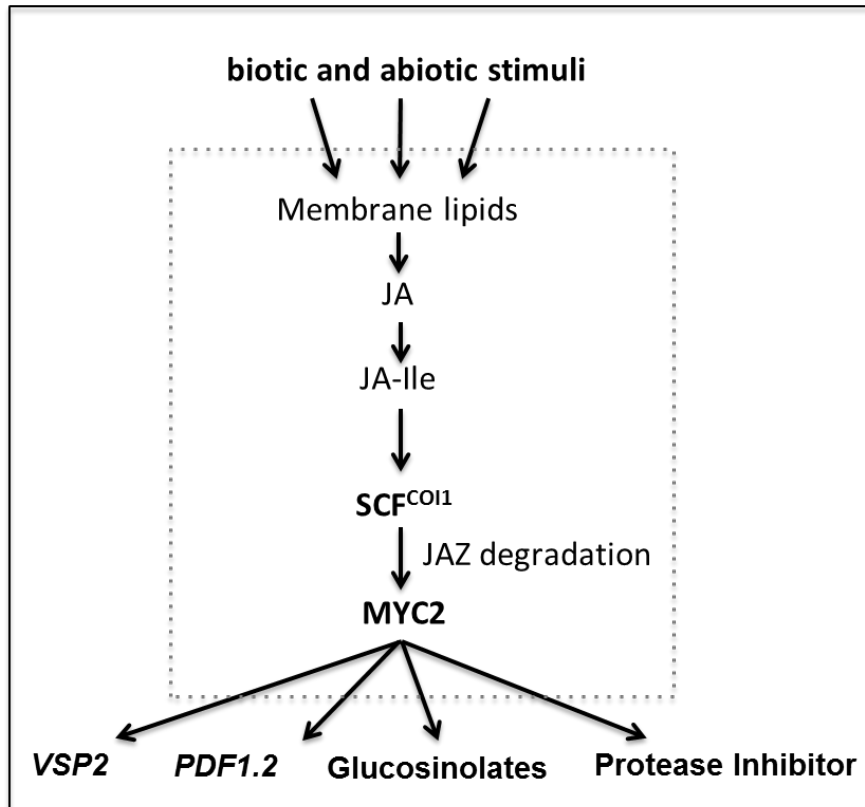


Figure 7: Induction and downstream JA signaling pathway in *Arabidopsis thaliana*. Accumulation of JA is induced by herbivory and other biotic stimuli as well as by abiotic stimuli. The active jasmonate JA-Ile is produced and interacts with the SCF-COI1 receptor complex. This interaction induces the degradation of JAZ repressor proteins and enables the MYC2-dependent gene expression (adapted from Howe and Jander 2008).

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