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# The role of early signaling events in plant-insect interactions

**Running title: early events in plant-insect interactions**

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1 **Abstract.**

2 The response of plants to the stress caused by herbivores involves several different defense  
3 mechanisms. These responses begin at the plant cell plasma membrane, where insect herbivores  
4 interact physically by causing mechanical damage and chemically by introducing elicitors or  
5 triggering plant-derived signaling molecules. The earliest plant responses to herbivore contact  
6 are represented by ion flux unbalances generated in the plant cell plasma membrane at the  
7 damaged site. Differences in the charge distribution generate plasma transmembrane potential  
8 ( $V_m$ ) variation, the first event that eventually leads to the initiation of signal transduction  
9 pathways and gene expression. Calcium signaling and the generation of reactive oxygen and  
10 nitrogen species are early events closely related to  $V_m$  variations. This review provides an  
11 update on recent developments and advances in plant early signaling in response to herbivory,  
12 with particular emphasis on the electrophysiological variations of the plasma membrane  
13 potential, calcium signaling, cation channel activity, the production of reactive oxygen and  
14 nitrogen species, and the formation of a systemically moving signal from wounded tissues. The  
15 roles of calcium dependent protein kinases and calcineurin signaling are also discussed.

16 **Key words** Biotic stress, Calcium signaling, Plant electrophysiology, Reactive oxygen and  
17 nitrogen species, Signal transduction, Transmembrane potential ( $V_m$ ).

18 **Introduction**

19 Over the course of millions of years of evolution, plants have developed defensive strategies  
20 against herbivorous insects that have led to myriad molecular interactions at both the genomic  
21 and metabolic levels. The ability for an organism to survive depends on its ability to respond  
22 quickly and efficiently to external stimuli and to develop effective and sustainable defenses.

1 These defenses are intended to directly protect the plant with toxic compounds (or using  
2 mechanical defense structures such as thorns and glandular trichomes) or to indirectly protect it  
3 through molecular interactions that may attract predators or parasitoids of the herbivorous  
4 attacker (Wu and Baldwin, 2010; Baldwin, 2010). Plants have evolved means to recognize and  
5 respond quickly to herbivory. These means include the perception of molecular patterns and  
6 defense effectors (Bos *et al.*, 2010; Bonaventure *et al.*, 2011; Maffei *et al.*, 2012), the elevation  
7 of cytosolic calcium ( $[Ca^{2+}]_{cyt}$ ) (Reddy *et al.*, 2011), the depolarization of the plasma  
8 transmembrane potential ( $V_m$ ) (Bricchi *et al.*, 2010), ion efflux/influx (Bricchi *et al.*, 2013),  
9 mitogen-activated protein kinase (MAPK) activation and protein phosphorylation (Arimura and  
10 Maffei, 2010; Arimura *et al.*, 2011), the activation of NADPH oxidase, and the production of  
11 reactive oxygen (ROS) and nitrogen (RNS) species (Miller and Mittler, 2006; Bricchi *et al.*,  
12 2010; Arimura *et al.*, 2011; Marino *et al.*, 2012). These cascades lead to a rise in the production  
13 of phytohormones jasmonic acid (JA) and salicylic acid (SA) (Zipfel, 2009; Consales *et al.*,  
14 2012; Erb *et al.*, 2012; Bricchi *et al.*, 2013), an increase in the production of ethylene (Arimura *et*  
15 *al.*, 2009; Onkokesung *et al.*, 2010; Diaz, 2011; Scala *et al.*, 2013), the expression of late defense  
16 response genes involved in the emission of volatile organic compounds (VOCs) (Wu and  
17 Baldwin, 2010; Baldwin, 2010; Maffei *et al.*, 2011; Karban *et al.*, 2011) and the production of  
18 toxic compounds (Karbon, 2010). These events start locally at the feeding site but can spread  
19 systemically throughout the plant (Wu and Baldwin, 2009; Maffei, 2010; Bricchi *et al.*, 2012).  
20 Although the individual responses that comprise these pathways have been widely catalogued,  
21 the connections between them and their interdependence have received little research attention to  
22 date.

1 Plant responses to herbivory have been previously reviewed both prior to gene expression  
2 changes (Ebel and Mithöfer, 1998; Garcia-Brugger *et al.*, 2006; Maffei *et al.*, 2007; Zipfel, 2009;  
3 Arimura *et al.*, 2011) and after gene expression changes (Howe and Jander, 2008; Mithöfer *et al.*,  
4 2009a; Wu and Baldwin, 2010; Baldwin, 2010; Bonaventure *et al.*, 2011; Bonaventure, 2012;  
5 Pearse and Karban, 2013). The aim of this review is to provide an update on recent developments  
6 and advances in our understanding of early plant signaling in response to herbivory, with  
7 particular emphasis on the electrophysiological variations of the plasma membrane potential,  
8 calcium signaling, cation channel activity, reactive oxygen and nitrogen species production and  
9 the presence of a systemically moving signal from the wounded tissues.

#### 10 **Defense in preparation for attack: the sensitivity of the plasma membrane and the role of** 11 **symplastic signaling**

12 The use of electrophysiology in the study of plant cells has witnessed a slow and steady increase  
13 for a number of purposes in recent years (Ochatt, 2013). Electrical signals are known to travel  
14 along the plant at different rates, carrying different messages (Gurovich and Hermosilla, 2009;  
15 Volkov *et al.*, 2010; Oyarce and Gurovich, 2011; Volkov, 2012; Volkov *et al.*, 2013). Vm  
16 depolarization is correlated to increases in the cytosolic calcium ion levels, ion channel activity  
17 and ROS and RNS bursts. All these events occur seconds to minutes after herbivory and are  
18 among the earliest plant defense responses.

19 The plasma membrane recognizes changes in the environment surrounding the cell, starting a  
20 cascade of electric signaling that eventually results in specific responses. Leaf damage by insect  
21 herbivores implies the direct delivery of elicitors or the indirect generation of plant cell wall-  
22 derived elicitors that may bind specific receptors at the plant plasma membrane. Emerging

1 evidence indicates that many high-affinity receptors for insect herbivores are located in the plant  
2 cell plasma membrane (Maffei *et al.*, 2012). The elicitor-receptor reaction produces variations in  
3 the  $V_m$ , which is defined as the difference in the electrochemical gradient between the interior  
4 and exterior of the plant cell. These variations can lead to either more positive (depolarization) or  
5 more negative (hyperpolarization)  $V_m$  values, and such events eventually lead to the generation  
6 of signaling cascades (Zebelo and Maffei, 2012a). In the *Spodoptera littoralis-Phaseolus lunatus*  
7 interaction, both direct herbivory and the insect's oral secretions (OS) have been demonstrated to  
8 induce a rapid  $V_m$  depolarization (Maffei *et al.*, 2007; Bricchi *et al.*, 2010; Bricchi *et al.*, 2012).  
9 The same response has been shown in higher plant species like *Arabidopsis thaliana* (Zebelo and  
10 Maffei, 2012b; Bricchi *et al.*, 2013) and *Ginkgo biloba* (Mohanta *et al.*, 2012), as well as in  
11 lower plant species like the fern *Pteris vittata* (Imbiscuso *et al.*, 2009). Interestingly, a significant  
12  $V_m$  depolarization was observed in response to almost every stylet puncture during *Myzus*  
13 *persicae* phloem feeding (Bricchi *et al.*, 2012).

14 It is known that systemic signaling induced by biotic stressors is transduced by either chemical  
15 or electrical signals (Masi *et al.*, 2009; Zimmermann *et al.*, 2009; Zebelo *et al.*, 2012; Zebelo and  
16 Maffei, 2012b; Baluska and Mancuso, 2013; Salvador-Recatala *et al.*, 2014; Mousavi *et al.*,  
17 2014).

18 OS from some insect herbivores contain effectors that overcome antiherbivore defenses.  
19 Herbivores possess diverse microbes in their digestive systems and salivary glands that can  
20 modify plant-insect interactions. For example, Colorado potato beetle (*Leptinotarsa*  
21 *decemlineata*) larvae exploit bacteria in their OS to suppress the anti-herbivore defenses of  
22 tomato plants (*Solanum lycopersicum*) (Chung *et al.*, 2013). Furthermore, applying bacteria  
23 isolated from larval OS to wounded plants confirmed that microbial symbionts are responsible

1 for this defense suppression. A further demonstration that salivary components are necessary to  
2 trigger plant responses to herbivore larvae has been recently provided. The ablation of the ventral  
3 eversible gland (VEG) of *S. littoralis* prompted a significant reduction in the Vm depolarization  
4 and significantly reduced both the cytosolic calcium concentration ( $[Ca^{2+}]_{cyt}$ ) and the  $H_2O_2$  burst  
5 (Zebelo and Maffei, 2012b). Moreover, VEG-ablated larvae induced a reduced defense-related  
6 enzyme expression and a reduced emission of plant volatiles (Zebelo *et al.*, 2014).

7 In general, three mechanisms are recognized for the transmission of electrical signals following  
8 herbivory: action potentials (APs), variation potentials (VPs) and system potentials (SPs). The  
9 OS of insect herbivores are known to cause both APs and VPs, but it is still unclear whether  
10 insect herbivory can cause SPs. SPs have been described as novel electrical long distance signals  
11 in plants that are induced by wounding, acting as the forerunners of slower chemical signals  
12 (Zimmermann *et al.*, 2009). SPs serve as back up APs and VPs and can remain overlapped with  
13 APs and VPs in some instances. Although SPs have been demonstrated only in mechanically  
14 damaged tissues, it is difficult to exclude the occurrences of SPs following herbivore feeding  
15 (Zebelo and Maffei, 2012a).

16 APs comprise a generic long distance signaling system that may act to potentiate a host response  
17 to subsequent signals delivered through alternative long distance information packages. An AP is  
18 a momentary change in the electrical potential of plant cells that sense stimuli from  
19 environmental stressors, eventually leading to intercellular and intracellular communication. A  
20 number of substances strongly depolarize the plasma membrane and thus presumably activate  
21 voltage-gated ion channels. Although in principle it is possible that (anion) channels are directly  
22 activated by depolarization, the temporal sequence of the ion flux kinetics of barley leaves shows  
23 that  $Ca^{2+}$  is lost from the apoplast well before the apoplastic anion concentration (measured as

1 Cl<sup>-</sup>) starts to increase (Felle and Zimmermann, 2007). Therefore, channel activity is involved in  
2 APs. The more the channels are activated, the more rapid the depolarization will be, eventually  
3 leading to an accelerated depolarization that is measured as a membrane potential ‘break-  
4 through’ typical of an AP. APs generated by herbivory propagate as a fast electrical signals that  
5 travel through the entire plant from the point of origin of the perceived input at a speed up to 40  
6 cm sec<sup>-1</sup> (Volkov, 2012). Zebelo and co-workers demonstrated that herbivore-induced plant  
7 volatiles (HIPVs) trigger APs and VPs on nearby receiver plants (Zebelo *et al.*, 2012).

8 It is generally accepted that wounded leaves communicate their damaged status to other leaves  
9 through a long-distance process. Using non-invasive electrodes, the surface potential changes in  
10 *A. thaliana* were mapped after leaf wounding, and it was found that membrane depolarization is  
11 correlated with JA signaling domains in undamaged leaves. Furthermore, of the 34 screened  
12 membrane protein mutant lines, the mutations in several clade 3 *GLUTAMATE RECEPTOR-*  
13 *LIKE* genes (GLRs 3.2, 3.3 and 3.6) attenuated wound-induced surface potential changes,  
14 showing a reduced JA-response gene expression in leaves distal to wounds in a *glr3.3 glr3.6*  
15 double mutant (Mousavi *et al.*, 2013). These results open new avenues for research in organ-to-  
16 organ wound signaling, demonstrating the existence of plant genes with functions related to  
17 those important for synaptic activity in animals. An open question remains: how are electrical  
18 signals propagated through the plant body? While animals have a nervous system that is  
19 specialized in the conduction of electrical signals, nothing similar is present in plants. Central to  
20 the success of these defenses is the need for local and systemic communication between cells.  
21 For plant cells, which are surrounded by cell walls, symplastic continuity is achieved through the  
22 presence of plasmodesmata (PD). These plasma-membrane-lined channels, bridging the cell  
23 wall, provide symplastic continuity and provide soluble and membrane environments for the

1 passage of small and large molecules and the potential for electrical conduction (Maule *et al.*,  
2 2011). PD-located proteins (PDLPs) are type-I membrane proteins with receptor-like properties,  
3 although the nature of their potential ligands is not known (Amari *et al.*, 2010). Using  
4 *Arabidopsis* plants mutated for *pdlp* genes, Bricchi and co-workers (2013) not only implicated  
5 PDs directly for their role in the defense against herbivory but also showed that some molecular  
6 responses to herbivory can be genetically distinguished from each other and from the overall  
7 defense response. However, although PDs have been correlated with gap junctions, no synaptic  
8 mechanisms of molecules are present to justify what has been demonstrated in the animal  
9 nervous system. Little is known about the electrophysiological responses of phloem sieve  
10 elements in wounding, and whether natural damaging stimuli induce propagating electrical  
11 signals in these tissues. Very recently, the use of living aphids and the direct current (DC)  
12 version of the electrical penetration graph (EPG) were used to detect changes in the membrane  
13 potential of *Arabidopsis* sieve elements (SEs) during caterpillar wounding. Feeding wounds in  
14 the lamina induced rapid depolarization waves in the affected leaf, increasing to the maximum  
15 amplitude (c. 60 mV) within 2 s. Major damage to the midvein induced fast and slow  
16 depolarization waves in unwounded neighbor leaves but only slow depolarization waves in non-  
17 neighboring leaves. The slow depolarization waves rose to a maximum amplitude (c. 30 mV)  
18 within 14 s. The distal electrical signals elicited by caterpillar cutting are indistinguishable from  
19 those elicited by a purely mechanical stimulus, demonstrating that the mechanical aspect of  
20 insect chewing is sufficient to induce the full electrophysiological response recorded in the  
21 phloem sieve elements of unwounded leaves (Salvador-Recatala *et al.*, 2014). Moreover, no  
22 remote electrical signals were recorded from the sieve elements of *glr3.3a glr3.6a* plants,  
23 indicating that GLR3.3 and/or GLR3.6 are necessary for the production of both the slow and fast

1 (action potential-like) remote depolarization waves. Therefore, it is conceivable that the  
2 depolarization waves produced by the sieve elements in response to remote wounding  
3 contributed to the global wound-activated surface potentials (Salvador-Recatala *et al.*, 2014).

4 Genetically encoded voltage-sensitive fluorescent proteins (VSFPs) are being used in  
5 neurobiology as non-invasive tools to study synchronous electrical activities in specific groups of  
6 nerve cells. This "light-based electrophysiology" has been recently adapted for use in plant  
7 systems. The production of transgenic plants engineered to express different versions of VSFPs  
8 that are targeted to the plasma membrane and internal membranes of root cells has allowed the  
9 recording of concurrent changes in the plasma membrane potential in populations of cells and at  
10 multiple membrane systems within single cells in response to various stimuli in living plants.  
11 Such coordinated electrical changes may globally orchestrate cell behavior to elicit successful  
12 reactions of the entire root to varying and unpredictable environments (Matzke and Matzke,  
13 2013).

14 Another interesting connection to electrical signaling could be the recent discovery that plants  
15 are able to discriminate between the vibrations caused by chewing and those caused by wind or  
16 insect song. A vibration-signaling pathway would complement the known signaling pathways  
17 that rely on volatile, electrical, or phloem-borne signals. It has been suggested that vibrations  
18 may represent a new long distance signaling mechanism in plant-insect interactions that might  
19 contribute to the systemic induction of chemical defenses (Appel and Cocroft, 2014). The  
20 mechanisms used by plants to detect and respond to mechanical vibration have received  
21 experimental attention from several groups (Gagliano *et al.*, 2012; Monshausen and Haswell,  
22 2013). Mechanoreception is thought to begin with the triggering of mechanosensors in the cell  
23 wall and/or plasma membrane, causing fluxes of calcium, ROS, and H<sup>+</sup>. These trigger

1 downstream responses involving many plant hormones and the rapid expression of genes that  
2 respond early to many plant stresses (Appel and Cocroft, 2014). Vm changes that occur  
3 following herbivory have also been associated with the same signaling events.

4 In the next section, we describe recent findings in early calcium signaling and ion concentration  
5 variations upon herbivory following Vm depolarization.

### 6 **Calcium and other ions act as second messengers in plant-insect interactions**

7 The candidate ion species responsible for Vm variations in plant cells following herbivory are  
8 calcium ( $\text{Ca}^{2+}$ ), protons ( $\text{H}^+$ ), potassium ( $\text{K}^+$ ) and chlorine ( $\text{Cl}^-$ ). Herbivore feeding causes a  
9 dramatic  $\text{Ca}^{2+}$  cytosolic ion influx limited to a few cell layers lining the wounded zone (Maffei *et*  
10 *al.*, 2004; Howe and Jander, 2008). This response is limited to herbivory or biotrophic activity;  
11 neither single nor repeated mechanical wounding induces such significant changes in the  
12 cytosolic  $\text{Ca}^{2+}$  ion influx (Bricchi *et al.*, 2010). The fact that single or repeated mechanical  
13 wounding alone is not sufficient to elicit significant  $[\text{Ca}^{2+}]_{\text{cyt}}$  variations points to oral factors [or  
14 herbivore-associated elicitors (Bonaventure *et al.*, 2011)] as triggers for a  $[\text{Ca}^{2+}]_{\text{cyt}}$  burst. Insect  
15 feeding and isolated insect-derived elicitors are known to cause changes in the  $\text{Ca}^{2+}$  homeostasis  
16 resulting from the tight regulation of protein channels and transporters located in the plasma  
17 membrane and organelle membranes (Jammes *et al.*, 2011) and  $\text{Ca}^{2+}$  sensors (Arimura and  
18 Maffei, 2010; Kudla *et al.*, 2010; Batistic and Kudla, 2012). These events have been associated  
19 with Vm depolarization (Bricchi *et al.*, 2013). In plants,  $[\text{Ca}^{2+}]_{\text{cyt}}$  is maintained in the nM range  
20 (100–200 nM), whereas in many organelles and in the apoplast,  $[\text{Ca}^{2+}]$  reaches the mM range  
21 (Dodd *et al.*, 2010). The dynamics of spatial and temporal  $\text{Ca}^{2+}$  changes in the cytosol and/or in  
22 other compartments of the plant cell are now accepted to generate “calcium signatures”, which

1 might be responsible for the initiation of specific downstream events that could eventually lead  
2 to the appropriate responses (Mithöfer *et al.*, 2009b; Batistic and Kudla, 2012; Short *et al.*,  
3 2012).

4 Several techniques have been used and developed to localize, measure and monitor  $[Ca^{2+}]_{cyt}$   
5 variations. A large number of fluorescent  $Ca^{2+}$  indicators are available for studying  $Ca^{2+}$  in plant  
6 cells (Mithöfer *et al.*, 2009b). The loading of  $Ca^{2+}$ -sensitive fluorescent probes into plant cells is  
7 an essential step in measuring the activities of cytoplasmic free  $Ca^{2+}$  ions with a fluorescent  
8 imaging technique. However, barriers to the loading of the test compounds or the  $Ca^{2+}$ -sensitive  
9 fluorescent dyes could be represented by the low permeability of the cell wall and by a massive  
10 cuticle. This would allow the penetration of only a limited number of cell layers, most likely near  
11 the infection zone. In addition to bio-luminescent techniques using aequorin, two fluorescent  
12  $Ca^{2+}$  indicators have been recently used to successfully demonstrate the induction of  $Ca^{2+}$   
13 signatures upon herbivory: Fluo-3 AM (Kanchiswamy *et al.*, 2010) and Calcium Orange™  
14 (Bricchi *et al.*, 2010; Mohanta *et al.*, 2012; Bricchi *et al.*, 2013). Despite their proven efficacy,  
15 these two indicators do not allow for a precise quantification of  $[Ca^{2+}]_{cyt}$  variations. Another way  
16 to fine-tune the  $Ca^{2+}$  variations is by using the Yellow Cameleon (YC)  $Ca^{2+}$ -sensor (Russell,  
17 2011). Recently, Maffei and co-workers (2014) used a Cameleon YC3.6 reporter protein  
18 expressed in *Arabidopsis thaliana* to quantify  $[Ca^{2+}]_{cyt}$  variations upon mechanical damage (MD)  
19 to leaves, herbivory by 3rd and 5th instar larvae of *Spodoptera littoralis* and *S. littoralis* OS  
20 applied to MD. YC3.6 allowed for a clear distinction between MD and herbivory and  
21 quantitatively discriminated calcium responses between the two larvae instars (Verrillo *et al.*,  
22 2014). The GFP probes in particular can be targeted to well-defined subcellular locales, enabling  
23 high-resolution mapping of these signals within the cell (Swanson *et al.*, 2011).

1 The development of various  $\text{Ca}^{2+}$  probes from the past six decades, the improvements that have  
2 been developed in this field, the limitations of each probe and important points to consider while  
3 planning ideal  $\text{Ca}^{2+}$  imaging experiments in plant science are all topics that have been recently  
4 reviewed (Kanchiswamy *et al.*, 2014).

#### 5 *Calcium sensors*

6 Calcium sensors play a major role in calcium signaling upon herbivory. In the standard model,  
7  $\text{Ca}^{2+}$ -sensor proteins, such as CaM (calmodulin), detect  $\text{Ca}^{2+}$  signals and subsequently regulate  
8 downstream targets to advance the signal transduction cascade (Du *et al.*, 2011). In *Arabidopsis*,  
9 7 genes encode the 4 CaM isoforms (*CaM1/4;CaM2/3/5;CaM6;CaM7*), which differ only in one  
10 to five amino acid residues (Batistic and Kudla, 2012). *Arabidopsis SIGNAL RESPONSIVE1*  
11 (*AtSR1* or *CAMTA3*) encodes a calmodulin (CaM)-binding transcription factor involved in the  
12 mediation of biotic stress responses (Galon *et al.*, 2008). AtSR1 is an important component of  
13 plant resistance to insect herbivory as well as one of only three described proteins involved in  
14  $\text{Ca}^{2+}$ /CaM-dependent signaling to function in the regulation of glucosinolate metabolism,  
15 providing a novel avenue for future investigations into plant–insect interactions (Laluk *et al.*,  
16 2012).  $\text{Ca}^{2+}$ /CaM-binding is also critical for the AtSR1-mediated herbivore-induced wound  
17 response. Interestingly, *atsr1* mutant plants are more susceptible to herbivore attack than wild-  
18 type plants. The complementation of *atsr1* mutant plants by overexpressing the wild-type AtSR1  
19 protein can effectively restore plant resistance to herbivore attack. However, when mutants of  
20 AtSR1 with impaired CaM-binding ability were overexpressed in *atsr1* mutant plants, plant  
21 resistance to herbivore attack was not restored, suggesting a key role for  $\text{Ca}^{2+}$ /CaM-binding in  
22 wound signaling (Qiu *et al.*, 2012).

1 In addition to CaM, plants possess many CaM-like (CML) proteins (50 in *Arabidopsis*) that are  
2 predicted to function as Ca<sup>2+</sup> sensors, but which remain largely uncharacterized (Vadassery *et*  
3 *al.*, 2012b). Nevertheless, it is known that most CMLs are cytoplasmic proteins and that some  
4 CMLs undergo lipid modifications resulting in membrane binding (Batistic and Kudla, 2012).  
5 Among the CMLs, two are particularly involved in the plant immune and biotic response:  
6 CML43 and CML42.

7 CML43 displays characteristics typical of Ca<sup>2+</sup> sensors, and its GUS reporter activity strongly  
8 increased when *Arabidopsis*-transformed plants were exposed to the defense compound SA  
9 (salicylic acid). Therefore, CML43 functions as a Ca<sup>2+</sup> sensor in the plant immune response as  
10 well (Bender *et al.*, 2014). The perception of microbe-associated molecular patterns (MAMPs) is  
11 closely connected to plant responses to insect herbivory. MAMPs typically induce a transient  
12 Ca<sup>2+</sup> burst, resulting in a rapid (within seconds) increase in the free cytosolic Ca<sup>2+</sup>, which  
13 subsequently (within minutes) declines to steady-state Ca<sup>2+</sup> levels (Frei dit Frey *et al.*, 2012).

14 Plant gene expression induced by OS revealed the up-regulation of a gene encoding the  
15 calmodulin-like protein CML42, which negatively regulates plant defense. CML42 is localized  
16 to the cytosol and nucleus. Its up-regulation is negatively regulated by the JA receptor  
17 Coronatine Insensitive1 (COI1), as a loss of functional COI1 results in prolonged CML42  
18 activation. CML42 thus acts as a negative regulator of plant defenses by decreasing COI1-  
19 mediated JA sensitivity and the expression of JA-responsive genes, independent of herbivory-  
20 induced JA biosynthesis. Furthermore, the results indicate that CML42 acts as a crucial signaling  
21 component connecting Ca<sup>2+</sup> and JA signaling (Vadassery *et al.*, 2012a). CML42 is also involved  
22 in abiotic stress responses, as kaempferol glycosides were down regulated in *cml42* and impaired  
23 in ultraviolet B resistance. Under drought stress conditions, the level of abscisic acid

1 accumulation was higher in *cml42* plants. Thus, CML42 might serve as a  $\text{Ca}^{2+}$  sensor, with  
2 multiple functions in insect herbivory defense and abiotic stress responses. A porin-like protein  
3 (PLP), most likely of bacterial origin, was identified in the collected OS of *S. littoralis* larvae.  
4 PLP exhibits channel-forming activity and up-regulates CML42 in *Arabidopsis*; however, it is  
5 not sufficient to elevate in vivo  $[\text{Ca}^{2+}]_{\text{cyt}}$ . Because membrane channel formation is a widespread  
6 phenomenon in plant-insect interactions, this PLP might represent an example for microbial  
7 compounds from the insect gut, which are initially involved in plant-insect interactions (Guo *et*  
8 *al.*, 2013).

9 In addition to calmodulin, plants have two other main families of  $\text{Ca}^{2+}$  sensors: calcineurin B-  
10 like (CBLs) and calcium dependent protein kinases (CPKs). After CBL proteins sense  $\text{Ca}^{2+}$   
11 signatures, these proteins interact selectively with CBL-interacting protein kinases (CIPKs),  
12 thereby forming the CBL/CIPK complexes involved in decoding calcium signals (Zhu *et al.*,  
13 2013; Kim, 2013). The CBL-CIPK system shows variety, specificity, and complexity in response  
14 to different stresses, and the CBL-CIPK signaling pathway is regulated by complex mechanisms  
15 in plant cells involving crosstalk with other signaling pathways (Yu *et al.*, 2014). In vivo and in  
16 vitro data have indicated that the kinase CIPK26, upon interaction with the calcium sensors  
17 CBL1 or CBL9, enhances the activity of the NADPH oxidase RBOHF via phosphorylation  
18 (Drerup *et al.*, 2013; Kimura *et al.*, 2013). Moreover, CBL/CIPK may control the activity of a  $\text{K}^{+}$   
19 channel from the Shaker family, VvK1.2 in grapevine (Cuellar *et al.*, 2013), whereas protein  
20 kinase CIPK9 interacts with the calcium sensor CBL3 and plays crucial roles in  $\text{K}^{+}$  homeostasis  
21 in *Arabidopsis* (Liu *et al.*, 2013). Both ROS and  $\text{K}^{+}$  are involved in plant responses to herbivory  
22 (see below). In tea (*Camelia sinensis*) leaves exposed to a mild infestation of green leafhopper  
23 (*Empoasca vitis*), a subtractive cDNA library was constructed using the suppression subtractive

1 hybridization strategy. Genes involved in the CBL–CIPK pathway were up-regulated by  
2 herbivory. The expression of a CBL-interacting protein kinase 19, *Cs-Ev9*, significantly  
3 increased in the Fuzao2 tea cultivar infested by the tea leafhopper (by approximately 18 times  
4 with respect to the controls), providing the first evidence for the involvement of the CBL–CIPK  
5 pathway in response to herbivory (Yang *et al.*, 2011).

6 CPKs are multifunctional proteins with  $\text{Ca}^{2+}$  binding and signaling capabilities in single proteins  
7 to directly translate  $\text{Ca}^{2+}$  signals into phosphorylation events (Tena *et al.*, 2011; Boudsocq and  
8 Sheen, 2013; Valmonte *et al.*, 2014). Recent studies have shown that CPKs play a significant  
9 role in herbivore-elicited signaling cascades. Upon herbivory, the plant responds by activating  
10  $\text{Ca}^{2+}$  signatures and corresponding CPKs such as CPK3 and CPK13 in *Arabidopsis*. These CPKs  
11 transcriptionally regulate the plant defensin gene (*PDF1.2*) independent of phytohormone  
12 signaling cascades in response to *S. littoralis* insect damage (Arimura and Maffei, 2010;  
13 Kanchiswamy *et al.*, 2010). This regulation occurs through the phosphorylation of the HSF2A  
14 transcription factor, which positively regulates the expression of *PDF1.2* independent of  
15 ethylene, JA and abscisic acid. CPK3 also induces the negative feedback regulation of herbivore-  
16 induced  $\text{Ca}^{2+}$  signals, indicating that CPKs can play redundant and specific roles in plant defense  
17 (Kanchiswamy *et al.*, 2010).

#### 18 *Ca<sup>2+</sup>-ATPases*

19 Herbivory induces extracellular stimuli that trigger increases in the cytosolic calcium levels,  
20 which are detrimental to plants. To cope with such stresses, plants need to develop efficient  
21 efflux mechanisms to maintain ionic homeostasis. The  $\text{Ca}^{2+}$ -ATPases are members of the P-type  
22 ATPase superfamily, which performs many fundamental processes in organisms by actively

1 transporting ions across cellular membranes (Bose *et al.*, 2011). Moreover, ATP-dependent P-  
2 type calcium ATPases can modulate the biotic stress response by activating the components of  
3 signaling pathways. The plasma membrane and endomembrane-bound  $\text{Ca}^{2+}$  channels regulate  
4 cytoplasmic  $[\text{Ca}^{2+}]$  levels, promoting  $\text{Ca}^{2+}$  homeostasis by mediating the influx and efflux of  
5  $\text{Ca}^{2+}$ . Furthermore, the type IIB  $\text{Ca}^{2+}$ -ATPases have a CAM-binding domain, which, upon  
6 binding with  $\text{Ca}^{2+}$ , forms a complex ( $\text{Ca}^{2+}/\text{CaM}$ ) and initiates many signal networks (Huda *et al.*,  
7 2013). Upon herbivory, the initial  $\text{Ca}^{2+}$  burst is followed by a consistent decrease in the cytosolic  
8  $[\text{Ca}^{2+}]$ , which implies the involvement of a  $\text{Ca}^{2+}$  efflux-mediated increased  $\text{Ca}^{2+}$ -ATPase activity  
9 (Maffei *et al.*, 2007).

#### 10 *Potassium channels*

11 Recently, the question on whether Vm depolarization was caused by a calcium-signaling  
12 pathway was addressed. An *Arabidopsis* line (*pdko3*) with a mutation in the genes encoding  
13 plasmodesmal proteins was found to be defective in some of the typical plant responses to  
14 herbivory. Following herbivory and the release of the *S. littoralis* OS containing a putative  $\beta$ -  
15 galactofuranose polysaccharide, the mutant line, unlike WT plants, shows an almost complete  
16 loss of Vm depolarization. However, both the *pdko3* and wild type (WT) plants show increased  
17 accumulation of cytosolic  $\text{Ca}^{2+}$ . Thus, mutations in genes for plasmodesmal proteins have  
18 provided valuable genetic tools for the dissection of the complex spectrum of responses to  
19 herbivory and have shown us that the responses to herbivory imply a  $\text{Ca}^{2+}$ -independent Vm  
20 depolarization (Bricchi *et al.*, 2013). To understand the cause of Vm depolarization, the  $\text{K}^{+}$   
21 channel activity was measured by confocal imaging when *Arabidopsis* plants were damaged by  
22 *S. littoralis* feeding or OS introduced to mechanically damaged leaves. Potassium represents the  
23 major osmotically active cation in plant cells and is known to be fundamental for plant functions

1 such as the control of membrane potential (Geiger *et al.*, 2009). Upon herbivory, the increased  
2 concentration of cytosolic  $\text{Ca}^{2+}$  triggers the opening of inward rectifier  $\text{K}^+$  channels, which are  
3 mainly responsible for the herbivore-induced  $V_m$  depolarization. In the *pdko3 Arabidopsis*  
4 mutant, the connection between increased calcium and  $\text{K}^+$  channel activation is broken, leading  
5 to significantly decreased  $V_m$  depolarization. However, the lower absolute levels of cytosolic  
6 calcium in the *pdko3* leaves open the possibility of a threshold effect in  $\text{K}^+$  channel activation  
7 (Bricchi *et al.*, 2013).

8 Elicitor-dependent accumulation of second messengers such as  $\text{Ca}^{2+}$  and reactive  
9 oxygen/nitrogen species are central to many signaling and regulation processes in plants.  
10 However, the mechanisms that govern the reciprocal interrelation of  $\text{Ca}^{2+}$ , ROS and RNS  
11 signaling are only beginning to emerge. NADPH oxidases of the respiratory burst oxidase  
12 homolog (RBOH) family are critical components contributing to the generation of ROS whereas  
13 Calcineurin B-like (CBL)  $\text{Ca}^{2+}$  sensor proteins together with their interacting kinases (CIPKs)  
14 have been shown to function in many  $\text{Ca}^{2+}$  signaling processes, including in the control of  $\text{K}^+$   
15 channels. Figure 1 shows the role of  $\text{Ca}^{2+}$  signaling pathways following herbivory.

## 16 **The second line of defense uses oxidative weapons: ROS and RNS**

17 The unraveling of the mechanisms connected to changes in the  $V_m$ ,  $[\text{Ca}^{2+}]_{\text{cyt}}$ , ROS and RNS  
18 production upon insect perception will undoubtedly shed critical light on the mechanisms used  
19 by plants to sense insect attacks (Bonaventure, 2012). ROS and RNS constitute key features  
20 underpinning the dynamic nature of cell signaling systems in plants. Despite their importance in  
21 many aspects of cell biology, our understanding of oxidative and especially of nitrosative  
22 signaling and their regulation remains poor (Molassiotis and Fotopoulos, 2011).

## 1 *The role of ROS*

2 Emerging evidence has begun to implicate ROS as pivotal redox-based signaling molecules in  
3 the plant defense response (Skelly and Loake, 2013). The modification of cysteines in key  
4 regulatory proteins by changes in redox homeostasis is appearing as a central responsive  
5 molecular switch that controls the function of a variety of regulators of plant defense responses  
6 (Yun *et al.*, 2012). ROS such as H<sub>2</sub>O<sub>2</sub> can also activate lipoxygenases to initiate the biosynthesis  
7 of oxylipins such as JA (Porta and Rocha-Sosa, 2002); indeed, JA treatment alone produces a  
8 H<sub>2</sub>O<sub>2</sub> burst (Ozawa *et al.*, 2009). Mechanically damaged Lima bean leaves react rapidly and  
9 dramatically to H<sub>2</sub>O<sub>2</sub> by inducing a strong V<sub>m</sub> depolarization. However, leaves wounded by *S.*  
10 *littoralis* already show a reduced starting V<sub>m</sub>, with the consequence of dramatically lower or  
11 even no responsiveness to H<sub>2</sub>O<sub>2</sub> application (Mithöfer *et al.*, 2009a). Thus, the calcium-induced  
12 potassium-dependent depolarization of the V<sub>m</sub> following herbivory is linked to a reduction in the  
13 downstream responses of the attacked leaves to signaling molecules such as H<sub>2</sub>O<sub>2</sub> (Bricchi *et al.*,  
14 2010).

15 The production of ROS such as H<sub>2</sub>O<sub>2</sub> depends on the activity of oxidases. Plant NADPH  
16 oxidases, also known as respiratory burst oxidase homologues (RBOHs), are the most thoroughly  
17 studied enzymatic ROS-generating systems. Our understanding of their involvement in various  
18 plant processes has increased considerably in recent years (Marino *et al.*, 2012). The ability of  
19 RBOHs to integrate calcium signaling and protein phosphorylation with ROS production,  
20 coupled with genetic studies demonstrating their involvement in many different biological  
21 processes in cells, places RBOHs at the center of the ROS network of cells and demonstrates  
22 their important function in plants (Suzuki *et al.*, 2011). In *Arabidopsis*, a Ca<sup>2+</sup>-signaling network  
23 regulates the formation of ROS. After interaction with the calcium sensors CBL1 or CBL9, the

1 kinase CIPK26 enhances the activity of the NADPH oxidase RBOH via phosphorylation. The  
2 CBL-interacting protein kinase CIPK26 specifically interacts with the N-terminal domain of  
3 RBOH in yeast two-hybrid analyses and with the full-length RBOH protein in plant cells. In  
4 addition, CIPK26 phosphorylates RBOH in vitro, and the co-expression of either CBL1 or CBL9  
5 with CIPK26 strongly enhances ROS production by RBOH in HEK293T cells. Together, these  
6 findings identify a direct connection between CBL-CIPK-mediated  $\text{Ca}^{2+}$  signaling and ROS  
7 signaling in plants and provide evidence for the synergistic activation of the NADPH oxidase  
8 RBOH by direct  $\text{Ca}^{2+}$ -binding to its EF-hands and  $\text{Ca}^{2+}$ -induced phosphorylation by  
9 CBL1/9CIPK26 complexes (Drerup *et al.*, 2013). Moreover, the direct binding of CIPK26 to  
10 RBOH negatively modulates ROS production and plays a role in the regulation of ROS signaling  
11 in plants (Kimura *et al.*, 2013). In *Arabidopsis*, extracellular ATP causes the production of ROS  
12 in intact roots, with the plasma membrane NADPH oxidase AtRBOHC being the major  
13 contributor. This resulted in the stimulation of plasma membrane  $\text{Ca}^{2+}$ -permeable channels,  
14 which contribute to the elevation of cytosolic free  $\text{Ca}^{2+}$ . The disruption of this pathway in the  
15 *AtrbohC* mutant impaired the extracellular ATP-induced increase in ROS, the activation of  $\text{Ca}^{2+}$   
16 channels, and the transcription of the *MAP kinase3* gene that is known to be involved in stress  
17 responses, showing that higher plants evolved a mechanism to transduce the ATP signal at the  
18 plasma membrane (Demidchik *et al.*, 2009). In the model plant *Nicotiana attenuata*, transcripts  
19 of the respiratory burst NADPH oxidase homolog NaRBOHD are rapidly and transiently induced  
20 by wounding and are amplified when *Manduca sexta* OS are added to the wounds. The *M. sexta*  
21 OS contain fatty-acid-amino-acid-conjugates (FACs), which are responsible for the increase in  
22 NaRBOHD transcripts. Silencing NaRBOHD significantly reduced the ROS levels after OS  
23 elicitation, without influencing the expression of the pivotal hormones that regulate plant

1 resistance to herbivores. In addition, NaRBOHD-silenced plants were more vulnerable to insect  
2 herbivores, especially to the larvae of the generalist *S. littoralis*. These results indicate that  
3 NaRBOHD-based defenses play an important role in late herbivore-elicited responses (Wu *et al.*,  
4 2013).

5 The evidence of ROS involvement in plant-insect interactions is growing. The accumulation of  
6 reactive oxygen species seems to play a major role in determining the extent of tissue alterations  
7 during gall morphogenesis (Carneiro *et al.*, 2014). The detection of enzymes related to ROS  
8 production has been described for galls induced by sucking insects (de Oliveira and dos Santos  
9 Isaias, 2010). The potential role of ROS in the defense systems of wheat (*Triticum aestivum*) and  
10 rice (*Oryza sativa*) against Hessian fly (*Mayetiola destructor*) larvae reveals a rapid and  
11 prolonged accumulation of H<sub>2</sub>O<sub>2</sub> in wheat plants at the attack site during incompatible  
12 interactions. Increased accumulation of both H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-•</sup> was detected in rice plants during  
13 non-host interactions with the larvae. Class III peroxidases might play a role in ROS generation  
14 in resistant wheat and non-host rice plants during the response to Hessian fly attacks (Liu *et al.*,  
15 2010). Moreover, insect infestation impacts the stress signaling network through the effects on  
16 the ROS and cellular redox metabolism, with particular emphasis on the roles of ROS in the  
17 plant responses to phloem-feeding insects (Kerchev *et al.*, 2012). Pea aphid (*Acyrtosiphon*  
18 *pisum*) causes oxidative stress conditions in pea leaves through the enhanced production of H<sub>2</sub>O<sub>2</sub>  
19 and O<sub>2</sub><sup>-•</sup>. The early, strong generation of H<sub>2</sub>O<sub>2</sub> was observed at 24 h in aphid-infested leaves,  
20 whereas the strong generation and continuous increase of O<sub>2</sub><sup>-•</sup> production in aphid-infested  
21 leaves from 0 to 96 h enhanced the defense potential to protect against aphid herbivory. The  
22 relative release of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-•</sup> was estimated with confocal microscopy by staining leaves  
23 with specific fluorochromes [dichlorodihydro-fluorescein diacetate (DCFH-DA) and

1 dihydroethidium (DHE)] to reveal the fine-tuned regulatory mechanisms involved in plant-aphid  
2 interactions (Van *et al.*, 2013).

3 The concentration of H<sub>2</sub>O<sub>2</sub> and malondialdehyde (MDA) and the activities of several ROS  
4 scavenging enzymes and related gene expression [e.g., superoxide dismutase (SOD), catalase  
5 (CAT), and peroxidase (POD)] were measured in different species damaged by mechanical and  
6 herbivore wounding. After infestation with chewing insects, the POD activity in the sap and total  
7 soluble protein of tomato, cowpea and cotton were enhanced. Similar results were observed with  
8 the feeding of sap sucking insects (Singh *et al.*, 2013). In poplar, the concentration of H<sub>2</sub>O<sub>2</sub> and  
9 MDA and the activities of SOD, CAT and POD were enhanced by herbivore wounding  
10 treatment, but not as much as by mechanical wounding, suggesting that H<sub>2</sub>O<sub>2</sub>, SOD, CAT and  
11 POD are associated with insect resistance (An *et al.*, 2010). In the “living fossil” plant *Ginkgo*  
12 *biloba*, a significantly higher H<sub>2</sub>O<sub>2</sub> production is observed 30 min after herbivory than in  
13 mechanically damaged leaves; however, by 4 h after feeding, the values dropped to the control  
14 levels. The use of DPI (diphenyleneiodonium, a suicide inhibitor of the phagocytic NADPH  
15 oxidase and an inhibitor of NADH-dependent H<sub>2</sub>O<sub>2</sub> production by peroxidase) inhibited  
16 herbivory-dependent H<sub>2</sub>O<sub>2</sub> production to the control levels, whereas verapamil (a voltage-gated  
17 Ca<sup>2+</sup> channel antagonist with a significant effect on herbivore-induced Ca<sup>+2</sup> release) significantly  
18 increases the level of H<sub>2</sub>O<sub>2</sub> upon herbivory. These data confirm the existence of an interplay  
19 between H<sub>2</sub>O<sub>2</sub> and calcium homeostasis (Mohanta *et al.*, 2012).

20 Another interesting question is how much insects can tolerate the increased ROS production in  
21 the attacked leaves. In the emerald ash borer (*Agrilus planipennis*), antioxidant genes quench  
22 ROS from both dietary and endogenous sources (Rajarapu *et al.*, 2011), whereas *Lymantria*  
23 *dispar* caterpillar larvae can tolerate elevated levels of ROS in their midguts without nutritionally

1 significant changes in the compositions of susceptible essential amino acids in their food  
2 (Barbehenn *et al.*, 2012). The abundance of vitamin C (VitC) in plants influences their  
3 susceptibility to insect feeding as VitC is an essential dietary nutrient that serves as an  
4 antioxidant in the insect midgut and is also a substrate for plant-derived ascorbate oxidase, which  
5 can lead to the generation of toxic reactive oxygen species. Herbivores appear to influence both  
6 the de novo synthesis and redox cycling of VitC in their host plants, thereby potentially altering  
7 the nutritional value of crops and their susceptibility to pests. The recent development of  
8 genetically modified crops with enhanced VitC content provides both an impetus and a tool for  
9 further studies on the role of VitC in plant-insect interactions (Goggin *et al.*, 2010).

#### 10 *Involvement of NRS*

11 The available descriptions of NO-mediated physiological processes are continuously increasing.  
12 Although it was initially associated with plant defense responses against pathogens, reports  
13 indicate that NO is also involved in plant responses to insects. *S*-nitrosoglutathione reductase  
14 (GSNOR) reduces the NO adduct *S*-nitrosoglutathione (GSNO), an essential reservoir for NO  
15 bioactivity involved in plant-herbivore interactions. Using a virus-induced gene silencing (VIGS)  
16 system, the activity of GSNOR was knocked down in *N. attenuata*. The silencing of GSNOR  
17 decreased the herbivory-induced accumulation of JA and ethylene without compromising the  
18 activity of two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase  
19 (SIPK) and wound-induced protein kinase (WIPK) (Wuensche *et al.*, 2011a). *M. sexta* feeding  
20 decreased the activity of trypsin proteinase inhibitors (TPIs), which were detected in GSNOR-  
21 silenced plants. Therefore, GSNOR plays an important role in plant resistance to herbivory and  
22 JA signaling and suggests the potential involvement of NO in plant-herbivore interactions  
23 (Wuensche *et al.*, 2011a).

1 Nitric oxide-associated protein 1 (NOA1) is required for plant resistance in plant-herbivore  
2 interactions. NOA1-silenced *N. attenuata* plants treated with mechanical wounding and *M. sexta*  
3 OS showed elevated levels of herbivory-induced JA but reduced concentrations of most carbon-  
4 based defensive compounds. These data suggest the involvement of NOA1 in *N. attenuata*'s  
5 defense against *M. sexta* attack, and highlight its role in photosynthesis and in the biosynthesis of  
6 jasmonates and secondary metabolites (Wuenschel *et al.*, 2011b).

7 *Nilaparvata lugens*, the brown plant hopper (BPH), is one of the most destructive phloem-  
8 feeding insects to attack rice throughout Asia. BPH feeding increases the level of endogenous  
9 NO in the leaf and sheath tissue of both resistant and susceptible rice cultivars within 1 h of  
10 infestation. The production of NO in response to BPH feeding appears to depend primarily on  
11 the activity of nitric oxide synthase (NOS). The application of exogenous NO stimulated the  
12 expression of certain drought stress-related genes, reduced the plant height, delayed leaf  
13 senescence and reduced the seedling mortality caused by BPH feeding. This suggests that NO  
14 signaling plays a role in the rice tolerance response to BPH feeding (Liu *et al.*, 2011). In the  
15 interaction between *S. littoralis* and Lima bean, both herbivory and robotic wounding induced  
16 marked accumulation of NO but with distinct temporal patterns. Herbivory caused a rapid and  
17 transient increase of NO levels, whereas the response to robotic wounding was of equal intensity  
18 but delayed (Bricchi *et al.*, 2010). Interestingly, the time course of NO production parallels the  
19 induction of the volatile compound emission observed in Lima beans (Arimura *et al.*, 2011),  
20 suggesting a possible involvement of NO in the signaling pathway leading to volatile emission.  
21 Figure 2 summarizes the changes in the ROS and RNS signaling pathways in response to  
22 herbivory.

23 **A rapid chemical signal provides a systemic warning**

1 Systemic responses are used by plants to prevent attacking herbivores from moving from  
2 wounded zones, where plant defenses are built up in response to feeding, to reach unattacked  
3 leaves. The existence of such a systemic signaling molecule was first reported in tomato plants in  
4 the pioneer studies of Ryan and co-workers (Green and Ryan, 1972). Since then, regulation of  
5 defense genes in systemic tissues upon herbivory has been increasingly reported (Champigny  
6 and Cameron, 2009), and considerable research effort has focused on the identification of  
7 systemic wound signals and the mechanisms by which they are generated, transported and  
8 perceived in distal leaves. The signaling species implicated in wound-induced systemic  
9 responses include oligosaccharides, reactive oxygen species, systemin, green leaf volatiles,  
10 hydraulic signals, electrical signals and various plant hormones (Koo *et al.*, 2009 and references  
11 cited therein). The nature of the systemic wound signal remains unknown. In the current model, a  
12 rapidly transmitted wound signal triggers the systemic synthesis of JA, which, upon conversion  
13 to JA-Ile, activates the expression of early response genes by the SCF<sup>COI1</sup>/JAZ pathway (Koo *et*  
14 *al.*, 2009). In addition to the responses in the attacked leaf, the enhanced levels of defense gene  
15 transcripts are found in unelicited leaves on the same plant. This between-leaf systemic signaling  
16 of defense responses occurs without MAPK activation in systemic leaves and suggests the  
17 existence of a mobile signal other than the one activating responses within the attacked leaves  
18 (Wu *et al.*, 2007). However, the nature of the signal and its means of propagation are still  
19 debated. Given how rapidly MAPKs are activated in undamaged regions, it may be that the  
20 signal is carried by electricity or rapidly propagated hydraulic signals. Another hypothesis is that  
21 relatively small molecules, such as FACs or FAC-elicited signal compounds, enter the xylem  
22 vessels through wounds and are carried to specific regions of the leaf (Wu *et al.*, 2007). Whether  
23 electric signaling or xylem transport is involved in activating MAPKs in undamaged regions

1 needs further investigation. Increasing evidence excludes the possibility that plant  
2 phytohormones (e.g., JA or its derivatives such as the JA isoleucine conjugate or its methyl ester)  
3 might be the travelling molecules because they are synthesized in the systemic leaves rather than  
4 transported (Wang *et al.*, 2008); moreover, insect elicitors induce a higher JA accumulation in  
5 distant tissues (VanDoorn *et al.*, 2010; Bricchi *et al.*, 2013).

6 Quite recently, evidence on the role of the symplastic connection for the transmission of the  
7 moving signal was provided. Hydroponically cultured *Arabidopsis* provides an ideal tool to  
8 study shoot-to-root signaling, allowing for changes in Vm in the root to be measured in real time  
9 following biotic or chemical stress on the shoots. When the root-Vm was measured in WT  
10 *Arabidopsis*, it was found to be significantly depolarized 7 - 8 min after either herbivory or the  
11 application of OS or FR $\alpha$ , a  $\beta$ -galactofuranose isolated from *S. littoralis* OS. On the other hand,  
12 the *pdko3* mutant showed no root-Vm depolarization after herbivory or the application of either  
13 OS or FR $\alpha$ . The rate of signal transduction was  $\sim 110$  cm h<sup>-1</sup>, which matches the rate of phloem  
14 transport (from 30 to 150 cm h<sup>-1</sup>). To test the phloem transport hypothesis, petioles of the treated  
15 leaves were subjected to either cold (4°C) or heat (60°). Both treatments completely abolished  
16 the WT herbivore-, OS- and FR $\alpha$ -induced root Vm depolarization, indicating the requirement of  
17 functional sieve elements. Therefore, because the *pdko3* mutant does not transmit the signal, it  
18 appears that that disruption of plasmodesmata function reduces the systemic signaling (Bricchi *et*  
19 *al.*, 2013). From all these studies, it is clear that elucidating the nature of the mobile signals in  
20 different species will contribute to an understanding of the rates of signal transmission and the  
21 speed of systemic plant responses to herbivory. In our laboratory, we tested different species and  
22 analyzed the time of root Vm depolarization upon leaf herbivory (*S. littoralis*). The rate of  
23 transmission was found to correspond to the phloem rate (Maffei *et al.*, unpublished results).

1 However, different biotic attacks may trigger different rates of plant responses. For instance, in  
2 *Arabidopsis*, the plasma membrane responds with a Vm depolarization depending on the nature  
3 of biotic attack. The Vm response is much more rapid upon *S. littoralis* (30 min -2 h) herbivory  
4 than following an attack by the aphid *Myzus persicae* (4-6 h). Furthermore, pathogenic infection  
5 with *Pseudomonas syringae* induces a very late Vm depolarization (16 h). Regardless of the  
6 nature of the biotroph and the rate of response, the Vm depolarization occurs at the same  
7 intensity, which in *Arabidopsis* corresponds to a Vm depolarization of approximately 40 mV.  
8 This suggests the occurrence of a Vm threshold, which is reached for successful  
9 herbivory/infection (Bricchi *et al.*, 2012). The question of why the Vm polarization occurs at  
10 different times appears to be associated with the mode of the biotic damage. The fast clipping  
11 and consistent plant tissue removal by chewing herbivores induces a "quantitative" response that  
12 is proportional to the level of tissue damage; on the other hand, the stylet probing and the phloem  
13 feeding of aphids induces a reduced level of damage that requires more time for a plant response.  
14 Finally, bacterial growth and tissue damage takes time, which appears to be proportional to the  
15 Vm depolarization. Therefore, besides the nature of the moving molecule, it will be important to  
16 evaluate the nature of the elicitor that induces its synthesis.

17 Because the use of plasmodesmata mutants in *Arabidopsis* suggests the involvement of phloem  
18 elements as the possible carriers of the moving signal(s), this hypothesis restricts the molecular  
19 size of the moving signal to plasmodesmata constraints, including the flux rate [ $3.3\text{-}8.5 \mu\text{m s}^{-1}$ ,  
20 (Rutschow *et al.*, 2011)], their specificity and directionality (Christensen *et al.*, 2009), their  
21 variable flux properties between species and tissue types (Maule *et al.*, 2011), the role of ROS as  
22 potent molecules that have an impact on the efficiency of PD trafficking (itez-Alfonso *et al.*,  
23 2011) and their size exclusion limits (Burch-Smith and Zambryski, 2012; Lee, 2014). Figure 3

1 illustrates a symplastic model for the transmission of the moving signal as well as for the effects  
2 of disrupting the symplastic continuity of the systemic response.

### 3 **Concluding remarks**

4 Depolarization of the  $V_m$  is one of the first responses of the plasma membrane. It depends  
5 mainly on the ion fluxes, including the opening of  $K^+$  channels, calcium release from internal  
6 stores or influx from the apoplast. The cytosolic calcium increase triggers protein calcium  
7 sensors including calmodulin, calcium-dependent protein kinases and calcineurin signaling,  
8 which activate a cascade of signals and eventually leads to transcription factor activation and  
9 transcriptional regulation. Calcium also triggers hydrogen peroxide production through the  
10 activation of CBL1/CBL9-CIPK26 interaction, which eventually phosphorylates RBOH and thus  
11 activates the production of ROS. Calcium homeostasis changes and cell redox status alterations  
12 also trigger NO production, which can either directly or indirectly affect the plant cell responses  
13 to herbivory. A growing body of evidence indicates the presence of mobile signaling molecules  
14 that travel from the wounded tissues towards systemic organs. Although the nature of this  
15 molecule remains unknown, its presence depends on the activity of insect's oral secretions,  
16 where specific elicitors (FACs or oligosaccharides) are necessary and sufficient to start the  
17 response process. Both the xylem and phloem are good candidates, given the path of the  
18 molecule toward the systemic tissues. In *Arabidopsis*, the elicitation with *S. littoralis*  $\beta$ -  
19 galactofuranose requires functioning plasmodesmata in order for the signaling molecule to reach  
20 the systemic tissues, justifying a model where the symplastic route is used.

21 Understanding fast responses of plants to the surrounding environment is of interest not only  
22 from an ecological and evolutionary perspective but also for the development of novel crop

1 protection strategies. Owing to the massive damage that herbivores cause to valuable crops, the  
2 deciphering of early signals from plants represents one of the most exciting areas research in the  
3 first line of defense.

4 Three areas where future efforts might result in major breakthroughs are related to the  
5 identification of herbivore-specific signal molecules, their recognition, and further signal  
6 transduction. The challenge for further research is to determine their mode of action, whether  
7 these signals are transduced by receptor-mediated processes or whether they simply interact with  
8 the plant membranes to initiate signal transduction pathways. One approach to achieve this goal  
9 is the use of plant mutants that are not responsive to a particular herbivory-related signal, as the  
10 *Arabidopsis pdko3* mutant or the several *N. attenuata* silenced plants (Yang *et al.*, 2012; Bricchi  
11 *et al.*, 2013; Woldemariam *et al.*, 2013; Wu *et al.*, 2013). The characterization and use of such  
12 mutants could result in the identification of genes encoding proteins involved in signal  
13 perception. Such studies can not only uncover individual signaling pathways but can also  
14 establish links in a network of alternative routes regulating the multitude of inducible plant  
15 defenses.

16

Accepted Article

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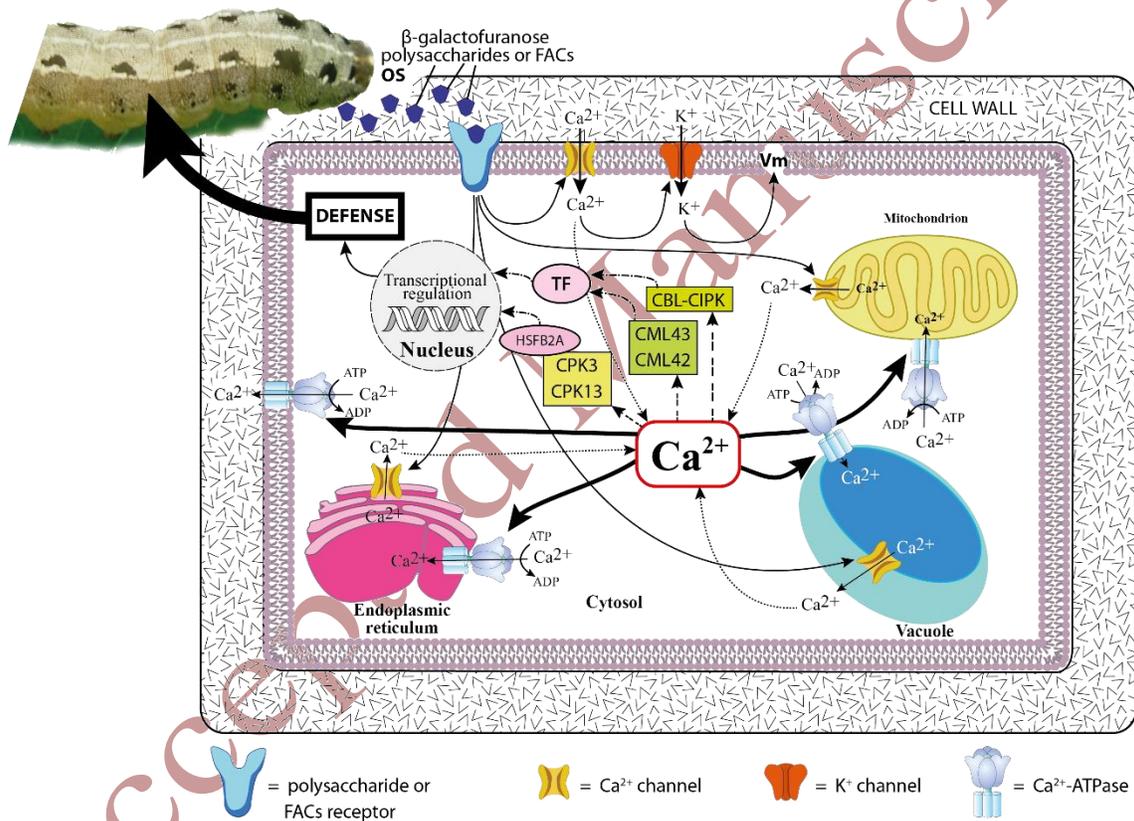
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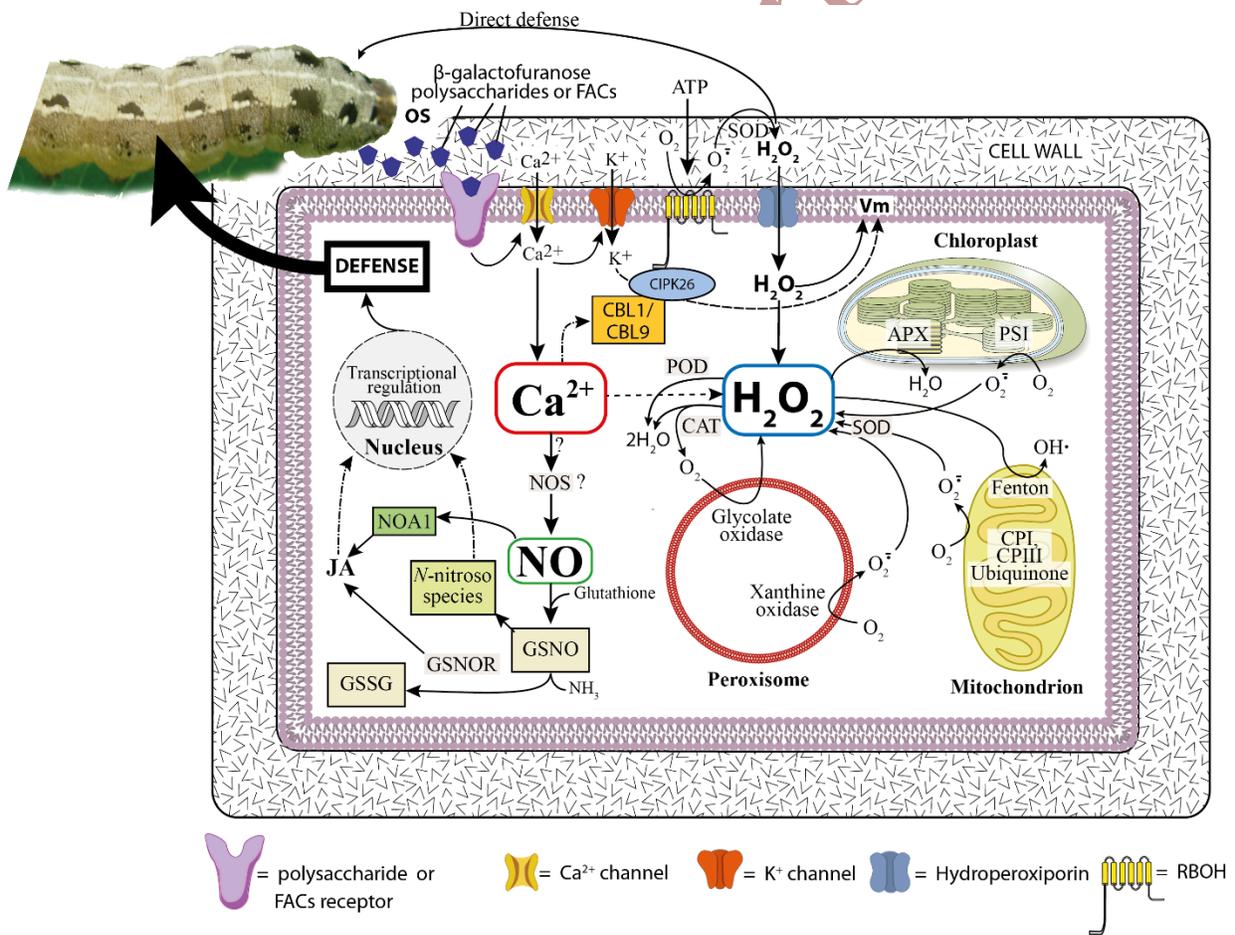
1 **Figure 1. Calcium signaling pathways after herbivory.** Following herbivory, insect-originated  
 2 elicitors bind on putative polysaccharide ( $\beta$ -galactofuranose) or FACs receptors, leading to  
 3 cytosolic  $\text{Ca}^{2+}$  homeostasis. This homeostasis is regulated by influx and efflux channels as well  
 4 as by ATP-dependent  $\text{Ca}^{2+}$  pumps. The cytosolic  $\text{Ca}^{2+}$  increase induces the release of  $\text{Ca}^{2+}$  from  
 5 cellular stores (mitochondria, vacuoles and the endoplasmic reticulum) via  $\text{Ca}^{2+}$  channels. The  
 6 cytosolic  $\text{Ca}^{2+}$  increase triggers two cascades of signaling events: 1) the activation of inward  $\text{K}^+$   
 7 channels, which cause  $V_m$  depolarization, and 2) the activation of CBL-interacting protein  
 8 kinases (CBL-CIPK), calmodulin-like protein (CML42 & CML43), and calcium dependent  
 9 protein kinases (CPK3 & CPK13) signaling pathways, which are involved in the activation of  
 10 transcription factors (e.g., HSF2A) that lead to the expression of defense genes in the nucleus.  
 11 The broken arrows indicate the calcium pathways contributing to the increased cytosolic calcium  
 12 concentrations.



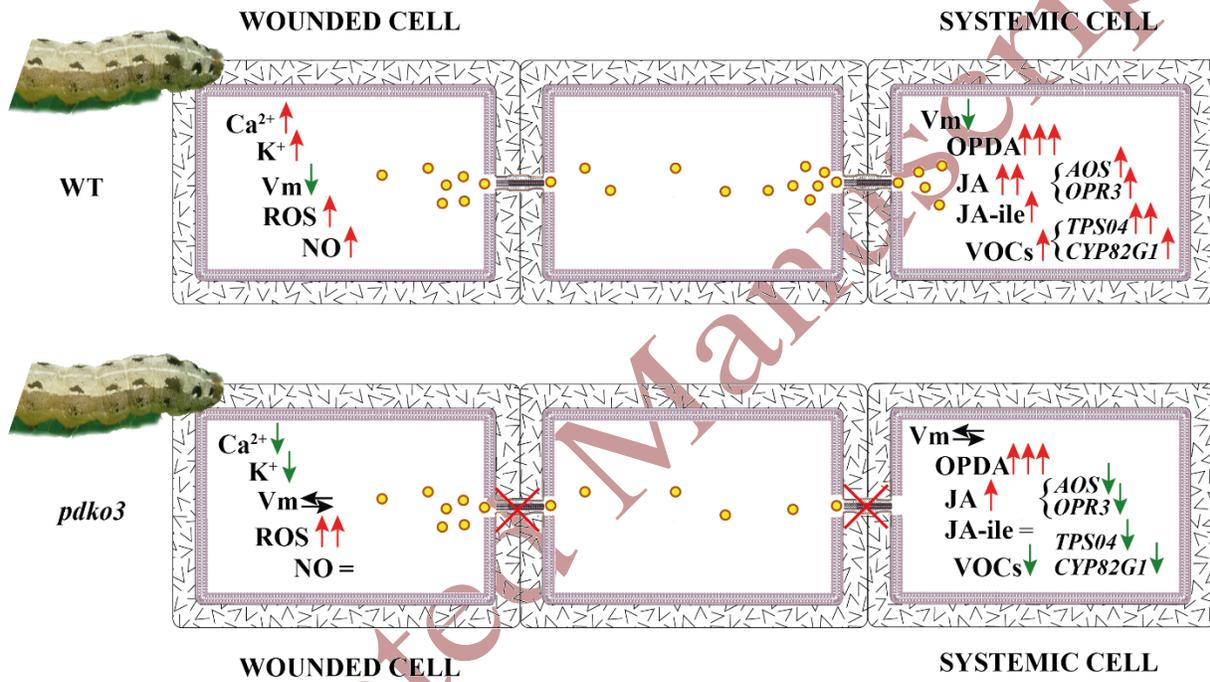
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1 **Figure 2. The role of ROS and NRS in plant-insect interactions.** Upon herbivory, insect  
 2 originated elicitors bind putative polysaccharide ( $\beta$ -galactofuranose) or FACs receptors, leading  
 3 to the cytosolic  $\text{Ca}^{2+}$  homeostasis (see Figure 1). The cytosolic  $\text{Ca}^{2+}$  increase triggers the CBL-  
 4 CIPK signaling pathways, which activate the plasma membrane NADP oxidase (ROBH). This in  
 5 turn generates the superoxide radical anion, which is eventually dismutated to hydrogen peroxide  
 6 ( $\text{H}_2\text{O}_2$ ) by the catalytic activity of superoxide dismutase (SOD). The activity of ROBH is also  
 7 regulated by external ATP.  $\text{H}_2\text{O}_2$  acts directly on herbivores or enters the cytosol through  
 8 hydroperoxidiporins, increasing the cytosolic  $\text{H}_2\text{O}_2$  concentration. By the action of mitochondrial  
 9 Fenton reaction and the activity of catalase (CAT) peroxidase (POD) and ascorbate peroxidase  
 10 (APX), the levels of  $\text{H}_2\text{O}_2$  are reduced. In contrast, the activity of peroxisomal xanthine oxidase,  
 11 mitochondrial CIP1/CIP3, Ubiquinone, and chloroplastic PSI catalyze the conversion of  $\text{O}_2$  to  
 12 superoxide radical anion ( $\text{O}_2^{\cdot-}$ ), which contributes to the elevation of the  $\text{H}_2\text{O}_2$  cytosolic  
 13 concentration by the action of SOD. Cytosolic  $\text{Ca}^{2+}$  increase may lead to nitric oxide synthase  
 14 (NOS) activity, thus increasing the cytosolic NO level. The NO adduct *S*-nitrosoglutathione  
 15 (GSNO) level is reduced by the activity of *S*-nitrosoglutathione reductase (GSNOR), producing  
 16 the oxidized glutathione disulphide (GSSG) and  $\text{NH}_3$ . Nitric oxide-associated protein 1 (NOA1)  
 17 and *N*-nitroso species, as well as the activity of GSNOR have been associated with the  
 18 phytohormone JA, triggering the transcriptional regulation of defense genes.



1 **Figure 3. *Arabidopsis* model for the transmission of a moving signal from wounded to**  
 2 **systemic tissues.** Upon herbivory and the release of factors present in insect oral secretions  
 3 (including a putative  $\beta$ -galactofuranose polysaccharide and/or FAC), both the *pdko3* and wild  
 4 type (WT) plants show an increased accumulation of  $[Ca^{2+}]_{\text{cyt}}$ , NO and  $H_2O_2$ . In contrast, unlike  
 5 WT plants, the mutant shows an almost complete loss of voltage-gated  $K^+$  channel activity and  
 6  $V_m$  depolarization, a loss of shoot-induced root- $V_m$  depolarization, a loss of gene expression  
 7 activation and regulation of the JA defense pathway and a much diminished release and altered  
 8 profile of the VOCs. These events implicate the symplastic route as probable candidate for the  
 9 means by which moving molecules reach systemic tissues.



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