Calcium Signaling Preceding the Emission of Plant Volatiles in Plant-Insect Interactions

Journal of the Indian Institute of Science
A Multidisciplinary Reviews Journal
ISSN: 0970-4140 Coden-JIISAD

REVIEWS

Calcium Signaling Preceding the Emission of Plant Volatiles in Plant–Insect Interactions

*Chidananda Nagamangala Kanchiswamy[a] and Massimo E. Maffei[b]**

Abstract | Plants react to herbivore attack by activating elaborate defence mechanisms. The success of plants in withstanding herbivory depends on their ability to quickly recognize, decipher the incoming signal, and adequately respond to a wide array of attacking insects. In contrast to the somatic adaptive immune system of mammals involving mobile defender cells, plant immune responses rely on the ability of each cell to recognize and respond to herbivore attack and on systemic signals originating from infected or wounded sites. Plant Ca^{2+} signals are involved in a sizable array of intracellular signaling pathways after pest invasion. A Ca^{2+} signal is defined by the balanced activation of Ca^{2+} channels at different cellular membranes, which is followed by the subsequent inactivation of channels and activation of efflux transporters to terminate Ca^{2+} influx and to rebalance cellular Ca^{2+} homeostasis. Upon herbivore feeding, there is a dramatic Ca^{2+} influx, followed by the activation of Ca^{2+}-dependent signal transduction pathways that include interacting downstream networks of kinases for defense responses. Like in animal cells, free intracellular Ca^{2+} ($[Ca^{2+}]_{cyt}$) variations in plant cells are key signals in many cellular regulatory functions, playing a major role in mediating various endogenous and exogenous signals. Ca^{2+}-binding sensory proteins such as Ca^{2+}-dependent protein kinases (CPKs) have been recently documented to mediate the signaling following Ca^{2+} influx after herbivory, in a phytohormone-independent manner. Here we review the sequence of signal transductions triggered by herbivory-invoked Ca^{2+} signaling leading to plant volatile emission by analyzing the connection between early events and the production of herbivore-induced volatile compounds.

Keywords: *calcium-dependent protein kinase, calcium signalling, calmodulin, insect–plant interactions, signal transduction pathway, volatile organic compounds*

1 Calcium Signaling in Plant–Insect Interactions

Calcium is a key regulator of plant responses to endogenous stimuli and stress signals of both biotic and abiotic nature.[1,2] Ca^{2+} is also involved in the control of many processes such as growth and differentiation, photomorphogenesis and embryogenesis, perception of symbiotic signals, hypersensitive responses induced by biotrophs, assembling and disassembling of cytoskeleton elements, perception of red and blue light and regulation of stomata.[3] In plant cells, Ca^{2+} plays a key physiological role as intracellular second messenger. It is especially important for the maintenance of cellular homeostasis and signal transduction pathways.[4–6] Cytosolic Ca^{2+} concentration is balanced by the presence of a large number of Ca^{2+} stores which can release Ca^{2+}, Ca^{2+} specific channels/pumps that regulate both Ca^{2+} influx and efflux in cells and subcellular compartments, and different (Ca^{2+}-binding) proteins that bind to Ca^{2+} either to sequester it or to perform some other

Second messenger: an intracellular signalling molecule released by the cell to trigger physiological changes such as proliferation, differentiation, migration, survival, and apoptosis. Second messengers are therefore one of the initiating components of intracellular signal transduction cascades. Besides calcium, examples of second messenger molecules include cyclic AMP, cyclic GMP, inositol triphosphate, and diacylglycerol.

[a]*Research and Innovation Centre Genomics and Biology of Fruit Crop Department, Fondazione Edmund Mach (FEM), Istituto Agrario San Michele (IASMA), Via Mach 1, 38010, San Michele all'Adige (TN), Italy.*

[b]*Department of Life Sciences and Systems Biology, Innovation Centre, University of Turin, Via Quarello 15/A, Turin 10135, Italy.*

**massimo.maffei@unito.it*

complex tasks.[7] All these components involved in regulation of Ca^{2+} concentrations at its equilibrium level constitute the complex network of the Ca^{2+} homeostasis system.[8] Intracellular calcium variations may depend on both the entry of Ca^{2+} in the cytoplasm upon release from cell organelles and the entry from the apoplasm[9–12] (Figure 1).

Calcium homeostasis: the mechanism by which the plant cell maintains adequate calcium levels. Disruptions of this mechanism lead to hypercalcemia or hypocalcemia, both of which can have important consequences for the plant cell.

In plants, cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) is maintained in the nM range (100–200 nM), whereas in many organelles and in the apoplast $[Ca^{2+}]$ reaches the mM range.[13] $[Ca^{2+}]_{cyt}$ can increase up to µM concentration in response to stress conditions.[14] This $[Ca^{2+}]_{cyt}$ variation is the result of a tight regulation of protein channels and transporters located in the plasma membrane and organellar membranes.[15] $[Ca^{2+}]_{cyt}$ variations occur in the form of transients, spikes and oscillations[1,16] that mostly happen within a timeframe of seconds. The dynamics of Ca^{2+} spatial and temporal changes either in the cytosol and/or in other compartments of the plant cell are now believed to generate "calcium signatures", which might be responsible for the initiation of specific downstream events, eventually leading to appropriate responses.[17–19] Since plant cells respond to extracellular stimuli with changes in cytosolic calcium concentration which ultimately controls many integrated physiological processes, the impact of herbivory on $[Ca^{2+}]_{cyt}$ has been investigated.[7,20–23] Usually stimulus-induced increases of Ca^{2+} concentration occur in the form of oscillations or in the form of spikes.[12,24,25] In the case of *Spodoptera littoralis* larvae feeding on lima bean leaves a spike is observed which depends on Ca^{2+} channel activity, since the response can be reduced by the calcium channel inhibitor verapamil,[26] EGTA (a calcium chelator) and ruthenium red (an inhibitor of calcium release from internal stores).[27]

Upon herbivory by chewing insects, plants respond with a cascade of events that lead to the activation of defense mechanisms.[28] These include perception of molecular patterns or effectors

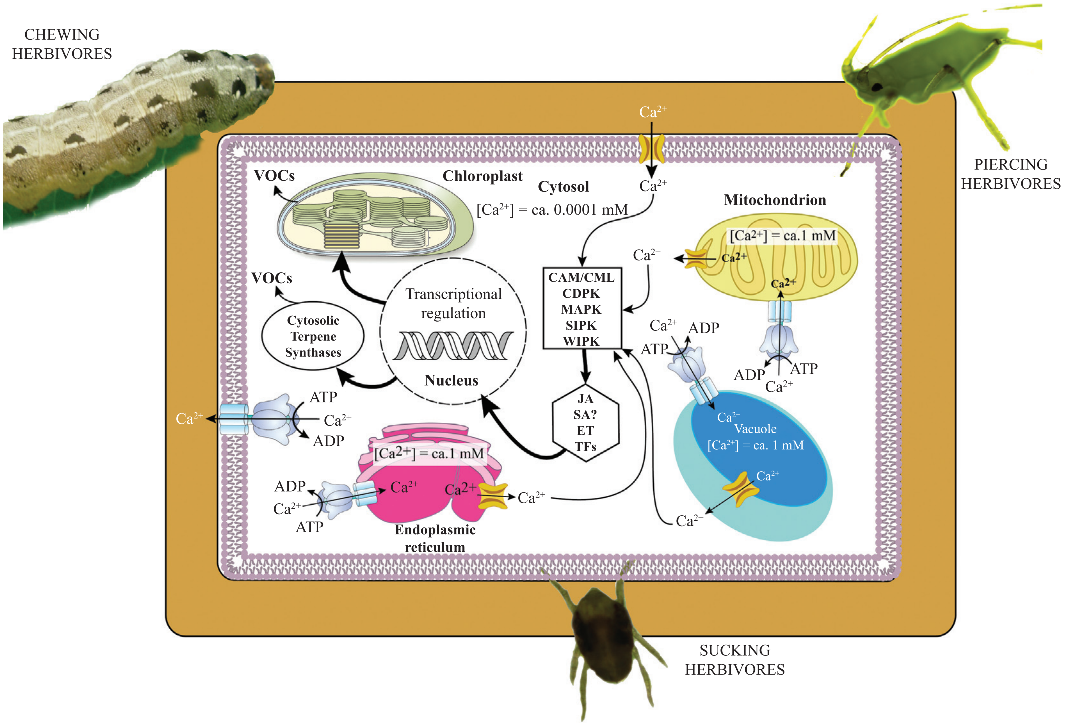

***Figure 1:* Calcium signaling and homeostasis upon herbivory**. Herbivory triggers different calcium responses. Ca^{2+} is actively pumped out of the cytosol by Ca^{2+}-ATPase, which determines the low concentration of the ion in the cytosol. The pump acts on the plasma membrane, mitochondrion, endoplasmic reticulum and vacuolar membranes. Ca^{2+} is also released into the cytosol by the action of different Ca^{2+} channels, which are activated by elicitors and other triggering events occurring upon herbivory by chewing herbivores, such as larvae of *Spodoptera littoralis*, piercing insects such as aphids and sucking mites such as spider mites. The increase of cytosolic Ca^{2+} is perceived by Ca^{2+} sensors and binding proteins that activate signal transduction pathways eventually leading to transcriptional regulation. The latter triggers indirect plant defense, such as the emission of volatile organic compounds (VOCs), produced either by chloroplastic or cytosolic terpene synthases. Direct defenses are also produced triggering other biochemical pathways (not shown).

of defense,[29,30] elevation of $[Ca^{2+}]_{cyt}$[31], plasma transmembrane potential (V_m) depolarization,[26] ion efflux/influx,[32] activation of NADPH oxidase and production of reactive oxygen species (ROS),[27,33] production of ethylene and jasmonate,[34] expression of late defense response genes,[23] changes in the proteome,[35] and emission of volatile organic compounds (VOCs).[36–39] These events start locally at the feeding site but can spread systemically throughout the plant.[40] Recently, the use of an Arabidopsis line (*pdko3*) mutated in genes encoding plasmodesmal proteins showed that $[Ca^{2+}]_{cyt}$ might not be directly involved in V_m depolarization. Rather the induced V_m depolarization appears to be associated with an increased voltage-gated K^+ channel activity.[41]

Early events upon biotic stress, like that inflicted by insect herbivores, include an immediate and dramatic Ca^{2+} influx limited to a few cell layers lining the damage zone.[20,22,40] In this context, $[Ca^{2+}]_{cyt}$ variations are triggered by oral secretions (OS) associated with herbivore feeding. The fact that single or repeated mechanical wounding alone is not sufficient to elicit significant $[Ca^{2+}]_{cyt}$ variations[42] points to oral factors [or herbivore-associated elicitors[30]] as triggers for a $[Ca^{2+}]_{cyt}$ burst.

Strictly connected to the variation of $[Ca^{2+}]_{cyt}$ is the induction of the oxidative burst, which has been demonstrated several times to be upstream of the ROS response;[43] however, H_2O_2 was found to elicit $[Ca^{2+}]_{cyt}$ release upon herbivory or the exogenous application of H_2O_2.[27] Despite ROS, other elicitors are known to trigger $[Ca^{2+}]_{cyt}$ release. Pathogen-associated molecular patterns (PaMPs)-induced $[Ca^{2+}]_{cyt}$ increase has been detected in several plants.[1,44–46] Also herbivore-associated molecular patterns (HAMPs) show remarkable similarities to PAMPs in Ca^{2+} responses. For example, the N-terminus of flagellin (flg22) and a peptide derived from the N-terminus of the bacterial Tu elongation factor (elf18), have been found to induce an increase of $[Ca^{2+}]_{cyt}$ in Arabidopsis,[47] just like it was found in the interaction between *S. littoralis* and its host plant lima bean.[27]

The effect of incubation of tomato leaves with some green leaf volatiles (GLVs) was a marked increase of $[Ca^{2+}]_{cyt}$. (*Z*)-3-hexenyl acetate was found to exert the highest activity with a strong $[Ca^{2+}]_{cyt}$ response. On the other hand, both the monoterpene α-pinene and the sesquiterpene β-caryophyllene showed a similar $[Ca^{2+}]_{cyt}$ response as observed for control leaves.[48]

Several techniques have been used and developed to localize, measure and monitor $[Ca^{2+}]_{cyt}$ variations and a large number of fluorescent Ca^{2+} indicators are available for studying Ca^{2+} in plant cells.[17] Loading of Ca^{2+}-sensitive fluorescent probes into plant cells is an essential step to measuring activities of cytoplasmic free Ca^{2+} ions with a fluorescent imaging technique. However, barriers to the loading of the test compounds or the Ca^{2+}-sensitive fluorescent dyes could be the low permeability of the cell wall as well as a massive cuticle. This would allow the penetration of only a limited number of cell layers probably near the infection zone.[26] Besides bio-luminescent techniques using aequorin,[32] two fluorescent Ca^{2+} indicators have been used several times to successfully demonstrate the induction of Ca^{2+} signatures upon herbivory: fluo-3 AM[26,27,49,50] and calcium orange™.[41,42,51] Despite their proved efficacy, these two indicators do not allow a precise quantification of $[Ca^{2+}]_{cyt}$ variations. Another way to fine-tune Ca^{2+} variations is by using the Yellow Cameleon (YC) Ca^{2+}-sensor.[52] Recently, Maffei and co-workers[53] used a Cameleon YC3.6 reporter protein expressed in *Arabidopsis thaliana* to quantify $[Ca^{2+}]_{cyt}$ variations upon leaf mechanical damage (MD), herbivory by *S. littoralis* 3rd and 5th instar larvae and *S. littoralis* oral secretions (OS) applied to MD. YC3.6 allowed a clear distinction between MD and herbivory and discriminated between the two larval instars.

The development of various Ca^{2+} probes over the past six decades, the improvements that have been developed in this field and the limitations of each probe and important points to consider while planning ideal Ca^{2+} imaging experiments in plant science, have been recently reviewed.[54]

2 Involvement of Calmodulin

In the standard model, Ca^{2+}-sensor proteins, such as CaM (calmodulin), detect Ca^{2+} signals and subsequently regulate downstream targets to advance the signal transduction cascade. In Arabidopsis, 7 genes encode for 4 CaM isoforms (*CaM1/4*; *CaM2/3/5*; *CaM6*; *CaM7*) which differ only in one to five amino acid residues.[18] Arabidopsis SIGNAL RESPONSIVE1 (*AtSR1* or *CAMTA3*) encodes a calmodulin (CaM)-binding transcription factor involved in the mediation of both biotic stress responses.[55] AtSR1 is an important component of plant resistance to insect herbivory as well as one of only three described proteins involved in Ca^{2+}/CaM-dependent signaling to function in the regulation of glucosinolate metabolism, providing a novel avenue for future investigations of plant–insect interactions.[56] Ca^{2+}/CaM-binding is also critical for AtSR1-mediated herbivore-induced wound

V_m depolarization: the opening and closing of ion channels can induce variations from the resting potential. This is called a depolarization if the interior voltage becomes less negative (say from –140 mV to –80 mV), or a hyperpolarization if the interior voltage becomes more negative.

Yellow Cameleon (YC) Ca^{2+}-sensors: genetically-encoded fluorescent indicators based on cyan fluorescent protein (CFP), a C-terminus of calmodulin (CaM), a Gly-Gly linker, a CaM-binding domain of myosin light chain kinase (M13), and a yellow fluorescent protein (YFP).

Pathogen-associated molecular patterns (PAMPs): molecules associated with groups of pathogens, that are recognized by cells of the innate immune system. These molecules are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in both plants and animals.

Calmodulin (CaM): an abbreviation for CALcium-MODULated protein; is a calcium-binding messenger protein expressed in all eukaryotic cells. CaM is a multifunctional intermediate messenger protein that transduces calcium signals by binding calcium ions and then modifying its interactions with various target proteins.

Green leaf volatiles: volatile organic compounds that are released when plants suffer tissue damage. Specifically, they refer to aldehydes, esters, and alcohols of 6-carbon compounds released after wounding.

response. Interestingly, *atsr1* mutant plants are more susceptible to herbivore attack than wild type plants. Complementation of *atsr1* mutant plants by overexpressing wild-type AtSR1 protein can effectively restore plant resistance to herbivore attack. However, when mutants of AtSR1 with impaired CaM-binding ability were overexpressed in *atsr1* mutant plants, plant resistance to herbivore attack was not restored, suggesting a key role for Ca^{2+}/CaM-binding in wound signalling.[57]

In addition to CaM, plants possess many CaM-like (CML) proteins (50 in Arabidopsis) that are predicted to function as Ca^{2+} sensors, but which remain largely uncharacterized.[58] Nevertheless, it is known that most CMLs are cytoplasmic proteins and that some CMLs undergo lipid modifications resulting in membrane binding.[18] Among CMLs, two of them are particularly involved in the plant immune and biotic response: CML43 and CML42. CML43 displays characteristics typical of Ca^{2+} sensors, and its GUS reporter activity strongly increased when Arabidopsis transformed plants were exposed to the defence compound salicylic acid (SA). Therefore, CML43 also functions as a Ca^{2+} sensor in plant immune response.[59] The perception of microbe-associated molecular patterns (MAMPs) is closely connected to plant responses to insect herbivory. MAMPs typically induce a transient Ca^{2+} burst, resulting in a rapid (within seconds) increase of free cytosolic Ca^{2+}, which subsequently (within minutes) declines to steady-state Ca^{2+} levels.[60]

Plant gene expression induced by oral secretions revealed up-regulation of a gene encoding a calmodulin-like protein, CML42, which negatively regulates plant defense. CML42 is localized to the cytosol and nucleus and its up-regulation is negatively regulated by the jasmonate receptor Coronatine Insensitive1 (COI1), as loss of functional COI1 resulted in prolonged CML42 activation. CML42 thus acts as a negative regulator of plant defense by decreasing COI1-mediated JA sensitivity and the expression of JA-responsive genes, and is independent of herbivory-induced JA biosynthesis. Furthermore, results indicate that CML42 acts as a crucial signaling component connecting Ca^{2+} and JA signalling.[61] CML42 is also involved in abiotic stress responses, as kaempferol glycosides were down-regulated in *cml42*, and impaired in ultraviolet B (UV-B) resistance. Under drought stress, the level of abscisic acid accumulation was higher in *cml42* plants. Thus, CML42 might serve as a Ca^{2+} sensor having multiple functions in insect herbivory defense and abiotic stress responses.

Calcineurin B-like proteins: after these proteins sense Ca^{2+} signatures, they interact selectively with CBL-interacting protein kinases (CIPKs), thereby forming CBL/CIPK complexes, which are involved in decoding calcium signals.

Calcium-dependent protein kinases: serine/threonine-specific protein kinases that are regulated by the Ca^{2+}/calmodulin complex. They are involved in many signaling cascades and are also necessary for Ca^{2+} homeostasis.

A porin-like protein (PLP), most likely of bacterial origin, was determined from collected OS of *S. littoralis* larvae. PLP exhibited channel-forming activity and up-regulates the calmodulin-like CML42 in *Arabidopsis thaliana*, however it is not sufficient to elevate in vivo $[Ca^{2+}]_{cyt}$. Because membrane channel formation is a widespread phenomenon in plant–insect interactions, this PLP might represent an example of microbial compounds from the insect gut which are initially involved in plant–insect interactions.[62]

3 Ca^{2+} ATPases

Ca^{2+} ATPases have also been shown to regulate defense responses by affecting programmed cell death.[63] Endoplasmic reticulum-localized Ca^{2+} ATPase contributes to pathogen-induced cell death and alters the MAMP-triggered Ca^{2+} burst.[64,65] The relevance of the Ca^{2+} influx in MAMP-elicited plant responses is underscored by the polysaccharides that are secreted by bacterial pathogens to chelate Ca^{2+} in the apoplastic space.[66] Similar Ca^{2+}-binding effects have been demonstrated in purified protein fractions of the watery and coagulable saliva of green rice leafhoppers (*Nephotettix cincticeps*).[67] Studies on two plasma membrane Ca^{2+} ATPases, ACA8 and ACA10, based on loss-of-function mutant data, show that they act as positive regulators of early MAMP responses, demonstrating the importance of coordinated and fine-tuned MAMP responses, including Ca^{2+} signaling, for plant immunity.[60]

4 Role of Calcium-Dependent Protein Kinases

Ca^{2+} mediates plant response to a wide range of external stimuli via Ca^{2+} signatures that are defined by spatio-temporal features including amplitude, frequency, duration and sub-cellular location, which determine their specificity along with the diverse proteins that are able to sense and decode the respective signals.[68–70] Besides calmodulin, there are two more main families of Ca^{2+} sensors in plants: calcineurin B-like (CBLs) and calcium-dependent protein kinases (CPKs). CBLs depend on Ca^{2+} for the conformational change of protein partners whereas CPKs are multifunctional proteins consisting of Ca^{2+} binding and signaling capabilities within a single protein to directly translate Ca^{2+} signals into phosphorylation events.[71–75] This unique characteristic enables CPKs to regulate multiple plant biological processes including growth, development and defence.[76–78]

In Arabidopsis, CPKs comprise a gene family with 34 members that are phylogenetically subdivided into four clades.[79] Considering their importance to plant defence, growth and development, these CPK families have also been studied in several economically important crop plants, including wheat, corn, rice, tomato and apple.[80–84] Numerous studies on CPKs from different crop species have shown their significant roles in biotic, abiotic, cold, salt, drought, heat, phytohormone-dependent or phytohormone-independent regulation of multiple signaling cascades.[84–87]

Recent studies have further shown that CPKs play a significant role in herbivore-elicited signaling cascades. Upon herbivory, plants respond by activating Ca^{2+} signatures and corresponding CPKs such as CPK3 and CPK13 in Arabidopsis. These CPKs transcriptionally regulate the plant defensin gene (PDF 1.2) independent of phytohormone signaling cascades in response to *S. littoralis* insect damage.[88,89] This regulation occurs through phosphorylation of the HSFB2A transcription factor, which positively regulates the expression of PDF1.2 independent of ethylene, JA and ABA. CPK3 also induces negative feedback regulation of herbivore-induced Ca^{2+} signals indicating that CPKs can play redundant as well as specific roles in plant defence.[88] CPK3 is also activated by the microbial elicitor flagellin (flg22)[90] and positively regulates the flg22-responsive gene *NHL10*, suggesting its possible role in MAMP signalling.[74,91] In contrast to these studies, the tomato CPK (LeCDPK2) phosphorylates the ethylene biosynthesis responsive enzyme LeACS2 upon wounding and thereby regulates ethylene production in response to wounding. Further, LeACS2 is also phosphorylated at a different site by mitogen-activated protein kinase (MAPK) upon wounding suggesting that both CPK and MAPK are involved in regulating ethylene biosynthesis upon wounding.[92] Wounding in tomato plants causes extracellular alkalinization regulated by another kinase LeCPK1 which inhibits plasma membrane H^+-ATPase activity.[93] Upon wounding in maize, ZmCPK11 is activated by the JA-dependent signaling pathway but its precise biological activity remains to be elucidated.[86] Recent studies have further shown that tobacco CPKs (NtCDPK4 and NtCDPK5) negatively regulate JA and defence metabolite accumulation upon herbivory independent of JA biosynthesis enzymes.[94]

Many of these current insights into the role of CPKs in modulating herbivore-elicited signaling cascades suggest that there might be multifaceted regulation of defence signaling cascades that are dependent or independent of phytohormone or transcription factor-mediated regulations; these findings reveal the complexity of fine-tuning that is possible in herbivore defence signaling cascades.[95]

5 Concluding Remarks

The availability of genome sequences of several economically important crop species combined with modern molecular tools provides significant breakthroughs to understand the versatile and evolutionarily conserved calcium signaling pathways (with particular reference to CPKs) among numerous crop species, their significant contribution in the regulation of most metabolic pathways of crop plants and in the control of plant defence, growth and development. Advanced molecular tools have helped in the understanding of the expression pattern, localization, phosphorylation and interacting proteins, Ca^{2+} sensitivity and substrate specificity in the regulation of the complex and sophisticated Ca^{2+} signaling network. Activation of Ca^{2+} sensing depends on Ca^{2+} signatures which are localized in the cell. Hence, future studies should focus on elucidation of wound- or herbivore-reactive Ca^{2+} channels, pumps and transporters using modern cellular, genetic and molecular tools in a spatio-temporal analysis. In particular, studies on the activation of CPKs and their translocation in response to specific Ca^{2+} signatures should be targeted to decipher the complex network of CPKs in crop plants. So far, although calcium signaling has always been observed to precede volatile emission, no studies have been conducted to link herbivore-induced Ca^{2+} signatures and Ca^{2+} sensor proteins and the production of plant volatiles as indirect defenses. Considering the importance of wound- or herbivore-induced plant volatiles for inter/intraspecies communication and their important role in multitrophic interactions and priming effects,[96,97] the deciphering of this possible signalling network is of considerable importance. To this end, the use of mutants would greatly benefit the role of early calcium signaling events in volatile production. Another important study area involves EF-hand domains and their importance for functional specificity to Ca^{2+} signatures via EF-hand mutant analysis and further identification of specific substrates for CPKs. The significance of EF-hand domains in regulating various biological responses would provide novel insights into Ca^{2+} signalling eventually leading to new discoveries for the genetic manipulation of economically important traits of crop plants.

Received 18 September 2014,
Revision accepted 14 October 2014.

Transcription factor: a protein that binds to specific DNA sequences, thereby controlling the rate of transcription of genetic information from DNA to messenger RNA.

Mitogen-activated protein kinases: protein kinases that are specific to the amino acids serine, threonine, and tyrosine. They are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, and heat shock. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.

Phytohormones: chemicals that regulate plant growth. They are signal molecules produced within the plant, and occur in extremely low concentrations. They regulate cellular processes locally in targeted cells and, when moved to other locations, also in other functional parts of the plant.

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Chidananda Nagamangala Kanchiswamy obtained a Ph.D. in Plant Biosensing at the University of Turin (Italy), studying the role of Arabidopsis calcium dependent protein kinases in herbivore response signaling cascades. He spent most of his PhD research at the Max Planck Institute for Chemical Ecology, Jena, and at the Center for Ecological Research Institute, Kyoto University (Japan). He now works as a postdoc (Marie Curie COFUND postdoc incoming 2010) at Foundation Edmund Mach, San Michele all'Adige, Trento, Italy, investigating microbial volatile organic compounds perception, signal transduction and their effect on model and crop plants.

Massimo Maffei is Professor of Plant Physiology, Department of Life Sciences and Systems Biology, at the University of Turin, Italy. His research activity is focussed on plant interactions with the surrounding biotic and abiotic environment, with particular reference to signal transduction pathways, gene expression and metabolomics. He has been the Coordinator of the Ministerial Centre of Excellence for Biosensing, Head of Department of Plant Biology and Deputy Dean of Faculty of Sciences. He is the Editor-in-Chief of the international *Journal of Plant Interactions* (Taylor & Francis, London) and member of the advisory board of the *Journal of Essential Oil Research*. He is reviewer for several international journals dealing with Plant Physiology, Phytochemistry, Natural Products Chemistry and Plant Biology.