

Before gene expression: early events in plant–insect interaction

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Successful defense depends on the ability of the plant to recognize an attacking ‘enemy’ as early as possible. Early defense responses require enemy-initiated signaling cascades. Their activation ensures an induced response that is quantitative, timely and coordinated with other activities of the host cells. Damage-induced ion imbalances and modulations of channel activities are the first events occurring in the plasma membrane and result in rapid perturbations of the plasma membrane potential (V_m) involving variations of cytosolic Ca^{2+} concentrations. Interacting downstream networks of kinases and phytohormones mediate the signal and result in concerted gene activation. Here we review and discuss early events occurring before herbivore attack-related gene expression that are responsible for cascades of events and signal transductions, eventually leading to indirect and direct plant responses.

Early events in plant–insect interactions

The success of plants to withstand biotic stresses depends on their ability to quickly recognize, decipher the incoming signal, and adequately respond to a wide array of attacking biotrophs. Current research on the plant–insect interaction is focused mainly on genomics and proteomics, which are late events induced by biotic stress. By contrast, events within the first seconds to minutes, which are responsible for recognition and triggering of signal transduction pathways, are still poorly understood. In this review, we focus on these early events in plant–insect interactions, starting from damage-induced ion imbalances, causing variations in membrane potentials, Ca^{2+} -signaling, production of reactive oxygen species, kinase activities, phytohormones, and their cross-talk up to processes that precede gene expression (Box 1).

Herbivory implies both wounding and chemical interaction

Mechanical wounding of plant tissues is an inevitable consequence caused by invading microorganisms and insect herbivory, but the speed and intensity of wounding are different. To dissect insect wounding and chemical interactions (oral secretions), control experiments have been performed using either a razor (blade) for scratching or cutting the leaf or a pattern wheel for puncturing leaf areas. Although it was apparent that wounding has an impact on

gene expression *in planta* [1], it remained questionable whether these treatments resembled insect feeding because herbivorous insects consume leaves by continuously clipping off and ingesting small pieces of tissue. This process can be considered as a series of sequential mechanical wounds, and is often accompanied by simultaneous introduction of saliva and foregut secretions (regurgitate). A single mechanical wound is generally unable to initiate a consistent response (e.g. emission of volatiles). However, in Lima bean (*Phaseolus lunatus*), continuous mechanical wounding is sufficient to elicit the same blend of volatiles as those induced by herbivores, albeit with different quantities [2]. These findings clearly demonstrate that herbivore-like wounding is necessary but not sufficient, and that the mutual chemical interaction between the protagonists is required to trigger the full response.

Electrical signaling is a sensing system for rapid responses to herbivore wounding

The plasma membrane of cells is the only compartment with a direct contact to the environment and represents the sensing element able to recognize changes and to initiate cascades of events eventually leading to specific responses. Changes in plasma transmembrane potential (V_m) or modulation of ion fluxes at the plasma membrane level are amongst the earliest cellular responses to biotic and abiotic stresses [3,4]. Herbivory-induced V_m changes are followed by a fast electrical signal (action potential) that travels through the entire plant from the point of origin of the perceived input [5]. Whereas the action potential is a momentary change in electrical potential on the surface of a cell that propagates up to 40 m sec^{-1} [6], which is too fast for the movement of a chemical signal along with either phloem or xylem [7], V_m changes are much slower: for a distance of 6–7 cm (dimensions of Lima bean leaves), the overall process takes about 5–6 min and involves signaling molecules traveling with the same speed ($\sim 1 \text{ cm min}^{-1}$).

Of particular importance in early recognition between the host and pathogen or herbivore is the role of signal molecules that can affect V_m either directly or via receptors [4]. For example, H_2O_2 is a strong depolarizing molecule that can be induced or introduced by the feeding insect [8,9]. In addition to H_2O_2 , elicitors such as the peptide pep 13 [10] or β -glucans [11] and other oligosaccharides [12] derived from the breakdown of pathogen cell walls induce V_m depolarization. Induction of ion fluxes [13] and V_m depolarization [14] have been demonstrated for oral secretions of herbivores, but not for known elicitors from

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Box 1. Early and late events in plant–herbivore interaction

Timed hierarchy of consecutive events detectable in plant tissues, which are initiated by feeding insects (Figure 1). The earliest events measurable are V_m changes at the plasma membrane, immediately followed by changes in the intracellular Ca^{2+} concentration and the generation of H_2O_2 . Within minutes, kinases and the phytohormones jasmonic acid (JA) and salicylic acid (SA) are detectable. Gene activations and subsequent metabolic changes are first seen after about 1 h.

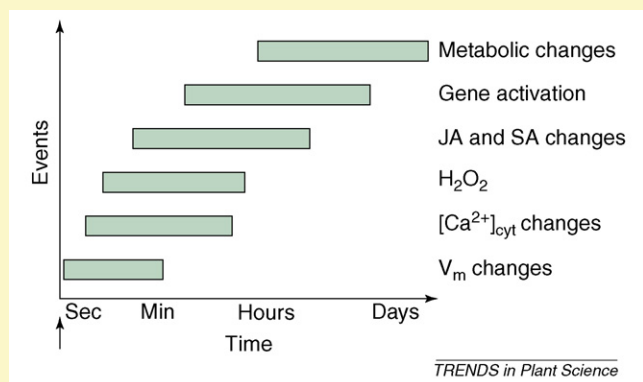


Figure 1.

the regurgitate of *Spodoptera* spp., such as volicitin [15] or inceptin [16]. Given that ion fluxes through channels directly influence V_m it seems reasonable to assume that unknown elicitors regulate the activities of various channels and, thus, might be considered as important factors inducing electrical signals. Among them, Ca^{2+} -channels are predominantly involved in cell signaling [17].

Ca^{2+} homeostasis and early signaling

The calcium ion is recognized as a second messenger in numerous plant signaling pathways. In healthy tissues, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is maintained at a level between 100 and 200 nM. This is 10^4 times less than that in the apoplasmic fluid and 10^4 to 10^5 times less than that in cellular organelles, providing the driving force for Ca^{2+} import into the cytosol, where it acts as a signal [18]. Calcium signatures is the term used to refer to spatial and temporal changes in $[Ca^{2+}]_{cyt}$ and might proceed as single calcium transients, oscillations, or repeated spikes with specific subcellular location, lag time, amplitude and frequency. Moreover, the same signal can induce different calcium signatures depending on the organ, the tissue, or the cell type in a tissue [19]. The information encoded in transient Ca^{2+} changes is decoded by an array of Ca^{2+} -binding proteins giving rise to a cascade of downstream effects, including altered protein phosphorylation and gene expression patterns [20]. The Ca^{2+} -binding proteins fall into two main classes, referred to as sensor relays and sensor responders, respectively [21]. Sensor relays such as calmodulin (CaM), CaM-related proteins and calcineurin B-like proteins (CBL) can function through bimolecular interactions [22]. Treatment with a chelator of extracellular Ca^{2+} suppressed the expression of defense-related genes both in Lima beans directly challenged with spider mites (*Tetranychus urticae*) [23] and in leaves receiving the induced volatiles from damaged plants [24].

Direct intracellular monitoring of calcium fluctuations *in vivo* has been done by either using fluorescent probes or the bioluminescence-based aequorin technology [25]. When lepidopteran larvae feed on plants, the cell membrane is depolarized in the vicinity of the bite zone followed by a transient increase of the cytosolic Ca^{2+} in the same region [25]. Upon interaction with herbivores, $[Ca^{2+}]_{cyt}$ elevation can principally result from an uptake of Ca^{2+} from the extracellular medium [14], from Ca^{2+} mobilization from organelles, and/or from both [25]. Insect salivary secretions significantly contribute to the increased $[Ca^{2+}]_{cyt}$, whereas mechanical damage does not [26]. In this context an involvement of insect elicitors such as volicitin and inceptin in Ca^{2+} influx remains to be established. By contrast, the connection between Ca^{2+} and reactive oxygen species (ROS; e.g. H_2O_2) has been already confirmed [25] (Box 2).

Besides the regulation of $[Ca^{2+}]_{cyt}$ levels, little is yet known about other putative ion channels and their regulation by insect-derived elicitors, the target proteins that are affected by the pharmacological agents, or the effector proteins that respond to changes in the Ca^{2+} level. There clearly is a need for a further characterization of signal-responsive ion channels, the coupling of signal perception with ion channel activity and with downstream elements in the putative signal cascades.

ROS production and short-term effects on V_m

The generation of ROS is a common response to pathogen invasion, and they have a dual role: at low concentration they act as second messengers involved in cell signaling and at high concentration they are part of the direct defense [27–33]. ROS are also generated in plant tissues in response to mechanical wounding [34] and in the interaction with herbivores [25,35,36].

Pathogen-induced ROS production, in particular H_2O_2 , is observed both locally and systemically in leaves of several plant species [37]. In response to herbivores, H_2O_2 levels increase as long as the attacks persist. Furthermore, the presence of H_2O_2 in the plant in response to herbivory could be advantageous preventing secondary microbial infection of wounded zones [37]. ROS can interact with ion channels and modify their activity [38]. In the interaction between the herbivore *Spodoptera littoralis* and Lima bean, it has been shown that H_2O_2 is released upon herbivore feeding and, to a lesser extent, mechanical damage [25]. H_2O_2 release causes V_m depolarization involving Ca^{2+} channels. Mechanically damaged Lima bean leaves (V_m approximately -140 mV) react fast to exogenous H_2O_2 by inducing a strong V_m depolarization. Leaves wounded by herbivores have a constant reduced V_m (approximately -90 mV) and show a dramatically lower or even no response to additional H_2O_2 application. The long-lasting depolarization of the V_m by H_2O_2 could be considered a strategy by the insect to silence plant responses [25] given that the insect actively introduces considerable amounts of H_2O_2 into the damaged tissue [39]. To cope with the enhanced levels of H_2O_2 , the activity of H_2O_2 scavenging enzymes in the plant, for example, catalase increases [25].

Besides H_2O_2 , nitric oxide (NO) functions as a signaling molecule in plant responses [40,41]. NO can function as a

Box 2. Early events in plant–herbivore interactions

The primary candidate for intercellular signaling in higher plants is the stimulus-induced change in plasma membrane potential (V_m). Herbivores aggressively attack leaves by continuous feeding (Figure 1a). False color confocal laser scanning micrographs of herbivore-wounded areas incubated with the membrane-permeant Ca^{2+} -selective fluorescent dye, Fluo-3 AM, show a dramatic Ca^{2+} influx limited to the few cell layers surrounding the area in which the insect has been feeding (green area in Figure 1b; chloroplast fluorescence is shown in red). $[\text{Ca}^{2+}]_{\text{cyt}}$ variations are followed by the production of H_2O_2 , which can be localized in the extracellular matrix by transmission electron microscopy by using CeCl_3 (the arrows in Figure 1c indicate a strong and evident CeCl_3 precipitation against cell walls facing intercellular spaces). 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) can be used to detect the presence of active peroxidases and the release of H_2O_2 from biological samples, with particular reference to mitochondria. The subcellular localization of H_2O_2 production in herbivore-wounded leaves following incubation with Amplex Red shows a clear localization in mitochondria or peroxisomes (stained in red in Figure 1d). Chloroplasts (Figure 1d stained in green) do not show any localization of H_2O_2 . $[\text{Ca}^{2+}]_{\text{cyt}}$ variations and H_2O_2 production are early events that are correlated with a strong V_m depolarization in the bite zone (Figure 1e), which is followed by a transient V_m hyperpolarization and, finally, a constant V_m depolarization throughout the rest of the attacked leaf (the broken line in Figure 1e represents the typical V_m value of a mechanically damaged Lima bean leaf).

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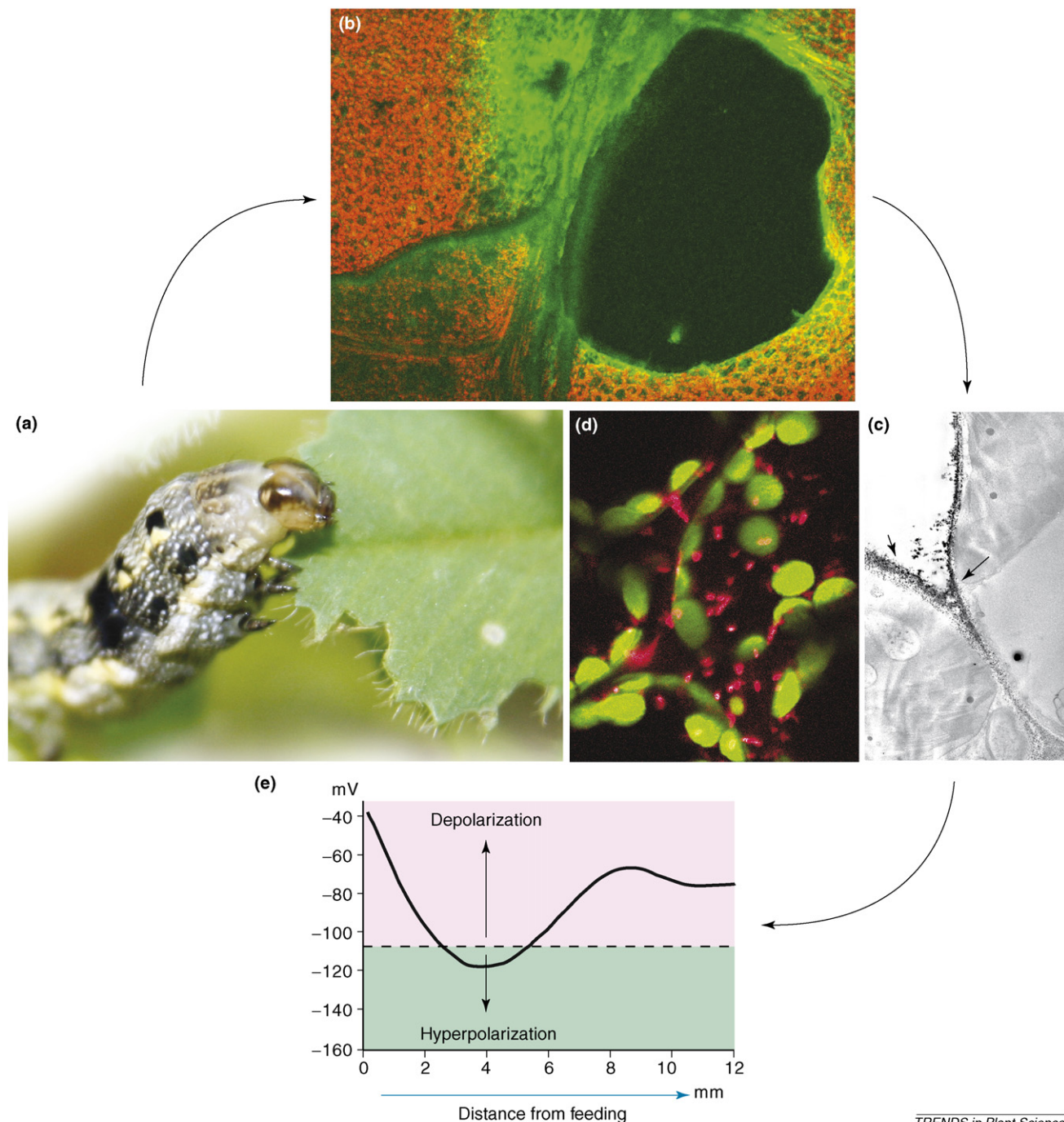


Figure 1.

negative regulator of some wound-related signals and can reduce the production of wound-induced H_2O_2 . In plant–pathogen interactions, jasmonic acid (JA) treatment results in a strong NO-production, suggesting a self-amplifying JA–NO loop [41]. NO is also known to induce the accumulation of salicylic acid (SA), the crucial mediator for the establishment of the systemic resistance response [41]. Therefore, the role of NO in responses to biotic stresses appears to be fine-tuned by SA, which might inhibit JA biosynthesis and antagonize the activation of the normal defense response.

Although the presence and function of NO has not been studied in plant–insect interactions, the JA-linked NO production [42] might also be relevant in this context given that jasmonates play the crucial role. More and detailed studies on the integration of NO in the network of signaling pathways involved in herbivory are necessary.

Early signal transduction, involvement of kinases

Mitogen-activated protein kinase (MAPK) cascades are important pathways downstream of sensors and receptors that regulate cellular responses to both external and endogenous stimuli in eukaryotes. In plants, there is evidence for MAPKs playing a role in the signaling of (a)biotic stresses, pathogens and plant hormones. cDNA was isolated from a species of tobacco encoding a mitogen-activated protein kinase (MAPK) whose transcript began to accumulate in the leaves only minutes after a single mechanical wounding event [43,44]. This protein kinase (WIPK) was essential for JA formation and JA-induced responses because silenced plants were unable to produce JA and methyl-JA after wounding. The loss of WIPK function resulted in an increased accumulation of SA and its β -glucoside after wounding. Moreover, WIPK has also been reported to elicit transcription of a gene for a ω -3 fatty acid desaturase (FAD7), which catalyzes the conversion of linoleic acid to linolenic acid, a precursor of JA [45]. In tobacco, the WIPK and the SA-induced protein kinase (SIPK) were found to share an upstream MAPKK, *NtMEK2* [46], and to interact with the calmodulin (CaM)-binding MAPK phosphatase (*NtMKP1*) [47]. *NtWIF* is a transcription factor activated upon phosphorylation by wound-induced protein kinase (WIPK) in tobacco plants. *NtMPK4*, a tobacco homolog of *Arabidopsis thaliana* MPK4, was activated by SA-induced protein kinase kinase (SIPKK) [48]. Transgenic tobacco plants overexpressing *NtWIF* exhibited constitutive accumulation of transcripts for the pathogenesis-related genes PR-1a and PR-2 and an enhanced level of SA. The results suggest that *NtWIF* is likely to influence SA biosynthesis, being located downstream of WIPK [49]. Because calcium-dependent protein kinases (CDPKs) are regularly involved in signal transduction of a variety of biotic and abiotic stresses, their involvement as active protein cascades in herbivore and wound responses cannot be excluded [50]. CDPKs compose a large family of serine/threonine kinases in plants (for example, 34 members in *Arabidopsis*) [51]. In potato (*Solanum tuberosum*) plants, JA has been reported to affect CDPK transcript level [52], and in tobacco a link between CDPKs (*NtCDPK2*), MAP kinases, and JA and ethylene cross-talks has been suggested [53]. It will be interesting to further address the question as to where the

regulated kinases are positioned within the different signal transduction chains in plant cells and, more specifically, whether they represent a component relaying herbivory-mediated changes in ion fluxes to specific transcription factors suspected to be involved in the activation of defense-related responses. Several kinases, CDPKs and MAPKs have been described to be activated in both *Nicotiana attenuata* and *Solanum nigrum* after attack by the solanaceous generalist herbivore *Manduca sexta* [54].

Ethylene, jasmonates and salicylates: how early do they respond?

A later element of the signal transduction pathway following tissue damage by chewing insects is represented by the network of phytohormones. By means of synergistic and antagonistic interactions (cross-talk), which depend on the herbivorous insect and the attacked plant, a fine-tuned response is achieved that regulates gene expression and, thus, controls the production of protective metabolites. For example, in many plants, herbivory stimulates the production of JA (\sim 10–15-fold), its precursor 12-oxophytodienoic acid (12-OPDA) (2–5-fold) and ethylene, whereas the level of abscisic acid and indole acetic acid is often decreased [55]. The time course for the individual phytohormones is different and can be assessed after extraction and derivatization by GC-MS methods [55,56]. The average resting level for JA has been found to be $10\text{--}40\text{ ng g}^{-1}$ FW; this increases 10–15-fold within the first 1–2 h after damage in various plants [55–57]. In Lima bean, the first increase of JA was observed as early as 10 min after damage and reached a maximum after 80–90 min. At the resting level, *trans*-JA comprised \sim 80% of the total JA, but immediately after wounding the biologically more active *cis*-JA dominated, comprising \sim 80% of the total JA [56]. During the feeding process, a clear spatial gradient of several oxylipins (including JA and 12-OPDA) developed. The highest concentration occurred directly at the feeding site and radiated from there \sim 10–20 mm into the undamaged tissue [58]. Whether or not local gradients around the biting-zone are typical of JA and 12-OPDA remains to be established. In the neighboring leaves, only an increase in the level of 12-OPDA, not of JA, was observed. Given that 12-OPDA is not only a precursor for JA but can also act as a JA-independent signal, its accumulation might be relevant for induction of a certain subset of defense responses. Other oxylipins that are simultaneously formed with JA might also play a role in plant defense, but their function is, as yet, less clear [56]. Silencing of the octadecanoid pathway in *N. attenuata* resulted in plants that were more vulnerable to insect attack, demonstrating the importance of this pathway for plant defense against insects [59].

Microbial infections generally caused much higher levels of SA than occurred as a result of insect feeding. In corn (*Zea mays*) [55] and *Arabidopsis* [60,61], the level of SA was raised only moderately. Enhanced ethylene production is another early and active response of plants to biotic stress. Ethylene synergizes volatile emission in corn [62] and modulates many other defense responses to insect herbivory [63–65]. Finally, in *Arabidopsis*, key elements for the integration of the signals resulting from JA and

ethylene (cross-talk) appear to be the transcription factor *AtMYC2* [66] and the ethylene response factor 1 (*ERF1*) [67], respectively.

Pathogens versus herbivores: common mechanisms?

Early signal transduction pathway studies with elicitors revealed striking similarities between plants and animals with regard to the molecules that perceive and transmit signals associated with invaders. In plant–pathogen and plant–herbivore interactions, early events, such as V_m changes, protein phosphorylation or activation of plasma membrane proteins, directly or indirectly trigger diverse signaling networks, interconnecting branch pathways that amplify and specify the physiological response [67] (Figure 1). A more complete understanding of trade-offs between resistance to pathogens and herbivores at the cellular level requires knowledge of the respective signal transduction pathways that are activated in response to insects and microbes [68]. In plant–pathogen interactions, plant receptor proteins perceive pathogen- or interaction-derived signals, initializing downstream signaling events. Elevation of $[Ca^{2+}]_{cyt}$ occurs almost instantaneously during the interaction between *Arabidopsis* and the pathogen *Pseudomonas syringae* [69], and Ca^{2+} appears to serve as a post-recognition molecular switch in plant–pathogen and in plant–herbivore interactions [23]. In both cases,

Ca^{2+} -activated kinases and phosphatases integrate the elevated Ca^{2+} signals into coordinated plant defense responses [47]. Apoplastic generation of superoxide ($O_2^{\bullet-}$), or its dismutation product H_2O_2 , has been documented following recognition of a variety of pathogens [70], and has also been suggested to be a common response after herbivore attack [5,25]. Accumulation of inducible antimicrobial compounds is often orchestrated through signal-transduction pathways linked to perception of the pathogen by receptors [71].

The same mechanisms are evident in plant–herbivore interactions. Recently, inceptins (proteolytic fragments of chloroplastic ATP synthase γ -subunit) have been demonstrated to function as potent indirect signals eliciting specific plant responses to insect attack, such as the induction of volatiles, phenylpropanoids, and protease inhibitor defenses [16]. JA, SA and ethylene are central players in mediating responses to pathogens and wounds. JA is usually associated with wounding pathways, whereas SA is most often thought to function in pathogen responses [68]; depending on plant and attacking organism, both pathways are additionally and differentially regulated by ethylene [72]. JA- or SA-dependent volatile emission by plants attacked by herbivores or microorganisms is another common response. Infection of pepper (*Capsicum annuum*) plants by incompatible *Xanthomonas* strains was

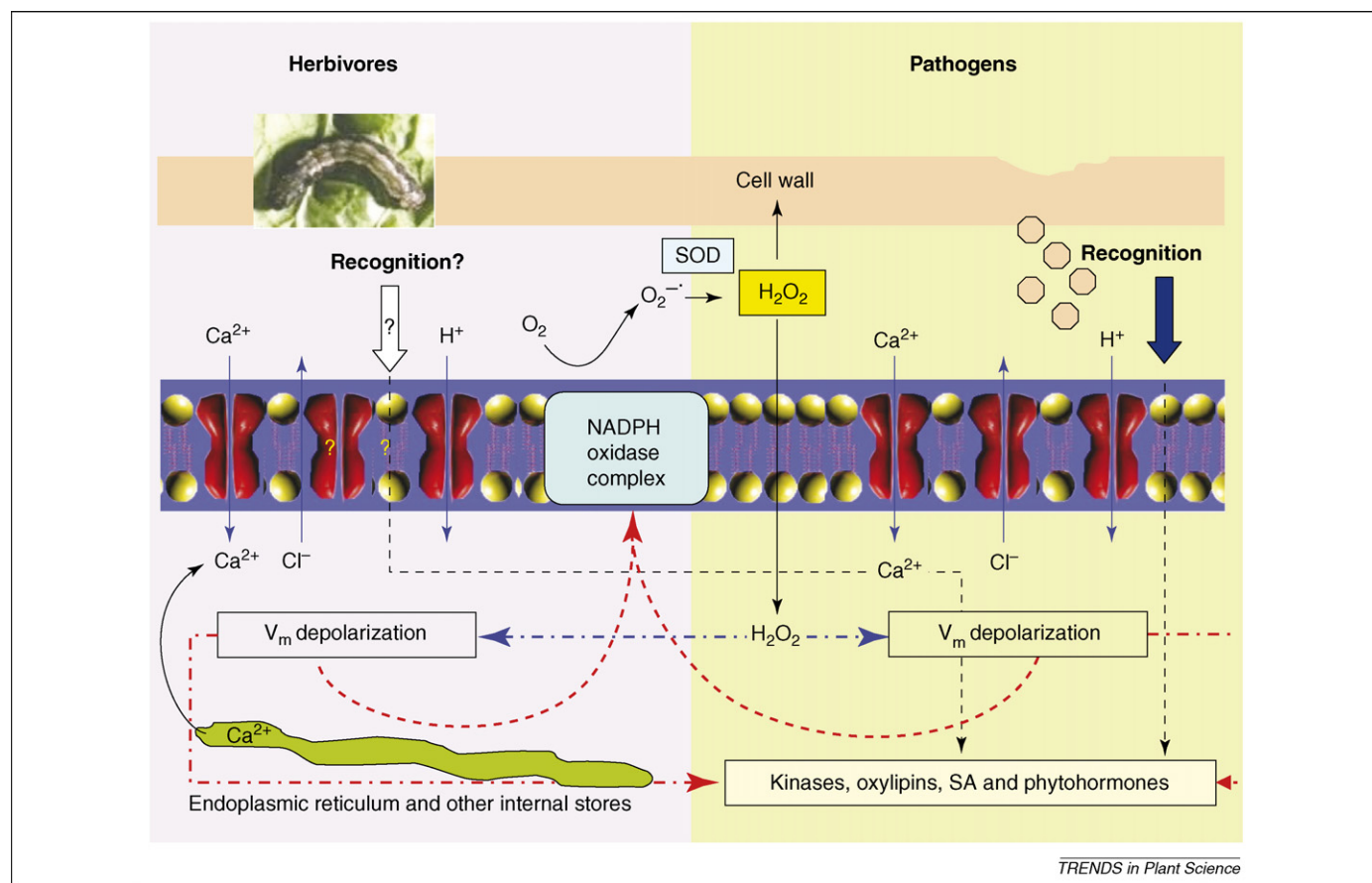


Figure 1. Early events in plant–pathogen and plant–herbivore interactions. In plant–pathogen interactions, plant receptor proteins perceive pathogen-derived or interaction-dependent signals, followed by downstream signaling events, including ion fluxes. Influx of H^+ and Ca^{2+} and efflux of Cl^- eventually induce V_m depolarization. Although Ca^{2+} influx has been demonstrated in plant–insect interactions, the role of anion and proton channels is not yet clear. Both pathogen and herbivores induce activation of the NADPH oxidase complex that generates the anion superoxide, which is quickly converted by the action of superoxide dismutase to H_2O_2 . H_2O_2 can accumulate in the extracellular matrix or enter the cell, where it prompts a concentration-dependent V_m depolarization.

demonstrated to up regulate SA production, resulting in reduced induction of volatiles and enhanced feeding activity of beet armyworm [73].

Conclusions and outlook

Recent studies have advanced our understanding of the mechanisms by which plants recognize herbivores and subsequently activate direct and indirect defense responses. Three areas where future efforts might result in major breakthroughs are related to the identification of herbivore-specific signal molecules, their recognition, and further signal transduction. The challenge for further research in this area is to determine their mode of action, whether these signals are transduced by receptor-mediated processes or if they simply interact with the plant membranes thereby initiating signal transduction pathways. One approach to achieve this goal might be the use of plant mutants that are not responsive to a particular herbivory-related signal. Characterization and the use of such mutants could result in the identification of both the genes encoding proteins involved in signal perception or proteins acting downstream in signal transduction and regulation of the defense response. Such an approach might yield important insight into the nature, the organization, and the integration of signal perception and transduction. There is a general need to identify putative components of signal transduction pathways by using biochemical and genetic methods, to study their possible interactions, and to analyze causal relationships. Not only could such studies uncover individual signaling pathways, but they could also establish links in a network of alternative routes regulating the multitude of inducible plant defenses.

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