Chapter 12
Plant Electrophysiology: Early Stages of the Plant Response to Chemical Signals

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Abstract Plant defence strategies start at the plant cell plasma membrane, where volatile organic compounds (VOCs) induced by insect herbivores or plant pathogens interact chemically and trigger plant signalling molecules. The earliest plant responses for the perception of VOCs are ion flux imbalances generated in the plant cell plasma membrane at the perception zone. This different charge distribution generates variation in the plasma transmembrane potential (V_m), which is the first event preceding the regulation of signal transduction pathways and gene expression. Change in the V_m can be through either an increase (hyperpolarization) or a decrease (depolarization) in the membrane potential. Here, we review recent advances in electrophysiological methods for the study of the early events of VOC perception and the correlation between V_m depolarization and plant signal transduction pathways leading to changes in gene expression.

12.1 Introduction

The plasma membrane represents the sensing element that recognizes changes in the cell-surrounding environment and starts cascades of electric signalling, eventually resulting in specific plant responses. Leaf damage, infection by plant pathogens, and feeding by insect herbivores induce the delivery of elicitors or the generation of plant cell wall-derived elicitors that may bind to specific receptors in the plant plasma membrane. Emerging evidence indicates that many high-affinity receptors for insect herbivores (Maffei et al. 2007a, 2012), plant pathogens (Elmore

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and Coaker 2011), and allelochemicals (Roshchina 2001) are located in the plant cell plasma membrane. The elicitor–receptor interaction results in variation in the plasma transmembrane potential (V_m), which is defined as the difference in the electrochemical gradient between the interior and exterior of the plant cell. This variation can lead to either more positive (depolarization) or more negative (hyperpolarization) V_m values, and these events eventually lead to the generation of signalling cascades.

Intercellular plasma membrane depolarization was recorded in *Nitella* sp. cells for the first time in 1930, earlier than the first intracellular electrical signal recording in animal cells (Nastuk and Hodgkin 1950; Tasaki 1952). Recently, most of the chemistry of the neuromotoric system of animals has been found in plants; for example, neurotransmitters such as acetylcholine and cellular messengers and cellular motors such as calmodulin and actin (Cao et al. 2006). Although this nerve-like cellular machinery never develops the same degree of complexity as in animal nerves, a simple plant neural network is formed, especially within phloem cells, which is responsible for the symplastic plasmodesmata-mediated communication over long distances (Fromm and Lautner 2007; Bricchi et al. 2013). Despite the direct effect of herbivore or microbial elicitors, plant membranes can also respond to VOCs either produced by the same plant or emitted by neighboring plants. Here, we summarize recent research on V_m variation as a common event of plant–VOC interactions. We will start this overview by describing the techniques currently used in plant electrophysiology to detect V_m variation.

12.2 Electrophysiological Methods for the Evaluation of V_m Variations

The system we currently use to measure V_m in leaf segments is the result of many technical tests, which eventually gave a useful set of electrical, electronic, and hydraulic instruments for conducting on-line (or real-time) recording of electrical variation in plant plasma transmembrane potential. This system was initially developed to measure membrane potential variations upon the effect of the phytoalexin isosakuranetin (5,7-dihydroxy 4′-methoxy flavanone) on potassium uptake in wheat root segments (Sacco and Maffei 1997) and the allelopathic effects of *Mentha* x *piperita* essential oil and monoterpenes on cucumber root membrane potential (Maffei et al. 2001). The system consists mainly of a homemade block constructed from Plexiglas, a polymethyl methacrylate (PMMA) polymer, or Teflon, a more inert polymer of polytetrafluoroethylene (PTFE) and some wells and sockets as shown in Fig. 12.1. We use this block to perfuse specific chemicals (including plant volatiles) dissolved in a buffer through a leaf segment, which allows electrophysiological measurements to be made from living tissues. A small square section of a leaf is incubated in a fresh buffer and then placed in the central socket of the block, and the V_m is measured as described in Fig. 12.1. A peristaltic pump—normally operating at a speed of 1 ml min^{-1}—pumps the buffer

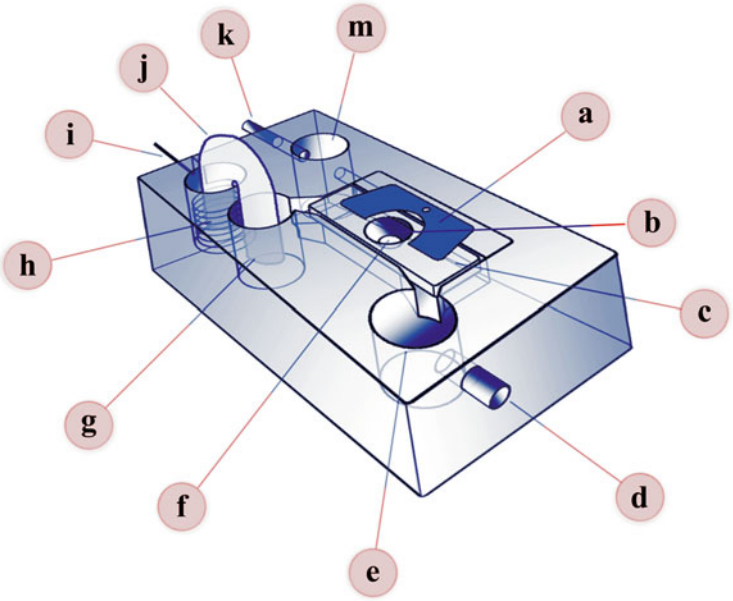

Fig. 12.1 Schematic representation of the system used for the evaluation of V_m in leaf segments. Modified from (Maffei and Bossi 2006). (**a**) Stainless steel holed blade that holds down the top and allows the probe to reach the leaf fragment and directly measure V_m; (**b**) hole giving access to the probe; (**c**) plastic lid with hole that fixes the leaf fragment for V_m detection; (**d**) excess buffered solution is extracted from the central socket through this exit with a vacuum pump; (**e**) well used to remove excess buffer; (**f**) central socket where a small square part of a leaf is placed; (**g**) well containing one of the two ends of the salt bridge; (**h**) well containing one of the two ends of the salt bridge and the silver wired solenoid; (**i**) wire solenoid connected to the ground; (**j**) salt bridge providing the electric link between the ground and the buffer solution; it is a curled glass pipe filled with agarose containing 3 M KCl; (**k**) inlet for buffer flowing from the peristaltic pump; (**m**) well where bubbles, if present, can easily emerge and dissolve

through the system. The core of the equipment is the electrical circuit, which allows V_m measurement. In order to measure V_m, we use very fine tipped (2–3 μm) borosilicate glass capillaries (WPI Inc, model 1B150F-4), which are made with a capillary puller (Nareshige model PE-21) and filled with a 3 M KCl solution diluted with ultrapure water (Millipore) (Maffei et al. 2004). Due to the very fine tip, the 3 M KCl solution in the inner part of the glass electrode permits an efficient electrical conductance with a very low (fM) loss of ions from the electrode to the cellular matrix. Glass microelectrodes are directly connected to a probe (WPI Inc.) by means of an electrode holder (WPI Inc.); this probe does the first step of cleaning and stabilizing the signal and is connected to a signal amplifier (WPI Inc.; e.g., model Electro 705). The amplifier takes the electrical signal coming from the cell and brings it amplified, cleaned, and stabilized to an oscilloscope (e.g., Tektronics model TDS 210), for further digital elaboration and data storage. The signal is measured and recorded in mV. The oscilloscope also allows visualizing the wave of

the electrical signal, the shape of which gives important information about cell condition, electrode integrity, and, in general, the electrical conditions of the entire system. The oscilloscope is also plugged to the ground, to complete the electrical circuit; ground is represented by the silver wire solenoid in the well. The ground is a special silver–silver chloride electrode, which acts as a signal transducer by converting ionic currents in a solution to an electric current within a wire, the same operation done by the probe attached to the glass electrode.

All instruments and electrical devices are very sensitive to both physical vibration and environmental electrical noises; thus all equipment is mounted on a stable work table, electrically grounded, and kept in a Faraday cage; all cables and wires, when not shielded properly, are wrapped with aluminum foil in order to reduce noise. Obtaining a good V_m is a very delicate operation, and the use of a micromanipulator, which can move in three directions, is fundamental. A stereo microscope or a special video camera is also needed in order to see exactly where to position the electrode onto the leaf tissue. Data recording is performed on a normal paper recorder or directly to a computer equipped with data logger software.

V_m can also be recorded in intact plant leaves. Therefore, we developed a chamber where molecules diffused in air can be focussed on leaves. Figure 12.2 shows how a plant cutting bearing entire leaves can be analyzed. The membrane potential is captured with a glass electrode that is positioned directly on a leaf that is

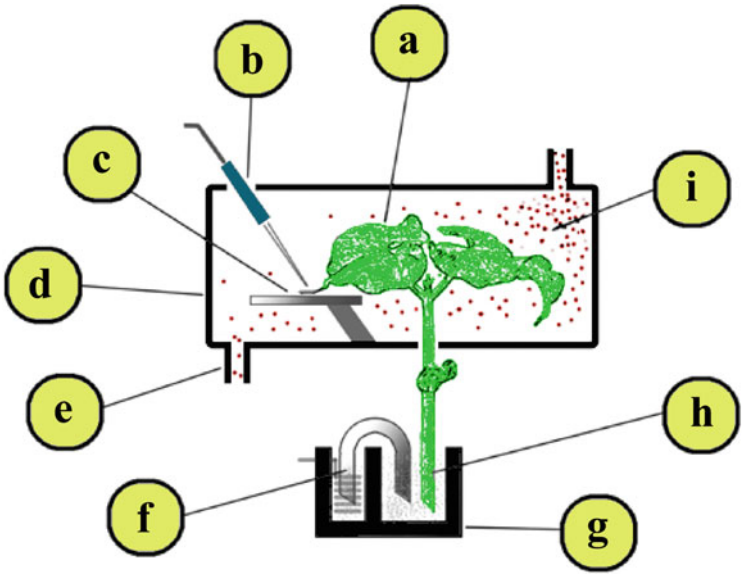

Fig. 12.2 Schematic representation of the system used for the detection of V_m in intact leaves of plant cuttings. Modified from (Maffei and Bossi 2006). (**a**) Leaves perceive and respond to volatile organic compounds that saturate the chamber; (**b**) V_m is detected with a probe; (**c**) holder; (**d**) Teflon chamber; (**e**) outlet for excess volatiles; (**f**) salt bridge (see also Fig. 12.1 for details); (**g**) Teflon box hosting the stem and the salt bridge; (**h**) stem immersed in a buffered solution; (**i**) inlet for volatile organic compounds

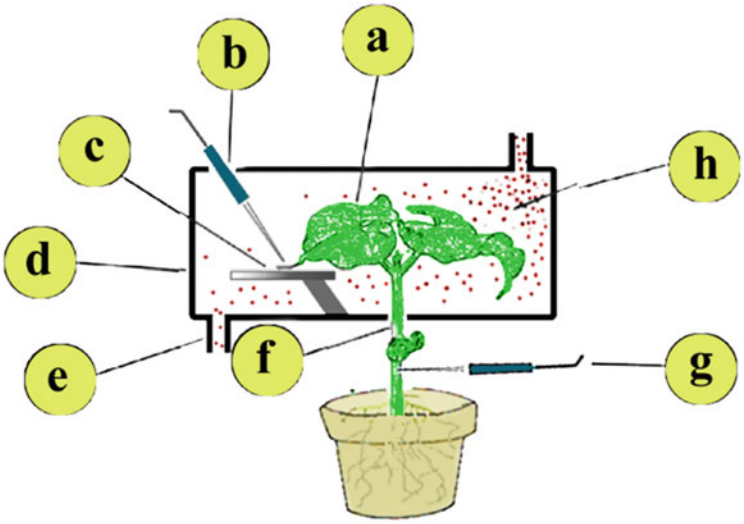

Fig. 12.3 Scheme of the system used for the detection of V_m in intact leaves of potted plants. Modified from (Maffei and Bossi 2006). (**a**) Leaves perceive and respond to volatile organic compounds that saturate the chamber; (b) V_m is detected with a probe; (**c**) holder; (**d**) Teflon chamber; (**e**) outlet for excess volatiles; (**f**) potted plant; (**g**) ground electrode inserted in the stem; (**h**) inlet for volatile organic compounds

fixed on a holder. The V_m of intact plant tissue is measured with two glass micropipettes filled with 3 M KCl and with a tip resistance of 4–10 MΩ. With the help of a micromanipulator, the first electrode is inserted into the mesophyll cells of the leaf. The electrode is then connected to a probe and to the amplifier. The position of the second electrode depends on the experimental setup. When the experiment utilizes a potted plant, the electrode is inserted into the plant stem phloem cells using a micromanipulator and is then connected to the ground port (Fig. 12.3) (Zebelo et al. 2012). When the experiment is with a plant cutting (Fig. 12.2), the plant cutting's stem is immersed in an ionic solution in a Teflon well. A salt bridge allows the plant to be in contact with the ground through a special coiled silver–silver chloride electrode, which acts as a signal transducer by converting ionic currents in the solution to an electric current within a wire connected to the ground port of the oscilloscope (Maffei and Bossi 2006).

12.3 Mechanisms of Electrical Signalling

Now that we have shown how to detect V_m variation in plants, we will turn our attention to the known electrical signalling mechanisms that plants adopt to respond to insect and pathogen attacks, and in the perception of VOCs. In principle, the

systemic signalling induced by biotic stressors and volatile signals is transduced by either chemical or electrical signals (Heil and Silva Bueno 2007; Maffei et al. 2007b; Mithöfer et al. 2009; Zebelo and Maffei 2012b; Zimmermann et al. 2009). Three broad mechanisms are recognized for the transmission of electrical signals, those involving action potentials (APs), variation potentials (VPs), and system potentials (SPs).

Action Potentials In higher plants, action potentials (APs) are defined generically as a long distance signalling system that may act to potentiate a host response to subsequent signals delivered through alternative long distance information packages. An AP is a momentary change in electrical potential of plant cells in response to environmental stimuli that eventually leads to intercellular and intracellular communication. A number of substances strongly depolarize the plasma membrane and thus presumably activate (voltage-gated) ion channels. Excitation of APs in plant cells depends on Ca^{2+}, Cl^{-}, and K^{+} ions (Zebelo and Maffei 2012a; Zimmermann et al. 2009; Felle and Zimmermann 2007). Felle and Zimmermann (2007) showed that although in principle it is possible that (anion) channels are directly activated by depolarization, the temporal sequence of the ion flux kinetics of barley leaves shows that Ca^{2+} is lost from the apoplast well before apoplastic anion concentration (measured as Cl^{-}) starts to increase. Therefore, channel activity is involved in APs, and the more the channels are activated the more rapid the depolarization will be, eventually leading to an accelerated depolarization that is measured as membrane potential "break-through," typical of an AP. When plasma membrane depolarization reaches a certain critical threshold level, AP is generated according to the "all or none" rule (Pyatygin et al. 2008). Like herbivores (and their oral secretions) and plant pathogens (and their elicitors), volatile signalling is known to cause APs, which usually appear as a single pulse; in rare cases, several repeated pulses are generated during VOC perception, and then APs propagate along the conducting bundles beyond the area of its generation. APs generated by VOCs propagate as fast electrical signals that travel through the entire plant from the point of VOC interception (Zebelo et al. 2012). APs generated by herbivory propagate at a speed up to 40 m s^{-1} (Volkov and Mwesigwa 2000). Using the aphid technique, Fromm and Bauer (1994) measured electric and cold-shock triggered APs in maize sieve tubes travelling at 3–5 cm s^{-1}. In the presence of leaf-feeding larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*), the speed at which insect-induced APs moved downward through the stem was about 0.05 cm s^{-1} (Fig. 12.4). Zebelo and coworkers demonstrated that herbivore-induced plant volatiles (HIPVs) trigger APs and VPs on nearby receiver plants (Zebelo et al. 2012).

Since APs are released by agents added in a realistic concentration range, it is suggested that they may serve as the first and fast systemic signals following attack from pathogens (Felle and Zimmermann 2007). Indeed, APs with a complete Ca^{2+} signature are required for activation of plant defence responses against pathogens (Blume et al. 2000). Similar Ca^{2+} signatures were recorded in tomato plants exposed to herbivore-induced plant volatiles and synthetic VOCs (Zebelo

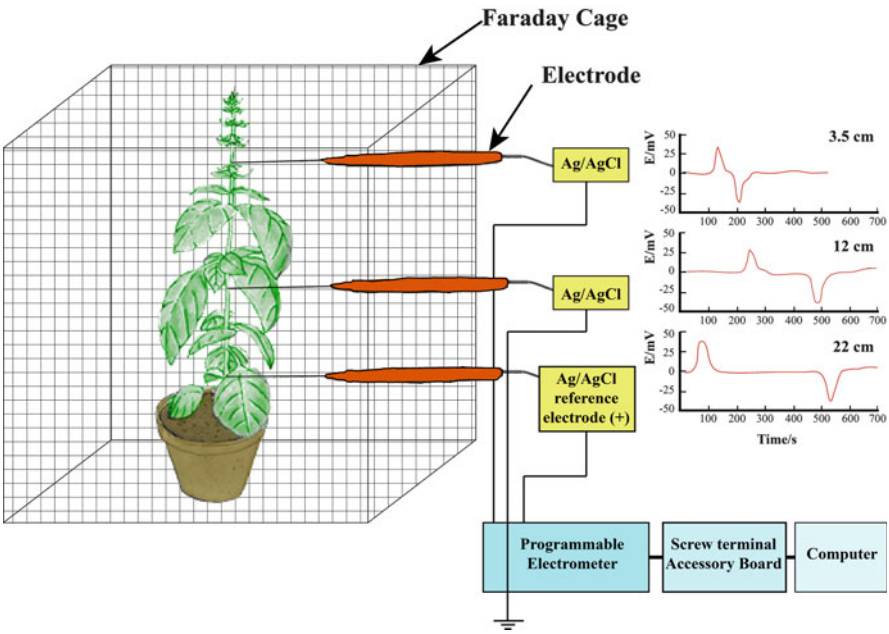

Fig. 12.4 Detection of action potentials in plants. All bioelectrochemical measurements are conducted at room temperature inside a Faraday cage. Ag/AgCl electrodes are connected to a programmable electrometer-amplifier with a high input impedance (>200 TΩ) through a triaxial cable. A computer with a multi-input–output plugin data acquisition board is interfaced with the electrometer and used to record the digital data. On the *right side* is an example of APs from measurements of plants challenged by the Colorado potato beetle on young terminal leaves. Distances between electrodes are indicated. Adapted from Volkov and Haack (1995)

et al. 2012). Figure 12.4 shows a typical configuration of apparatus for measurement of APs upon herbivory.

Variation Potentials Variation potentials (VPs) are defined as slow oscillations of the plasma transmembrane potential. In plants, VPs are characterized by amplitudes and speeds that decrease with increasing distance from the site injured by insect herbivores (Maffei and Bossi 2006) or plant pathogen infection (Elmore and Coaker 2011; Bricchi et al. 2012). While APs do not carry much information about the nature or intensity of the damage caused by the insect herbivore or plant pathogen infection, VPs are modulated in amplitude as well as in their interdependent ion fluxes, from which the plant or the affected organ may be able to gain information about the nature and intensity of the biotic injury (Zebelo and Maffei 2012a). V_m changes upon biotic stress have been associated with other important signalling events such as intracellular Ca^{2+} concentration variations (see below) and oxidative stress caused by either H_2O_2 or NO (Maffei et al. 2004; Maischak et al. 2007; Zebelo and Maffei 2012a; Bricchi et al. 2010). Mechanically damaged Lima bean leaves react fast and dramatically to H_2O_2 by undergoing a strong V_m

depolarization. However, leaves wounded by the herbivore *Spodoptera littoralis* already show a reduced starting V_m with the consequence of a dramatically lower or even no responsiveness to H_2O_2 application (Maffei et al. 2006). Thus, the depolarization of the V_m by the action of herbivory is linked to a reduction in downstream responses of attacked leaves to signalling molecules such as H_2O_2 and NO (Bricchi et al. 2010).

Manipulation of VPs by pathogens might result in significant lowering of host tissue responses and activation of defence. For instance, in *Arabidopsis thaliana*, significant VP variations were recorded after sixteen hours of *Pseudomonas syringae* DC3000 infection. At the same time, a large number of defence genes were downregulated including the respiratory burst oxidase protein C (RbohC)/NADPH oxidase (At5g51060), NADP-dependent oxidoreductase (At5g16960), universal stress protein (USP) family protein (At3g62550), mechanosensitive ion channel domain-containing protein (At1g53470), and a MATE efflux protein (At1g58340) (Bricchi et al. 2012).

Herbivorous insects and their oral secretions, plant pathogens and their elicitors, as well as volatile signalling are known to cause both APs and VPs, but it is still unclear whether herbivorous insects and volatile signalling can cause SPs (Bricchi et al. 2013; Maffei et al. 2007b; Zebelo and Maffei 2012b). SPs have recently been described as novel electrical long distance apoplastic signals in plants induced by wounding and acting as a forerunner of slower travelling chemical signals (Zebelo and Maffei 2012a; Zimmermann et al. 2009). SPs serve as a backup to APs and VPs and overlap with APs and VPs in some instances. Having this brief background about SPs, it is hard to exclude the occurrence of SPs during volatile signalling (Zebelo and Maffei 2012a).

12.4 Generation of Electrical Signals Upon Biotic Stress

12.4.1 The Plant Plasma Transmembrane Potential (V_m) Responds to Biotic Stress

Plant defence strategies against biotic stressors are generally orchestrated as a network of perception systems, which start at the front line of damage (the plasma membrane) where insect herbivores or plant pathogens interact with plants physically (by mechanical damage) and chemically (by introducing elicitors or triggering plant-derived signalling molecules). There a fast state change (the V_m variation) triggers gene expression-independent reactions, eventually leading to the activation of signalling pathways able to affect the cell, its surroundings, and the whole plant, with concerted local and systemic responses. In contrast to hydraulic, mechanical, and hormonal signal transduction, electrical signals are able to deliver fast information over long distances. To better understand the role of V_m variation as a triggering event, we will address some key questions on (i) how the plasma

transmembrane potential (V_m) responds to biotic stress and (ii) how ion channels and transporters are involved in the perturbation of the chemiosmotic balance.

As stated above, the cell plasma membrane is the only cellular component with 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 V_m or modulation of ion fluxes at the plasma membrane level are among the fastest cellular responses to biotic and abiotic stresses (Shabala and Bose 2012; Maffei et al. 2007a). The candidate ion species responsible for V_m variations in plant cells upon biotic stress are calcium (Ca^{+2}), protons (H^+), potassium (K^+), and chlorine (Cl^{-1}).

Herbivory-Dependent V_m *Variations* Herbivory-dependent V_m variations result from the combination of two concomitant processes: (1) the mechanical damage of the plant tissues and (2) the introduction of oral secretion (OS) of the feeding insect into wounded tissues. Thus, the attacked plant faces both a mechanical and a chemical challenge (Bricchi et al. 2010; Mithöfer et al. 2009). Application of insect OS to a mechanical injury can mimic most plant responses to herbivory (Reymond et al. 2004), suggesting that OS elicitors are major contributing factors to a plant recognizing insect attacks (Bricchi et al. 2012; Consales et al. 2012; Maffei et al. 2012). Indeed, several elicitors have been isolated from insect OS that trigger plant defences against herbivory, such as β-glucosidase (Mattiacci et al. 1995), volicitin (a fatty acid–amino acid conjugate) (Halitschke et al. 2001), caeliferins (Alborn et al. 2007), inceptins (Schmelz et al. 2006), and a still uncharacterized polysaccharide elicitor (Bricchi et al. 2013). Lepidopteran OS consists of mandibular and other labial secretions, glandular secretions from the ventral eversible gland, and regurgitate from the digestive tract (Felton 2008; Zebelo and Maffei 2012b). In the *S. littoralis–Phaseolus lunatus* interaction, both direct herbivory and the insect's oral secretions have been demonstrated to induce a fast plant cell V_m depolarization (Bricchi et al. 2010, 2012; Maffei and Bossi 2006; Maffei et al. 2004), and the same response was shown when *S. littoralis* was feeding on other higher plant species such as *A. thaliana* (Zebelo and Maffei 2012b; Bricchi et al. 2013) and *Ginkgo biloba* (Mohanta et al. 2012) and lower plant species such as the fern *Pteris vittata* (Imbiscuso et al. 2009). Interestingly, a significant V_m depolarization was observed at almost every stylet puncture during phloem feeding by *Myzus persicae* (Bricchi et al. 2012).

During insect-elicited V_m variations, the plant cell responds with a V_m depolarization, the duration of which depends on the nature of the biotic attack. For instance, the V_m depolarization is much more rapid in response to feeding by *S. littoralis* (30 min) than by the aphid *Myzus persicae* (4–6 h) (Bricchi et al. 2012). Irrespective of the nature of the biotroph, this event occurs at the same intensity, which in Arabidopsis corresponds to a V_m depolarization of about 40 mV. The different timing of V_m depolarization appears to be associated with the mode of biotic damage. The fast clipping and consistent plant tissue removal by chewing herbivores evidently induces a "quantitative" response that is proportional to the amount of tissue damage; however, stylet probing and phloem feeding by

aphids induces less damage, which requires more time for a plant response. This implies that the speed of the V_m response allows setting a time scale for conducting comparative genome-wide analyses. In fact, in Arabidopsis, a clear relationship between V_m depolarization and gene expression was found. When genome analyses were conducted at the point of V_m depolarization, feeding by the aphid *M. persicae* regulated a wider array of Arabidopsis genes than feeding by *S. littoralis* (Bricchi et al. 2012).

Pathogen-Dependent V_m *Variations* In plant–pathogen interactions, V_m depolarization is a reliable early indicator of the leaf hypersensitive response (HR) (Pike et al. 2005). *P. syringae* DC3000 infection is also able to induce a V_m depolarization in Arabidopsis with the same magnitude of V_m depolarization as recorded for aphid and herbivore attacks, suggesting the presence of a V_m threshold that has to be reached for a successful herbivory/infection response (Bricchi et al. 2012; Pike et al. 2005). Bacterial flagellin represents one of the best studied pathogen-associated molecular patterns (PAMP) that induce V_m depolarization in mesophyll cells and in root hair cells of Arabidopsis (Jeworutzki et al. 2010). Elicitors isolated from the fungus *Cladosporium fulvum* were also demonstrated to induce V_m depolarization in tomato plants (Gelli et al. 1997). Plasma membrane ion channels are rapidly activated by pathogen infection or by elicitor treatment of plant cells (Fisahn et al. 2004). However, bacterial growth and tissue damage take time, which appears to be proportional to the timing of V_m depolarization (14–16 h) (Bricchi et al. 2012). At the time of V_m depolarization, an almost completely opposite state of regulation was observed for Arabidopsis damaged by the aphid *M. persicae* and the pathogen *P. syringae* DC3000, with the former suppressing and the latter activating defence responses (Bricchi et al. 2012). In parsley cells treated with Pep-13, an oligopeptide fragment of a 42-kDa *Phytophthora sojae* cell wall glycoprotein, extracellular alkalinization, Ca^{2+} influx, and efflux of K^+ and Cl^- lead to V_m depolarization (Fisahn et al. 2004; Blume et al. 2000).

Abiotic and biotic stresses, including mechanical wounding and insect attack, elicit signals that trigger a phosphorylation cascade leading to jasmonic acid (JA) biosynthesis. JA is a key regulatory component in defence-signalling pathways. The JA level increases in corn (*Zea mays*) and hybrid poplar (*Populus deltoides* × *nigra*) seedlings after exposure to green leaf volatiles (Engelberth et al. 2004).

12.4.2 V_m Changes and Ion Variations in Response to Biotic Stress

Plant ion channels are transmembrane proteins located in the plant cell membranes that mediate ion fluxes across the cell compartments (Wang 2012). The hydrophilic pore structure of plant ion channels allows the passage of ions through the

membranes at extremely high rates (106–108 ions per second through one channel protein) driven by transmembrane electrochemical potentials (Maathuis et al. 1997). Different plant ion channels are selectively expressed in specific tissues or cells, where they perform the appointed functions to facilitate the physiological processes of those tissues or cells (Wang 2012). Recall that V_m variations are caused by an unbalanced ion distribution across the plasma membrane and that depolarization occurs when cations (such as K^+ and Ca^{2+}) are allowed to enter the cell or anions (e.g., Cl^-) are allowed to exit the cell. Furthermore, hyperpolarization depends mainly on the activity of the plasma membrane H^+-ATPase or when inward anion channels or outward cation channels are opened (Maffei et al. 2004).

Herbivory-Dependent Ion Fluctuations and Their Effects on the V_m An electrophysiological approach called the planar (black) lipid bilayer technique (BLM) is widely used to elucidate the molecular mechanisms and activities of various biologically active substances (Winterhalter 2000). Using BLM, Boland and coworkers demonstrated that insect-derived elicitors (e.g., fatty-acid–glutamine conjugates, LeaGln) and the *Spodoptera exigua*-derived OS directly interact with artificial lipid bilayers to generate channel-like activities that are highly conductive and selective for certain ions (Maischak et al. 2007).

In general, upon herbivory, the increase in cytosolic calcium precedes membrane depolarization (Maffei et al. 2007a). Ca^{2+} is one of the principal intracellular messengers and during electric signal generation and propagation, Ca^{2+} enters the cytoplasm through voltage-gated Ca^{2+} channels located at the plasma membrane and other membranes of intracellular stores (Arimura and Maffei 2010). Herbivore feeding causes a dramatic Ca^{2+} cytosolic ion influx limited to a few cell layers lining the wounded zone (Zebelo and Maffei 2015). This response is limited to herbivory or biotrophic activity, since neither single nor repeated mechanical wounding events are able to induce significant changes in cytosolic Ca^{2+} ion influx (Bricchi et al. 2010). Insect feeding and isolated insect-derived elicitors are known to cause activation of Ca^{2+} channels (Arimura and Maffei 2010; Maffei et al. 2007a), and these events have been associated with V_m depolarization (Maffei et al. 2004).

However, upon herbivory, the Ca^{2+} signalling depends on the symplastic continuity granted by functioning plasmodesmata. We have recently made the surprising observation that an Arabidopsis line (*pdko3*) mutated in genes encoding plasmodesmal proteins is defective in some of the typical plant responses to herbivory. Following herbivory and the release of OS, both the *pdko3* and wild-type (WT) plants showed increased accumulation of cytosolic Ca^{2+}, but, unlike WT plants, the mutant line showed an almost complete loss of V_m depolarization. Thus, the mutations in genes for plasmodesmal proteins have provided valuable genetic tools for the dissection of the complex spectrum of responses to herbivory and shown us that the responses to herbivory imply a Ca^{2+}-independent V_m depolarization (Bricchi et al. 2013).

Increased K^+ channel activity was observed when Arabidopsis plants were damaged by *S. littoralis* feeding, or OS was introduced to mechanically damaged

leaves (Bricchi et al. 2013). Potassium represents the major osmotically active cation in plant cells and is fundamental for plant functions such as control of membrane potential (Geiger et al. 2009; Lebaudy et al. 2007). Upon herbivory, the increased concentration of cytosolic Ca^{2+} triggers the opening of inward rectifier K^+ channels that are the major factor responsible for herbivore-induced V_m depolarization. In the *pdko3* Arabidopsis mutant, it appears that the connection between increased calcium and K^+ channel activation is broken leading to significantly decreased V_m depolarization, although lower absolute levels of cytosolic calcium in *pdko3* leaves opens the possibility of a threshold effect on K^+ channel activation (Bricchi et al. 2013).

Microbe-Induced Variation in Ion Homeostasis and the Effect on V_m Receptor-mediated recruitment of calcium stores and activation of plasma membrane anion channels represent initial steps in pathogen recognition and innate immunity-related signalling (Elmore and Coaker 2011). The increased cytoplasmic Ca^{2+} concentration observed in response to stress induced by bacteria and fungi involves extracellular polysaccharides, peptidoglycans, lipo-oligosaccharides, or chitin and appears to be an essential, common event after recognition of microbes by plant cells (Aslam et al. 2009; Lecourieux et al. 2005; Miya et al. 2007; Nurnberger and Scheel 2001; Romeis et al. 2001).

The use of ion-selective electrodes and imaging of simultaneous changes in cytosolic-free Ca^{2+} concentration allowed identification of the ion species involved in membrane depolarization following bacterial flagellin (flg22) treatment (Jeworutzki et al. 2010). Application of 10 nM flg22 to Arabidopsis leaves and roots induced a strong V_m depolarization after a lag phase of 1–3 min, with the magnitude and velocity of the depolarization dependent on the concentration at which flg22 was applied. Aequorin is a calcium-activated photoprotein; its apoprotein is called apoaequorin, and its prosthetic group is called the luciferin. Stimulation of Arabidopsis plants constitutively expressing apoaequorin to monitor the cytosolic Ca^{2+} concentration after application of flg22 resulted in a dose-dependent transient rise in cytosolic Ca^{2+}. When membrane potential recordings and Ca^{2+} imaging experiments were performed simultaneously, flg22 addition was found to cause a V_m depolarization and a cytosolic Ca^{2+} increase, which occurred within the same time frame (Jeworutzki et al. 2010).

Cryptogein is a 10-kD protein secreted by the oomycete *Phytophthora cryptogea* that induces a hypersensitive response in tobacco (*Nicotiana tabacum*) plants and systemic acquired resistance against various pathogens. Within the first 5 min after infection, cryptogein induces an anion efflux and a Ca^{+2} influx, which gives rise to a fast and large V_m depolarization. The latter, in cryptogenin-treated plants, has been associated with the inhibition of glucose uptake, which is symported with H^+ and, thus, depends on the transmembrane electrochemical potential difference (Bourque et al. 2002). Elicitors from *C. fulvum* activate tomato plasma membrane Ca^{2+} permeable channels resulting in increased cytosolic Ca^{2+} concentrations (Gelli et al. 1997).

Alamethicin is an elicitor from the fungus *Trichoderma viride* and is well known to produce pores in artificial membranes as well as in animal and plant cell membranes (Duclohier et al. 2003). Alamethicin induces pore formation in the tonoplast membrane of the plant *Chara corallina*, and these pores are conductive depending on the polarity of the voltage applied in the plasma membranes (Luhring et al. 2007).

Unlike other plant–pathogen interactions and in a similar fashion to plant responses to herbivory, Ca^{2+} signalling appears to be nonessential to the recognition of the early stages of viral infection. Shabala et al. (2010) observed significant changes in K^+ fluxes as early as 10 min after viral inoculation, which were mediated by depolarization-activated outward-rectifying K^+ channels. This may suggest that viral infections trigger a different mechanism of plant defence signalling compared to signals derived from other microbial pathogens; hence, altered Ca^{2+} fluxes across the plasma membrane may not be a common prerequisite for all elicitor-activated defence reactions.

VOC-Dependent Ion Fluctuations and Their Effects on V_m Compared to herbivore- and microbe-dependent ion fluctuations, there is limited information on the role of VOC-dependent ion fluctuations and their effects on the V_m. Zebelo and coworkers showed that VOCs emitted by tomato plants damaged by the herbivore *Spodoptera littoralis* trigger a significant V_m depolarization in the mesophyll cells of receiver tomato plants (Zebelo et al. 2012). In the same study, VOCs from mechanically damaged and control unwounded tomato plants did not exert significant differences in the V_m of receiver tomato plants compared to the effects of clean air. In a real-time experiment using confocal laser scanning microscopy (CLSM), tomato plants treated with the fluorescent calcium indicator Calcium orange were exposed to herbivore-induced plant VOCs and showed an increase in cytosolic calcium concentration $[Ca^{2+}]_{cyt}$ (Zebelo et al. 2012). These results show that plant perception of volatile cues from the surrounding environment is mediated by early events, occurring within seconds and involving the alteration of the plasma membrane potential and increases in the cytosolic calcium. GLVs are produced as long as herbivory is present, therefore the continuous emission of these molecules signals to neighbor plants the persistency of herbivore attack. Although the volatile language is still difficult to decipher, we found that low molecular weight VOCs such as GLVs prompt a faster and stronger V_m and calcium response when compared to higher molecular weight compounds such as monoterpenes and sesquiterpenes, which appear to act only on the V_m component of the plant cell response (Zebelo et al. 2012).

12.4.3 Effects of Biotic Stress on Transporter Activity

Transporters are specialized proteins that assist in the movement of small molecules, ions, macromolecules, peptides, and lipids across a biological membrane.

The plasma membrane H^+-ATPases are the primary pumps responsible for the establishment of cellular membrane potential in plants (Boller and Felix 2009). Plasma membrane H^+-ATPases use energy derived from ATP hydrolysis to pump protons to the extracellular space and inside the vacuole, thus creating and maintaining a negative V_m potential (Elmore and Coaker 2011). Therefore, any alteration in H^+-ATPase activity leads to V_m variation, with a depolarizing effect caused by the reduction of its ability to expel protons from the cytosol. In addition to regulating basic aspects of plant cell function, these enzymes contribute to signalling events in response to diverse environmental stimuli. H^+-ATPases are dynamically regulated during plant immune responses, and quantitative proteomics studies have demonstrated complex spatial and temporal modulation of plasma membrane H^+-ATPase activity during early pathogen recognition (Keinath et al. 2010; Nuhse et al. 2007) and insect herbivore recognition (Bricchi et al. 2013; Schaller and Oecking 1999). For instance, in response to pathogens, an increase in H^+-ATPase activity (i.e., V_m hyperpolarization) has been demonstrated in tomato (Veraestrella et al. 1994) and barley (Knogge 1996), whereas the inhibition of the H^+-ATPase (i.e., V_m depolarization) is exploited by pathogens in order to overcome plant resistance (Zhou et al. 2000). The pea pathogen, *Mycosphaerella pinodes*, inhibits H^+-ATPase activity in the pea plasma membrane through the action of the suppressor of phytoalexin accumulation, supprescin B (Kato et al. 1993).

H^+-ATPases and Ca^{2+}-ATPases are members of a class known as P-type ATPases. In response to insect herbivory, Ca^{2+} ions are released into the cytosol via channel proteins and pumped back into their stores (organelles) and the apoplast via Ca^{2+}-ATPase pumps (Bricchi et al. 2010; Maffei et al. 2004). Ca^{2+}-ATPases are regulated by herbivore OS (Maischak et al. 2007). Given the numerous roles of P-ATPase activity in plant cell physiology, it is no wonder that pathogens have evolved mechanisms to target these enzymes (Elmore and Coaker 2011).

Fusicoccin is produced by the fungal pathogen *Fusicoccum* (*Phomopsis*) *amygdale* and functions by locking the interaction between 14-3-3 proteins and the C-terminal regulatory domain of the plasma membrane H^+-ATPase, which leads to constitutive activation of this hydrogen pump and causes a constant V_m hyperpolarization (Baunsgaard et al. 1998). The mechanism for the reverse effect, where some still unknown biotic elicitors may interfere with the activation of the H^+-ATPase rendering the pump unable to repolarize a depolarized V_m, is particularly intriguing.

12.5 Concluding Remarks

Depolarization of the V_m is one of the first responses to biotic stress of the plasma membrane, which mainly depends on ion fluxes, including the opening of K^+ channels and the release of calcium from internal stores or through influx from the apoplast. These early events are followed by the activation of signalling pathways, eventually leading to plant responses to biotic stress (Fig. 12.5).

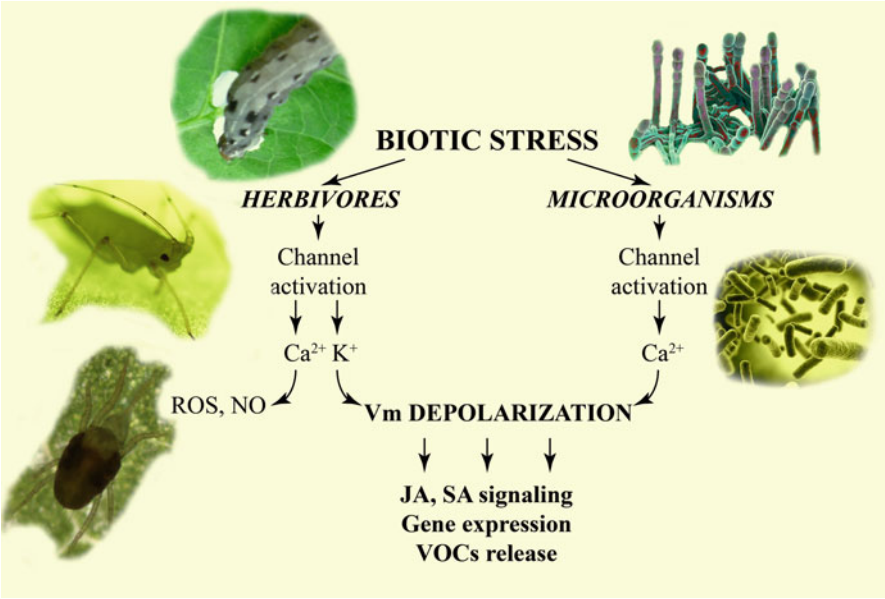

Fig. 12.5 V_m depolarization is a common event upon biotic stress. Insect herbivores induce the opening of both calcium and potassium channels. Potassium channel activity is the main factor responsible for V_m depolarization, whereas increased cytosolic calcium activates ROS and NO (Bricchi et al. 2013). Microorganisms activate calcium channels inducing plant cell V_m depolarization through the concerted action of anion and cation channels. V_m depolarization is then followed by activation of jasmonic acid (JA) and salicylic acid (SA) signalling pathways eventually leading to plant responses to biotic stress, including gene expression and the release of herbivore-induced and microbe-induced volatile organic compounds (VOC)

Thus, electrophysiology is indeed a valuable tool to study and understand early events in plant interactions with other organisms (including other plants), and V_m evaluation, more than the single patch analysis, gives a tissue image of cooperative interplay among wounded and unwounded cells.

Understanding fast responses of plants to the surrounding environment is of interest not only from an ecological and evolutionary perspective but also for the development of novel crop protection strategies. Owing to the massive damage that herbivores and pathogens cause to valuable crops, the deciphering of early signals from plants represents one of the most exciting fields of research in the first line of defence.

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 is to determine their mode of action, whether these signals are transduced by receptor-mediated processes or simply interact with the plant membranes and thereby initiate 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;

indeed, the Arabidopsis *pdko3* mutant was instrumental to dissecting early and late events in plant responses to herbivory (Bricchi et al. 2013). Characterization and the use of such mutants could result in the identification of genes encoding proteins involved in signal perception. Not only can such studies uncover individual signalling pathways, but they can also establish links in a network of alternative routes regulating the multitude of inducible plant defences. Much more has to be done in this field, but the promising results obtained in intact rooted plants following biotic and abiotic stress may lead to interesting new discoveries.

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