8 Electrophysiological Characterization of Plant Cation Channels

VADIM DEMIDCHIK,[1] ANATOLY SOKOLIK,[2] VLADIMIR YURIN[3]

8.1 Introduction

Cation channels are macromolecular protein pores in bio-membranes that catalyze passive cation influx and efflux (MacKinnon 2004). They do not use ATP energy to transport cations as opposed to active transporters such as pumps and carriers. Since cation channels are not limited by the rate of metabolic interactions, they saturate at much higher concentrations than active transporters and demonstrate low Q_{10} coefficients (<2.0). Cation channels consist of several transmembrane alpha helices that are also called transmembrane spans or transmembrane domains. These transmembrane domains form a pore region with a selectivity filter that selects cations over anions. Rearrangement of transmembrane domains causes pore opening (activation) or closing (deactivation). Different cation channels have different activators and inhibitors, including membrane voltage (V_m), H^+, divalent cations, G-proteins, ATP, cyclic nucleotides, hormones, ROS, amino acids, stretching and gravity. Specific chemical sites in the channel macromolecule are responsible for interactions with activating and inhibiting factors. Some cation channels have fixed anion surface charges outside and/or inside of the channel entry. These charges increase a local cation concentration and modify voltage-dependence, gating and selectivity of the channel (Green and Anderson 1991; Miedema 2002). Protons and divalent cations effectively screen surface charges and cause significant changes in the channel function. Cation channels are sensitive to a range of specific and non-specific blockers. Experiments with blockers, or so-called pharmacological analysis, are necessary for the selection between several groups of channels. For example, tetraethylammonium (TEA^+) is a specific blocker of K^+ channels that does not affect other cation channels (reviewed by Demidchik et al. 2002a). Blockers can be of "natural" origin, such as Ca^{2+}, Mg^{2+}, Zn^{2+} or H^+, or xenobiotics, for example Ba^{2+}, TEA^+, Cs^+, lanthanides, dihypropiridines, phenylalkylamines

[1] Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB23EA, UK

[2] Laboratory of Plant Cell Physiology, Biological Faculty, Belarusian State University, 4 Skaryna Avenue, 220050, Minsk, Belarus

[3] Department of Physiology and Biochemistry of Plants, Biological Faculty, Belarusian State University, 4 Skaryna Avenue, 220050, Minsk, Belarus

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among others. Analysis of blockage provides important information about molecular determinants of the channel (Hille 1994).

Cation channels play multiple physiological roles in plants. They catalyze nutritional uptake of N (taken up as NH_4^+), macronutrient and micronutrient cations such as K^+, Ca^{2+}, Na^+, Fe^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+}. Cation channels are responsible for the generation of negative resting V_m and action potentials. This is necessary for maintaining structural and functional integrity of the membrane, signaling processes and polarity. Cation channels are directly involved in osmotic balance and regulation of the turgor. This property of cation channels underlies stomata opening and closing. Calcium-permeable cation channels trigger Ca^{2+} signaling in plants that is involved in tissue and organ coordinated growth, development and stress responses. ROS, amino acids, purines, elicitors, hormones, gravity, different stresses and stretching act through activation of cationic channels. Having multiple physiological roles in plants, cation channels have been a subject of extensive study. Different physiological and molecular techniques have been employed for examination of physiology and structure of cation channels. Unfortunately, the crystal structure of plant cation channels remains unknown.

Significant progress has been achieved in our understanding of the molecular nature of cation channels in the last decade (reviewed by Davenport 2002; Demidchik et al. 2002a; White et al. 2002; Véry and Sentenac 2003). Many genes encoding plant cation channels have been identified and characterized molecularly. Analyses of knock-out plants and plants over-expressing K^+ channels showed for the first time physiological consequences of the lack or abundance of the particular channel. Nevertheless, molecular studies do not provide information regarding physiological characteristics of cation channels in intact cells. Electrophysiological techniques should be employed to establish channel properties in in vivo conditions. Here, we briefly review some of the most important electrophysiological techniques and provide examples of their use for studies of cation channels in plants.

8.2 Overview of electrophysiological techniques

The main question of the physiology of cation channels is how cations are transported through the channel and how this transport is regulated by internal and external factors. To investigate this, electrical currents or net fluxes mediated by cation movement through the channel should be measured.

There are two main types of electrophysiological recordings in plant cells: extracellular and intracellular recordings. Techniques for extracellular electrophysiological recordings include extracellular electrodes and “microelectrode ion flux estimation” (MIFE®). Intracellular recordings can be performed by impaling a cell with one or more fine-tipped electrodes (so-called “impalement techniques”), or by sticking a cell to the glass micropipette (so-called

patch-clamp technique). Intracellular recordings allow measuring V_m and/or membrane current (I_m). All these techniques have played significant roles in the characterization of plant cation channels. They have many advantages and disadvantages that are discussed below. Plant cation channels can also be characterized electrophysiologically by incorporation of purified channels into the planar lipid bilayers that mimic the cell membrane. This technique is not discussed here because it is reviewed elsewhere (Tester 1990; White 1998).

8.2.1 Extracellular recordings

In recording with extracellular electrodes, an electrode is placed in the extracellular medium and field-potentials contributed by the action potentials of many small cells or one giant alga cell are measured (Zawadzki 1980). This technique is useful for examination of generalized electrical responses of higher plant tissue or large cells, for example giant alga internodes. Despite their frequent use in early works, extracellular electrodes have not found much application in recent studies. For example, Favre et al. (2001) have applied extracellular electrodes for studying action potentials induced in *Arabidopsis thaliana* leaves by wounding and externally applied high concentration of KCl.

Another extracellular technique, namely MIFE®, has revolutionized the field of non-invasive plant membrane biology during the last decade (Shabala et al. 1997; Shabala 2000; Newman 2001; Babourina et al. 2002; Demidchik et al. 2002b, 2003; Hush et al. 1992; reviewed in this issue by Shabala). In this technique extracellular ion-selective microelectrodes "vibrate" (move forward/backward) with a period of few seconds near the surface of plant tissue or protoplast, with the simultaneous recording activities of several ions. Measuring ionic activity at two points at given distances (usually at about 50 and 100 μM from the tissue surface) provides a difference in ionic activity near the cell surface. Using the magnitude of this difference and taking into account the geometry of the surface, a special software calculates net ionic flux across the membrane surface. In contrast to extracellular electrodes MIFE® provides information about the ion selectivity of fluxes and numeric data on these fluxes. MIFE® can be applied to different types of tissues (including different root and leaf tissues), protoplasts (Shabala et al. 1998), root hairs (Babourina et al. 2001) and pollen tube (Tegg et al. 2005).

Apart from its many advantages, MIFE® also has limitations. Firstly, MIFE® does not allow recording ion activities higher than 0.2–1 mM. For example, this limitation renders impossible measurement of the toxic Na^+ influx under salinity conditions when [Na^+] is too high. Secondly, MIFE® recordings require a pause of about 2 min after addition of test substances to the assay chamber for mixing of solutions. This limitation is crucial when fluxes show fast kinetics of activation. Demidchik and Shabala (unpublished) have recently tried to decrease this pause and have developed a new MIFE®-based

approach to study fast changes in plant cation fluxes. They have minimized the volume of the assay chamber, changed the system for addition of solutions and decreased the time of mixing. This reduced the pause after mixing by several times. Thirdly, some dyes for MIFE® electrodes are not very selective between cations. For example, Na^+-selective dye also senses K^+. Therefore, corrections should be made if several cations are present in the assay solution. Fourthly, using MIFE® is strictly limited by surfaces that are directly exposed to the bathing medium. This technique does not allow measurements of fluxes in internal tissues that are covered by several layers of cells.

A pioneering combination of MIFE® with patch-clamp technique has been recently developed in Australia (Tyerman et al. 2001). This novel approach allows simultaneous measurements of cationic conductance and corresponded fluxes that makes it possible an accurate identification of ion-specificity of the conductance. Work on coupling MIFE with other electrophysiological (two-electrode voltage-clamp) and cellular (confocal imaging) techniques is now in progress in our laboratories and in the laboratory of Dr. Sergey Shabala. Successful combinations of these techniques will open new horizons in non-invasive technologies for the membrane biology of plants. Combination of MIFE®, two-electrode voltage-clamp and confocal microscopy will be particularly important for studying membrane systems that lack activities in patch-clamp configurations, for example plant neurotransmitter-activated receptors (Demidchik et al. 2004).

8.2.2 Intracellular recordings

8.2.2.1 Measurement of membrane potential by impaling with single electrode

Ideas of selective permeability to cations of the plant plasma membrane were developed in the beginning of the twentieth century by Osterhout in Harvard University (Osterhout 1908). This was followed by the first measurements of the plasma membrane potential in sea green alga *Valonia macrophysa* using impalements with sharp glass microelectrodes (Osterhout et al. 1927). In the next 30–40 years, membrane potentials were examined in *Valonia, Halicystis*, *Nitella, Chara,* and *Nitellopsis* (reviewed by Osterhout 1958; Hope and Walker 1975). In these experiments, giant plant cells were impaled with one electrode and V_m was measured between this electrode and indifferent electrode in the bathing solution. Studies of V_m in different conditions played significant roles in the plant physiology, particularly for understanding membrane selective permeability, electrogenic processes and action potentials. These studies predicted existence of cation channels in plant cell membranes. These days, measurement of V_m remains a useful tool in plant electrophysiology. For

example, using this technique, Ehrhardt et al. (1992) discovered Nod-factor-induced depolarization of the plasma membrane of plant root cells that later on was shown to be an effect of activation of inwardly rectifying K^+ channels (Kurkdjian et al. 2000; Ivashikina et al. 2001). Measuring V_m response to purines, Lew and Dearnaley (2000) have found depolarizing effect of these substances on the root plasma membrane. In 2003, Demidchik et al. showed that purine-activated cation channels that are similar to animal purinoceptors probably catalyze this depolarization effect. Dennison and Spalding (2000) found that glutamate depolarizes the plasma membrane of *Arabidopsis* root cells. This finding has led to the discovery of plant glutamate-activated cation channels (Demidchik et al. 2004). Impalement with sharp microelectrodes was a basis for development of intracellular ion-selective electrodes—a powerful experimental tool allowing measurements of activities of different cations in the cytosol and other cellular compartments (reviewed by Miller et al. 2001).

8.2.2.2 Two-electrode voltage-clamp technique

Measurement of V_m changes can indirectly show that cation channels exist in the cell membrane, but it does not inform on properties of cationic currents flowing through these channels. The nature of plant cationic conductances remained unclear until the first application on plants of two-electrode voltage-clamp technique by Findley (1961) and Hope (1961). This technique was previously successfully used in animal physiology by Hodgkin and Huxley (1952) for creation of "ion channel theory". Two-electrode voltage-clamp technique "clamps" or maintains V_m at a value the experimenter specifies. Voltage control is established using feedback through an operational amplifier circuit (see details in Halliwell et al. 1994). The main value of the voltage-clamp technique is that it allows one to measure the amount of ionic current crossing a membrane at any given voltage at a given time. Using two-electrode voltage-clamp, Belarusian and Russian physiologists characterized for the first time plant K^+ channels (Sokolik and Yurin 1981, 1986), Ca^{2+} channels (Plaks et al. 1979, 1980; Lunevsky et al. 1983) and non-selective cation channels (NSCC) (Yurin et al. 1991). Results that were obtained in experiments with giant alga cells have been summarized and reviewed by Tester in 1990.

A particular success in characterization of cation channels in alga was achieved due to the development of a very efficient voltage-clamp method that required only one electrode impalement (Sokolik and Yurin 1981, 1986; see Demidchik et al. 1997 for details). Impaling with one electrode decreased the damage of cells and significantly increased a percentage of successful preparations. In this method a second electrode was placed in extracellular space in 100 mM KCl solution (as in the cytosol) near nodes, which were isolated electrically from the middle of the cell by white petroleum jelly. The plasma membrane in

nodes has very low resistance. Therefore, extracellular electrodes are in contact with the cytosol, providing high quality voltage-clamping. An exciting development of this electrophysiological method was combined voltage-clamp and "turgescent" perfusion of the alga cell (Plaks et al. 1979, 1980) that allowed the study of tonoplast cation channels in vivo (without damaging cytoplasm). In this method, the perfusion of vacuole sap was carried out without rupture of the plasma membrane and cytoplasm. This was achieved by impaling glass microneedles into the vacuole through both nodes. Solution flow between two microneedles allowed changing the vacuole solution.

Two-electrode voltage-clamp technique is much less invasive than patch-clamp technique because it does not damage cell wall, membranes and the cytosol. However, this technique has not been extensively used for characterization of cation channels in higher plants. The main reasons of this include: inconvenience of using small cells, preparation problems in highly organized and specialized multi-cellular tissues and organs, impossibility to manipulate the intracellular solution and electrolyte leakage from the electrode to the cytosol that was significant in cells smaller than giant alga. Experimental conditions allowed using two-electrode voltage-clamp for studying cation channels only in a few higher plants. For example, this technique was used for the characterization of K^+ channels in guard cells (Blatt 1988; Langer et al. 2004) and root hairs (Lew 1991). In experiments on large cells of higher plants, for example root hairs, another significant limitation is current dissipation along the length of the cells (Meharg et al. 1994). This result in distortions of the current–voltage (I–V) curve, including consistent underestimation of the membrane current, linearization of the I–V and masking of conductance changes in the presence of transported substrates.

Current recording by two-electrode voltage-clamp technique has been recently coupled with the measurements of cytosolic Ca^{2+} activity in application on guard cells (Levchenko et al. 2005). Three-barreled electrodes were used: two barrels clamped voltage and third barrel loaded fluorescent dye FURA into the cytoplasm. Recordings were carried out on intact leaves that were not excised from the plant. This novel technique has a great potential for studying plant cation channels, particularly Ca^{2+} channels. This technique has already shown that data on ABA regulation of Ca^{2+} channels in protoplasts derived from guard cell should be revised (Levchenko et al. 2005).

Two-electrode voltage-clamp is also extensively used for characterization of plant cation channels heterologously expressed in *Xenopus laevis* oocytes (reviewed by Dreyer et al. 1999; Miller and Zhou 2000). Probably the most successful functional expression in *Xenopus* oocytes was carried out in the case of *Arabidopsis* K^+ channels (reviewed by Véry and Sentenac 2002).

In the late 1980s, two-electrode voltage-clamp was replaced by patch-clamp technique that brought electrophysiological measurements to a new quality by allowing the testing of protoplasts derived form small cells and different plant tissues.

8.2.2.3 Patch-clamp technique

Patch-clamp technique was developed in the 1970s by Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991. With this technique a glass micropipette with large diameter at the tip (> 1 μm) forms a high resistance contact (so-called gigaohm seal) with a protoplast derived from plant cells. Only one electrode measures voltage and injects current to clamp the voltage at constant level. Types of patch-clamp modes are shown in Fig. 8.1. They include: cell attached, whole cell, inside-out and outside-out modes that have advantages and disadvantages as shown in Fig. 8.1. Inside-out and outside-out modes are also called excised patches. Whole-cell and outside-out modes are the most frequently used patch-clamp configurations for studies plant cation channels. Patch-clamp can also be used in combination with imaging techniques (Taylor et al. 1997; Pei et al. 2000) and MIFE® (see above).

The first experiments with patch-clamp technique on plant cells were carried out in parallel by German and Israeli laboratories in middle eighties

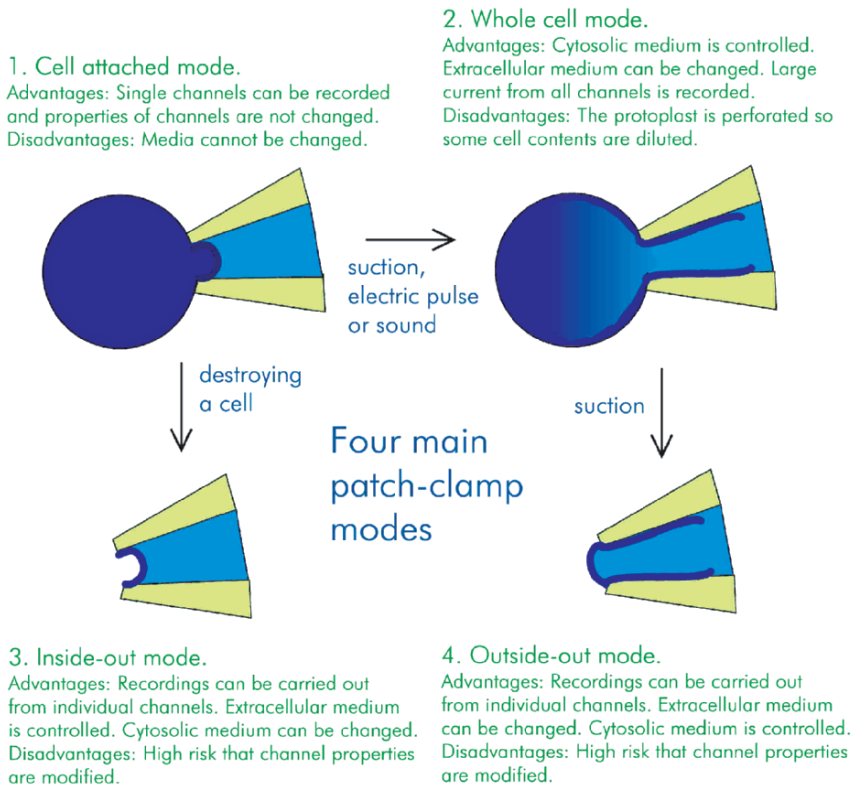

Fig. 8.1. Patch-clamp modes

(Moran et al. 1984; Schroeder et al. 1984). Two types of cation channels were characterized in these early studies. After these pioneering works, many laboratories worldwide employed patch-clamping for studying cation channels. Our modern knowledge about cation channels in higher plants is based on patch-clamp studies that were carried out in the last 20 years (reviewed by Hedrich and Schroeder 1989; Tester 1990; Tyerman and Schachtman 1992; Barkla and Pantoja 1996; Tanner and Caspari 1996; White 1998; Tyerman and Skerrett 1999; Zimmermann et al. 1999; Demidchik et al. 2002b; Véry and Sentenac 2002, 2003; White and Broadley 2003; Scholz-Starke et al. 2005).

Data accumulated in patch-clamp studies led to the functional classification of plant cation channels. They can be divided into three main groups. The first identified group is K^+ channels that includes two large classes of outwardly and inwardly rectifying K^+ channels, or so-called KOR and KIR, respectively. These channels are selective for K^+ and NH_4^+ over other cations and catalyze passive influx and redistribution of these cations. There is also physiological-molecular evidence that K^+ channels regulate stomata function (Hosy et al. 2003). Calcium channels are the second group of plant cation channels. This group include two main types including depolarization- (Thion et al. 1996, 1998) and hyperpolarization-activated Ca^{2+} channels (Gelli and Blumwald 1997; Hamilton et al. 2000, 2001; Kiegle et al. 2000; Véry and Davies 2000; Demidchik et al. 2002b; Stoelzle et al. 2003) (DACC and HACC, respectively). Calcium channels catalyze hormone-, elicitor-, and cyclic nucleotide-induced Ca^{2+} influx, and elevated Ca^{2+} uptake by growing cells. There is strong experimental evidence that these channels are crucial for plant signaling, stress responses, growth, development, mineral nutrition and other physiological functions (White 1998; White and Broadley 2003). It is still unclear whether plant Ca^{2+} channels are highly selective for Ca^{2+} over all other cations (like animal Ca^{2+} channel) or not. Genes encoding classic voltage-gated Ca^{2+} channels that are similar to animal counterparts have not been identified in plants (White et al. 2002; White and Broadley 2003). Therefore, many plant Ca^{2+}-permeable channels probably belong to non-selective cation channels (NSCC). NSCC are the third group of plant cation channels. They have about equal permeability for K^+ and Na^+ ($P_K/P_{Na} < 3$) and higher Ca^{2+} permeability ($P_{Ca}/P_K > 0.1–0.2$) (Demidchik et al. 2002a). Plant NSCC exist in the plasma membrane and in the tonoplast. Tonoplast NSCC are well studied at the physiological level in many species. There are two main classes of tonoplast NSCC: "slow-activating vacuolar channels" (SV channels) and "fast-activating vacuolar channels" (FV channels). SV channels show slow kinetics and activation at $[Ca^{2+}]_{cyt.} > 0.5–5$ μM. FV channels rapidly activate and function at physiological $[Ca^{2+}]_{cyt.}$ ($< 0.3–1$ μM). The laboratory of Professor Dale Sanders (University of York) has recently characterized, for the first time, tonoplast NSCC at the molecular level (Peiter et al. 2005). They have shown that TPC1 homologue is a part of SV channel in the tonoplast of *Arabidopsis* leaf cells and that this channel is involved in the regulation of stomata closing at high extracellular Ca^{2+} concentrations. Plasma membrane NSCC embrace

four main classes: (1) constitutive NSCC; (2) reactive oxygen specie-activated (ROS-activated) NSCC; (3) ligand-activated NSCC; and (4) mechanosensitive NSCC. Constitutive NSCC are active in the plasma membrane without activating factors. For opening ligand-activated NSCC interact with a chemical ligand. ROS-activated NSCC require H_2O_2 or $OH^{\bullet}$ for the activation. Mechanosensitive NSCC require plasma membrane stretching for activation. All these channels vary in their voltage-dependence, pharmacology and characteristics of unitary conductances (see review by Demidchik et al. 2002a).

Apart from its many advantages, the patch-clamp technique also has some problems. On of the main problems is that treatment of plant cells by cellulytic enzymes damages native channels and their regulation. For example, the treatment by cellulases during protoplast isolation induces a oxidative burst in plant cells and activations of hydrolytic enzymes (Brudern and Thiel 1999; Kennedy and de Filippis 2004). This can lead to degradation of membrane and cytosolic proteins and to changes of cation channel properties or even disappearance of some cation channels (Demidchik, unpublished data). Treatment of plants with SH-reducing agents is a possible way to avoid undesirable artifacts from the oxidative damage. However, it is not a "panacea" because in natural conditions SH-groups are not maintained as permanently reduced, so SH-reducing agents may cause artifacts too.

Another major problem of patch-clamp technique is the replacement of the cytosolic native solution by artificial solution in whole cell mode. This provides the control of intracellular medium but, on the other hand, it also damages cytosolic regulation of the channels. This problem underlies the lack of successful patch-clamp characterization of plant ionotropic receptors (Demidchik et al. 2004). Experiments with plant purine-activated conductances have recently shown that purines cause reliable Ca^{2+} influx (as measured by aequorin luminometry and MIFE®) in all root cells and root-derived protoplasts; however, they are 5 times less effective in patch-clamped protoplasts (Demidchik et al., unpublished).

The quality of voltage-clamping in patch-clamp experiments is good if protoplast membrane resistance is higher than resistance of the patch pipette. The technical problem arises when the ratio between the resistance of patch pipette and protoplast membrane increases. If the resistance of the patch pipette is higher than the resistance of the patched membrane, the voltage is manly clamped on the tip of the pipette but not on the membrane. This particularly affects I-V curve rectification and currents recorded under maximally depolarized or hyperpolarized voltages. The study of vacuolar cation channels are particularly in danger of artifacts due to very low resistance of isolated vacuoles. For example, this phenomenon explains the absence of rectification in many early recordings made on tonoplast cationic conductances. Smaller patches, diluted solutions and lower resistance of the pipette help to avoid artifacts.

Another obvious problem of patch-clamp technique is that not all plant cells and tissues give protoplasts suitable for patching. It is also difficult to

select between protoplasts that are derived from different tissues. Specific GFP labeling of plant tissues was successfully used for the selection of tissue-specific protoplasts in *Arabidopsis* (Kiegle et al. 2000; Demidchik et al. 2003). Using laser ablation in combination with plasmolysis/deplasmolysis step is another approach to isolate protoplasts from specific tissues (Taylor et al. 1997; Véry and Davies 2000; Foreman et al. 2003). Using this method protoplasts were isolated from *Fucus* pollen tube and *Arabidopsis* root hairs that allowed characterization of Ca^{2+} channels in these species.

8.3 Conclusions and perspectives

Electrophysiological characterization of cationic conductances and cation channels in plants has been progressing during almost 80 years. It adapted many methods from animal physiology but also developed plant-specific approaches, for example MIFE® and cell wall laser microsurgery for protoplast isolation. Application of voltage-clamp techniques resulted in the characterization of cation channels in many plant species and in different tissues and functional classification of plant cation channels. Electrophysiological techniques should be used with care and experimenters must take into account possible artifacts and problems. The future of plant electrophysiology is in developing new non-invasive techniques and approaches that cause less damage to the cell and channels than patch-clamping. Combining different physiological methods with electrophysiological techniques also have good perspectives.

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