Signaling and Communication in Plants

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Editors

Ion Channels and Plant Stress Responses

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"This book is dedicated to Vladimir Yurin and Anatoliy Sokolik who pioneered research into plant potassium channels"

Preface

Plants live in a constantly changing environment from which they cannot physically escape. Plants therefore need signalling and response mechanisms to adapt to new local conditions. The efficacy of such mechanisms underlies the plant performance during stress and therefore also impacts greatly on agricultural productivity. Modulation of ion channel activity not only provides a means for rapid signal generation but also allows adjustment of cellular physiology. For example, Ca^{2+} permeable ion channels can transduce environmental stimuli into Ca^{2+}-encoded messages which can modify the gene expression. Furthermore, ion channel activity is essential to control cellular ion homeostasis that impacts on plant responses to drought, salinity, pathogens, nutrient deficiency, heavy metals, xenobiotics and other stresses.

This volume focuses on the crucial roles of different types of ion channel in plant stress responses. Functions of ion channels are discussed in the context of mechanisms to relay external and endogenous signals during stress and as mechanisms to regulate cellular ion homeostasis and enzymatic activities in the context of biotic and abiotic stress. The chapters presented cover cation and anion channels located in various cellular compartments and tissues.

Colchester, September 2009 — Vadim Demidchik
York, September 2009 — Frans Maathuis

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Ion Channels and Plant Stress: Past, Present, and Future

Nobuyuki Uozumi and Julian I. Schroeder

Abstract Perhaps the most significant change in plant electrophysiological studies that began some 25 years ago was a shift in focus from more basic electrical and biophysical properties of plant membranes to pursuing the understanding of the plant physiological and cell biological functions of individual plant ion channel types. In the 1990s, ion channels were characterized as targets of upstream signal transduction mechanisms, and in the later 1990s powerful combined molecular genetics, patch clamp, and plant physiological response analyses further manifested the importance of ion channels for many biological and stress responses of plants. Essential metals and ions in the intracellular and intraorganellar spaces of plant cells contribute to the activities of regulatory proteins, signal transduction, and to the maintenance of turgor pressure, osmoregulation, toxic metal chelation, and membrane potential control. A large number of studies on mineral nutrition have sustained the profitable cultivation of plant growth and development, and provided important knowledge on plant physiological mechanism of absorption of minerals from soils. Abiotic stress and biotic stresses are a global problem for plant growth in agricultural and noncultivated lands. Ion channels in plant cells play crucial functions in adapting to and overcoming abiotic and biotic stresses. Plant membrane transport systems play an important role not only in the uptake of nutrients from the soil but also in the adaptation to stress and environmental change.

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A revolution has taken place in the understanding of cell physiological, biophysical, molecular, and interaction network properties of plant ion channels and transporters as summarized here. However, many exciting and stimulating questions remain open to discovery, promising that research on plant ion channels will continue to be a vibrant area of research for many years to come.

1 Introduction

Among plant nutrients, potassium or K^+, is the most abundant cellular cation controlling cellular homeostasis, plant movements, cell expansion, guard cell turgor, membrane potentials, and many other processes. Potassium ions also counteract toxic effects of cations such as sodium (Na^+). Potassium transport properties have served as a classical model for understanding mechanisms of plant ion transport (Epstein et al. 1963). Studies indeed show that principles learned from K^+ transport and K^+ channel analyses can be applied to other transport systems.

Characterization of ion channel functions in plant stress responses led to the formulation of models of how multiple ion channels and transporters can function together in mediating a response. Studies in guard cells led to an early model for the interplay of a network of ion channels and proton ATPases in mediating stomatal opening and closing (Shimazaki et al. 1986; Schroeder and Hagiwara 1989; Schroeder and Hedrich 1989; Thiel et al. 1992; Lemtiri-Chlieh and MacRobbie 1994; Ward and Schroeder 1994; Davies and Sanders 1995; Blatt et al. 1999). Remarkably, studies of rapid changes in plant pathogen responses and other rapid stimulus-responses show ion transport behaviors that, at least in general terms, show similarity to those mediating stomatal closing (Nurnberger et al. 1994; Jabs et al. 1997). In guard cells, cytosolic Ca^{2+} activates anion efflux channels and inhibits K^+ uptake channels (Schroeder and Hagiwara 1989), which together with Ca^{2+} inhibition of plasma membrane proton pumps (Kinoshita et al. 1995) causes anion and K^+ efflux and depolarization of the plasma membrane to reduce the turgor pressure of guard cells.

Calcium (Ca^{2+}) concentrations are tightly controlled at low submicromolar concentrations in the cytosol. Increases in Ca^{2+} concentrations and stimulus-induced enhancement in Ca^{2+} sensitivity (Young et al. 2006) function as an effective signal which modulates calcium-binding proteins thus transmitting signals in signal transduction pathways. Ion channels that mediate Ca^{2+} influx into the cytosol from the extracellular space and from organelles have been characterized in electrophysiological studies (Miller et al. 1990). However, the genes encoding these ion channels still remain mostly uncharacterized in plant cells, probably due to the presence of large gene families with overlapping functions (Shimazaki et al. 1986; Blatt 2000; Ward et al. 2009). Anion channels in the plasma membrane have also emerged as major mechanisms regulating signal transduction and ion transport. Two types of

anion channel currents (slow (S)-type and rapid (R)-type) have been characterized extensively in guard cells and in hypocotyl cells (Keller et al. 1989; Schroeder and Hagiwara 1989; Marten et al. 1992; Colcombet et al. 2001), and genes encoding the anion conducting subunits of slow-type anion channels have been identified using *Arabidopsis* mutants (Negi et al. 2008; Vahisalu et al. 2008). Recent genetic approaches have led to identification of two additional gene families that encode anion conducting channel subunits that play major roles in aluminum resistance (Sasaki et al. 2004; Furukawa et al. 2007; Magalhaes et al. 2007). Yet another class of proteins exists, which shows similarity to mammalian chloride channels, AtCLCs. Functional characterization of the AtCLCa membrane protein showed that it encodes a nitrate-proton exchanger in the vacuolar membrane, rather than an anion channel (De Angeli et al. 2006) and additional CLC proteins are targeted to other organelle membranes (Marmagne et al. 2007).

In this chapter, we will provide an overview of the classes of different ion channels that have been characterized and their underlying gene families. In several cases, we discuss examples of their physiological functions in guard cells as well as in other cell and tissue types. The relevance of these channels in stress responses in many plant cell types is discussed throughout this book. Figures 1 and 2 summarize progress over the past 25 years in the identification of plant ion channel classes, technical advances, and major genes encoding plant membrane transport systems. Figure 1 exemplifies the accelerating pace of discovery in this thriving field.

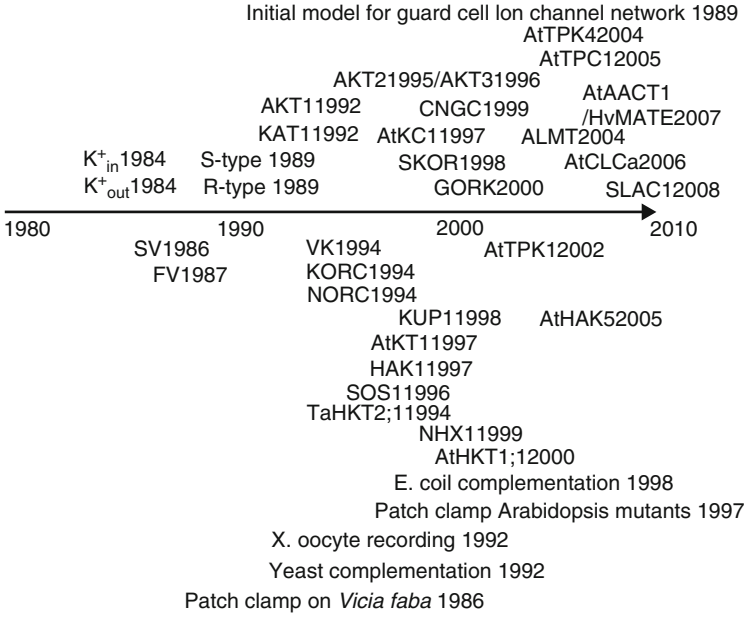

Fig. 1 Time-line of progress on the identification of individual plant ion channel classes, the genes encoding these plant ion transporters, and introduction of new techniques

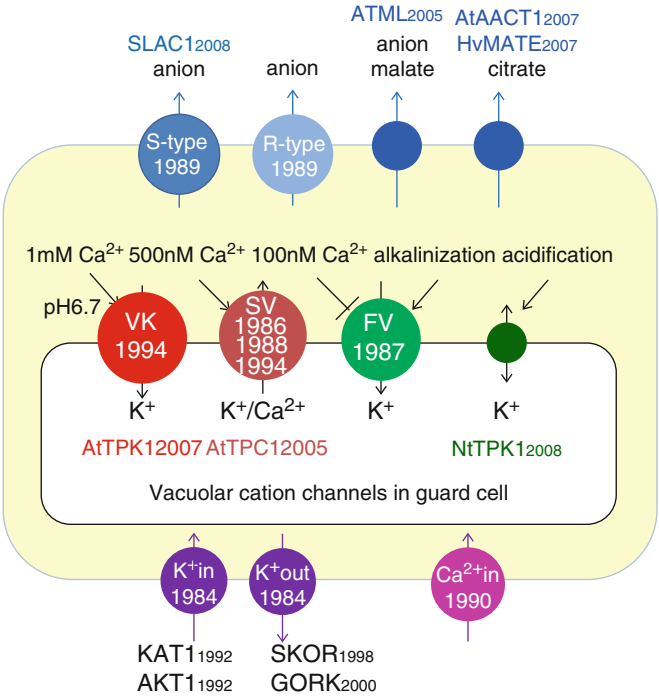

Fig. 2 Simplified scheme of several of the cation and anion channels in the plasma membrane and in the vacuolar membrane of plant cells, which were identified and characterized in patch clamp studies. Genes encoding some of these ion channels have been cloned and characterized (see text)

2 Plasma Membrane K^+ Channels in Guard Cells

The first characterizations of single plant ion channels were reported in 1984 in the analyses of leaf cells (Moran et al. 1984) and guard cells (Schroeder et al. 1984). These successful applications of patch clamp techniques for the measurement of plant ion channels opened the door to electrophysiological characterizations of ion channels in plant membranes of land plant cells, which are usually orders of magnitude smaller than the classically analyzed giant algae cells (Curtis and Cole 1938; Tazawa 1968, 1972). They also reported the measurement of sodium, potassium, and chloride ions in protoplasm of algal cells, which may be more difficult to measure than plant cells. Two major classes of voltage-dependent K^+ channels were characterized in guard cells; hyperpolarization-activated "inward-rectifying" K^+ channels and depolarization-activated "outward-rectifying" K^+ channels (Schroeder et al. 1984, 1987; Blatt 1988). Inward-rectifying K^+ channels are activated by hyperpolarization via electrogenic proton pumps controlled by blue light signals (Assmann et al. 1985; Shimazaki et al. 1986). The opening of stomatal pores is regulated by the accumulation of K^+ in guard cells. Both inward- and

outward-rectifying K^+ channels were proposed to contribute to the physiological transport of K^+ into and out of guard cells during stomatal movements (Schroeder et al. 1987). Subsequent studies in many different plant cell types including coleoptiles, root hair cells, aleurone, root cortex, and xylem parenchyma cells showed that these types of K^+ channels are widely distributed and were proposed to have important functions in K^+ transport and membrane potential control (Bush et al. 1988; Kourie and Goldsmith 1992; Gassmann and Schroeder 1994; Wegner and Raschke 1994; Maathuis et al. 1997; de Boer and Volkov 2003).

2.1 Characterization of K^+ Channel and Transporter cDNAs

In 1992, two distinct K^+ channel genes, KAT1 and AKT1, were isolated from *Arabidopsis thaliana* by complementation of K^+ uptake deficient yeast mutants (Anderson et al. 1992; Sentenac et al. 1992). Both genes encode six putative transmembrane regions and a predicted voltage sensor domain, and resemble Shaker K^+ channels in *Drosophila* neurons. For the isolation of these genes, both groups used yeast mutants which are unable to grow at low concentrations of K^+ in the medium. The use of yeast expression systems provides a powerful method for isolation of channel and transporter cDNAs and for structure-function analyses of these transporters (Frommer and Ninnemann 1995; Uozumi et al. 1995; Hoth et al. 1997; Nakamura et al. 1997).

Electrophysiological characterization of the KAT1-encoded protein in *Xenopus* oocytes showed that KAT1 functions as a heperpolarization-activated K^+ channel (Schachtman et al. 1992). Thus these studies led to the first isolation and characterization of eukaryotic inward-rectifying K^+ channel genes (Anderson et al. 1992; Schachtman et al. 1992; Sentenac et al. 1992), as hyperpolarization-activated K^+ channels genes had not yet been identified in animal genomes (Kubo et al. 1993; Ward et al. 2009).

AKT1 expression in *Xenopus* oocytes failed to show ion channel activities, but insect cells (Sf9 cell line) expressing AKT1 displayed an inwardly rectifying K^+ conductance (Gaymard et al. 1996). Other types of *Arabidopsis* K^+ channel genes have been isolated after this; a weakly inward-rectifying K^+ channel, AKT2 (Cao et al. 1995; Ketchum and Slayman 1996), depolarization-activated K^+ channels, SKOR and GORK (Gaymard et al. 1998; Ache et al. 2000), and a silent channel, AtKC1 which is likely to modulate other K^+ channels (Dreyer et al. 1997; Reintanz et al. 2002). The role of the silent regulatory subunit has been confirmed for the carrot AtKC1 homolog, KDC1 (Bregante et al. 2008). The cytosolic regulatory components, calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs) are closely associated with several ion channels and transporters that function in adaptation to salinity or ion stress in plant cells. The complex of CBL1/CIPK23 directly controls AKT1-mediated K^+ uptake in roots and enhances K^+ uptake when ambient K^+ becomes deficient (Li et al. 2006; Xu et al. 2006).

Interestingly, *Escherichia coli* was shown to be another heterologous expression system suitable for functional expression of both plasma membrane-located and

organelle membrane-located plant channels/transporters (Uozumi 2001). Using this system, K^+ uptake activities of KAT1, AKT2, HKT-type transporters, and KUP-type transporters were measured (Kim et al. 1998; Uozumi et al. 2000; Uozumi 2001). Moreover, the transmembrane topologies of the *Shaker*-type K^+ channel KAT1 and the Na^+/K^+ transporter, HKT1 (TaHKT2;1) were determined by means of a bacterial alkaline phosphatase fusion approarch (Kim et al. 1998; Uozumi et al. 1998, 2000; Kato et al. 2001; Uozumi 2001).

KUP/HAK/KT genes encode a separate class of important plant K^+ uptake transport proteins and were isolated after earlier genomic EST sequencing showed plant isoforms with homology to *E. coli* Kup and yeast HAK transporters (Quintero and Blatt 1997; Santa-Maria et al. 1997; Fu and Luan 1998; Kim et al. 1998). The *Arabidopsis* genome sequence shows the presence of 13 genes KUP/HAK/KT genes in the *Arabidopsis thaliana* genome (Mäser et al. 2001; Ahn et al. 2004), and the physiological role of AtKUP4 and AtHAK5 has been reported (Rigas et al. 2001; Gierth et al. 2005). AKT1 and AtHAK5 likely together mediate K^+ uptake from soil. The transport mechanism by which these KUP/HAK/KTs mediate K^+ uptake into plants cells remains unknown (Maathuis and Sanders 1994). An important question for future research will be the characterization of the interplay of several different K^+ transporter/channel classes in mediating K^+ transport.

3 Critical Roles of Plasma Membrane Anion Channels in Plant Stress Responses

Stomatal closing is mediated by the release of ions and organic solutes from guard cells. Electrophysiological studies led to a model for the mechanisms that can drive K^+ release from guard cells. Electrophysiological research on outward-rectifying K^+ channels indicated that inhibition of proton pumps would not suffice for depolarization-activation of K^+ channels (Schroeder et al. 1987; Schroeder 1988). Elevation of the cytosolic Ca^{2+} concentration in guard cells led to the activation of a novel class of plant ion channels – S-type anion channels (Schroeder and Hagiwara 1989). Due to the electrochemical gradient of anions across the plasma membrane of guard cells, activation of anion channels causes anion efflux leading to depolarization. Anion channels were therefore proposed as drivers of ion efflux, thus controlling stomatal closing (Schroeder and Hagiwara 1989). Further research revealed additional types of anion channel in guard cells with properties different from those of S-type anion channels (Keller et al. 1989). These so-called R-type anion channels can also mediate anion efflux leading to stomatal closing.

Anion channels in guard cells are permeable to chloride, nitrate, sulfate, and malate (Keller et al. 1989; Schroeder and Hagiwara 1989; Schmidt and Schroeder 1994). Patch clamp analyses of the plasma membrane of *Vicia faba* guard cells revealed that these two types of anion channel conductances coexist in the membrane (Schroeder and Keller 1992). R-type anion channels are characterized as rapidly activating with kinetics that are time- and voltage-dependent and that show

inactivation (Keller et al. 1989; Hedrich et al. 1990). The other class of depolarization activated anion channels exhibits extremely slow voltage dependent activation and deactivation properties – the S-type anion channels (Schroeder and Hagiwara 1989; Schroeder and Keller 1992). It has been proposed that R-type and S-type anion channels may be encoded by the same channel protein (Linder and Raschke 1992), despite their relatively significant differences in some biophysical and regulatory properties. The plant hormone abscisic acid, which is induced in response to drought stress, activates both S-type and R-type anion channels (Grabov et al. 1997; Pei et al. 1997, 1998; Raschke 2003; Raschke et al. 2003; Roelfsema et al. 2004). S-type and R-type anion channels have also been characterized in hypocotyl cells of *Arabidopsis* and were also shown to co-exist in the same cells (Colcombet et al. 2005). Studies in the *Arabidopsis* hypocotyls also suggested that these two anion channels can be clearly distinguished in these cells (Colcombet et al. 2005). Nevertheless, it is possible that these two very different anion currents share molecular components (Raschke 2003).

4 Roles of Anion Channels in Stress Responses and Identification of Anion Channel Gene Families

SLAC1 (slow anion channel-associated 1) encodes a homologue of bacterial dicarboxylate/malic acid (C4-dicarboxylate) transport proteins and was identified as an S-type slow anion channel (Vahisalu et al. 2008). The plasma membrane protein SLAC1 plays an essential role in stomatal closure in response to CO_2, ABA, ozone, darkness, humidity reduction, Ca ions, hydrogen peroxide, and nitric oxide (Negi et al. 2008; Vahisalu et al. 2008). Loss-of-function mutations in *SLAC1* are accompanied by an overaccumulation of osmoregulatory anions in guard cell protoplasts (Negi et al. 2008). T-DNA insertion and point mutations in the *SLAC1* gene led to abrogation of S-type anion channels in guard cells (Vahisalu et al. 2008). Interestingly however, R-type anion channels were intact in *slac1* mutant guard cells. SLAC1 shows homology to a yeast and a bacterial malate transporter. The permeability of S-type anion channels to anions and the increased trapping of malate in *slac1* guard cells suggest that SLAC1 encodes the anion conducting subunit of S-type anion channels (Negi et al. 2008; Vahisalu et al. 2008). *Slac1* mutants provide strong evidence for the model that anion channels represent central mechanisms in mediating stomatal closing. Interestingly, a different type of malate transporter, AtABC14 has been identified as a malate import protein mediating malate uptake from the cell wall into guard cells (Lee et al. 2008) and thus distinct channels and transporters are now known that mediate anion efflux and uptake in guard cells.

Aluminum is the third most abundant element in the Earth's crust. In acidic soils aluminum (Al^{3+}) is solubilized and Al^{3+} is toxic to plants. However, plants release organic acids, including malate and citrate from their roots, to chelate free aluminum (Al^{3+}) in acidic soil (Ma et al. 2001; Kochian et al. 2004). Al^{3+} activates anion

channels in the plasma membrane of wheat roots (Ryan et al. 1997). Genes were identified in genetic studies and named *ALMTs* for Al^{3+}-activated malate transporters, since they play important roles in this Al^{3+} resistance response (Sasaki et al. 2004). ALMT expression in *Xenopus* oocytes is sufficient for Al^{3+}-activated anion channels, showing that ALMTs appear to function as a type of Al^{3+} receptor (Pineros et al. 2008). TaALMT1 mediates transport of malate, and to a lesser extent nitrate/chloride based on electrophysiological measurements (Pineros et al. 2008; Zhang et al. 2008).

Furthermore, Al^{3+}-activated citrate transporters (HvAACT1) (Furukawa et al. 2007) and (SbMATE) (Magalhaes et al. 2007) belong to the multidrug resistance transporter family and also function in aluminum tolerance in acid soils. The Al^{3+} resistance-associated anion transporters show no homology to the above SLAC1 anion channel from guard cells.

In animals, chloride channels of the ClC family have been characterized. Bacterial CLC homologues however function as 2 $Cl^-/1H^+$ exchangers (Accardi and Miller 2004; Picollo and Pusch 2005; Miller 2006). The functions of the homologous genes in *Arabidopsis* and tobacco have largely remained unknown (Hechenberger et al. 1996; Lurin et al. 1996). However, in 2006 the AtCLCa transporter was characterized as a NO_3^-/H^+ exchanger in the vacuolar membrane of *Arabidopsis* cells (De Angeli et al. 2006). *Atclca* knockout mutants provide evidence that AtCLCa functions in nitrate accumulation into vacuoles in *Arabidopsis thaliana* (Geelen et al. 2000). AtCLCd and AtCLCe are targeted to the thylakoid membranes in chloroplasts and AtCLCf was localized in Golgi membranes (Marmagne et al. 2007). Further studies on the subcellular localizations of AtCLCs may illuminate intracellular anion transport mechanisms in plant cells.

5 Ca^{2+} Channels and Intracellular Ca^{2+} Elevations

Stimulus-induced changes in the Ca^{2+} concentration in the cytoplasm of plant cells are triggered by many diverse stimuli (Hetherington and Brownlee 2004). Intracellular Ca^{2+} concentration changes in guard cells were identified using fluorescent Ca^{2+} indicators, Fura-2 (McAinsh et al. 1990; Schroeder and Hagiwara 1990), and Fluo-3 (Gilroy et al. 1990). Patch clamp analyses showed the presence of Ca^{2+}-permeable channels in the plasma membrane of guard cells (Schroeder and Hagiwara 1990; Hamilton et al. 2000; Pei et al. 2000). ABA-induced intracellular Ca^{2+} elevations have been extensively studied (Allan et al. 1994; Grabov and Blatt 1998; Allen et al. 1999a; Staxen et al. 1999). The pH-independent, green florescent protein-based Ca^{2+} indicators yellow cameleon 2.1 and 3.6 were applied for monitoring cytoplasmic free Ca^{2+}, $[Ca^{2+}]_{cyt}$, in *Arabidopsis thaliana* (Allen et al. 1999b; Miyawaki et al. 1999; Yang et al. 2008). Studies using low concentration cameleon or fura2-based Ca^{2+} reporters have revealed that repetitive spontaneous Ca^{2+} transients occur in plant cells (Grabov and Blatt 1998; Allen et al. 1999a; Staxen et al. 1999; Wais et al. 2000; Young et al. 2006; Yang et al. 2008). Furthermore, experimentally imposing Ca^{2+} oscillations, by repetitive depolarizations and

hyperpolarizations of the plasma membrane, showed that independent of the Ca^{2+} elevation pattern, Ca^{2+}-induced a rapid stomatal closure which was named the "Ca^{2+} reactive" stomatal closing response (Allen et al. 2001). In addition to this Ca^{2+} reactive response, it was revealed that the pattern of experimentally-induced $[Ca^{2+}]_{cyt}$ elevations controls the ability of stomata to re-open after the initial stomatal closing response, even when the $[Ca^{2+}]_{cyt}$ elevations are terminated (Allen et al. 2001; Li et al. 2004). This long-term Ca^{2+} pattern inhibition of re-opening of stomatal pores, was named the "Ca^{2+} programmed" response and is impaired in glutamate receptor overexpressing guard cells (Cho et al. 2009). Thus $[Ca^{2+}]_{cyt}$ oscillation kinetics in guard cells can function in maintaining steady-state stomatal closing. Organelles in plant cells serve as intracellular stores for Ca^{2+}. A Ca^{2+} sensing receptor, CAS, was isolated via a functional expression screening approach using heterologous expression (Han et al. 2003). Recent work shows that CAS1 is localized in thylakoid membranes (Nomura et al. 2008; Weinl et al. 2008) and functions in extracellular Ca^{2+}-induced, transient cytosolic Ca^{2+} increases, which lead to stomatal closure (Han et al. 2003; Nomura et al. 2008; Weinl et al. 2008).

6 Gene Candidates for Plasma Membrane Ca^{2+} Channels

Several classes of Ca^{2+} permeable channels have been characterized in the plasma membrane of plant cells, including depolarization-activated Ca^{2+} channels (Thuleau et al. 1994a, b; Miedema et al. 2008) and hyperpolarization-activated Ca^{2+} influx channels (Gelli and Blumwald 1997; Hamilton et al. 2000; Pei et al. 2000; Demidchik et al. 2002). In general, plant Ca^{2+} channels are not entirely Ca^{2+} selective but also show permeabilities to other cations (Schroeder and Hagiwara 1990; Thuleau et al. 1994a, b; Pei et al. 2000; Demidchik et al. 2002). However, the genes encoding plasma membrane Ca^{2+} channels remain less well-clarified. Two gene families are likely to provide possible candidates. One family includes 20 genes in the *Arabidopsis* genome and encodes homologs to "ionotropic" glutamate receptors, which encode receptor ion channels in animal systems (Lam et al. 1998; Kim et al. 2001). Research has shown that glutamate application to roots causes $[Ca^{2+}]_i$ elevations that are disrupted in knock-out mutants in the *Glr3.3*, glutamate receptor gene (Qi et al. 2006). A second candidate family of plant Ca^{2+} permeable channels is cyclic nucleotide-gated channel homologs. In *Arabidopsis*, 20 different cyclic nucleotide-gated channel genes (*CNGCs*) are present, and several individual channels have been analyzed. Voltage dependent K^+ channels, including KAT1 and AKT1 have corresponding cyclic nucleotide binding sites in the C-terminal regions (Hoshi 1995). However, CNGC channels do not include the typical "GYG" K^+ selectivity signature sequence of K^+ channels (Ward et al. 2009). Studies analyzing CNGC functions after heterologous expression in yeast indicate that they may encode Ca^{2+} permeable channels (Kohler et al. 1999; Leng et al. 1999), although this may not apply to all members of the CNGC family. Genetic analysis showed that both AtCNGC11 and AtCNGC12 are positive mediators of resistance signaling pathways activated by pathogen infection (Yoshioka et al. 2006). Future research

into the physiological functions of this large gene family may reveal new and unexpected ion channel functions.

7 Properties of Vacuolar Cation Channels

Plant vacuoles often take up more than 90% of the cell volume, and thus the channels mediating K^+ transport across the vacuolar membrane (tonoplast) may be of relevance to cell volume regulation and storage of this nutrient. Three classes of cation channel, SV (Slow Vacuolar), VK (Vacuolar K), and FV (Fast Vacuolar), have been named based on the endogenous K^+ channel activities identified by patch clamp studies. FV channels mediate K^+ transport at very low concentrations of cytosolic Ca^{2+} (Hedrich and Neher 1987; Allen and Sanders 1996). SV channels are activated by elevation in the cytosolic Ca^{2+} concentration (Hedrich and Neher 1987; Pei et al. 1999). SV channels were initially reported to be anion permeable channels (Hedrich et al. 1986). However, later studies revealed that SV channels are Ca^{2+} permeable cation channels that do not significantly conduct anions (Ward and Schroeder 1994; Ward et al. 1995; Allen and Sanders 1996). A third class of vacuolar cation channels are the Ca^{2+}-activated channels, named VK channels, which are highly K^+ selective channels (Ward and Schroeder 1994). The determination of genome sequences of *Arabidopsis* and reverse genetic approaches have led to the identification of the genes encoding SV channels (Peiter et al. 2005) and VK channels (Gobert et al. 2007). The AtTPC1 protein is targeted to the vacuolar membrane and these proteins encode SV channels (Peiter et al. 2005). The genes encoding two-pore K^+ channels (TPKs) include two repeats of membrane-pore-membrane domains (Czempinski et al. 1997, 2002; Kaplan et al. 2007). AtTPK1, 2, 3, and 5 are tonoplast K^+ channels (Voelker et al. 2006), whereas AtTPK4 is located in the plasma membrane (Becker et al. 2004). AtTPK1 was shown to encode the VK channel (Gobert et al. 2007). Functional characterization of NtTPK1, located in tobacco tonoplasts, shows K^+ currents induced by cytosolic acidification, indicating the presence of other types of vacuolar K^+ channels that differ from the above vacuolar channel types (Hamamoto et al. 2008).

8 Sodium Transport Systems in Plants

Sodium (Na^+) is not categorized as an essential nutrient in higher plants, and excessive Na^+ leads to detrimental effects on plant growth. Several distinct classes of Na^+ transporters mediate Na^+ homeostasis (Fig. 3). After Na^+ entry into the cytoplasm of root cells, Na^+ is loaded into the xylem (de Boer 1999). The presence of a Na^+/H^+ exchange activity at the xylem/symplast interface of soybean roots (Lacan and Durand 1996) and Na^+-permeable nonselective ion channels in the plasma membrane of barley root xylem parenchyma cells (NORC) (Wegner and

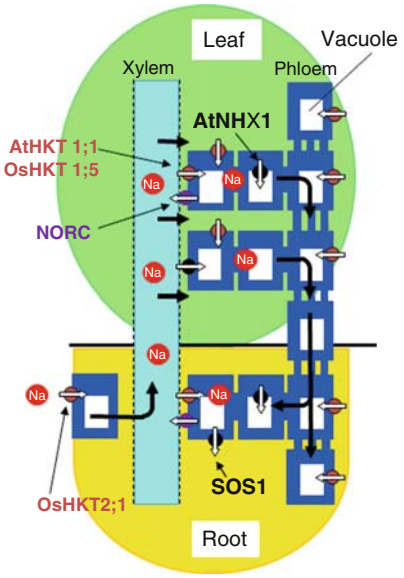

Fig. 3 Simplified model for mechanisms of Na^+ absorption, recirculation, and extrusion by different classes of Na^+ channels/transporters, including Na^+ loaded into xylem vessel by non-selective outwardly rectifying cation conductance, NORC (Wegner and De Boer 1997), Na^+ influx mediated by HKT transporters (Uozumi et al. 2000; Mäser et al. 2002a; Sunarpi et al. 2005), plasma membrane Na^+ extrusion via SOS1 antiporters (Shi et al. 2000), and tonoplast Na^+ sequestration by NHX antiporters (Apes et al. 1999). AtHKT1;1, and OsHKT1;5 are present in the plasma membrane of xylem parenchyma cells, and mediate unloading of Na^+ from xylem vessels into xylem parenchyma cells, thus protecting leaves from Na^+ overaccumulation and Na^+ damage (leaf Na^+ exclusion) (Berthomieu et al. 2003; Sunarpi et al. 2005; Ren et al. 2005). In the case of K^+ starvation in soils, rice roots take up Na^+ at low extracellular Na^+ levels via OsHKT2;1 (Horie et al. 2007). Na^+ is sequestered in vacuoles by AtNHX1. Excessive Na^+ in the cytosol is transported out of cells by SOS1

De Boer 1997) and in wheat and Arabidopsis root cortex and epidermis (NSC) (Tyerman et al. 1997; Buschmann et al. 2000; Davenport and Tester 2000; Demidchik and Tester 2002) have been reported.

The exclusion of Na^+ from plant cells and the sequestration of Na^+ in vacuoles alleviate sodium stress under saline conditions. The plasma membrane Na^+/H^+ antiporter named SOS1 (Shi et al. 2000), was identified in an *Arabidopsis* mutant, *sos1*, that shows a salt oversensitive phenotype (Wu et al. 1996). SOS1-mediated Na^+/H^+ transport activity is modulated by a Ca^{2+} sensor/protein kinase complex CBL4 (SOS3)/CIPK24 (SOS2) (Wu et al. 1996; Shi et al. 2002; Zhu 2002). Na^+/H^+ antiporters were also identified which are targeted to the vacuole-membrane. The first functionally-characterized member of this gene family, AtNHX1, contributes to Na^+ and monovalent cation sequestration in plant vacuoles. Overexpression of AtNHX1 was shown to increase salt tolerance in *Arabidopsis* (Apse et al. 1999).

In contrast to these Na^+ transporters that remove Na^+ from the cytoplasm, molecular identification of plasma membrane Na^+ influx systems into plant cells has also been achieved. Na^+ uptake transporters in wheat HKT1 also named, TaHKT1 (TaHKT2;1) (Schachtman and Schroeder 1994; Rubio et al. 1995; Gassmann et al. 1996) and in *Arabidopsis thaliana* AtHKT1 (AtHKT1;1) were identified (Uozumi et al. 2000). The first HKT gene, TaHKT1 (TaHKT2;1), was originally cloned from wheat and shown to mediate K^+ and Na^+ co-transport in yeast and *Xenopus* oocytes (Schachtman and Schroeder 1994; Rubio et al. 1995; Gassmann et al. 1996). Further extensive studies on HKT structure and function demonstrated that HKTs include 4 domains that resemble the K^+ permeation pore of a K^+ channel tetramer (Durell et al. 1999; Kato et al. 2001; Mäser et al. 2002a; Tholema et al. 2005; Gambale and Uozumi 2006) and HKT transporters have indeed been proposed to mediate channel-like transport (Gassmann et al. 1996; Corratge et al. 2007). Note that the term, transporter or channel has been used interchangeably for HKT transporters, and HKTs provide an interesting model to explore the shrinking distinctions between co-transporters and ion channels. Whereas some HKT transporters change their K^+ and Na^+ selectivities depending on the ionic conditions, similar to multi-ion channel pores (Schachtman and Schroeder 1994; Rubio et al. 1995; Gassmann et al. 1996; Horie et al. 2001), the only HKT transporter encoded in the *Arabidopsis* genome, AtHKT1, was found to be more Na^+ selective (Uozumi et al. 2000). Further studies showed that HKT transporters fall into either of these two cation selectivity HKT subfamilies (Horie et al. 2001, 2006). Research identified an amino acid residue that contributes to the distinction of these two cation selectivities of HKT transporters: AtHKT1;1 has a Ser instead of Gly in the first pore loop region which reduces K^+ selectivity. In contrast, TaHKT1 lacks this residue and is more Na^+ selective (Durell et al. 1999; Mäser et al. 2002a; Tholema et al. 2005; Gambale and Uozumi 2006). The nomenclature of HKT transporters cloned from various plants has been divided into two distinct groups, which also largely separate these subfamilies by their Ser or Gly in the selectivity filter, with the exception of OsHKT2;1 (Horie et al. 2001). Bacterial HKT homologs, Trk, or Ktr transporters, function as major K^+ uptake systems (Gaber et al. 1988; Ko et al. 1990; Schlosser et al. 1995; Nakamura et al. 1998; Matsuda et al. 2004). K^+ uptake is stimulated by Na^+ in the cyanobacterial Ktr homologues of this family and significantly contributes to adaptation to hyperosmolar shock (Matsuda et al. 2004).

The question why plants express Na^+ selective Na^+ influx transporters such as AtHKT1;1 remained. Null mutations or those that reduce activity in the Na^+ transporter AtHKT1;1 (Mäser et al. 2002b; Gong et al. 2004; Berthomieu et al. 2003) resulted in Na^+ overaccumulation in leaves of these plants. The AtHKT1;1 transporter was immuno-localized in the plasma membrane of xylem parenchyma cells (Sunarpi et al. 2005). The Na^+ hypersensitive phenotype of *Athkt1;1* mutants (Mäser et al. 2002b) is due to the lack of Na^+ retrieval from xylem vessels by AtHKT1;1, leading to toxic Na^+ overaccumulation in leaves (Sunarpi et al. 2005). Mapping of a salt tolerance quantitative trait locus (QTL) from rice led to the isolation of OsHKT1;5, which is expressed in xylem parenchyma cells (Ren et al. 2005) and thus AtHKT1;1 and OsHKT1;5 have analogous functions in Na^+

retrieval from the xylem sap (Ren et al. 2005; Sunarpi et al. 2005). Interestingly, this HKT transporter-mediated exclusion of Na^+ accumulation in *Arabidopsis* and rice leaves via Na^+ removal from the xylem, has more recently been found to be the underlying mechanism of three major salinity tolerance QTLs in wheat (Byrt et al. 2007), providing an example of transfer of knowledge from model plants such as *Arabidopsis* and rice (Uozumi et al. 2000; Mäser et al. 2002b; Ren et al. 2005; Sunarpi et al. 2005), to applications in the field.

In contrast to the above discussed sodium toxicity at high Na^+ concentrations, low concentrations of Na^+ (e.g. < 5 mM) support growth of many plant species when K^+ is deficient. The Na^+ transporter OsHKT2;1 (previously named OsHKT1) is strongly induced in rice roots in response to K^+ starvation (Horie et al. 2001). Three loss of function mutant lines in *OsHKT2;1* exhibited substantial reduction in Na^+ influx into plant roots, showing that rice plants use Na^+ as a nutrient in the medium for their survival and growth under K^+ starvation and low Na^+ conditions (Horie et al. 2007). Thus several classes of Na^+ transporters and exchangers exist in plants and each class has unique roles in mediating sodium tolerance.

9 Future Prospects

Starting 25 years ago the study of plant transport moved into the era of identifying and characterizing individual ion channels and transporters. Such studies have benefited from several independent technical innovations including patch clamping, heterologous expression in yeast, oocytes, *E coli* and animal cells, ion sensitive fluorophores for imaging, biophysical structure-function analyses, forward and reverse genetic analyses, and the sequencing of reference plant genomes. However, the genes encoding some of the known channels/transporters remain to be identified. Additional approaches will aid in their identification including genetic studies of natural variation, systems biology, in silico analyses and proteomics. Abiotic stress and biotic stress continuously influence the plant body. Plants have developed an adaptive response to them; for example, reactive oxygen species have been used as intracellular and extracellular signals, which regulate membrane transport system, and coregulate Ca^{2+} signaling (McAinsh et al. 1996; Pei et al. 2000; Foreman et al. 2003; Demidchik et al. 2007).

Interestingly, almost every characterized plant ion channel and transporter class was found to have unique and intriguing properties, which have required new concepts and interdisciplinary analyses for their characterizations. These unique properties are often intimately related to their physiological functions and remain a basis for further analyses in the future. These advances are also contributing to the derivation of fundamental principles on the relationship of channels and transporters in all organisms. Moreover, many of the identified plant ion channels and transporters are linked to major environmental stresses that are directly relevant for the challenges facing humanity in the present century, including drought resistance, desiccation

avoidance, salt tolerance, aluminum resistance, pathogen responses, and water use efficiency. These pressing global needs will require further creative, interactive, and dynamic research efforts by the community of plant ion transport researchers. In particular, new knowledge will lead to the selection and generation of elite crops.

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The Role of Ion Channels in Plant Salt Tolerance

Anna Amtmann and Mary J. Beilby

Abstract Salinisation of agricultural land threatens world food production because it exposes crops to low water potential and high concentration of toxic ions in the soil. In particular, all major crops are sensitive to high concentrations of sodium (Na^+). Due to the negative electrical potential inside cells Na^+ influx into plant roots can occur through ion channels or other membrane transport proteins that facilitate passive diffusion of Na^+ across the plasma membrane. In this chapter, we discuss the contribution of different types of ion channels to Na^+ influx. In the first part of the chapter, we recapitulate the basic properties of different types of plant ion channels such as voltage-dependence of gating and relative selectivity for Na^+ and potassium and build a simple model to assess how these channels contribute to whole-cell ionic current and Na^+ uptake. In the second part of the chapter, we describe a number of experimental studies that have investigated Na^+ flux and ion channel currents in different plant species. The combined evidence suggests that salt tolerance in plants is based on the restriction of Na^+ influx through voltage-independent ion channels.

Abbreviations

VIC	Voltage-independent channel
IRC	Inward-rectifying channel
ORC	Outward-rectifying channel
GHK	Goldman-Hodgkin-Katz

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I–V	Current–Voltage
P_K:P_{Na}	Relative Na^+/K^+ permeability
CNGC	Cyclic nucleotide gated channel

1 Introduction

Salinisation of agricultural land hampers food production in many areas of the world (Amtmann et al. 2004; Munns and Tester 2008). Most land plants, including all major crops, are unable to grow if salt concentration of the soil solution exceeds 100 mM. However, a small and diverse range of plant species, so-called halophytes, is able to grow and even thrive on high salt concentrations. Salt tolerance in plants is often linked to the restriction of Na^+ accumulation and maintenance of a high K^+/Na^+ ratio in the shoots (Maathuis and Amtmann 1999; Moller and Tester 2007). The rate of Na^+ accumulation in plant shoots is determined by the net uptake of Na^+ into roots and its net translocation from roots to shoots. Net uptake of Na^+ into roots is the sum of unidirectional Na^+ influx and unidirectional Na^+ efflux across the plasma membrane of epidermal and cortical cells. Net root-shoot translocation of Na^+ is the result of net Na^+ flux from roots to shoots in the xylem and Na^+ recycling from shoots to roots in the phloem (Tester and Davenport 2003). Na^+ flux in the xylem involves Na^+ efflux from root parenchyma cells into the xylem and the recovery of Na^+ from the xylem. Na^+ recycling requires the loading of Na^+ into the phloem in the leaves and its recovery in the roots (Fig. 1).

Several transporters have been identified that mediate Na^+ transport across the plasma membranes of the cells involved in this complex system of whole-plant Na^+

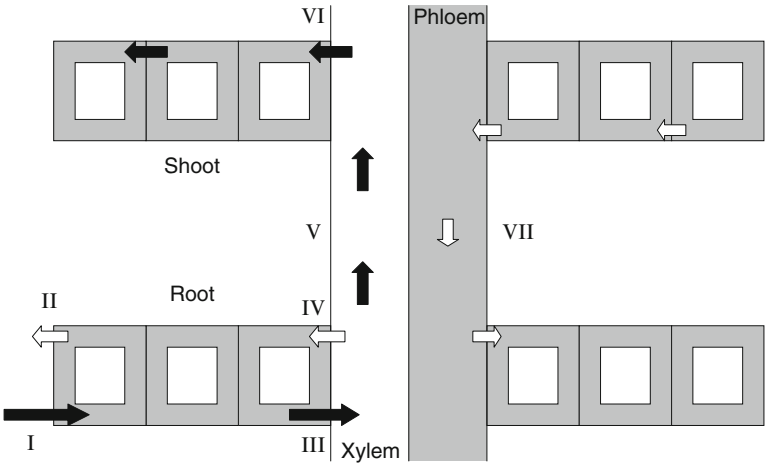

Fig. 1 Flux of sodium (Na^+) through the plant. I: unidirectional Na^+ influx into root cells, II: unidirectional Na^+ efflux from roots to the external medium, III: Na^+ loading into the root xylem, IV: recovery of Na^+ from the root xylem, V: root-shoot translocation of Na^+ in the xylem, VI: Na^+ unloading from the xylem in the shoot, VII: Na^+ recycling through the phloem

fluxes (Amtmann et al. 2004; Munns and Tester 2008). For example, the Na^+/H^+ antiporter SOS1 mediates the efflux of Na^+ from root cells into the soil or cortical apoplast (Wu et al. 1996; Shi et al. 2000; Rus et al. 2004). Together with HKT and CHX-type transporters, it also provides a means for Na^+ transport into and out of the xylem (Shi et al. 2002; Hall et al. 2006; Huang et al. 2006; James et al. 2006; Byrt et al. 2007). Much less is known about the transporters responsible for Na^+ uptake into root epidermal and cortical cells. This chapter discusses the roles of different types of ion channels in root Na^+ uptake. The first part of the chapter considers the relevance of different types of ion channels for Na^+ uptake based on theoretical current–voltage curves, which are generated for a combination of ion channels differing in selectivity and voltage dependence. The second part of the chapter describes a number of specific experimental studies that have investigated the roles of ion channels in Na^+ uptake and salt tolerance. It is hoped that the chapter contributes to the understanding of a fundamental aspect of plant adaptation to saline environments.

2 The Role of Ion Channels in Na^+ Uptake: A Simple Model

2.1 Electrochemical Gradients and Fluxes

The driving force for Na^+ into root cells is the combined gradient of voltage and chemical activity across the plasma membrane (electrochemical gradient). In a typical plant cell, the difference in electrical potential between the cytoplasm and the apoplast (membrane potential) is in the order of –120 to –180 mV. According to the Nernst equation, this provides a driving force for 100–1000-fold accumulation of Na^+ in the cytoplasm. Measurements of Na^+ in the cytoplasm are sparse and accompanied by considerable error since both X-ray measurements (Binzel et al. 1985; Hajibagheri and Flowers 1989; Flowers and Hajibagheri 2001) and radiotracer flux analyses (Kronzucker et al. 2006) have underlying problems with the exact assignment of the determined values to intracellular compartments, while the use of Na^+-sensitive microelectrodes and dyes is restricted to certain cell types (Carden et al. 2003; Kader and Lindberg 2005; Anil et al. 2007). Nevertheless, the combined evidence suggests that cytoplasmic Na^+ concentrations are generally in the low millimolar range. This is in accordance with the notion that cytoplasmic Na^+ concentrations above 100 mM are toxic due to the detrimental effects of a high Na^+ environment to protein stability (Serrano et al. 1999) and displacement of K^+ from essential co-factor binding sites on K^+-dependent enzymes (Wyn Jones and Pollard 1983). Thus in both low and high salt environments, living cells have to balance passive influx of Na^+ with Na^+ efflux, either across the plasma membrane back into the apoplast or across the tonoplast into the vacuole. The energy requirement for Na^+ efflux is considerable; approximately −5.7 kJ/mol per tenfold concentration gradient or per −60 mV of membrane potential. In addition to energy, time is an important factor for salt tolerance because the *rate* of Na^+ uptake will determine

how quickly Na^+ reaches toxic levels inside the cell. It is clear then that limiting Na^+ influx into root cells is a fundamental requisite for plant life in high salt conditions.

Balancing Na^+ influx with Na^+ export from the cytoplasm back into the apoplast (also sometimes termed 'futile cycling') is one way of reducing the Na^+ load (Britto and Kronzucker 2006; Malagoli et al. 2008). The importance of Na^+ export from root cells for salt tolerance is evident in the salt over-sensitivity of mutants that are impaired in the plasma membrane Na^+/H^+ antiporter SOS1 (Wu et al. 1996; Shi et al. 2000). This system seems to be similarly crucial in salt-sensitive and salt-tolerant species (Oh et al. 2007). Futile cycling occurs to a varying degree in all plants investigated so far with 78–98% of Na^+ taken up transported back into the environment (Kronzucker et al. 2006, 2008; Wang et al. 2006, 2009; Malagoli et al. 2008). A second strategy for removing Na^+ from the cytoplasm is to compartmentalise it in the vacuoles. Na^+ uptake into the vacuole also requires energy but has a dual benefit in saline conditions; it avoids Na^+ build-up in the apoplast (Oertli 1968) and enhances the intracellular solute potential thereby contributing to turgor adjustment. The importance of Na^+ allocation into vacuoles is evident in the fact that over-expression of NHX-type vacuolar Na^+/H^+ antiporters enhances salt tolerance in plants (Apse et al. 1999; Zhang et al. 2001; Ohta et al. 2002). One point that is rarely discussed in this context is that vacuolar Na^+ storage as a means to remove Na^+ from the cytoplasm relies on growth. Only if the vacuolar lumen is constantly enlarged can rapid saturation of this mechanism be avoided. Or, putting it the other way round, when the vacuolar storage space is exhausted Na^+ will accumulate in the cytoplasm, and its toxic effect will slow down growth thereby exacerbating the problem. The ability of plants to cope with high Na^+ concentrations in the soil therefore relies on maintaining a positive balance between the rate of growth (enlargement of the vacuolar lumen) and the rate of Na^+ uptake across the root plasma membrane.

Two important conclusions can be drawn from the above considerations:

- The rate of Na^+-uptake (the size of Na^+-influx) is critical for the ability of plants to avoid the build-up of toxic Na^+ concentrations in the cytoplasm.
- The driving force for Na^+ uptake into roots is directed inward and therefore Na^+ uptake can proceed through passive transport.

Ion channels represent the most common pathway for passive ion flux across biological membranes and will therefore be at the centre of this review. However, it should be noted that other transport systems could contribute to passive influx of Na^+ into plant cells. For example, some members of the HKT family transport Na^+, either in a high-affinity mode coupled to K^+ uptake or in a low-affinity affinity mode as an Na^+–Na^+ co-transport system (Rubio et al. 1995; Uozumi et al. 2000; Horie et al. 2001; Liu et al. 2001). Additionally, Na^+ influx may occur through proteins mediating proton-coupled transport of K^+, amino acids, sugars, etc. The resulting cumulative 'leakage' of Na^+ into cells cannot readily be distinguished from Na^+ currents through voltage-independent channels (see below) but due to low transport rates of the above mentioned systems compared to ion channels their contribution is likely to be very small.

2.2 Fundamental Characteristics of Different Channel Types

Before one can assess which channel types are relevant for Na^+ uptake, it is important to understand the basic properties of ion channel proteins. Ion channels are generally characterised by three features: (a) their conductance, which determines the amount of current that flows through an open channel at any given voltage, (b) their open probability, which determines how many channels are active at any given voltage and (c) their selectivity, which determines the relative flux of different ions through an open channel. The specific properties of ion channels are best explored in voltage clamp experiments, in which the movement of ions through ion channel proteins is monitored as a current that flows across a single channel (e.g. in excised membrane patches, single-channel current) or a population of channels (e.g. all channels in the plasma membrane of one cell, whole-cell current). The driving force for ion flux through the channels can be experimentally manipulated by clamping the membrane to different voltages. Voltage-clamp experiments have established that some components of the whole-cell current are 'time-dependent', which means that they require a certain time to reach a new steady state after a sudden change of voltage. This behaviour can be explained by the fact that the open probability of the underlying channels is voltage-dependent. Upon a change in voltage, a certain number of channels change from an inactive into an active state or *vice versa*, until the total number of active channels complies with the new voltage. The time that is required for this change is in the range of milliseconds and when essayed over a large number of individual channel proteins the activation or inactivation is visible as a change in macroscopic current over time. So-called inward rectifying channels (IRCs) increase their open probability (activate) if the voltage is clamped to more negative (hyperpolarised) voltages while so-called outward rectifying channels (ORCs) activate if the membrane is depolarised. The open-probability (P_o) of voltage-dependent ion channels is usually well described with a Boltzman distribution (Fig. 2a), in which the half activation potential, V_{50}, is the voltage at which half of the maximal number of channels are open, and the gating charge, z_g, is the steepness of the voltage dependence. In addition to time-dependent currents, the whole-cell current usually comprises a current component that responds instantaneously to a sudden change in voltage, which indicates that the open-probability of the underlying channels is voltage-independent. Note that the term voltage-independent channel (VIC) refers to the open probability alone; the current through any open channel will of course change with voltage according to Ohm's law ('open-channel conductance'). Both the open-channel conductance and the voltage-dependence of the open probability are best described by current–voltage (*I–V*) curves in which the current through the channel (or an ensemble of channels) is plotted against the voltage. By convention negative currents represent influx of cations into the cytoplasm, while positive currents represent cation efflux from the cytoplasm. *I–V* curves determined for an entire cell are the sum of individual *I–V* curves for different channel types, each of which is the product of the *I–V* curve of a single channel with the total number of channels in the cell and the open probability. Decomposing the whole-cell

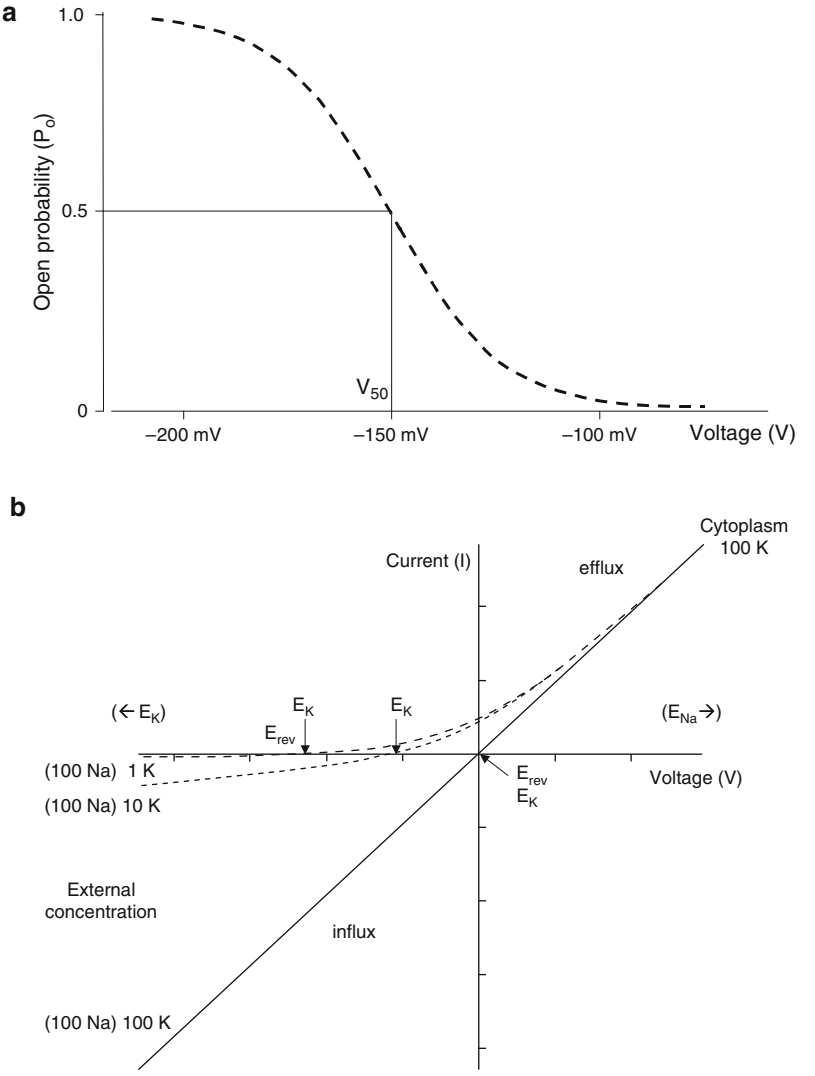

Fig. 2 Open probability and selectivity of ion channels. (**a**) Typical voltage-dependence of an inward rectifying K^+-channel. In this example the half-activation potential (V_{50}) is –150 mV and the gating charge is 2. (**b**) Current (I) flowing through an open K^+-selective channel at any given voltage (V). The cytoplasmic K^+ concentration is set as 100 in all curves, the external K^+ concentration as 100 (*solid curve*), 10 (*dotted curve*), or 1 (*dashed curve*). In these cases the reversal potential E_{rev} is equal to E_K, as calculated with the Nernst equation: $E_K = -60$ mV log ($[K]_{cyt}/[K]_{ext}$). The same curves are produced with the Goldman-Hodgkin-Katz (GHK) model if the external medium contains 100 Na^+ instead of K^+, and the permeability of the channel is equal for K^+ and Na^+ (*solid curve*), 10 times higher for K^+ than Na^+ (*dotted curve*) or 100 times higher for K^+ than Na^+(*dashed curve*). In these cases E_K is very negative and E_{Na} is very positive

current–voltage relationship into individual channel types is a considerable challenge that relies on detailed characterisation of the properties of individual channels in excised patch clamp experiments.

While the measurement of currents in voltage-clamp experiments reveals the voltage-dependence and activation kinetics of channels it does not directly tell us which ions carry the current. If only one permeable ion species is present on both sides of the membrane, the membrane potential at which the current is nil (the reversal potential, E_{rev}) is equal to the equilibrium potential of this ion (E_{ion}) given by the Nernst equation (Fig. 2b). The relative permeability of ion channels for different ions is usually established by exposing the outer and inner side of the membrane to different ion concentrations. E_{rev} can then be compared with E_{ion} of each ion; if E_{rev} is closer to E_A than E_B the permeability of the channel is higher for A than for B. The Goldman-Hodgkin-Katz (GHK) equation provides the simplest approach to calculate the relative permeability from the reversal potential (Hille 2001). However, the GHK model makes two important assumptions; (a) that ions move independently through the channel and (b) that the electrical field across the membrane is constant. In other words, it only applies if no ion–ion or ion–protein interactions occur in the channel, neither of which is usually the case (Hille 2001). It therefore remains difficult to predict the exact contribution of different ions to a current when the membrane is exposed to a mixture of ions. Nevertheless the GHK model is usually a good approximation of the actual currents, and until now it has not been replaced by a satisfactory general model that takes into account different types of molecular interactions. More importantly, the GHK model helps us to conceptually understand ion fluxes across cell membranes and provides a useful guidance for experiments. In the following section of this chapter we will apply existing knowledge on channel selectivity and voltage-dependence in a model based on Nernst, GHK and Boltzman equations, to assess which channel types are most likely to contribute to Na^+ uptake in saline conditions.

2.3 Contribution of Different Channel Types to Na^+ Uptake

In all plant species investigated so far the plasma membrane of root epidermal and cortex cells displays all three types of currents described above (Table 1). Inward-rectifying currents activating upon hyperpolarisation (V_{50} of −150 to −190 mV) usually show a relatively steep voltage-dependence (z_g around 2) and are highly selective for K^+ over Na^+ (relative permeability, P_K:P_{Na} around 50). Outward rectifying currents activating upon depolarisation (V_{50} around 0 mV) display weaker voltage-dependence (z_g below 2) and are less selective for K^+ over Na^+ (P_K:P_{Na} around 10). The channels underlying these two types of currents are well characterised with respect to their electrical properties at the single-channel level and the structure–function relations within the channel proteins (Véry and Sentenac 2003; Dreyer et al. 2004). Much less is known about the molecular nature of the transporters underlying the instantaneous currents across root plasma membranes

Table 1 Equations and parameters used to generate current–voltage (I/V) curves shown in Figs. 3 and 4

General equation:

$$I(V) = P_o NP(F^2/RT)V(S_i - S_e \exp(-FV/RT)/(1 - \exp(-FV/RT))$$

with P: permeability of the channel for ion S, S_i, S_e: cytoplasmic and external concentration of S, R: gas constant, T: temperature[a], F: Faraday constant, P_o: open probability.

$P_o = (1 - 1/(1 + \exp(-z_g(F/RT)(V - V_{50}))))$ for IRC

$P_o = 1/(1 + \exp(-z_g(F/RT)(V - V_{50})))^2$ for ORC

$P_o = 1$ for VIC

with z_g: gating charge, V_{50}: half activation potential.

Parameters:

Channel	Ion (S_i/S_e in mM)	z_g	V_{50} (mV)	NP (m^3s^{-1})	Figure (curve)
IRC	K^+ (100/2)	2	−150	2×10^{-17}	Fig. 3a (dotted)
					Fig. 4b (dotted)
IRC	Na^+ (2/100)	2	−150	4×10^{-19}	Fig. 3a (dashed)
					Fig. 4b (dashed)
IRC	Na^+ (2/100)	2	−150	2×10^{-19}	Fig. 3a (solid)
IRC	Na^+ (2/100)	2	−150	2×10^{-18}	Fig. 4a (dash-dotted)
ORC	K^+ (100/2)	1	0	1×10^{-17}	Fig. 3a (dotted)
					Fig. 4b (dotted)
ORC	Na^+ (2/100)	1	0	1×10^{-18}	Fig. 3a (dash-dotted)
					Fig. 4b (dashed)
ORC	Na^+ (2/100)	1	0	2×10^{-19}	Fig. 3a (dashed)
ORC	Na^+ (2/100)	1	0	1×10^{-19}	Fig. 3a (solid)
ORC	K^+ (100/2)	1	−60	1×10^{-17}	Fig. 3b (dotted)
ORC	K^+ (100/2)	1	−120	1×10^{-17}	Fig. 3b (dotted)
ORC	Na^+ (2/100)	1	−60	1×10^{-18}	Fig. 3b (solid)
ORC	Na^+ (2/100)	1	−120	1×10^{-18}	Fig. 3b (dash-dotted)
VIC	K^+ (100/2)	n.a.[b]	n.a.	1×10^{-18}	Fig. 3c (dotted)
					Fig. 4b (dotted)
VIC	Na^+ (2/100)	n.a.	n.a.	1×10^{-18}	Fig. 3c (dashed)
					Fig. 4b (dashed)
VIC	Na^+ (2/100)	n.a.	n.a.	5×10^{-19}	Fig. 3c (dash-dotted)
VIC	Na^+ (2/100)	n.a.	n.a.	1×10^{-19}	Fig. 3c (solid)
IRC	K^+ (100/1)	2	−150	2×10^{-17}	Fig. 4a (dotted)
ORC	K^+ (100/1)	1	0	1×10^{-17}	Fig. 4a (dotted)
VIC	K^+ (100/1)	n.a.	n.a.	1×10^{-18}	Fig. 4a (dotted)
IRC	Na^+ (1/1)	2	−150	4×10^{-19}	Fig. 4a (dashed)
ORC	Na^+ (1/1)	1	0	1×10^{-18}	Fig. 4a (dashed)
VIC	Na^+ (1/1)	n.a.	n.a.	1×10^{-18}	Fig. 4a (dashed)

[a] 18°C for Figs. 3 and 4

[b] n.a.: not applicable

although they are electrophysiologically well characterised (Amtmann and Sanders 1999; Demidchik et al. 2002; Demidchik and Maathuis 2007). A common feature of these currents in most plant species is that they display weak selectivity for K^+ over

other cations, in particular Na^+, and they have therefore also been termed 'non-selective cation channels' (NSCCs, see Demidchik and Maathuis 2007 for review). In this chapter we will continue to call them voltage-independent channels (VICs) since their selectivity is the topic of discussion here. Another common feature of VICs is that they are insensitive to ions that typically block voltage-dependent currents such as Cs^+ and TEA^+. By contrast, VICs are partially inhibited by external Ca^{2+} with a K_d in the range of a few hundred micromolar (Amtmann et al. 1997; Roberts and Tester 1997; Tyerman et al. 1997; Demidchik and Tester 2002; Volkov and Amtmann 2006). It is this latter feature that has indicated that VICs may play an important role in Na^+ uptake because unidirectional influx of Na^+ measured in radiotracer flux experiments is inhibited by external Ca^{2+} in a similar fashion (Davenport and Tester 2000; Tester and Davenport 2003). Reports on other regulatory features of VICs are less consistent. For example, inhibition by cyclic nucleotides (CNs) or activation by glutamate was observed in some studies but not others (Maathuis and Sanders 2001; Demidchik and Tester 2002; Demidchik et al. 2004; Volkov and Amtmann 2006). The assignment of instantaneous currents to members of the 'cyclic-nucleotide gated channel' (CNGC; Talke et al. 2003) or 'glutamate receptor' (GLR; Davenport 2002) gene families is therefore still under question. In addition to IRC, ORC and VIC, hyperpolarisation and depolarisation activated Ca^{2+} channels (HACC and DACC; Miedema et al. 2008) might contribute to Na^+ uptake in certain cell types such as root hairs. Again, the genes encoding these channels have not yet been identified. In the following model calculations we concentrate on IRC, ORC and VIC as the main potential pathways for Na entry into plant root cells but the model can easily be extended to evaluate the contribution of additional pathways.

Based on known general features of ORCs, IRCs and VICs, we can calculate the K^+ and Na^+ current through each channel type for a given concentration of K^+ and Na^+ at either side of the membrane. To generate 'whole-cell' *I–V* curves, the channel current given by the GHK equation is multiplied with the channel's open probability, P_o, determined by the Boltzman equation and the total number of channels, N (Amtmann and Sanders 1999). For VICs P_o is set to 1, for IRCs and ORCs P_o is calculated using a Boltzman distribution with typical values for V_{50} and z_g. P, the permeability of the single channel for a specific ion, is often not known and neither is N, the total number of channels in the membrane, but the product NP can be chosen so that it mimics the whole-cell current produced by a specific type of channel. The values chosen for P also have to reflect the relative Na^+/K^+ permeability (P_K:P_{Na}) of the particular channel (e.g. P is set 10 times higher for K^+ than for Na^+ to account for a channel P_K:P_{Na} of 10). The equations and values used to generate the *I–V* curves shown in Figs. 3 and 4 are listed in Table 1.

Figure 3 demonstrates the effect of relative permeability and voltage-dependence on the amount of K^+ and Na^+ current through the different channel types in a hypothetical 'high-salt' situation (cytoplasmic/extracellular concentrations are 100/2 mM for K^+ and 2/100 mM for Na^+). Figure 3a shows K^+ and Na^+ currents through IRCs with a V_{50} of −150 mV and a gating charge of 2, and ORCs with a V_{50} of 0 mV and a gating charge of 1. Na^+ current through the IRC (apparent at voltages

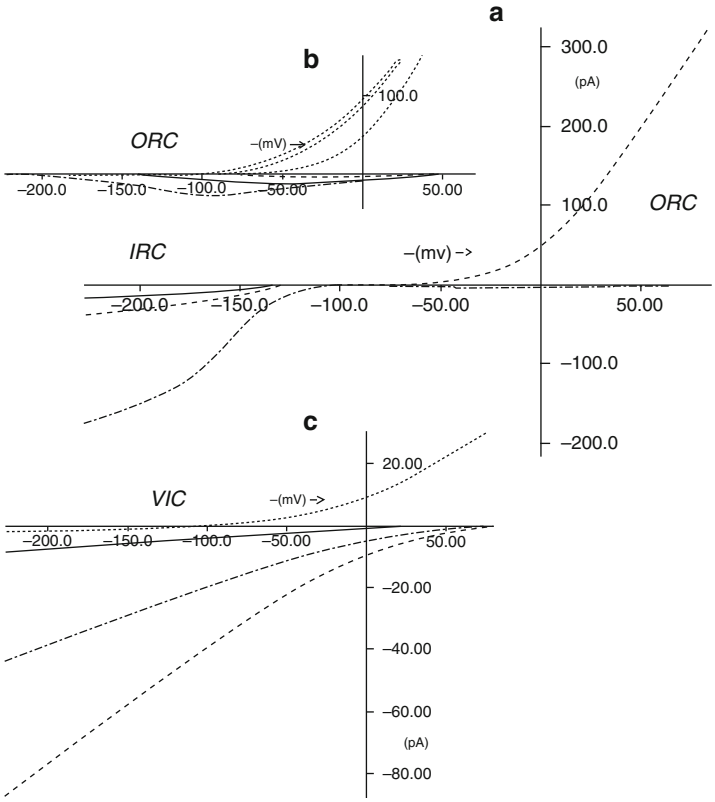

Fig. 3 Theoretical whole-cell current–voltage curves describing the net flux of K^+ (*dotted lines*) and Na^+ (*other lines*) through plasma membrane ion channels. K^+ flux is represented by dotted lines, Na^+ flux by other lines. (**a**) Effect of relative permeability of IRC and ORC on Na^+ inward current. P_K/P_{Na} is set to 10 (*dash-dotted curve*), 50 (*dashed curve*), or 100 (*solid curve*). Note the dotted curve representing K^+ inward current identical to the dashed curve for Na^+ inward current. (**b**) Effect of the half activation potential V_{50} of the ORC on Na^+ inward current; V_{50} is set to 0 mV (*dashed curve*), −60 mV (*solid curve*) or −120 mV (*dash-dotted curve*). Note that dashed and solid curves are indistinguishable from the X-axis. (**c**) Effect of the relative permeability of VICs for K^+ and Na^+ on Na^+-inward current. P_K/P_{Na} was set to 1 (*dashed curve*), 2 (*dash-dotted curve*) or 10 (*solid curve*). Ion concentrations: 100 mM K^+, 2 mM Na^+ in cytoplasm, 2 mM K^+, 100 mM Na^+ outside. Equations and all other parameters used to generate *I–V* curves are given in Table 1

below –100 mV) is equal to K^+ inward current through this channel if P_K:P_{Na} of the IRC is 50 (dashed and dotted curves overlap at negative voltages). The current is reduced by half when P_K:P_{Na} is 100 and increases 5 times if P_K:P_{Na} is 10. Although these results are rather trivial due to the simple conditions chosen here (external K^+/Na^+ ratio is 1/50) they illustrate two important facts: Firstly, a channel with a very high selectivity for K^+ over Na^+ will still transport a considerable amount of Na^+ if the external Na^+ concentration is very high and the K^+/Na^+ ratio is low (note that sea water has approximately 50 times more Na^+ than K^+). Secondly, a high P_K:P_{Na} of the IRC is essential to avoid very large influx of Na^+ because this channel

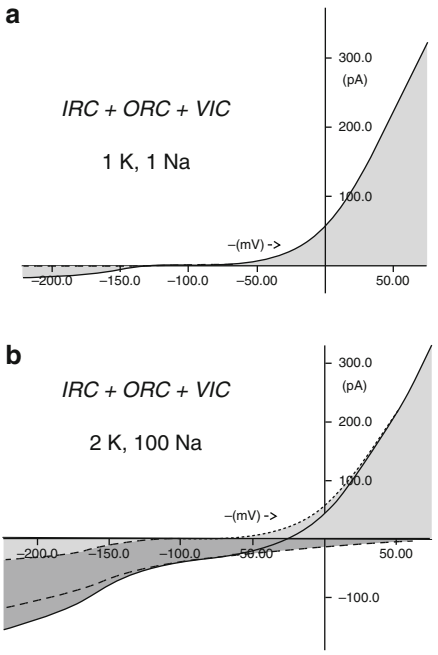

Fig. 4 Theoretical whole-cell current–voltage curves describing the total net flux of K^+ and Na^+ through an ensemble of IRC, ORC and VIC. Total K^+ flux is shown in light grey, total Na^+ flux in dark grey. Equations and all parameters used to generate the I–V curves are given in Table 1. Total current is given in solid lines. (**a**) Ion concentrations are 1 mM K^+ and 1 mM Na^+ outside the cell, and 100 mM K^+ and 1 mM Na^+ in the cytoplasm. Note that the dash-dotted curve indicated in Table 1 is to small to be seen, and that the dotted curve is hidden under the solid curve. (**b**) Ion concentrations are 2 mM K^+ and 100 mM Na^+ outside the cell, and 100 mM K^+ and 2 mM Na^+ in the cytoplasm. Note that the dotted curve for K^+-inward current is hidden under the identical dashed curve

operates at membrane potentials that are much more negative than E_{Na} (E_{Na} = +100 mV in our example). It should be noted that a study of young *Arabidopsis thaliana* seedlings found blockage of the IRC by cytoplasmic Na^+ (Qi and Spalding 2004), which would eliminate both K^+ and Na^+ influx through the IRC when the Na^+ concentration in the cytoplasm rises. However, no such inhibition was found for the IRC in roots of more mature Arabidopsis plants or other species (Volkov and Amtmann 2006).

Changing the P_K:P_{Na} of the ORC between 10 and 100 has little effect on Na^+ influx through the ORC (apparent as an inward current between +60 and –60 mV). The ORC-mediated Na^+ inward current is generally very small because the activity range of the channel is close to E_{Na}. Figure 3b explores how the voltage-dependence of the ORC influences Na^+ influx through this channel type. Shifting V_{50} negative (from 0 to –60 and –120 mV) increases the Na^+ inward current and moves it into a range of physiological membrane potentials (solid and dash-dotted curves). However, even with a V_{50} of –120 mV the ORC-mediated Na^+ inward current is relatively small (compared to IRC and VIC currents, compare Fig. 3a and c). Very negative V_{50} values are unlikely to occur in saline conditions as the activation potential of ORCs is usually coupled to E_K (Blatt and Gradmann 1997; Maathuis et al. 1997; Amtmann and Blatt 2009). Considering that accumulated salt in the soil will always contain some K^+, the external K^+ concentration accompanying Na^+

concentrations of more than 50 mM will rarely be lower than 1 mM, representing an E_K of approximately −120 mV. We conclude that in most conditions the ORC will not make a major contribution to Na^+ uptake. Experimental evidence for blockage of the ORC by Na^+ from the cytoplasmic and/or external side (Thiel and Blatt 1991; Shabala et al. 2006), further argues against a role of ORC in Na^+ uptake.

Figure 3c shows the effect of the relative permeability of VICs on Na^+ inward current through this type of channel. Whole-cell instantaneous currents are usually small compared to time-dependent currents and this has been taken into account by assigning a whole-cell K^+ conductance (*NP*) that is smaller than the respective values for IRC and ORC (10^{-18} m^3s^{-1} for VIC, 10^{-17} m^3s^{-1} for ORC, 2×10^{-17} m^3s^{-1} for IRC, Table 1). In accordance with experimental evidence the VIC-mediated K^+ inward current in our model is very small (dotted curve). However, in most plant species this channel type does not discriminate between K^+ and other cations. With a P_K:P_{Na} of 1 (dashed curve) the VIC-mediated Na^+ inward current is considerably larger than IRC-mediated Na^+ current (dashed curve in Fig. 3b). Furthermore, because VICs are voltage-independent, Na^+ inward currents are still apparent in a voltage range where the IRC is closed (here between 0 and –120 mV). Increasing P_K:P_{Na} of the VICs to 2 and 10 strongly reduces the amount of Na^+ influx (dash-dotted and solid curves).

Calculating separate *I–V* curves for each ion and each channel under consideration has the advantage that the model can be easily extended for additional ions or channels, and that different subsets of *I–V* curves can be combined to address different questions. For example, we can add K^+ and Na^+ currents through IRCs to visualise the total current through this channel type, or we can add Na^+ currents through several channel types and compare the resulting total Na^+ current with the total K^+ current carried by the same ensemble of channels. We can thus easily establish how much each channel type contributes to Na^+ influx in any combination of K^+ and Na^+ concentrations inside and outside the cell, and how this contribution is influenced by different channel features.

Figure 4 shows total K^+ and Na^+ currents through an ensemble of IRC, ORC and VICs for hypothetical low-salt and high-salt conditions. The three channel types considered here have again basic features that represent most cation channels recorded in plant root cells (Table 1). From the range of available experimental data, values for individual channel features were chosen that are typical and, in case of doubt, promote Na^+ influx rather than minimise it. Figure 4a shows that in a situation with 1 mM K^+ and 1 mM Na^+ outside the cell, K^+ inward flux is almost entirely mediated by IRCs and Na^+ influx is very small. Increasing the external salt concentration to 100 M Na^+ and 2 mM K^+ (Fig. 4b) produces large Na^+ influx (dark grey) most of which is mediated by VICs. The IRCs make an increasing contribution at negative voltages below –140 mV to a maximum of approximately 30%. In a living cell, the inward current through ion channels will be counteracted by proton efflux through the plasma membrane proton pump resulting in a membrane potential that is more negative than the reversal potential of the ion channel-mediated cation currents. However, it is also clear that in saline conditions the Na^+ current through the VIC will shift E_{rev} to a considerably more positive voltage (compare

E_{rev} in Fig. 4a and b), which explains why cells are often more depolarised in high salt conditions than in low salt conditions, in which the K^+ inward current through IRCs is the only major counterpart to the proton pump. Most importantly, the salt-induced depolarisation shifts the membrane potential into a voltage range where the IRC is inactive and therefore K^+ influx is impaired. This, in turn, results in a high ratio of Na^+ versus K^+ influx imposing further strain on the cytoplasmic K^+/Na^+ ratio. As illustrated in Fig. 4c even a 50% reduction of VIC Na^+ permeability (either by reducing the number of channels to half or by increasing P_K:P_{Na} to 2) has a strong effect not only on Na^+ influx but also on E_{rev} and the K^+/Na^+ influx ratio. It can be concluded from this exercise that

- VICs make the strongest contribution to Na^+ influx in high salt conditions despite their relatively low number,
- Na^+ influx through non-selective VICs depolarises the membrane in high salt conditions, and
- down-regulating VICs (decreasing the number of active channels) or increasing their selectivity for K^+ over Na^+ could be an important means to reduce Na^+ influx and maintain K^+ influx into cells in high salt conditions.

In the remainder of this chapter we will describe a number of experimental studies that have investigated the role of ion channels in Na^+ influx and salt tolerance, and discuss the outcome of these studies in the context of the predictions made by our model calculations.

3 The Role of Ion-Channels in Salt Tolerance: Experimental Evidence

3.1 Ion Channels and Salt Tolerance in Crops

Voltage-independent, non-selective cation channels in plants were first described for several crop species. Characterisation of discrete single channel currents in excised membrane patches from root cells of rye, wheat and maize and cultured cells of barley proved that at least part of the whole-cell 'leak' current was mediated by channel proteins (White and Lemitiri-Clieh 1995; Amtmann et al. 1997; Roberts and Tester 1997; Tyerman et al. 1997). Based on theoretical *I–V* curves as the ones shown here it was suggested that these channels could constitute a pathway for Na^+ influx in saline conditions (Amtmann and Sanders 1999). A subsequent study analysed single channels from wheat roots in lipid bilayers and compared their characteristics with whole-cell currents from wheat root protoplasts and ^{22}Na influx into wheat roots (Davenport and Tester 2000). One channel type identified in lipid bilayers was similar to the one described in membrane patches with respect to conductance, permeability sequence and relative permeability for K^+ and Na^+ (P_K:P_{Na} of approx. 0.8). Most importantly,

inward Na^+ current through the channel was not inhibited by known inhibitors of voltage-dependent K^+ channels (TEA^+, Cs^+, verapamil) but showed partial inhibition by external Ca^{2+} with a K_i of 0.65 mM. Similarly, unidirectional influx of Na^+ into wheat root segments was insensitive to K^+-channel inhibitors but was inhibited by external Ca^{2+} with a K_i of 0.61 mM. This study provided good evidence that Na^+-influx in wheat is indeed mediated by non-selective VICs. To date no electrophysiological studies have been carried out on rice, but analysis of fluxes indicates that rice differs from other plant species insofar as an important part of Na^+ uptake proceeds through apoplastic leakage that breaches the cell wall barriers of exodermis and endodermis (so-called by-pass flow, Yadav et al. 1996). Apoplastic linkage between the external solution and the stele could occur in root apices (before the Casparian strip is formed) or at the sites of lateral root emergence (Yadav et al. 1996).

3.2 *Ion Channels and Salt Tolerance in* Arabidopsis thaliana

The interest of plant scientists in non-selective ion channels was fuelled by the discovery that the *Arabidopsis* genome contains two large families of genes that are homologous to so-called cyclic nucleotide gated channels (CNGCs) (Talke et al. 2003) and glutamate receptors (GLRs) (Davenport 2002). In animals these channels are non-selective for cations and, in many cases, voltage-independent, thus exhibiting features that are similar to those of plant VICs. The sensitivity of plant VICs to CNs was explored in a study with root protoplasts from *A. thaliana* (Maathuis and Sanders 2001). cGMP and cAMP reduced the open probability of VICs in excised outside-out patches almost to zero and a membrane-permeable cGMP analog reduced the instantaneous current recorded in the whole-cell configuration. The authors went on to investigate the effect of membrane-permeable CNs on unidirectional ^{22}Na influx into *A. thaliana* roots and on the growth of *A. thaliana* seedlings in saline condition (100 mM NaCl). Unidirectional Na^+ influx was indeed lower in plants exposed to cGMP or cAMP and treatment with either of the CNs considerable improved growth on high salt. The study provided experimental proof for a link between VICs, Na^+ influx and salt tolerance but, as is so often the case, it raised as many questions as it answered. For example, CNGCs from animals and *A. thaliana* (at least those that have been characterised) are activated rather than de-activated by CNs. Currents through *A. thaliana* heterologously expressed CNGC proteins also differ in other features from VIC-type currents. For example, CNGC1 is inward-rectifying and CNGC2 is selective for K^+ (Leng et al. 1999, 2002). Another question relates to the observation that CN-dependency of VICs was only found in some protoplasts. It would be interesting to know whether this subset of protoplasts derives from a specific cell type within the root. Finally, if CNs have a regulatory role in salt adaptation then why do plants require experimental application of CNs to take advantage of this role? Clearly down-regulation of VICs by endogenous CNs is either not optimised for salt stress, or it is limited to maintaining other essential functions of the channels. These questions apart, the

study provides strong evidence that inhibition of VIC-mediated currents reduces Na^+ influx and improves salt tolerance in *A. thaliana*. A separate study investigated the pharmacological profile of ^{22}Na radiotracer flux into living *A. thaliana* plants to assess the contribution of different transporters to Na^+ influx (Essah et al. 2003). Na^+ influx was not affected by inhibitors of voltage-dependent K^+ channels (Cs^+, TEA^+), Cl^- channels (Zn^{2+}), Ca^{2+} channels (Gd^{3+}, La^{3+}, verapamil) or Na^+/H^+ antiporters (amiloride), and was unchanged in *akt1* and *hkt1* knockout mutants. Na^+-influx was partially inhibited by external Ca^{2+} as well as cGMP, but stimulated by glutamate and 4-amino butyric acid (GABA). This pharmacological spectrum agreed well with the one determined for non-selective ion channels in *A. thaliana* root protoplasts (Demidchik and Tester 2002). While these data further confirmed that VICs are the main contributor to Na^+ influx they also suggested that more than one pathway was involved without providing clear indication which genes may encode these pathways.

3.3 Ion Channels and Salt Tolerance in Halophytic Higher Plants

The research described above provided firm evidence that VICs provide the major pathway for in Na^+ uptake in many salt-sensitive plant species, and that inhibition of this pathway increases salt tolerance, but the question whether successful evolutionary adaptation to a saline environment involved restriction of this pathway, remained an open question. *Thellungiella halophila* (often called *salsuginea*) a salt-tolerant close relative of *A. thaliana* provides a convenient system to address this question (Inan et al. 2004; Amtmann et al. 2005; Amtmann 2009). *T. halophila* still grows in an external salt concentration comparable to sea water (e.g. 500 mM NaCl) and, unlike most plant species, it survives a sudden drastic increase in Na^+ concentration (salt shock; Inan et al. 2004). Several laboratories have shown that *T. halophila* (Shandong ecotype) accumulates considerably less Na^+ in its leaves than *A. thaliana* at a similar external salt concentration, both in the short term (hours) and in the long run (weeks) and also maintains a higher K^+/Na^+ ratio (Inan et al. 2004; Volkov et al. 2004; Wang et al. 2006; Aleman et al. 2009a). Radiotracer flux analysis of entire plants showed that unidirectional ^{22}Na influx into the roots of *T. halophila* exhibits similar characteristics as those found in *A. thaliana* and other salt-sensitive species, in particular inhibition by external Ca^{2+} and insensitivity to the IRC and ORC-blockers Cs^+ and TEA^+ (Wang et al. 2006). However, ^{22}Na influx was approximately 2 times smaller in *T. halophila* than in *A. thaliana*. Rather unexpectedly, ^{22}Na efflux was also smaller in *T. halophila*. Comparison of ^{22}Na flux with net shoot Na^+ accumulation suggested a scenario in which (a) differences in unidirectional Na^+ influx into roots can account for the different Na^+ accumulation in the two species, (b) both species operate a similar degree of futile Na^+ cycling with approximately 78% of the Na^+ taken up being exported back into the environment, and (c) less energy-dependent Na^+ efflux is required in *T. halophila* than *A. thaliana* to balance Na^+-influx (Fig. 5). Further support for this model was provided by a patch-clamp study comparing VIC-mediated currents in the

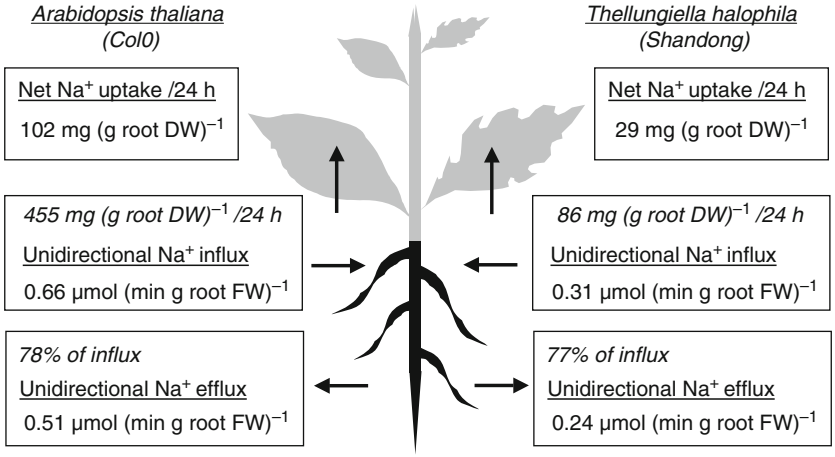

Fig. 5 Unidirectional flux and net uptake of Na^+ in *A. thaliana* and *T. halophila*. Measured values are given in normal font, calculated values in italic font. All data taken from (Wang et al. 2006)

plasma membrane of root protoplasts from *T. halophila* and *A. thaliana* (Volkov and Amtmann 2006). VIC currents showed similar pharmacological profiles as in other species, most notably inhibition by external Ca^{2+} and lack of inhibition by Cs^+ or TEA^+, and this profile was reflected in net Na^+ accumulation in the shoots. However, the reversal potential of *T. halophila* VIC currents shifted negative when K^+ in the external medium was replaced with Na^+, which indicates that VICs in *T. halophila* (unlike their counterparts in *A. thaliana* and other salt-sensitive species) are selective for K^+ over Na^+. Interestingly the whole-cell conductance was similar for K^+ and Na^+, indicating that ion movement through the channel does not satisfy the GHK model. Nevertheless the negative E_{rev} resulted in a considerably smaller Na^+ inward current in *T. halophila* compared to *A. thaliana* over a wide range of voltages. Impalements of epidermal root cells in their native root environment showed that *T. halophila* root cells are much less depolarised in response to NaCl than those of *A. thaliana* (Volkov and Amtmann 2006). This finding supports the notion that the size of VIC-mediated Na^+ influx strongly impacts on the membrane potential (see Sect. 2.3). Na^+ influx calculated from the measured VIC currents at the respective membrane potentials also agreed well with unidirectional ^{22}Na influx previously measured in both species (see above). The combined results from the studies with *T. halophila* suggest that salt tolerance in this species is at least in part due to increased K^+/Na^+ selectivity of root VICs, which reduces unidirectional Na^+ influx. The dual benefit of this strategy consists in (a) maintenance of hyperpolarized membrane potential and thus selective uptake capacity for K^+ through the IRC and the high-affinity transporter HAK5 (Aleman et al. 2009b), and (b) the reduced energy requirement for Na^+ export.

Limitation of Na^+ influx into roots is also evident in other salt-tolerant species. For example, it was shown that *Pucinella tenuiflora*, a monocotyledonous

halophyte, achieves a higher K^+/Na^+ accumulation ratio in the shoot than wheat (Wang et al. 2009). As in the example of *T. halophila* and *A. thaliana*, both unidirectional Na^+ influx into the roots and unidirectional Na^+ efflux from the roots were smaller in *P. tenuiflora* than in wheat while the relative amount of futile Na^+ cycling was the same in both species (efflux being 80% of influx).

In contrast to the 'salt excluders' *T. halophila* and *P. tenuiflora*, the halophyte *Suaeda maritima* is a 'salt includer' which accumulates large amounts of Na^+ during growth. Interestingly, this halophyte also seems to employ different pathways for Na^+ uptake than the other two halophytes. A recent study showed that cAMP, Ca^{2+} or Li^+ had no effect on unidirectional Na^+ influx or net Na^+ accumulation in *S. maritima*, whereas Ba^{2+} decreased both parameters (Wang et al. 2007). Most strikingly, at high external NaCl (150 mM) unidirectional Na^+ influx into roots, net Na^+ accumulation in the shoot and growth were all inhibited by TEA^+ and Cs^+, suggesting that in saline conditions K^+-inward rectifying channels mediate Na^+ influx. The main question arising from this study is how *S. maritima* manages to maintain a membrane potential in high salt that is negative enough to operate the IRC. One possible explanation is that *S. maritima* has no VICs, thereby reducing the depolarising effect of Na^+, and a very high activity of the plasma membrane proton pump, which counteracts Na^+-influx through the IRC. An obvious benefit of restricting Na^+-uptake through IRCs is that it will always be accompanied by considerable K^+ uptake (see IRC-mediated K^+ and Na^+ current in Figs. 3 and 4), which ensures a high K^+/Na^+ accumulation rate in the plant. Seen in this light, *S. maritima* and *T. halophila* follow the same general strategy which consists in the restriction of Na^+-uptake through VICs and the maintenance of a negative membrane potential for K^+-uptake (Fig. 6). One could even argue that the elimination of Na^+ influx through VICs is necessary for substantial Na^+ uptake through IRCs, and hence a pre-requisite for 'Na^+-inclusion' in halophytes. The fact that experimental inhibition of VICs increases salt-tolerance in a glycophyte (Maathuis and Sanders 2001; see Sect. 3.2) further advocates VICs as a primary target for improving salt tolerance in crops. Figure 6 summarises the proposed involvement of different ion channels in Na^+ uptake in different plant species.

3.4 Ion Channels and Salt Tolerance in Charophytes

A four-gene phylogenetic analysis locates embryophyte land plants phylogenetically within the Charophyta, and identifies the Charales as the closest living aquatic relatives of land plants (Karol et al. 2001; McCourt et al. 2004). Fossil evidence tells us that the Devonian charophytes (living 416 – 359 million year ago) could survive in marine or brackish environments (Kelman et al. 2004; Edgell 2003). Modern charophytes contain both salt-sensitive (*Chara australis*) and salt-tolerant genera (*Lamprothamnium succinctum*). The salt-tolerance of *Lamprothamnium* is impressive: every single cell in the plant can survive at salinities greater than seawater and can tolerate large shifts in salinity (Beilby and Shepherd 2006). Cells of *C. australis*, on the other hand, die within days of exposure to 50 mM

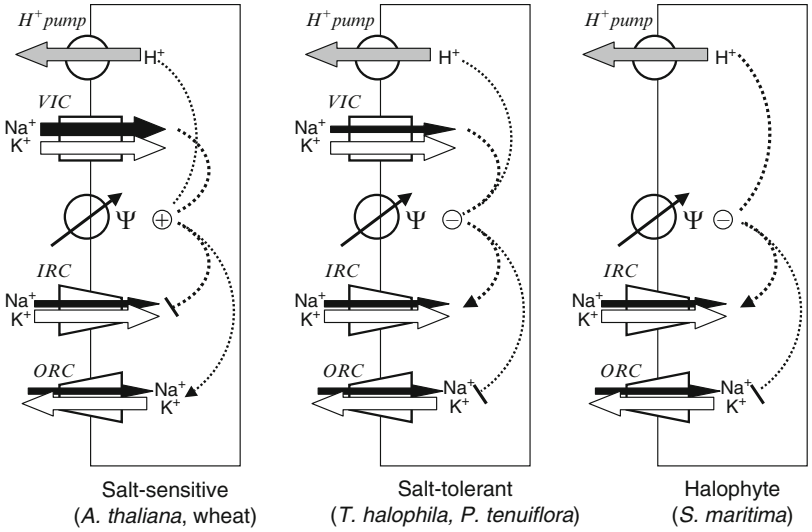

Fig. 6 Proposed scenarios of Na^+ and K^+ flux across plasma membrane ion channels in roots of salt-sensitive and salt-tolerant plant species. *Left panel*: In salt-sensitive plants a large Na^+ influx through the voltage-independent channel (VIC) leads to depolarisation of the membrane potential, which in turn inactivates the K^+-inward rectifying channel (IRC) and activates the K^+-outward rectifying channel (ORC). Proton pump activity is not large enough to fully compensate for the depolarising effect of the VIC. *Centre panel*: In some salt-tolerant plants Na^+ influx through the VIC is reduced (e.g. through higher K^+/Na^+ selectivity in *T. halophil*a), and the membrane potential remains negative enough to activate the IRC. *Right panel*: In the halophyte *S. maritima*, the VIC appears to be absent and Na^+ influx occurs only through the IRC. Large pump activity hyperpolarises the membrane

NaCl if the medium is low in calcium. Thus, comparing the electrophysiology of these closely related plants of ancient lineage is likely to identify a minimal ensemble of factors that influence salt tolerance.

The electrophysiology of charophytes is well explored using the voltage clamp technique (Beilby 1989, 1990). The *I*/*V* characteristics are very similar to those of *A.thaliana* and crop plants (Chen et al. 2007; Munns and Tester 2008) and contain the outward current from the proton pump, inward and outward rectifying currents through IRCs and ORCs and a background (leak) current thought to flow through non-selective VICs.

The two components of salt stress, reduction in turgor and sodium toxicity, were distinguished experimentally by exposing the cells to sorbitol medium and saline medium of equivalent osmolarity. In both *Chara* and *Lamprothamnium*, the background conductance does not change upon mild (non plasmolysing) turgor decrease but it increases in a Ca^{2+} dependent manner in saline medium. The proton pump in salt tolerant charophyte cells is activated by a decrease in turgor (Al Khazaaly and Beilby 2007) and must therefore sense the pressure change or receive information from a pressure sensor. This activation is transient, as *Lamprothamnium* cells regulate their turgor (Bisson and Kirst 1980). The proton pump is also activated

by an increase in Na^+ concentration (Beilby and Shepherd 2001), so cells must be able to monitor Na^+ concentration. This activation persists as long as the cells stay in the high salt medium. The smaller *Lamprothamnium* plants in more saline environments are presumed to have less energy for growth (Shepherd et al. 1999). The proton pump in salt-sensitive charophyte cells does not respond to decrease in turgor (Beilby and Shepherd 2006). It is transiently activated by an increase in Na^+ concentration if Ca^{2+} concentration in the medium is sufficiently high. It is rapidly inactivated when Na^+ concentration is high and Ca^{2+} concentration is low (Beilby and Shepherd 2006; Shepherd et al. 2008). Thus, in charophytes higher Ca^{2+} content of saline media exerts its protective influence not only by blocking VICs, but also by keeping the pump running.

In salt-sensitive *Chara,* the inactivation of the pump brings the membrane potential to the E_{rev} of the background current, which is near −100 mV and rather insensitive to changes in ionic composition or pH of the medium. (This is puzzling from thermodynamic considerations and needs more research.) Spontaneous repetitive transient depolarisations (action potentials, APs) are often observed with long duration in low calcium saline media, further depleting the cell of K^+ and Cl^- (Shepherd et al. 2008). The involvement of APs in signalling saline stress from root to shoot may also be important in land plants (Felle and Zimmermann 2007).

Recently, another parameter of salt stress was found that distinguishes *Chara* from *Lamprothamnium*: *Chara* exhibits salinity-induced noise in the membrane potential upon exposure to saline medium (Al Khazaaly et al. 2009). At frequencies between 1 and 500 mHz classical noise analysis shows ($1/f^2$) rise of noise power as frequency falls, and a marked increase in noise power when the cell is exposed to high salinity (but not to equivalent osmotic stress). Inspection of the time domain shows that as well as initiating depolarisation, exposure to high Na^+ concentrations usually initiates a continuous but random series of small rapid depolarisations with a slower recovery. It is postulated that high Na^+ concentration activates proton (or hydroxyl) channels. After longer exposure to high salinity, the membrane potential of *C. australis* cells continues to depolarize toward zero, while the noise diminishes (suggesting that progressively larger numbers of proton/hydroxyl channels are activated). The *I*/*V* data after several hours of saline stress can be simulated with the action of proton/hydroxyl channels (Beilby and Al Khazaaly 2009). The activation of these channels at the time of exposure to salt would be disastrous for plant cells, as both the negative membrane potential and the pH gradients between the cytoplasm, vacuole and the medium are necessary for the cell to survive in high salt. Interestingly, proton/hydroxyl channels are also present in roots of wheat where they mediate circulating currents similar to those observed in charophyte cells (Raven 1991; Tyerman et al. 2001).

In summary, the salt-tolerant *Lamprothamnium* senses a decrease of turgor and an increase of Na^+ in the medium, and responds by pumping protons faster to maintain a negative membrane potential while keeping proton/hydroxyl channels closed. The turgor is thus regulated. Salt-sensitive *Chara* does not respond to turgor decrease, does not regulate turgor, loses the pump function and negative membrane potential and undergoes spontaneous repetitive APs. The opening of proton/

hydroxyl channels speeds up the irreversible decline by further decreasing the membrane potential and promoting K^+ loss through ORCs.

4 Summary and Conclusions

Salt sensitivity in plants is often accompanied by high Na^+ accumulation and a low Na^+/K^+ ratio in the shoot. These parameters are ultimately determined by the rate of Na^+ uptake into root cells. Theoretical current–voltage relationships based on existing knowledge on selectivity and gating properties of plant ion channels point to an important role of voltage-independent channels (VICs) in root Na^+ uptake. In salt-sensitive plant species, including *A. thaliana* and important crops like wheat, maize and barley, experimental evidence supports the notion that non-selective VICs are the main pathway for Na^+ influx into roots. Inhibition of VIC-mediated Na^+ influx with cyclic nucleotides increases salt tolerance in *A. thaliana*. Rice seems to differ from the above mentioned plant species insofar as apoplastic Na^+ influx makes an important contribution to Na^+ accumulation. In many salt-tolerant plant species unidirectional Na^+ influx into roots is smaller than in related salt-sensitive species. In *T. halophila*, a close relative of *A. thaliana*, this is achieved by increased K^+/Na^+ selectivity of VICs. Restriction of Na^+ influx through VICs has the additional benefit of maintaining a negative membrane potential enabling selective K^+-uptake through inward rectifying K^+ channels (IRCs), which supports K^+/Na^+ homeostasis in the plant. In the halophyte *S. maritima* Na^+ influx into roots occurs through IRCs. IRC activity depends on a hyperpolarised membrane potential, which is probably achieved through elimination of VIC-mediated Na^+ influx and strong activity of the plasma membrane proton pump. The importance of the proton pump for salt tolerance is also evident in ancestral aquatic plants of the genus Charales. Recent evidence suggests that salt-sensitivity in *Chara* is due to uncoupling of the proton pump by proton (or hydroxyl) channels under salt stress. This channel type has also been described for wheat, and its role for salt-sensitivity in land plants requires further attention in the future. We conclude that inhibition/modification of VICs and activation of the proton pump should be at the centre of biotechnological efforts to improve salt tolerance in crops.

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Cation Channels and the Uptake of Radiocaesium by Plants

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Abstract Caesium (Cs) is not required by plants and rarely reaches toxic concentrations in the natural environment. However, two harmful, long-lived, radioisotopes of Cs (^{134}Cs and ^{137}Cs) are produced anthropogenically. These radioisotopes enter the terrestrial food chain through plants and thereby impact on human health and commerce. Since radiocaesium enters plants across the plasma membranes of root cells, reducing radiocaesium influx to root cells is expected to reduce its concentration in edible tissues. Theoretical models indicate that, in K-replete plants, most Cs (30–90%) enters root cells through voltage-independent cation channels (VICCs), with K^+/H^+ symporters (KUPs) contributing the remainder. This conclusion is consistent with the pharmacology of Cs^+ influx to K-replete plants, which is identical to that of VICCs, and the phenotypes of *Arabidopsis* mutants lacking particular VICCs, such as cyclic nucleotide gated channels (CNGCs), which have lower shoot Cs concentrations than wild-type plants. During K-starvation, the expression of genes encoding KUPs, such as *AtHAK5*, increases, resulting in increased Cs^+ uptake and an increased contribution of KUPs to total Cs^+ uptake, as witnessed by changes in the pharmacology of Cs^+ influx to roots and the phenotype of *Arabidopsis* mutants lacking AtHAK5, which accumulate less Cs than wild-type plants. Unfortunately, the absence of CNGCs and KUPs has pleiotropic effects on plant growth, and manipulation of their cationic selectivity may be required to develop crop genotypes with reduced radiocaesium accumulation.

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Abbreviations

CNGC	Cyclic nucleotide gated channel
CPA	Cation/proton-antiporter family
DACC	Depolarisation-activated Ca^{2+} channel
GLR	Glutamate receptor
HACC	Hyperpolarisation-activated Ca^{2+} channel
KIRC	Inward-rectifying K^+ channel
KORC	Outward-rectifying K^+ channel
KUP	'High-affinity' K^+/H^+ symporter
NORC	Non-specific outward-rectifying cation channel
NSCC	Non-specific cation channel
QTL	Quantitative trait locus
TPK	Tandem pore K^+ channel
VICC	Voltage-insensitive cation channel

1 Background

Caesium (Cs) is an alkali metal element with chemical properties similar to rubidium (Rb) and potassium (K). It is found naturally as the stable isotope ^{133}Cs, which may reach concentrations of 25 mg g^{-1} dry soil and low micromolar concentrations in the soil solution (White and Broadley 2000). Caesium is not required by plants and, although Cs^+ can perturb cellular biochemistry by competing with K^+ (Cline and Hungate 1960; Hampton et al. 2004; Le Lay et al. 2006; Qi et al. 2008), it is rarely present at toxic concentrations in the natural environment (White and Broadley 2000). However, two anthropogenic radioisotopes of Cs (^{134}Cs and ^{137}Cs) produced in nuclear reactors and thermonuclear explosions are of environmental concern (White and Broadley 2000). These radioisotopes migrate rapidly in an aqueous environment, emit harmful β and γ radiation during their decay, have relatively long half-lives (2.06 and 30.17 years, respectively) and are rapidly incorporated into biological systems (White and Broadley 2000). They enter the terrestrial food chain through plants, and their presence in foodstuffs impacts upon both health and commerce.

Agricultural land in Belarus, Russia and Ukraine is still contaminated by ^{137}Cs originating from the Chernobyl accident in 1986 (Smith et al. 2000; Beresford et al. 2001). Two strategies are available to return this land to safe agricultural production. The first is to cleanse the soil of radiocaesium. The second is to grow crops that do not accumulate radiocaesium in their edible portions. Since Cs accumulation by plants is a heritable trait (Payne et al. 2004), plants with extreme phenotypes could be developed in breeding programs. Growing plants with an increased ability to accumulate ^{137}Cs accelerates the cleansing of contaminated soils (Entry et al. 1996;

Dushenkov 2003; White et al. 2003), whilst plants accumulating less ^{137}Cs in their edible tissues can be used to develop "safer" crops (White and Broadley 2000; White et al. 2003, 2004). Cultivation of safer crops complements other agricultural countermeasures to reduce the radiation dose to populations inhabiting areas contaminated by ^{137}Cs (Alexakhin 1993; Beresford et al. 2001). A recent survey of over 130 potential countermeasures for managing land contaminated with radiocaesium suggested that selective crop breeding was one of only six strategies worthy of further exploration (http://www.strategy-ec.org.uk). This chapter will focus on strategies to reduce Cs influx to plant roots and, thereby, radiocaesium accumulation by crops.

Plants acquire Cs from the soil solution. It is taken up by epidermal and cortical cells of the root as the monovalent cation, Cs^+, which is transported symplastically, through the interconnected cytoplasms of root cells, across the root to the stele, where it is loaded into the xylem (White and Broadley 2000; White et al. 2004). Only about 20% of the Cs delivered to the shoot via the xylem is retained by the shoot, and most is returned to the root via the phloem for recirculation within the plant (Buysse et al. 1995; Hampton 2005). Thus, it is argued that the physiological process impacting most on Cs accumulation by plants are the uptake of Cs from the rhizosphere and the delivery of Cs to the xylem (White and Broadley 2000; Hampton et al. 2005). These processes are catalysed by transport proteins in the plasma membrane of root cells, and control of their activities are, therefore, fundamental to the development of safer crops for soils contaminated by radiocaesium.

2 Historical Studies

It has long been known that the fluxes of monovalent cations across lipid membranes must be catalysed by transport proteins. Based upon the chemical similarity of Cs^+, Rb^+ and K^+, the concentration-dependencies for their uptake, and competition between these cations for uptake by plant roots, researchers proposed that they shared the same uptake mechanisms: high affinity mechanisms at micromolar rhizosphere concentrations and low affinity mechanisms at millimolar rhizosphere concentrations (Bange and Overstreet 1960; Handley and Overstreet 1961; Shaw and Bell 1989; Zhu and Smolders 2000). In addition, it was proposed that the high-affinity mechanisms catalyzing Cs^+ uptake were unconditionally energy-dependent, whereas the low affinity mechanisms catalyzing Cs^+ uptake could occur through nonspecific cation channels utilizing the Cs^+ electrochemical gradient alone (Bange and Overstreet 1960; Shaw and Bell 1989). However, molecular mechanisms of cation transport cannot be inferred solely from kinetic parameters: it is well known that inward-rectifying K^+ channels catalyse K^+ influx to plant cells from solutions with extremely low K^+ concentrations, provided there is a supporting electrochemical gradient, and that H^+/K^+-cotransporters contribute to K^+ influx to plant cells across a wide range of extracellular K^+ concentrations (White and Broadley 2000; Gierth and Mäser 2007; Britto and Kronzucker 2008; Karley and White 2009).

The uptake of Cs^+ by plant roots is not only reduced by the presence of monovalent cations in the rhizosphere, with an apparent effectiveness of $K^+ \geq Rb^+ > NH_4^+ > Na^+ \geq Li^+$ (Bange and Overstreet 1960; Handley and Overstreet 1961; Shaw and Bell 1989; Hampton et al. 2004), but is also partially inhibited by millimolar concentrations of divalent cations, with an apparent effectiveness of $Ba^{2+} > Mg^{2+} > Ca^{2+}$ (Bange and Overstreet 1960; Handley and Overstreet 1961; Sze and Hodges 1977; Broadley et al. 2001; Hampton et al. 2004) and trivalent cations, such as La^{3+} and Gd^{3+} (Broadley et al. 2001; Hampton et al. 2004).

3 Caesium Transport Proteins in Root Cells

Several types of transport protein are able to catalyse Cs^+ transport across the plasma membrane of root cells. Inward-rectifying K^+ channels (KIRCs), voltage-insensitive cation channels (VICCs), voltage-dependent Ca^{2+} channels (HACCs and DACCs) and "high-affinity" K^+/H^+ symporters (KUPs) can catalyse Cs^+ influx to root cells, whilst outward-rectifying cation channels (KORCs and NORCs) can catalyse Cs^+ efflux from root cells (White and Broadley 2000; White et al. 2004; Hampton et al. 2005; Qi et al. 2008). These transport proteins have contrasting abilities to discriminate between Cs^+ and K^+ and their relative abundance and activities vary with cell type, plant species and environmental conditions. It has, therefore, been postulated that differences in the complement of these transport proteins can account for the observations that both Cs^+ uptake and shoot Cs/K quotients vary (a) with plant species and (b) with plant K status (White and Broadley 2000; White et al. 2003, 2004; Hampton et al. 2005; Qi et al. 2008; Wiesel et al. 2008). Since Cs is not an essential element, nor is toxic to plants at the concentrations found in the natural environment, it is unlikely that there has been any evolutionary pressure to select for protein structures that permit or exclude Cs^+ transport. Thus, differences in the Cs/K selectivity of transport proteins are likely to have arisen serendipitously, as a consequence of the requirements for the transport of other, physiologically important, cations.

Several K^+-selective channels belonging to the "Shaker" superfamily are present in the plasma membrane of *Arabidopsis* root cells (Table 1). These include the KIRCs AtAKT1, which appears to be the dominant K^+ channel involved in K^+ nutrition (Hirsch et al. 1998; Spalding et al. 1999; Broadley et al. 2001; Gierth et al. 2005; Xu et al. 2006), AtKC1/AtKAT3/AtAKT4, which appears to be a regulatory subunit for AtAKT1 (Reintanz et al. 2002; Pilot et al. 2003; Fizames et al. 2004), and (possibly) AtKAT1, and the KORCs AtSKOR, which is implicated in loading K^+ into the xylem for transport to the shoot (Gaymard et al. 1998), and AtGORK, which is present in cells throughout the root, where it is thought to be involved in osmotic regulation and the maintenance of a negative cell membrane potential (Ivashikina et al. 2001; Reintanz et al. 2002; Fizames et al. 2004). Orthologues of genes encoding these channels have been found in roots of all plant species studied to date (Zimmermann and Chérel 2005; Ashley et al. 2006; Gambale and Uozumi

Table 1 Cation channels able to catalyse Cs^+ fluxes across the plasma membranes of root cells. The putative *Arabidopsis* genes encoding these transporters, and evidence for their expression in root cells and regulation by K starvation (in parentheses), are indicated

Transporter	Selectivity	Gene family	Expression pattern in root	Key references
KIRC	KIRC: $P_{Cs}/P_K = 0.07–0.43$	Shaker (7 members)	AtAKT1: Epidermis, cortex, endodermis (Unaffected) AtKAT1: Vasculature. AtKC1: Root hairs, epidermis, cortex, endodermis (Unaffected)	Wegner and Raschke (1994), Maathuis and Sanders (1995), White and Broadley (2000), Reintanz et al. (2002), Véry and Sentenac (2003), Maathuis et al. (2003), Pilot et al. (2003), White et al. (2004); Zimmermann and Chérel (2005)
KORC	KORC: $P_{Cs}/P_K = 0.12–0.31$ AtSKOR: $P_{Cs}/P_K = 0.15$	Shaker (2 members)	AtSKOR: Pericycle, xylem parenchyma (Transient decrease) AtGORK: Root hairs (Unaffected)	Maathuis and Sanders (1995), Roberts and Tester (1997a), Gaymard et al. (1998), White and Broadley (2000), Ivashikina et al. (2001), Reintanz et al. (2002), Véry and Sentenac (2003), Pilot et al. (2003), Maathuis et al. (2003), White et al. (2004); Zimmermann and Chérel (2005), Volkov and Amtmann (2006)
NORC	$P_{Cs} = P_K$	Unknown	–	Wegner and Raschke (1994)
VICC	ScVICC: $P_{Cs}/P_K = 0.85$ AtVICC: $P_{Cs}/P_K \approx 1.00$ ThVICC: $P_{Cs}/P_K = 0.43$ AtCNGC2: $G_{Cs}/G_K = 0.64$	AtCNGC (20 members)	AtCNGC1, AtCNGC2, AtCNGC3, AtCNGC5, AtCNGC6, AtCNGC8, AtCNGC9, AtCNGC10, AtCNGC12, AtCNGC13, AtCNGC14, AtCNGC15, AtCNGC17, AtCNGC18, AtCNGC19: Root (Unaffected)	White and Tester (1992), White and Lemtiri-Chlieh (1995), White and Broadley (2000), Maathuis and Sanders (2001), Demidchik and Tester (2002), Demidchik et al. (2002b), Leng et al. (2002), White et al. (2002, 2004), Balagué et al. (2003), Talke et al. (2003), Hampton et al. (2005), White (2005), Zimmermann and Chérel (2005), Volkov and Amtmann (2006), Gobert et al. (2006), Borsics et al. (2007), Christopher et al. (2007), Frietsch et al. (2007), Urquhart et al. (2007)

(*continued*)

Table 1 (continued)

Transporter	Selectivity	Gene family	Expression pattern in root	Key references
		AtGLR (20 members)	All expressed in roots. AtGLR1.1: Collet, lateral roots (Unaffected) AtGLR1.2, AtGLR1.3: Root (Upregulated) AtGLR2.1: Throughout root, except tip (unaffected) AtGLR2.3, AtGLR2.4, AtGLR2.8: Root (Unaffected) AtGLR3.1: Vasculature. AtGLR3.2: Stele. AtGLR3.3, AtGLR3.5, AtGLR3.6: Root (Unaffected)	Zhu et al. (2001), Chiu et al. (2002), Davenport (2002), Demidchik et al. (2002b, 2004), White et al. (2002, 2004), Hampton et al. (2004, 2005), Meyerhoff et al. (2005), Roy et al. (2008)
DACC	ScDACC: $P_{Cs}/P_K = 0.85$	Unknown	–	White (2000, 2005), White and Broadley (2000), White et al. (2002)
HACC	Unknown	Annexins (7 members)	All expressed in roots. AnnAt1: Throughout root. AnnAt2: Collet endodermis, initiating laterals, tip epidermis	Clark et al. (2001) White et al. (2002) Mortimer et al. (2008)

2006; Lebaudy et al. 2007). Although KIRCs are permeable to Cs^+, they transport little Cs^+ into root cells because increasing extracellular Cs^+ reduces cation permeation through them (Wegner and Raschke 1994; Maathuis and Sanders 1995; White and Lemtiri-Chlieh 1995; Bregante et al. 1997; White 1997; White and Broadley 2000). The KORCs are also permeable to Cs^+ and are relatively insensitive to inhibition by extracellular Cs^+ (Maathuis and Sanders 1995; Roberts and Tester 1995, 1997b; White and Lemtiri-Chlieh 1995; Vogelzang and Prins 1995; White 1997; Gaymard et al. 1998), although there is evidence that KORCs are inhibited by cytoplasmic Cs^+ in a voltage-dependent manner (Maathuis and Sanders 1995).

Caesium-permeable VICCs in the plasma membrane of root cells have been characterized using a variety of electrophysiological techniques. These channels are a subset of the non-specific cation channels (NSCCs; Demidchik et al. 2002b; Demidchik and Maathuis 2007). They were first observed as a "leak conductance" in the plasma membrane of green algae (Yurin et al. 1991; Demidchik et al. 1997). Their counterparts in higher plants were initially characterized following incorporation of plasma membrane vesicles from rye roots into artificial planar lipid bilayers (White and Tester 1992) and their presence was subsequently confirmed in protoplasts from rye roots (White and Lemtiri-Chlieh 1995). Since then they have been observed in plasma membrane fractions from wheat roots (Davenport and Tester 2000; White 2005) and appear to be ubiquitous in protoplasts from plant roots (White 1997, 1999; Roberts and Tester 1997b; Buschmann et al. 2000; Maathuis and Sanders 2001; Demidchik and Tester 2002; Demidchik et al. 2002a, b; Volkov and Amtmann 2006; Demidchik and Maathuis 2007). It is thought that VICCs are encoded by members of the cyclic-nucleotide gated channel (CNGC) and glutamate receptor (GLR) gene families (White and Broadley 2000; Davenport 2002; Demidchik et al. 2002b; White et al. 2002, 2004; Talke et al. 2003; Hampton et al. 2005; Demidchik and Maathuis 2007; Kaplan et al. 2007; Roy et al. 2008), most of which are expressed in roots (Table 1). Direct evidence that AtCNGCs and AtGLRs transport Cs^+ is scarce. However, both AtCNGC2 and AtCNGC4 mediated cyclic-nucleotide-dependent Cs^+ influx when expressed in oocytes (Leng et al. 2002; Balagué et al. 2003), the expression of *AtCNGC10* in *Escherichia coli* LB650 (*ΔtrkH*, *ΔtrkG*) resulted in Cs toxicity (Li et al. 2005), and the addition of cAMP to inside-out membrane patches from protoplasts of *Arabidopsis* root cells reduced the activity of VICCs permeable to Cs^+ (Maathuis and Sanders 2001). Similarly, AtGLR3.4 mediated Cs^+ influx when expressed in oocytes (Meyerhoff et al. 2005) and glutamate-activated, voltage-independent Cs^+ currents sensitive to quinine, La^{3+} and Gd^{3+} have been recorded in protoplasts from *Arabidopsis* root cells (Demidchik et al. 2002b, 2004). This pharmacological profile is consistent with that of GLR-mediated phenomena in plants (White et al. 2002).

Although NORCs transport Cs^+ (Wegner and Raschke 1994) they are unlikely to contribute significantly to Cs^+ efflux across the plasma membrane, since they open only at extremely positive membrane potentials and unphysiologically high cytosolic Ca^{2+} concentrations (Wegner and Raschke 1994; White 1997; Wegner and De Boer 1997). Similarly, although DACCs are permeable to Cs^+ (White 2000, 2005; White et al. 2002), it is thought that the Ca^{2+} concentrations found in the rhizosphere will

prevent Cs^+ permeating these channels (White and Broadley 2000). No genes encoding DACCs are known for certain. One candidate in some plant species appears to be *TPC1* (Hashimoto et al. 2005), although AtTPC1 is present in the tonoplast of *Arabidopsis* (Peiter et al. 2005; Ranf et al. 2008; Gradogna et al. 2009). Several HACCs have been recorded in the plasma membrane of root cells (White 2000; Demidchik et al. 2002a, 2007; White et al. 2002; Foreman et al. 2003; Miedema et al. 2008). The HACCs are thought to be encoded by members of the annexin gene family, all of which are expressed in roots (Clark et al. 2001; White et al. 2002; Mortimer et al. 2008). The permeability to Cs^+ of neither HACCs nor annexins appears to have been confirmed. However, a Cs^+-permeable, hyperpolarisation-activated NSCC activated by reactive oxygen species has been observed in the plasma membrane of protoplasts of *Arabidopsis* root cells (Demidchik et al. 2003).

The "high-affinity" K^+/H^+ symporters present in the plasma membrane of root cells are thought to be encoded by members of the KUP gene family (Rodríguez-Navarro and Rubio 2006; Gierth and Mäser 2007; Grabov 2007; Karley and White 2009) and it has been shown that AtHAK5 is present in the plasma membrane of *Arabidopsis* root cells (Qi et al. 2008). Plant KUPs are expected to transport Cs^+, as do their homologues from fungi and bacteria (White and Broadley 2000), but this has rarely been tested. However, the expression of a modified *AtHAK5*, with a leucine changed to a histidine at position 776, in a mutant yeast strain (CY162: Δ*trk1*, Δ*trk2*) with reduced K^+ uptake allows it to accumulate both K^+ and Cs^+ (Rubio et al. 2000; Qi et al. 2008), and Cs uptake and accumulation by *Arabidopsis* parallels the expression of *AtHAK5* (Hampton et al. 2004; Qi et al. 2008). Similarly, heterologous expression of barley, rice or pepper orthologs of *AtHAK5* (*HvHAK1*, *OsHAK1*, *CaHAK1*) promotes Cs^+ uptake in yeast, and their expression in roots of K-starved plants is correlated with increased high-affinity Cs^+ uptake (Santa-María et al. 1997; Rubio et al. 2000; Bañuelos et al. 2002; Martínez-Cordero et al. 2005).

Caesium influx to the vacuole is likely to be catalysed by cation/H^+-antiporters, whereas Cs^+ release from vacuoles probably occurs through Cs^+-permeable cation channels. Members of the CPA cation/H^+-antiporter family, which in *Arabidopsis* comprises eight *AtNHX* genes, 28 *AtCHX* genes, six *AtKEA* genes and two *AtNHD* genes resembling *NhaD*, are likely to catalyse Cs^+ transport into vacuoles of root cells, although this has not been proven (Sze et al. 2004; Zimmermann and Chérel 2005; Ashley et al. 2006; Pardo et al. 2006; Gierth and Mäser 2007). Indirect assays based on the ability of cations to dissipate a pH gradient held in liposomes containing AtNHX1, which is found in the tonoplast of root cells, or LeNHX2, an ortholog of AtNHX5 that is present in Golgi and pre-vacuolar compartments of tomato roots, suggest that these proteins transport Cs^+ but at lower rates than K^+ (Venema et al. 2002, 2003).

The electrical activities of several distinct Cs^+-permeable cation channels have been recorded in vacuoles from root cells, of which the two most frequently observed are the tonoplast SV and FV channels (Lebaudy et al. 2007; Pottosin and Schönknecht 2007). The gene encoding the *Arabidopsis* SV channel appears to be *AtTPC1* (Peiter et al. 2005; Ranf et al. 2008; Gradogna et al. 2009), but an

intracellular location of TPC1 orthologues is not observed in all plant species (Hashimoto et al. 2005). The SV channel has a significant permeability to Cs^+ (White 2000). The genetic identity of the FV channel is not yet known (Demidchik and Maathuis 2007). It is possible that Cs^+ fluxes across the tonoplast might be mediated by cation channels encoded by members of the tandem pore K^+ channel (*TPK*/*KCO*) and Kir-like channel (*KCO3*) gene families (Véry and Sentenac 2003; Zimmermann and Chérel 2005; Lebaudy et al. 2007). In *Arabidopsis*, *AtTPK1*, *AtTPK2*, *AtTPK3* (=*AtKCO6*), *AtTPK5* and *AtKCO3* are all expressed in roots and located at the tonoplast (Schönknecht et al. 2002; Zimmermann and Chérel 2005; Voelker et al. 2006; Latz et al. 2007). However, *AtTPK1* appears to encode a channel that has little permeability to Cs^+ and resembles the K^+-selective, VK channel (Bihler et al. 2005; Gobert et al. 2007; Latz et al. 2007; Lebaudy et al. 2007). Intriguingly, some KUPs, such as OsHAK5, are also found in the tonoplast (Bañuelos et al. 2002), and it has been suggested that these cation/H^+-symporters might catalyse the efflux of monovalent cations from the vacuole (Rodríguez-Navarro and Rubio 2006).

4 Molecular Mechanisms for Cs Uptake by Roots of Non-Mycorrhizal Plants

4.1 K-Replete Plants

The kinetic parameters of proteins able to transport Cs^+ across the plasma membrane have been incorporated into a theoretical model to predict their contributions to Cs^+ influx to a stereotypical root cell (White and Broadley 2000; Hampton et al. 2005). This model suggests that, under K-replete conditions, (a) Cs^+ influx through KIRCs is negligible, (b) VICCs mediate most (30–90%) Cs^+ influx, with KUPs mediating the remainder, and (c) KORCs load Cs^+ into the xylem. These predictions have been tested using *Arabidopsis*. First, the pharmacology of Cs^+ influx to roots of intact *Arabidopsis* was compared with that of transport proteins that could mediate Cs^+ influx (White and Broadley 2000; Broadley et al. 2001; Hampton et al. 2004; Qi et al. 2008). Second, Cs accumulation by mutants lacking specific transport proteins was assayed, with the expectation that mutants lacking transport proteins mediating Cs^+ influx to roots would have reduced Cs^+ uptake and shoot Cs concentrations (Broadley et al. 2001; White et al. 2004; Hampton et al. 2005; Qi et al. 2008). Third, genetic loci impacting on Cs^+ accumulation in K-replete plants were identified (Payne et al. 2004).

The prediction that VICCs catalyse significant Cs^+ influx to root cells is supported by the observation that both VICCs and Cs^+ uptake by roots of K-replete plants, are partially inhibited by submillimolar concentrations of Gd^{3+}, La^{3+}, Ba^{2+}, Mg^{2+} and Ca^{2+}, but not by TEA^+ or 10 μM Br-cAMP (White and Lemtiri-Chlieh 1995; White 1997, 1999; White and Broadley 2000; Broadley et al. 2001;

Demidchik et al. 2002a, b; Hampton et al. 2004, 2005; Volkov and Amtmann 2006). The prediction, that Cs^+ influx to root cells through KIRCs is negligible, is supported by the observations that both Cs^+ influx to roots and shoot Cs concentrations of *Arabidopsis* lacking AtAKT1 are often greater than those of wild-type plants (Broadley et al. 2001; White et al. 2004; Qi et al. 2008). Two explanations for the increased Cs uptake in *akt1* mutants have been suggested: (a) that the expression of genes encoding Cs^+-permeable transporters contributing to cellular K-homeostasis, such as AtHAK5, are upregulated in plants lacking AtAKT1, which is consistent with transcriptional analyses of *akt1* mutants (Zimmermann and Chérel 2005; Qi et al. 2008), and (b) that the loss of AtAKT1 results in a more negative cell membrane potential and, thereby, increases the activity of other Cs^+-permeable transporters (White et al. 2004).

Evidence that AtCNGCs underlie the VICC-mediated Cs^+ influx to roots of K-replete plants is based on measurements of Cs accumulation by *Arabidopsis* mutants lacking individual AtCNGCs. However, although some *Arabidopsis* mutants lacking AtCNGCs, such as *cngc2*, *cngc3*, *cngc16*, *cngc19* and *cngc20*, have lower shoot Cs concentrations than wild-type plants, mutants lacking other AtCNGCs, such as *cngc1*, *cngc9*, *cngc10* and *cngc12*, have greater shoot Cs concentrations than wildtype plants (White et al. 2004; Hampton et al. 2005). Again, it has been suggested that increased Cs accumulation in *Arabidopsis* mutants lacking particular AtCNGCs is a consequence of functional compensation in gene expression (White et al. 2004; Hampton et al. 2005). Thus, the expression of genes encoding plasma membrane K^+-transporters might be altered to compensate for the absence of AtCNGCs that contribute significantly to cellular K homeostasis and/or the expression of genes encoding Ca^{2+}-transporters might be altered to compensate for the absence of AtCNGCs that contribute to cytoplasmic Ca^{2+}-homeostasis or intracellular Ca^{2+} signalling (White et al. 2004; Hampton et al. 2005). This hypothesis is consistent with the upregulation of genes encoding AtKUPs in the *cngc4* mutant (Hampton 2005), and the observation that a greater fraction of Cs^+ influx to roots of *cngc1* and *cngc4* mutants is inhibited by extracellular NH_4^+ than in wild-type plants (Hampton et al. 2005). Interestingly, only the lack of AtCNGC1 decreased shoot K concentration significantly, which may attest to functional compensation by other K^+ transport proteins to maintain K^+ homeostasis in mutants lacking other AtCNGCs, and shoot Ca concentration was not affected by the absence of any AtCNGC (Hampton et al. 2005). *Arabidopsis* mutants lacking AtHAK5 (*hak5-1*, *hak5-2*) or AtKUP4 (*trh1*), and *Arabidopsis* mutants with aberrant AtKUP2 activity (*shy3.1*), have lower shoot Cs concentrations than wild-type plants (White et al. 2004; Qi et al. 2008). This is consistent with the prediction that KUPs catalyse Cs^+ influx to root cells. The prediction that Cs^+ is delivered to the xylem by a KORC, AtSKOR, is supported by the observation that shoot Cs concentrations are generally reduced in the *skor* mutant (White et al. 2004).

When the L*er* x Col genetic mapping population of *Arabidopsis* was grown on agar containing subtoxic levels of Cs, four chromosomal loci (QTL) impacting on shoot Cs concentration were identified, accounting for $> 80\%$ of the genetic contribution to the trait variation (Payne et al. 2004). These QTL were located on

Chromosomes I, II, IV and V. Significantly, the QTL on the top of Chromosomes I and V co-localised with QTL impacting shoot Cs concentration in the L*er* x CVI genetic mapping population of *Arabidopsis* (Payne et al. 2004). A cursory glance at these chromosomal regions reveals the presence of genes encoding putative Cs^+ transporters. For example, genes encoding a putative plasma membrane K^+-channel (*AtTPK4*) on Chromosome I and a putative plasma membrane VICC (*AtGLR3.1*) on Chromosome II are found within a genomic region of 100,000 bp (c. 25 genes) on either side of the marker where a significant allelic effect on shoot Cs concentration was observed in the L*er* x Col population (Payne et al. 2004).

4.2 K-Starved Plants

The intrinsic cationic selectivity of KIRCs, KUPs and VICCs differs, and the fluxes of Cs^+ and K^+ that they catalyse are influenced uniquely by both the absolute and relative concentrations of these cations in the rhizosphere (White and Broadley 2000). The expression of genes encoding these transporters is also affected differently by plant K status. White et al. (2004) suggested that these phenomena could account for: (a) the lack of correlation between the shoot Cs:K ratio and the Cs^+:K^+ ratio in the soil solution when plants were grown in media with contrasting K^+ concentrations (Cline and Hungate 1960; Smolders et al. 1996a, b), (b) differences in the relative uptake of Cs^+ and K^+ by plants of different K-status (e.g. Qi et al. 2008) and (c) increased Cs^+ uptake and accumulation by K-starved plants (e.g. Zhu and Smolders 2000; Hampton et al. 2004; Qi et al. 2008).

In *Arabidopsis*, K-starvation, but not Cs-toxicity, increases the expression of *AtHAK5*, occasionally *AtKUP3*, and both *AtGLR1.2* and *AtGLR1.3* in roots (Kim et al. 1998; Maathuis et al. 2003; Ahn et al. 2004; Armengaud et al. 2004; Hampton et al. 2004, 2005; Shin and Schachtman 2004; Gierth et al. 2005; Sahr et al. 2005; Amtmann et al. 2006; Cao et al. 2008; Qi et al. 2008). Potassium starvation also reduces the expression of *AtSKOR* (Maathuis et al. 2003; Pilot et al. 2003), but rarely affects the expression of genes encoding KIRCs, CNGCs or TPK/KCOs in *Arabidopsis* roots (Maathuis et al. 2003; Pilot et al. 2003; Hampton et al. 2004, 2005; Shin and Schachtman 2004; White et al. 2004; Zimmermann and Chérel 2005). The increased expression of *AtKUP*s, and in particular *AtHAK5*, results in an increased capacity for Cs^+ uptake, and changes in the pharmacology of Cs^+ uptake by roots of K-starved plants (Hampton et al. 2004, 2005; Qi et al. 2008). The fraction of Cs^+ uptake inhibited by NH_4^+ is greater in K-starved *Arabidopsis* than in K-replete *Arabidopsis*, which is consistent with the pharmacology of KUPs (Bañuelos et al. 2002; Martínez-Cordero et al. 2005; Nieves-Cordones et al. 2007; Fulgenzi et al. 2008; Qi et al. 2008) and the hypothesis that AtKUPs mediate more Cs^+ influx to roots of K-starved plants (Hampton et al. 2004, 2005; Qi et al. 2008). Thus, during K-starvation, K^+ uptake by *Arabidopsis* roots changes from being dominated by AtAKT1 to being dominated by AtHAK5, whilst Cs^+ uptake

changes from being dominated by VICCs to being dominated by AtHAK5. This results not only in greater Cs accumulation, but also in a greater Cs/K quotient in tissues of K-starved plants.

4.3 *Differences between Plant Species*

There is considerable variation among plant species in their ability to take up Cs and accumulate it in their shoots (e.g. Andersen 1967; Broadley and Willey 1997; Broadley et al. 1999a, b; White et al. 2003, 2004; Willey et al. 2005; Willey and Tang 2006; Watanabe et al. 2007). In general, among the Angiosperms, monocot species (e.g. Lilliales, Poales) have the lowest shoot Cs concentrations and species from the eudicot orders Asterales, Brassicales and Caryophyllales accumulate the highest shoot Cs concentrations (Broadley et al. 1999a; Willey et al. 2005). This suggests that the complement, abundance and/or kinetics of Cs-transport proteins differ between plant species. In addition, the Cs/K quotients in shoot tissues of different plant species grown under identical conditions vary widely (Andersen 1967; White et al. 2004). Since different transport proteins have contrasting abilities to discriminate between Cs^+ and K^+, this varying shoot Cs/K quotient suggests that a different complement of transport proteins is present in different plant species (White et al. 2004; Hampton et al. 2005; Wiesel et al. 2008). Shoot Cs concentrations and shoot Cs/K quotients are positively linearly correlated among plant species, suggesting that shoot Cs and K concentrations vary independently (Fig. 1). A plausible explanation for this observation is that all plants express constitutively an essential, selective K^+ transporter, such as AKT1, but differ in their complement of proteins catalyzing the non-specific uptake (or efflux) of Cs^+ and K^+, such as CNGCs or KUPs. Thus, plants with higher shoot Cs concentrations and shoot Cs/K quotients are likely to have higher *CNGC*: *AKT1* and/or *KUP*:*AKT1* expression ratios than plants with lower shoot Cs concentrations and shoot Cs/K quotients.

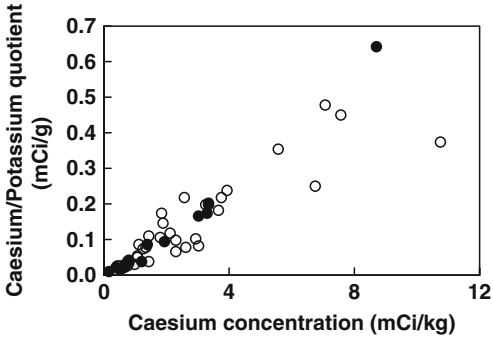

Fig. 1 Relationship between Cs concentrations and Cs/K quotients in shoots of 44 plant species grown on soil contaminated with 10 μCi carrier-free ^{137}Cs (Andersen 1967). Data for monocot (*filled circles*) and eudicot (*open circles*) species are shown

5 Molecular Mechanisms for Cs Uptake by Roots of Mycorrhizal Plants

Most plants live in symbiosis with mycorrhizal fungi. In this symbiosis, the fungi gain carbohydrates from plants and, in return, they help supply plants with water and essential mineral nutrients. There are several types of mycorrhizal symbioses, the two most common being arbuscular mycorrhizae and ectomycorrhizae. More than 80% of vascular plants live in symbiosis with arbuscular mycorrhizal (AM) fungi, which belong to the monophyletic phylum of Glomeromycota (Smith and Read 1997; Schüβler et al. 2001; Morgan et al. 2005). Because of the role of AM fungi in plant nutrition, it has been suggested that they might affect Cs uptake by plants (Entry et al. 1996). Studies in which only the AM fungi had access to radiocaesium have demonstrated that they are able to deliver it to their plant symbionts (Dupré de Boulois et al. 2006) and it has also been shown that AM fungi can also transfer Cs from one plant to another (Meding and Zasoski 2008). However, there is no consistent information about the influence of AM fungi on Cs accumulation by plants (Table 2). Similarly, there is no consistent information on the influence of ectomycorrhizal (EM) fungi on Cs accumulation by their tree symbionts. For example, Brunner et al. (1996) and Riesen and Brunner (1996) found that the EM fungus *Hebeloma crustuliniforme* decreased Cs uptake by *Picea abies*, whilst Ladeyn et al. (2008) found that symbiosis with *Rhizopogon roseolus* increased Cs uptake by *Pinus pinaster*.

The inconsistent effects of mycorrhizal fungi on Cs accumulation by their plant symbionts might be explained by a number of factors. First, the availability of Cs to organisms depends on the physical and chemical properties of the substrate (Entry et al. 1996), and both plant and fungal species differ in their ability to access different soil Cs pools (Berreck and Haselwandter 2001). Second, mycorrhizal associations could influence plant Cs accumulation directly, by altering the expression of genes encoding Cs-transporters, and/or indirectly by improving plant

Table 2 Effects of arbuscular mycorrhizal fungi on Cs accumulation by plants

Reference	Influence	Plant species	Fungal species
Entry et al. (1999)	Increase	*Paspalum notatum*	*Glomus mosseae*/ *Glomus intraradices*
Entry et al. (1999)	Increase	*Sorghum halepense*	*G. mosseae*/ *G. intraradices*
Entry et al. (1999)	Increase	*Panicum virgatum*	*G. mosseae*/ *G. intraradices*
Rogers and Williams (1986)	Increase	*Melilotus officinalis*	*Glomus* sp.
Rosén et al. (2005)	Increase	*Allium porrum*	Soil fungi
Joner et al. (2004)	No effect	*Medicago truncatula*	*G. intraradices*
Rogers and Williams (1986)	No effect	*Sorghum sudanense*	*Glomus* sp.
Rosén et al. (2005)	No effect	*Lolium perenne*	Soil fungi
Berreck and Haselwandter (2001)	Decrease	*Agrostis tenuis*	*G. mosseae*
Dighton and Terry (1996)	Decrease	*Trifolium repens*	Soil fungi

nutritional status, which would increase plant growth rates and root exploration of the substrate (Joner et al. 2004). It is possible for AM fungi to influence Cs uptake, and Cs redistribution within the plant, by altering the expression of genes encoding VICCs, KUPs and KORKs. It has been speculated that if mycorrhizae contribute to improved plant K status, then the complement of K transporters in roots of mycorrhizal plants would reflect that of K-replete plants (Wiesel et al. 2008). This implies that Cs uptake by roots of mycorrhizal plants would occur mainly through VICCs and that associations with AM fungi would reduce the accumulation of Cs by plants in K-limited environments. Interestingly, Liu et al. (2007) showed the upregulation of a gene similar to *AtCNGC1* of *Arabidopsis* in roots of *Medicago truncatula* during symbiosis with *Glomus intraradices*.

6 Prospects for the Generation of Safer Crops

It is impractical to remove large areas of agricultural land from crop production to cleanse them of radiocaesium. To reduce the radiation dose to populations inhabiting contaminated areas, the cultivation of crop genotypes accumulating less radiocaesium in their edible portions will complement other agricultural countermeasures (Alexakhin 1993; Beresford et al. 2001). Such genotypes might be obtained through phenotypic selection, plant breeding or genetic manipulation (White et al. 2003; Payne et al. 2004). Knowledge of the genes that impact on Cs accumulation by plants can inform all three strategies, by providing molecular markers for selection and breeding, and target genes for genetic manipulation. Since Cs^+, like K^+, is transported symplastically to the xylem, restricting Cs^+ uptake by root cells is an attractive option to reduce the entry of radiocaesium to plants and the food chain (White and Broadley 2000; White et al. 2003).

Caesium is a nonessential element and enters plants serendipitously through transporters that are primarily responsible for Ca^{2+} and/or K^+ uptake. Transport proteins that can facilitate Cs^+ influx to root cells have been identified, and their likely contributions to Cs^+ uptake under a variety of environmental conditions have been assessed in cation-flux modeling studies (White and Broadley 2000; Hampton et al. 2005). In K-replete plants VICCs, such as CNGCs, are likely to mediate most Cs influx to root cells, whilst Cs influx to roots of K-deficient plants is likely to be dominated by KUPs and, in particular, orthologs of AtHAK5 (White and Broadley 2000; Hampton et al. 2005). However, phenotypes of plants lacking proteins that contribute significantly to Cs^+ influx to root cells are often agronomically unattractive. For example, although *Arabidopsis* plants lacking certain AtCNGCs have reduced shoot radiocaesium concentrations, these mutants exhibit other detrimental phenotypes, including reduced growth rates and infertility, possibly because these channels are components of cytosolic Ca^{2+} signaling cascades (White 2000; Hampton et al. 2005). Similarly, when grown in media containing low K^+ concentrations, *Arabidopsis* lacking AtHAK5 have reduced rates of Cs^+ influx and

accumulation, but they also grow more slowly than wild-type plants, presumably because they lack sufficient K^+ (Qi et al. 2008). Thus, it appears that more subtle genetic manipulation of transport activities, such as altering the cationic selectivity of transport proteins, is required. A targeted mutagenesis strategy could be followed, similar to ones that produced variants of the KIRC AtKAT1 with reduced Cs^+ sensitivity (Ichida and Schroeder 1996; Ichida et al. 1999), to generate alleles of transport proteins with greater Ca^{2+}:Cs^+ and/or K^+:Cs^+ selectivity.

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Ion Channels in Plant Development

Anuphon Laohavisit and Julia M. Davies

Abstract Ion channels function at several levels in plant development to effect control of membrane voltage, facilitate generation of turgor, and integrate signalling. Despite the experimental problems associated with multi-gene channel families and redundancy, molecular approaches are now revealing the extent of channel function in development. Here, we review the key categories of channels implicated in development with a focus on channel involvement in polar growth and nodulation. Comparison of root hairs with pollen tubes as paradigms of polar growth reveals similar assemblies of channels involved in controlling membrane voltage and cytosolic free calcium.

Abbreviations

CNGC	Cyclic nucleotide gated channel
DACC	Depolarisation-activated Ca^{2+} channel
GORK	Guard cell outwardly rectifying K^+ channel
GLR	Glutamate receptor
HACC	Hyperpolarization-activated Ca^{2+} channel
KIR	Inward-rectifying K^+ channel
KOR	Outward-rectifying K^+ channel
MscS	Small conductance-like proteins
NSCC	Nonselective cation channel
PM	Plasma membrane
ROS	Reactive oxygen species
Rhd2-1 or RHD	Root hair deficient
TPK	Tandem pore K^+ channel

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1 Ion Channels in Plant Development

To increase cell size and develop form, plants require strict regulation and co-ordination of ion channel activities by growth regulators, with effects of biotic and abiotic stress as "higher tiers" of regulation. Despite a wealth of channel studies on native membranes, delineating channel function in developmental programmes has been limited by characterization of channel mutants. In addition to the problem of redundancy, assigning roles in development is complicated by possible nutritional roles for channels and pleiotropic mutational effects. Nevertheless, the first decade of this century has seen seminal contributions by "channel" laboratories to the field of plant development. Here, we review the key categories of channels implicated in development with evidence primarily from molecular studies. Channels are then placed into context for polar growth and nodulation as developmental paradigms.

1.1 Molecular Identification of K^+- and Anion Channels in Plant Development

Potassium (K^+) is a key osmoticum for turgor-driven cellular elongation. Together with anions, it also has an important role in regulating channels and active transporters through the control of membrane potential. Three families of K^+-selective channels are recognized in plants (Kir-like, Shaker and TPK; Tandem Pore K^+) and their functions have been reviewed recently by Lebaudy et al. 2007. Shaker K^+ channels for plasma membrane (PM) K^+ uptake are implicated in *Daucus* embryogenesis and polarity patterning (Formentin et al. 2006). Recently, the role of outward K^+ rectifiers in cell division and expansion has been re-addressed. Activation of a PM outward rectifier (NTORK1) can induce cell division of tobacco BY-2 cells while suppressing its expression promotes elongation (Sano et al. 2009) Vascular K^+ channels are also implicated in cell cycle control (Sano et al. 2009). Proliferative, tumerous growth in *Arabidopsis* involves the PM K^+ influx Shaker channels AKT1 and AKT2/3 (Deeken et al. 2003).

In *Arabidopsis,* the root epidermal PM AKT1 Shaker α-subunit is essential for root development in the presence of NH_4^+ (Hirsch et al. 1998). AKT1 activity is enhanced on phosphorylation by CIPK23, which lies downstream of the calcineurin B-like Ca^{2+} sensors CBL1 and CBL9 (Li et al. 2006a). The latter can be activated by increasing cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) which in turn could be caused by the reactive oxygen species (ROS) that are produced in response to K^+ deficiency (Shin and Schachtman 2004). The overall result of this signaling cascade would be an increased root K^+ uptake. Auxin-regulated expression of the maize Shaker K^+-uptake channel gene *ZMK1* has been linked to coleoptile elongation (Philippar et al. 1999) while K^+ channels also have critical roles to play in polar growth (see Sects. 1 and 2 in Chapter "The Role of Ion Channels in Plant Salt Tolerance").

Plant anion channels are variously regulated by membrane voltage, stretch, and light. They are often associated with nutrient acquisition, membrane stabilization, and initiating membrane depolarisation. PM anion channels in the hypocotyl epidermis are implicated in blue light- and auxin-regulated elongation (Cho and Spalding 1996; Colcombet et al. 2001, 2005). Other hormones such as brassinosteroids can also regulate PM anion channels in *Arabidopsis* suspension cells to promote the membrane hyperpolarisation which is necessary for K^+ uptake and cell expansion (Zhang et al. 2005). At the other end of a cell's life, mitochondrial voltage-regulated anion channels now appear to be involved in elicitor-induced programmed cell death (Tateda et al. 2009).

Genetic identities of the anion channels described electrophysiologically remain largely obscure. *AtSLAC1* (Slow Anion Channel-Associated 1) probably encodes a subunit for anion channel activity or regulation in *Arabididopsis* guard cell PM but no specific developmental phenotype has been described for a loss of function mutant (Vahisalu et al. 2008). Members of the *Ch*l*oride C*hannel (CLC) gene family are present in both *Arabidopsis* and rice, and could play roles in nutrition and development (reviewed by de Angeli et al. 2009; Lv et al. 2009). These transporters are present in endomembranes and may also translocate NO_3^- (de Angeli et al. 2009; Lv et al. 2009). Loss of vacuolar OsClC1 and OsClC2 function resulted in reduced rice growth, but knockouts of their counterparts in *Arabidopsis* did not result in a developmental phenotype (Nakamura et al. 2006; de Angeli et al. 2009). Given the importance of anion transport for membrane voltage control and therefore the command of cation transport in development, further exploration of anion channel families is eagerly awaited.

1.2 Molecular Identification of Mechanosensitive Channels

In *Arabidopsis*, *AtMCA1* encodes a putative mechanosensitive (MS) Ca^{2+} channel (Nakagawa et al. 2007). Its transcript is abundant in root tissue, leaves, and stem. AtMCA1-GFP protein is PM-localised in roots (Nakagawa et al. 2007). When *AtMCA1* is expressed in Chinese hamster ovary cells, stretching induces Ca^{2+} influx (observed using ratiometric Ca^{2+} imaging) suggesting that AtMCA1 forms a Ca^{2+} conductance. AtMCA1 is thought to have a role in root mechanosensing and growth as the loss of function mutant cannot penetrate hard agar (Nakagawa et al. 2007). AtMCA2 is 73 % similar to AtMCA1, suggesting that it too could be an MS channel.

The *AtMSLs* family encodes MscS (MS channel of small conductance)-like proteins. MscS is important in pressure perception and osmotic shock response in *E. coli* (Haswell and Meyerowitz 2006; Haswell et al. 2008). *AtMSL2* and 3 are likely to contribute to plastid development (controlling division, size, and shape) during plant development (Haswell and Meyerowitz 2006). The proteins are localised at the plastid envelope and the *Atmsl2* and *3* insertional mutants exhibit abnormal plastid development. Furthermore, AtMSL3 can complement osmotic-shock

response deficient *E. coli* cells that lack MS ion channel activity, suggesting that AtMSL3 is also an MS channel (Haswell and Meyerowitz 2006). However, there is no direct electrophysiological evidence for this (Haswell and Meyerowitz 2006). In contrast, AtMSL9 and AtMSL10 have been characterised electrophysiologically (Haswell et al. 2008). They are both localised to the PM of root cells, while there is a small fraction which is also localised to endomembranes. The *Atmsl* mutant root protoplasts lack the normal PM MS channel activity (Haswell et al. 2008). These MS channels are weakly permeant to Ca^{2+} and their ability to deliver physiologically significant Ca^{2+} influx remains unknown (Haswell et al. 2008). More work on this channel family is now required.

1.3 Glutamate Receptor-Like Channels and Cyclic-Nucleotide Gated Channels

Ionotropic glutamate receptors (GLR) form Ca^{2+}-permeable cation channels in animals and are essential for central nervous system function. Plant GLR structure has been reviewed by Forde and Lea (2007) and Demidchik and Maathuis (2007). There are 20 genes encoding putative GLR sub-units in *Arabidopsis* that are expressed throughout the plant and all are expressed in roots (Lam et al. 1998; Forde and Lea 2007; Roy et al. 2008). Native membrane electrophysiology and heterologous expression are now revealing the transport capacities of plant GLRs that are generally held to be present at the PM (summarized in Table 1 with references therein). AtGLR1.1, 1.4, 3.4, and 3.7 appear able to translocate Ca^{2+} while analysis of mutant plants implicates AtGLR 3.2 and 3.3 in Ca^{2+} transport and hence stimulation of exocytosis or growth-related signaling (Table 1; Chiu et al. 2002; Demidchik et al. 2002a; Meyerhoff et al. 2005; Stephens et al. 2008; Qi et al. 2006). Developmental phenotypes are also emerging from mutant analyses (Table 1 and references therein) with AtGLR 1.1implicated in control of root growth. The rice OsGLR3.1 also affects root growth (Li et al. 2006b). It is possible that GLRs are involved in co-ordinating root architecture with intracellular and extracellular glutamate/nitrogen status to optimise nutrient uptake and allocation (Forde and Lea 2007).

Cyclic nucleotide-gated channels (CNGCs) in animals form Ca^{2+}-permeable conductances and their homologues in plants have been identified (reviewed by Kaplan et al. 2007; Demidchik and Maathuis 2007). There are 20 genes encoding CNGC sub-units in *Arabidopsis* and their structure is addressed in Chapter "New Approaches to Study the Role of Ion Channels in Stress Induced Signalling; Measuring Calcium Permeation in Plant Cells and Organelles Using Optical and Electrophysiological Techniques". Developmental phenotypes for *Arabidopsis* CNGCs are summarised in Table 2 and addressed in Sect. 1 in Chapter "The Role of Ion Channels in Plant Salt Tolerance". The emerging pattern is of PM-localised channels that upon activation by cyclic nucleotide binding would

Table 1 Expression and properties of AtGLRs implicated in development

Gene name	Developmental phenotype	Ion transport ability	Expression	References
AtGLR1.1	Mutant's germination affected when the C:N altered in medium (1). Produces more ABA, hypersensitive to ABA (2), with shorter root length compared to WT.	Ion pore domain for AtGLR1.1 (and AtGLR1.4) shown to be permeable to Na^+, K^+ and Ca^{2+} ions as demonstrated by ion pore transplantation experiment and patch clamping of *Xenopus* oocytes (3).	RT-PCR revealed that it is highly expressed in root, some expression in leaf but not a lot in silique and flower (4).	Kang and Turano 2003 (1) Kang et al. 2004 (2) Tapken and Hollmann 2008 (3) Chiu et al. 2002 (4)
AtGLR3.1	Overexpressor impaired in long term 'Ca^{2+} programmed' stomatal closure but no clear developmental phenotype (1)	No transport activity of AtGLR3.1 has been demonstrated to date.	Expression abundant in root tissue (2). Also specifically expressed in guard cell (1)	Cho et al. 2009 (1) Chiu et al. 2002 (2)
AtGLR3.2	Overexpressor develops abnormally (deformation of leaf, stunted growth, death of shoot apex), consistent with Ca^{2+} deficiency phenotype (1).	No transport activity of AtGLR3.2 has been demonstrated to date	*AtGLR3.2* present in vasculature (1). Likely to be integral membrane protein (2)	Kim et al. 2001 (1) Turano et al. 2002 (2)

Table 2 Expression and properties of AtCNGCs implicated in development

Gene name	Developmental phenotype	Ion transport ability	Expression	References
AtCNGC1	Gravitropism-induced root curvature decreases in mutant, correlated with lower NO production. Root longer than WT, seedlings contain less Ca^{2+} (1)	Contributed to Ca^{2+} uptake in yeast mutant (1, 3). Expression in *Xenopus* oocytes showed that AtCNGC1 supports an inwardly rectifying K^+ conductance (2).	Immunoblotting showed that AtCNGC1 is localised in the root tissue. GFP tagged protein localises in yeast PM (1).	Ma et al. 2006 (1) Leng et al. 2002 (2) Ali et al. 2006 (3)
AtCNGC2	Mutant is dwarf and hypersensitive to high external Ca^{2+} (1, 3).	Heterologously expressed AtCNGC2 can transport Ca^{2+} and K^+ but not Na^+ (2). Patch clamping of mutant guard cell PM revealed that hyperpolarisation-activated Ca^{2+} conductance was absent (3).	RNA is evident in inflorescence, stem and leaves but not roots (4).	Chan et al. 2003 (1) Leng et al. 1999 (2) Ali et al. 2007 (3) Köhler et al. 2001 (4)
AtCNGC3	Mutant showed reduced germination under cation stress (1).	Supported K^+ and Na^+ accumulation when expressed in yeast (1).	Localised in PM by GFP tagging (1). Expressed in root tissue, root hair as well as shoot and leaves (1), demonstrated by GUS fusion.	Gobert et al. 2006 (1)
AtCNGC10	Antisense plant (40 % mRNA, no detectable protein) flowers early, reducedleaf thickness, surface area, palisade parenchyma cell length. Responds slowly to gravitropism (1).	Present at PM when expressed in HEK293 cell line and supported large inward K^+ conductance (2). Rescued K^+ channel mutants of *E. coli*, yeast and *Arabidopsis* (3).	Immunolocalised to PM (1). *AtCNGC10*-EYFP shows protein localised to PM of leaf protoplast (2). In ER, golgi, PM of root. Transcript highest in root. (2).	Borsics et al. 2007 (1) Christopher et al. 2007 (2) Li et al. 2005 (3)
AtCNGC11/12	Mutant has stunted growth, curly leaves (1).	Supported K^+ uptake in yeast; glutamate 519 is important residue in conferring transport function (2).	The chimeric protein is PM localised (2).	Yoshioka et al. 2006 (1) Baxter et al. 2008 (2)
AtCNGC18	Pollen tube growth is abnormal in overexpressor (2). T-DNA insertional mutant plant is male sterile with abnormal pollen tube germination and growth (1, 2).	Expression of *AtCNGC18* in *E. coli* causes Ca^{2+} accumulation (1).	PM-localised with GFP and YFP (1, 2), post golgi vesicles (2). GUS staining showed expression in pollen grain (1, 2)	Frietsch et al. 2007 (1) Chang et al. 2007 (2)

allow K^+ and Ca^{2+} influx. Elevation of $[Ca^{2+}]_{cyt}$ would cause Ca^{2+}/CaM formation and binding of that complex to the CNGC would restrict further channel activity to end the $[Ca^{2+}]_{cyt}$ signal (Kaplan et al. 2007). One of the most exciting findings of recent years is that the *cngc2* mutant is dwarfed, implicating this PM hyperpolarisation-activated Ca^{2+}-permeable cation channel (HACC) in growth and development (Ali et al. 2007; see Sect. 1 in Chapter "The Role of Ion Channels in Plant Salt Tolerance").

2 Ion Channels Acting in Concert

To appreciate the importance of ion channels in development, their roles are best considered in a well-established system. Thus, in this second part of the review, the role of ion channels in root hair development, pollen tube development, algal development, and nodule formation will be discussed.

2.1 Ion Channels in Root Hair Development

Root hairs are single cells that undergo polarised growth at the apex, regulated by hormones and environmental conditions. Apical PM fluxes of H^+, K^+, Cl^-, and Ca^{2+} are associated with elongative growth. Proteomics have so far failed to identify the channels involved (Brechenmacher et al. 2009), but root hair transcript analysis has revealed expression of 3 K^+ channel genes in *Arabidopsis* root hairs (Ivashikina et al. 2001). AtAKT1 and a modulatory PM α-subunit (AtKC1) are involved in root hair development (Ivashikina et al. 2001; Reintanz et al. 2002; Desbrosses et al. 2003). KDC1 was also identified as a Shaker-like, inwardly rectifying PM K^+ channel in carrot root hairs (Downey et al. 2000) and recent split ubiquitin assays indicate that it may form a hetero-tetrameric K^+ channel with AKT1 (Bregante et al. 2008). While it is readily envisaged that combinations of inward rectifiers govern K^+ uptake for expansion under a range of conditions (particularly PM voltage), the apparently sole PM K^+ outward rectifier in *Arabidopsis* root hairs (GORK; Guard Cell Outward Rectifying K^+ channel) (Ivashikina et al. 2001) may play a role in controlling voltage. However, loss of GORK does not produce a hair growth phenotype (Hosy et al. 2003).

Apical Ca^{2+} uptake by elongating root hairs is essential for generating the apex-high $[Ca^{2+}]_{cyt}$ gradient that may regulate exocytosis and signalling (Monshausen et al. 2008). Patch clamp analysis of the *Arabidopsis* root hair apical PM has revealed the co-existence of two Ca^{2+} channels that are differentially regulated by voltage (Miedema et al. 2008; Fig. 1). Hyperpolarisation-activated Ca^{2+}-permeable cation channels (HACCs) permit Ca^{2+} influx at voltage more negative than −100 mV but could operate at less negative voltage when $[Ca^{2+}]_{cyt}$ increases (Véry and Davies 2000). This could form a local positive feedback system for

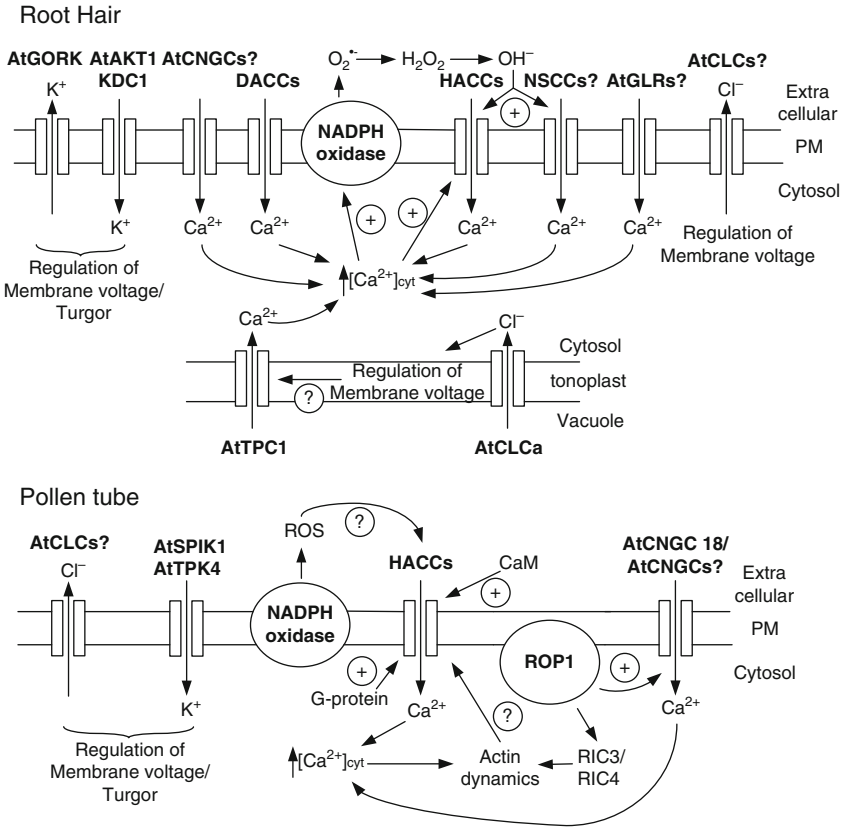

Fig. 1 *Simplified diagram of ion channels which may be involved during root hair and pollen tube development*. In root hair tip growth, only the localisation of HACCs and DACCs at the root hair apex is well established. Others are shown to be expressed in the root hair although whether they are localised at the apex remains unknown. In pollen tube growth, channels which are thought to be expressed in the pollen tube are shown. However, the evidence that these channels are localised at the pollen tube apex remains elusive. For details, see main text. In both cases, transport of K^+ and Cl^- may regulate membrane voltage and turgor-driven tip growth. Bold letters represent channels. Arrows with positive sign represent activation whilst arrows with question mark represent possible interactions or interaction with uncertain outcome

influx. Depolarisation-activated Ca^{2+} channels (DACCs) open at more depolarised voltage, with peak activity around −80 mV to −120 mV (Miedema et al. 2008). The presence of DACCs may enable Ca^{2+} uptake over the membrane voltage range in which HACCs are not operating. Together with HACCs, DACCs may enhance Ca^{2+} influx in response to different stimuli and could allow continued Ca^{2+} uptake in drought stress, which depolarises the root hair PM (Dauphin et al. 2001). HACCS and DACCS also co-exist in the PM of elongating epidermal cells of the main root where HACC activity may be "primed" by Ca^{2+} influx through non-selective cation channels (Demidchik et al. 2002b, 2003b). Chloride channels are likely to be key

components in voltage regulation at the apical PM, but although their activities have been described electrophysiologically in *Arabidopsis* and *Medicago*, their molecular identities remain unknown (Kurkdjian et al. 2000; Dauphin et al. 2001; Diatloff et al. 2004). Of the ClC family, *ClCa* is most strongly expressed in *Arabidopsis* root hairs but is likely to be localised at endomembranes (Lv et al. 2009).

The root hair apical PM contains an NADPH oxidase (encoded by Respiratory Burst Oxidase Homologue C in *Arabidopsis*) that can generate apoplastic superoxide anions (Foreman et al. 2003; Takeda et al. 2008; Fig. 1). These would readily form H_2O_2 which in turn could source apoplastic $OH^{\bullet}$ by reaction with extracellular Cu or Fe. An apical HACC activated by apoplastic $OH^{\bullet}$ has been identified in *Arabidopsis* root hairs that could enable Ca^{2+} influx, increase $[Ca^{2+}]_{cyt}$, and stimulate NADPH oxidase activity through that enzyme's EF hands to maintain the $[Ca^{2+}]_{cyt}$ gradient (Foreman et al. 2003; Takeda et al. 2008). A similar mechanism could operate in diffuse growth of the main root (Foreman et al. 2003). At present it is not clear whether the root hair $OH^{\bullet}$-activated HACC is a Ca^{2+}-permeable NSCC (as identified in the root epidermis; Demidchik et al. 2003b) or the constitutive HACC (which shows greater Ca^{2+} permeation) identified by Véry and Davies (2000). Extracellular ATP, ADP, ABA and ethylene are now known to be upstream regulators of NADPH oxidases and HACCs in a variety of plant cells, suggesting that these mechanisms may regulate root hair growth (Murata et al. 2001; Demidchik et al. 2003b, 2009; Zhao et al. 2007).

Molecular identities of HACCs and DACCs are unknown but it is feasible that (in *Arabidopsis*) GLRs 1.1, 3.3, 3.4, 3.7 and CNGCs 1, 3, 10 contribute to apical Ca^{2+} influx (see Table 2 and references therein; Chiu et al. 2002; Demidchik et al. 2002a; Meyerhoff et al. 2005; Stephens et al. 2008; Qi et al. 2006). AtGLR 3.4 is a particularly compelling candidate as it is expressed in root hairs, is PM-localised and appears Ca^{2+} transport-competent (Demidchik et al. 2002a; Meyerhoff et al. 2005). In rice, the *TPC1* (Two Pore Channel 1) gene was proposed to encode a DACC (albeit not specifically in hairs) with a role in Ca^{2+} uptake and development (Kurusu et al. 2004, 2005; Hashimoto et al. 2005). However, *AtTPC1* is a vacuolar Ca^{2+}-permeable channel (Peiter et al. 2005). A key question is whether CNGC2 (which forms a HACC in guard cells; Ali et al. 2007) is the root hair HACC as there are contradictory reports of its expression in root cells (Ma et al. 2006; Dinneny et al. 2008). Identification of PM and endomembrane root hair channels will not only unlock growth mechanisms but also aid understanding of nod signalling (see Sect. 4 in Chapter "The Role of Ion Channels in Plant Salt Tolerance").

2.2 Ion Channels in Pollen Tube Growth

Pollen presents an opportunity to track channel expression and function from grain maturation to polar pollen tube growth *in vitro*. *Arabidopsis* pollen grain expresses *SPIK1* (Shaker Pollen Inward K^+ channel; pollen-specific), *TPK1* and *TPK4, AKT2*, *KAT1*, *KAT2*, *GORK*, and *SKOR* (Stelar K^+ Outward Rectifier; a PM channel), the

putative chloride channel gene *CLC-c* and *CNGC 7, 8, 16*, and *18* (Mouline et al. 2002; Becker et al. 2003, 2004; Pina et al. 2005; Wang et al. 2008). In common with root hairs, growing pollen tubes maintain apical PM fluxes of H^+, K^+, Cl^-, and Ca^{2+} (Michard et al. 2008 and references therein). Loss of tube PM AtSPIK1 perturbs pollen germination and tube development (Mouline et al. 2002). AtTPK4, the only TPK channel present at the PM rather than tonoplast, is present in pollen tubes but loss of function does not result in a phenotype. Unlike Shaker channels such as SPIK1, AtTPK4 is weakly voltage-dependent and could permit K^+ uptake at less negative voltages where SPIK1 would be closed (Becker et al. 2004). Differential modulation of SPIK1 and TPK4 opening by Ca^{2+} and H^+ would further "fine-tune" K^+ influx (Mouline et al. 2002; Becker et al. 2004). Although apical Cl^- fluxes are linked to pollen tube growth, associated channel genes have yet to be identified.

Apical oscillatory Ca^{2+} influx is intimately related to pollen tube growth (Cárdenas et al. 2008a; Michard et al. 2008). An MS Ca^{2+}-permeable channel is localised to a small region of the *Lillium* pollen grain PM where pollen tube growth will occur, as well as at the tip of the growing tube (Dutta and Robinson 2004). Pollen tube apical PM of *Pyrus* (Qu et al. 2007) contains a HACC while PM HACCs have been identified in pollen protoplasts of *Arabidopsis* and *Lillium* that could be present at the tube apex. These are regulated by heterotrimeric G proteins or external calmodulin (Shang et al. 2005; Wu et al. 2007). PM Ca^{2+}-permeable channel activity in *Arabidopsis* pollen protoplasts is stimulated by actin depolymerisation (Wang et al. 2004) and this could relate to the inter-relationship of apical actin and $[Ca^{2+}]_{cyt}$ dynamics if these channels were present in the pollen tube. However, actin depolymerisation was recently reported to lower apical $[Ca^{2+}]_{cyt}$ in *Lilium* tubes (Cárdenas et al. 2008a).

Apical actin dynamics are regulated by the PM-localised ROP1 (Rho-related GTPase) which lies upstream of the ROP effector proteins RIC3 and RIC4 (ROP-interactive CRIB motif-containing proteins) in *Arabidopsis* (reviewed by Yang 2008). RIC3 is presently thought to lie upstream of actin-regulated PM Ca^{2+} channels. ROP activity also governs NADPH oxidase activity in root hairs (reviewed by Yang 2008; see Sect. 1 in Chapter "The Role of Ion Channels in Plant Salt Tolerance"), but while NADPH oxidase inhibitors or lowered *RBOH* expression negates pollen tube growth (Cárdenas et al. 2006; Potocký et al. 2007), interaction of ROS and PM Ca^{2+} channels has yet to be delineated. However, *AtCNGC18* is now firmly implicated in pollen tube polarity (Chang et al. 2007; Frietsch et al. 2007). The protein's localisation to the apical PM is promoted by ROP1 (Chang et al. 2007; Frietsch et al. 2007). *AtCNGC18* expression in *E. coli* results in Ca^{2+} accumulation, suggesting that *AtCNGC18* could be involved in pollen tube Ca^{2+} influx (Frietsch et al. 2007; Iwano et al. 2004). The *Atcngc18* insertion mutant exhibits aberrant pollen tube growth (Frietsch et al. 2007) while the overexpressor has wider and shorter tubes (Chang et al. 2007). The study of other *AtCNGCs* (such as *AtCNGC7* and *AtCNGC16* that are up-regulated on tube growth; Wang et al. 2008) is now necessary to elucidate the function of other potential Ca^{2+}-permeable channels and place their activity in the context of oscillating apical $[Ca^{2+}]_{cyt}$.

2.3 Ion Channels in Algal Development

Rhizoid outgrowth from algal embryos is also a polar growth process. That of the brown alga (*Fucus serratus*) shares a $[Ca^{2+}]_{cyt}$-ROS signalling system reminiscent of root hairs. The apical PM of this alga harbours a Ca^{2+}-permeable cation channel that is activated by exogenous H_2O_2 and is likely to contribute to growth and osmoregulation (Coelho et al. 2002, 2008). This channel may also be stretch-activated (Taylor et al. 1996). NADPH oxidase inhibition or addition of catalase abolishes the apex-high $[Ca^{2+}]_{cyt}$ gradient and suppresses rhizoid polar growth (Coelho et al. 2008). Buffering intracellular $[Ca^{2+}]_{cyt}$ also abolished the apex-high intracellular [ROS] which suggests a similar positive feedback loop to root hairs (Coelho et al. 2008). Algae, like plants, also use ion channels to regulate their membrane potentials. *Coccolithus pelagicus* harbours a PM inward-rectifying Cl^- conductance to regulate membrane voltage and hence the calcification process necessary for producing the external plates of this marine phytoplankton (Taylor and Brownlee 2003). Ion channel studies in algae will be advanced significantly by the sequencing of the *Chlamydomonas* genome. *Chlamydomonas* has a vertebrate-like voltage-dependent Ca^{2+} channel (CAV2) which is localised toward the distal part of the flagella and operates in deflagellation (Fujiu et al. 2009). The transport ability of CAV2 has yet to be demonstrated as its expression in heterologous systems has proved challenging (Fujiu et al. 2009).

2.4 Ion Channels in Nodule Development

Nod factors are rhizobial lipochitin oligosaccharide signalling molecules required to establish nodulation in legumes (Oldroyd and Downie 2008). They evoke specific changes in root epidermal $[Ca^{2+}]_{cyt}$ to initiate the symbiotic relationship between N-fixing rhizobia and legumes (reviewed by Oldroyd and Downie 2008). Nod factors cause transient Ca^{2+} influx at the root hair apex which, as it occurs before membrane depolarization (Ehrhardt et al. 1992; Felle et al. 1998, 1999), could initially be mediated by PM HACCs. The depolarized state can last for 15–30 min and perhaps there is a role for DACCs during this period. Increased $[Ca^{2+}]_{cyt}$-mediated Cl^- efflux also contributes to membrane depolarization (Felle et al. 1998, 1999; Kurkdjian et al. 2000). Upon Nod factor perception, there is a transient increase in intracellular ROS (possibly resulting ultimately from PM NADPH oxidase activity) at the tip of growing root hairs (Cárdenas et al. 2008b) and ROS efflux declines (Shaw and Long 2003). Although activation of root hair PM Ca^{2+} channels by intracellular ROS has not yet been demonstrated, intracellular H_2O_2 activates a PM HACC in *Arabidopsis* root epidermis (Demidchik et al. 2007) and it is feasible that such a mechanism could contribute to $[Ca^{2+}]_{cyt}$ elevation in nod signalling. Additionally, legume root hairs and epidermis could also contain the $OH^{\bullet}$ -activated PM HACC characterised in *Arabidopsis* (Foreman et al. 2003). It is

also feasible that an increase in $[Ca^{2+}]_{cyt}$ could also be due to Ca^{2+} release from apical internal stores. Efflux of K^+ is also observed after Cl^- efflux at the root hair apex, which could promote membrane repolarisation (Felle et al. 1998).

The genes encoding the channels involved in nod-induced apical $[Ca^{2+}]_{cyt}$ elevation have yet to be identified, but the *NodO* product (a peptide produced by *Rhizobium leguminosarum* biovar *viciae*) has *in vitro* monovalent cation-permeable channel activity and could contribute to plant ionic fluxes (Sutton et al. 1994). While Ca^{2+} influx can occur within a minute of nod perception, perinuclear Ca^{2+} oscillations ("Ca^{2+} spiking") are observed 10–30 min later and are essential for the plant's transcriptional response (reviewed by Oldroyd and Downie 2008). Spiking is not contingent on the initial Ca^{2+} influx and the two responses are genetically distinct (Oldroyd and Downie 2008). The CASTOR and POLLUX nuclear envelope proteins of *Lotus japonicus* are essential for spiking and are members of a novel class of cation channels with relatives in non-leguminous plants such as *Arabidopsis* (Charpentier et al. 2008). CASTOR forms a cation channel with weak preference for K^+ over Na^+ and Ca^{2+} in planar lipid bilayers. The mutant *castor-2* (Ala substituted for Thr at position 264 in the amino acid sequence) no longer exhibits Ca^{2+} spiking in response to Nod factor and the protein itself has altered channel characteristics (Charpentier et al. 2008). POLLUX is able to complement a K^+ transport-deficient yeast mutant and overexpressing POLLUX in a *castor* null mutant can restore nodulation, suggesting that both proteins have similar function *in planta* (Charpentier et al. 2008). It has been proposed that CASTOR and POLLUX act as voltage-regulating K^+ channels *in vivo*, the activities of which regulate an unidentified nuclear envelope Ca^{2+} channel (Charpentier et al. 2008; Oldroyd and Downie 2008). Nodule formation results in down-regulation of several root channel transcripts including *CNGCs1, 2 and 6, GLRs3.1* and *3.3, TPC1*, and *ClC-e* (Benedito et al. 2008).

3 Conclusions

Plants use discrete assemblies of co-localised ion channels to control development. In general, Ca^{2+}-permeable channels are associated with signal transduction (which may alter developmental gene expression) with K^+- and anion channels acting to regulate membrane voltage during these events. K^+ channels play critical roles in regulating turgor and membrane voltage to drive expansion. Channel modulation by ROS to regulate polar growth may be conserved from *Fucus* rhizoids to higher plant pollen tubes and root hairs. Molecular identities of channels (particularly for anions and Ca^{2+}) still require resolution and not just for those at the PM – the advances in nod signalling have demonstrated the clear need to keep studying endomembrane systems.

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Potassium and Potassium-Permeable Channels in Plant Salt Tolerance

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Abstract Salinity causes billion dollar losses in crop production around the globe and has also a significant social impact on rural communities. To breed salt tolerant crops, a better understanding of mechanisms mediating plant adaptive responses to salinity is needed. Over the last years, evidence has been accumulated suggesting that plants' ability to maintain a high cytosolic K^+/Na^+ ratio appears to be critical to plant salt tolerance. This paper reviews molecular and ionic mechanisms contributing to potassium homeostasis in salinized plant tissues and discuss prospects for breeding for salt tolerance by targeting this trait. We show that K^+ channels are instrumental to nearly all aspects of salinity stress signaling and tolerance, and the plant's ability to control intracellular K^+ homeostasis is arguably the most important feature of salt-tolerant species. The molecular identity of key genes, mediating plant adaptive responses to salinity, is analyzed, and the modes of their control are discussed. It is suggested that the major focus of plant physiologists and breeders should be on revealing the specificity of K^+ channel regulation under saline conditions and a "fine tuning" of all mechanisms involved in the regulation of K^+ homeostasis in plants, including both plasma- and endomembrane channels and transporters.

Abbreviations

CAX	Cation/H^+ antiporter
CBL	Calcineurin B-like proteins
CHX	Chloroplast envelope K^+/H^+ exchanger

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CNGC	Cyclic nucleotide-gated channel
CIPK	CBL-interacting protein kinases
FACC	Fast activating cation channel
FV	Fast vacuolar channel
GLR	Glutamate receptor
KIR	Inward-rectifying K^+ channel
KOR	Outward-rectifying K^+ channel
KUP/HAK/KT	H^+/K^+ symporter
NHX	Vacuolar Na^+/H^+ exchanger
NSCC	Non-selective cation channel
PCD	Programmed cell death
PM	Plasma membrane
ROS	Reactive oxygen species
SV	Slow vacuolar channel
TEA	Tetraethylammonium chloride
TPK/KCO	Two-pore K^+ channel
Trk/HKT	Na^+/K^+ symporter

1 Introduction

1.1 Salinity as an Issue

Global food production will need to increase by approximately 50% by 2050 to match the projected population growth (Flowers 2004; Rengasamy 2006). As most suitable land has already been cultivated, this implies a need for expansion into new areas to meet the above target. Most of these areas are either severely affected by salinity, or require extensive irrigation and, hence, are at risk to become saline (Flowers 2004). Over 800 million hectares of land worldwide are affected by salinity (Munns 2005), comprising nearly 7% of the world's total land area. Irrigation systems are particularly prone to salinization, with nearly one-third of irrigated land being severely affected (Munns 2002). The economic penalties are in the billion dollar range. Salt tolerant crops, or plant species able to remove excessive salt from the soil while lowering the water table, may contribute significantly to managing this problem. The key to engineering plants for salt tolerance lies in a better understanding of the key physiological mechanisms underlying the adaptive responses of plants to salinity. Numerous strategies are used by different species to deal with excessive NaCl content in the soil. Most of them are attributed to better regulation of Na^+ uptake and compartmentation (e.g., Na^+ exclusion from uptake and sequestration in vacuoles, regulation of Na^+ transport to the shoot at the xylem/root parenchyma boundary, retranslocation in phloem, or compartmentation within the shoot; reviewed by Tester and Davenport 2003). In addition, plant's ability to retain K^+ in its tissues under saline conditions appears to be central to salinity tolerance (Shabala and Cuin 2008). This chapter addresses the latter mechanism.

1.2 Physiological Constraints Imposed by Salinity

Traditionally, growth inhibition and poor plant performance under saline conditions are attributed to osmotic stress imposed by salinity and to specific ion (Na^+ in most cases) toxicity. As will be shown below, detrimental effects of each of these factors are crucially dependent on a plant's ability to maintain K^+ homeostasis and control K^+ transport across cellular membranes.

To deal with osmotic stress problem and maintain sufficient turgor pressure required to drive extension growth in roots and shoots, plants must increase their cell sap osmolality in a process called osmotic adjustment. The traditional view is that plants are doing this by increased *de novo* synthesis of a variety of organic osmolytes (so-called "compatible solutes") (Bohnert and Jensen 1996; Sakamoto and Murata 2000). However, being an energetically expensive process (Raven 1985), such *de novo* synthesis draws on a substantial portion of the ATP pool, thus making it less available for other metabolic functions and imposing growth penalties. Alternatively, plants can also achieve osmotic adjustment by increased accumulation of inorganic ions (mainly, Na^+, Cl^-, and K^+) in the cell. However, being accumulated in high quantities, both Na^+ and Cl^- are toxic to cell metabolism. Such specific ion (particularly Na^+) toxicity is often considered as a primary reason for detrimental effects of salinity (Tester and Davenport 2003). In this review, we show that an optimal Na^+ management by plants crucially depends on K^+ transport and Na^+ compartmentation.

Recently, a third component, an ROS-induced damage to key macromolecules and proteins, has been added to the list of detrimental effects of salinity (Zhu 2003; Tester and Davenport 2003). Moreover, several papers have shown that salinity-induced increases in ROS production may often lead to programmed cell death (PCD), and that the cytosolic K^+ "status" is critical in triggering salinity-induced PCD (reviewed in Shabala 2009). This issue is also discussed briefly in this chapter (see Sect. 1.2 in Chapter "Cation Channels and the Uptake of Radiocaesium by Plants").

2 Potassium Homeostasis in Plants

2.1 Potassium Essentiality and Functions in Plants

Being the second (after nitrogen) most abundant mineral nutrient in plants (Marschner 1995), potassium is crucial to cell metabolism. The typical potassium concentration in shoot tissue varies between 4 and 8% of the plant's dry weight. Importantly, the concentration of *free* potassium is highest among all essential mineral nutrients, which determines its crucial role in cellular osmo- and turgor regulation. In addition to this, K^+ activates over 50 key metabolic enzymes, including those involved in photosynthesis, oxidative metabolism, and protein synthesis (Marschner 1995).

Potassium is also widely used as a charge-balancing ion and is essential for all types of plant movements, including stomatal opening. In addition, K^+ neutralizes the soluble (e.g., organic acid anions and inorganic anions) and insoluble macromolecular anions and stabilizes cytosolic pH at the level optimal for most enzymatic reactions (pH ~ 7.2) (Cuin and Shabala 2006). Thus, cytosolic K^+ homeostasis is crucial to optimal cell metabolism. To a large extent, detrimental effects of salinity can be explained by the existing competition between Na^+ and K^+, as Na^+ substitutes K^+ at major binding sites in key metabolic processes in the cytoplasm (Marschner 1995). It is becoming increasingly evident that it is not the absolute quantity of Na^+ *per se*, but rather the cytosolic K^+/Na^+ ratio that determines cell metabolic competence and ultimately, the ability of a plant to survive in saline environments (Gorham et al. 1991; Gaxiola et al. 1992; Maathuis and Amtmann 1999; Cuin et al. 2003; Colmer et al. 2006; Shabala and Cuin 2008), and the difference in salt sensitivity between some species was attributed to enhanced K^+/Na^+ discrimination (Gorham et al. 1991; Dvořák et al. 1994; Dubcovsky et al. 1996; Volkov et al. 2004).

2.2 Tissue- and Organelle-Specific Potassium Compartmentation

The two major pools of potassium in plant cells are in the vacuole and in the cytosol. Cytosolic K^+ concentrations are maintained at a constant level of ~100 mM and do not differ between root and leaf cells (Walker et al. 1996; Cuin et al. 2003). Such strict cytosolic K^+ homeostasis is explained by the fact that both activation and protein biosynthesis rely on high and stable K^+ concentrations within the cytoplasm (Maathuis and Sanders 1994; Leigh et al. 1999). On the contrary, vacuolar K^+ content may vary dramatically between different cell types, ranging from ~120 mM in root cell vacuoles (Walker et al. 1996) to ~230 mM in mesophyll cell vacuoles (Cuin et al. 2003). Under K^+ deficiency conditions, cytosolic K^+ is maintained at a constant level at the expense of vacuolar potassium (Walker et al. 1996) where it can drop essentially to zero. However, given the important role of vacuolar K^+ in maintaining cell turgor, other cations (e.g., Na^+, Mg^{2+}, or Ca^{2+}) or organic solutes (e.g., sugars) must substitute for the osmotic functions of potassium in vacuoles. A failure to do this causes an immediate arrest of plant growth.

Salinity stress results in significant membrane depolarization, favoring passive K^+ efflux from the cytosol. As a result, a rapid decline in cytosolic K^+ is measured (Shabala et al. 2006). This decline, however, is only transient, as shown by direct electrophysiological measurements using impaled K^+ selective microelectrodes (Cuin et al. 2003; Shabala et al. 2006). The rapid recovery of cytosolic K^+ in these acute NaCl stress experiments may be interpreted as evidence for the restoration of the cytosolic K^+ pool at the expense of the vacuole (see Sect. 2 in in Chapter "Cation Channels and the Uptake of Radiocaesium by Plants"). It is obvious that such a process can have only a limited time span and, unless further K^+ leakage across the plasma membrane is prevented, the vacuolar K^+ pool will eventually become depleted. This is the case for prolonged salinity treatment (Cuin et al. 2003).

K^+ also plays an important role in charge balance in thylakoid membranes (Junge and Jackson 1982), as well as in enzymatic control of leaf photochemistry in stroma (Demmig and Gimmler 1983; Pier and Berkowitz 1987). The impact of salinity of K^+ transport and homeostasis in chloroplasts is discussed in Sect. 3 in Chapter "Cation Channels and the Uptake of Radiocaesium by Plants".

2.3 Major Potassium Transport Systems: A Brief Overview

Over millions of years, plants have evolved a sophisticated network of potassium transport systems. In *Arabidopsis*, seven major families of cation transporters are known, comprising 75 genes in total. These include (Mäser et al. 2001; Véry and Sentenac 2002, 2003; Shabala 2003) the following:

- Shaker-type family of K^+ channels (9 genes in total);
- "Two-pore" potassium channels (TPK; 5 genes in total);
- KUP/HAK/KT transporters (H^+/K^+ symporter, 13 genes in total);
- Trk/HKT transporters (Na^+/K^+ symporter; one gene);
- K^+/H^+ antiporter homologs (NHX/CHX; around 26 genes);
- Cyclic-nucleotide-gated channels (CNGC; 20 genes in total);
- Glutamate receptors (GLRs; 20 genes in total).

It is also important to note that not only are these transporters specifically expressed within various cell compartments and tissues, but also their expression patterns are strongly affected by environmental conditions and, specifically, by salinity. Thus, K^+ transport activity may be adjusted in various cells, independently in each organ/tissue, to match the plant's demands in a challenging environment. As a result, cytosolic K^+ homeostasis can be maintained to enable optimal plant function.

2.4 Potassium and Potassium-Permeable Channels

Shaker-type potassium channels include nine members in *Arabidopsis* and are further subdivided into three major functional groups, based on their voltage dependency (Véry and Sentenac 2002, 2003):

(a) Inward-rectifying channels (AKT1, KAT1, KAT2 and SPIK) mediating potassium uptake and activated by membrane hyperpolarization;
(b) Weakly-inward-rectifying channels (AKT2/3) which mediate both potassium uptake and release depending on the local potassium electrochemical gradients;
(c) Outward-rectifying channels (SKOR and GORK) mediating potassium release from the cell and activated by membrane depolarization.

Shaker channels are ubiquitously expressed in various plant tissues, providing a possibility for the rapid redistribution of K^+ between various plant parts and cellular compartments.

"Two-pore" TPK/KCO potassium channels have five members in *Arabidopsis* (Czempinski et al. 2002). Only one of them, AtTPK4, is targeted to the plasma membrane (in pollen tubes) and forms a functional K^+ channel there. All others encode tonoplast proteins (Voelker et al. 2006); see Sect. 1 in Chapter "Cation Channels and the Uptake of Radiocaesium by Plants" for further details. TPKs play roles in pollen tube growth, stomatal closure and radical development (Becker et al. 2004; Gobert et al. 2007).

Non-selective cation channels (NSCCs) form a large (40 putative members in *Arabidopsis*) heterogeneous group of channels. As the name suggests, NSCC typically show a high selectivity for cations over anions but differ widely in their ability to conduct mono- and divalent cations (Demidchik et al. 2002; Demidchik and Maathuis 2007). These channels are ubiquitous at the plasma and tonoplast membranes of plant cells and vary greatly in their voltage dependence and permeability ratios. Accordingly, they are further classified as depolarization activated, hyperpolarization activated, voltage insensitive, calcium activated, mechanosensitive, cyclic nucleotide-gated, and glutamate-gated (Demidchik et al. 2002). So far, no proteins responsible for non-selective cation currents have been identified at the molecular level. Likely candidates for NSCC forming proteins belong to two families, namely CNGCs and GluRs.

Cyclic nucleotide-gated channels (CNGCs) are ligand-gated channels that are regulated by cAMP or cGMP (Leng et al. 2002). Twenty and sixteen CNGC family members are identified in *Arabidopsis* (Köhler et al. 1999; Mäser et al. 2001) and rice (Yuan et al. 2003), respectively. In contrast to animal CNGCs, the domains binding cyclic nucleotide and calmodulin overlap in plant CNGCs (Köhler et al. 1999) enabling cross-talk between cyclic nucleotides and calmodulin signaling (Arazi et al. 2000). At least some CNGCs show equal permeability for K^+ and Na^+ (Balague et al. 2003; Bridges et al. 2005) and may thus impact on cytosolic K/Na ratios under saline conditions (Maathuis and Sanders 2001), and one specific channel (AtCNGC2) is probably highly selective for K^+ (Hua et al. 2003).

Ionotropic Glutamate receptors (iGluRs) form NSCCs in animals; whether they form functional channels *in planta* has yet to be established. In *Arabidopsis*, 20 genes are reported to encode putative glutamate receptor subunits (Lacombe et al. 2001). The high expression levels of all *AtGLR* genes in *Arabidopsis* roots imply that they are important in regulating ion (including K^+) uptake from the soil (Chiu et al. 2002). A possible signaling role has been also postulated (Lam et al. 1998).

3 Regulation of K^+ Channel Activity Under Saline Conditions

3.1 K^+ Channels and "Osmotic" and "Ionic" Components of Salt Stress

Salinity causes a plethora of physiological responses including a deceleration of the growth of root tips, shoots, and young leaves, as well as stomatal closure (Munns and Tester 2008). All these effects may be attributed to the impact of

salinity on K^+ transport across the plasma membrane (PM). Not only do high concentrations of Na^+ in the soil reduce the activity of K^+, making it less available for plants, but K^+ uptake is also significantly reduced as a result of the direct competition between Na^+ and K^+ for uptake sites at the PM, including both low- (e.g., NSCC) and high- (e.g., KUP and HKT) affinity transporters (Fig. 1; see also Shabala and Cuin 2008 for more details). Even more important is NaCl-induced PM depolarization. Depending on NaCl concentration and plant species, 40–80 mV PM depolarization was reported in both root (Shabala et al. 2005a; Chen et al. 2007b; Cuin et al. 2008) and leaf (Shabala 2000; Shabala et al. 2005b) tissues. Such depolarization makes passive K^+ uptake through inward-rectifying K^+ channels thermodynamically impossible and, at the same time, dramatically increases K^+ efflux through depolarization-activated outward-rectifying K^+ channels (Fig. 1). According to the modern view, a major portion of Na^+ influx in the root occurs via NSCC channels (see Demidchik and Maathuis 2007 for a review). This results in a significant PM depolarization. At the same time, changes in solution osmolality are sensed by a putative osmosensor and are translated into increased activity of the electrogenic H^+-ATPase (reviewed in Shabala and Cuin 2008), resulting in membrane hyperpolarization. The downstream targets of each of these components are voltage-dependent hyperpolarization- (KIR) and depolarization (KOR)-activated Shaker-type K^+ channels. Depending on the severity of the salt stress, one of components dominates, resulting in either increased K^+ uptake (at mild salinity levels), or in a massive K^+ loss (at more severe salt concentrations) from the cell. Increased H^+-pump activity might also provide an additional driving force for the high-affinity K^+ uptake via HAK/KUP transporters. This issue is discussed further in Sect. 2 in Chapter "Cation Channels and the Uptake of Radiocaesium by Plants".

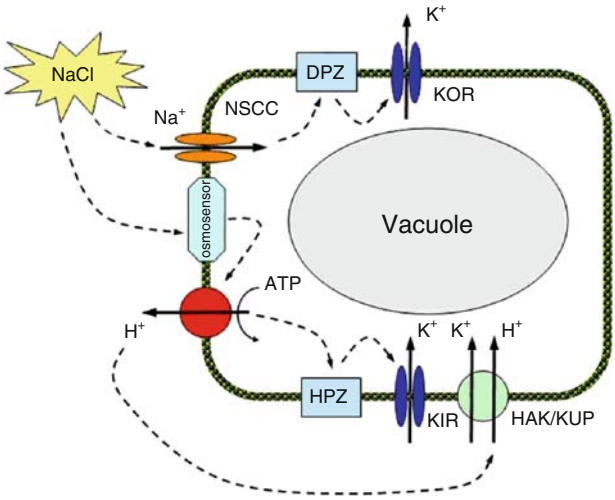

Fig. 1 Potassium transporters and cellular mechanisms of perception of ionic and osmotic components of salt stress. See text for explanations. Reproduced from Shabala and Cuin 2008, with permission of *Physiologia Plantarum*

3.2 GORK and AKT Channels as Downstream Targets of Salinity Effects

The model shown in Fig.1 highlights the importance of voltage gating and implicates Shaker-type K^+ channels as possible downstream targets during salinity stress signaling. This model was validated in direct electrophysiological experiments using a range of *Arabidopsis* transport mutants (Shabala and Cuin 2008) as well as concurrent measurements of net K^+ fluxes, membrane potential changes, and intracellular cell turgor pressure (Shabala and Lew 2002). No significant difference in NaCl-induced K^+ efflux was found between WT Columbia and the *akt1* mutant, while *gork* roots showed a much more attenuated response to salinity treatment (Shabala and Cuin 2008). At the same time, hyperosmotic mannitol treatment caused similar K^+ uptake in WT and *gork* roots, but had essentially no impact on K^+ fluxes in *akt1* roots. Also, clamping the membrane voltage at values positive and negative of E_K led to switching of the K^+ flux from net efflux to influx and *vice versa* in *Arabidopsis* root hairs (Shabala and Lew 2002). Given the strong correlation between the ability of the root to retain K^+ and plant salinity tolerance (Chen et al. 2005, 2007a, 2007b; Cuin et al. 2008; Smethurst et al. 2008), these findings point to the GORK channel as a main downstream target of the detrimental effects of salinity. In addition, GORK activity may impact on salinity tolerance via its modulation by both exogenous and endogenous factors. Four major lines of evidence support the above statement:

(1) In addition to NSCC (Demidchik et al. 2002), GORK channels appear to be the key target for ameliorative effects of supplementary Ca^{2+} and some other divalent cations as revealed in patch-clamp (Shabala et al. 2006) and pharmacological (Shabala et al. 2003, 2005b) studies.
(2) NaCl induced K^+ leak from roots occurs mainly via GORK channels; decreases in its activity are associated with a lower salt-induced depolarization and thus confers salt tolerance (Chen et al. 2005, 2007b; Cuin et al. 2008).
(3) Detrimental effects of salinity may be also ameliorated by either exogenous application of compatible solutes (Harinasut et al. 1996) or by overexpressing genes responsible for biosynthesis of various compatible solutes (Bohnert et al. 1995; Bray 1997). The recent work in our laboratory has shown that compatible solutes prevent NaCl-induced K^+ leakage from the cell, because of both the enhanced activity of the H^+-ATPase (Cuin and Shabala 2005, 2007a) and a decreased induction of TEA^+-sensitive K^+ leak by ROS (Demidchik et al. 2003; Cuin and Shabala 2007b). The specific mechanisms by which ROS stress activates K^+-permeable ion channels in plant membranes are discussed in this volume in chapter 11 by Demidchik.
(4) In animal tissues, caspase activity is significantly increased by a low cytosolic K^+ content (Hughes and Cidlowski 1999), and a decrease in cytosolic K^+ pool was suggested as a trigger for the PCD in plant cells (Demidchik, personal communication; Shabala 2009). Consistent with this notion, expression of the

animal CED-9 anti-apoptotic gene significantly increases plant salinity and oxidative stress tolerance by blocking K^+ efflux via KORs and NSCCs (Shabala et al. 2007a).

3.3 Voltage Gating and the Role of H^+-ATPases

Salt stress rapidly stimulates H^+ pumping by plasma membrane ATPases (Shabala 2000), most likely because of its modulation by 14-3-3 protein binding (Babakov et al. 2000). Such activation will tend to reduce membrane depolarization, attenuating or preventing K^+ efflux. In this context, salt-tolerant barley varieties had 2–2.5 fold intrinsically higher rates of H^+-ATPase pumping (Chen et al. 2007a). As a result, these varieties were capable of maintaining ~ 10 mV more negative membrane potential in root epidermal cells and displayed ~3-fold less NaCl-induced K^+ efflux. In addition, stimulation of H^+-pumping will acidify the apoplast, thus improving the K^+/Na^+ selectivity of the ion channel mediated transport. Indeed, such acidification will cause opposite effects on K^+-selective channels and NSCC, activation and inhibition, respectively (Amtmann et al. 2004).

It is not quite clear how long the NaCl-induced plasma membrane depolarization lasts and when the potential becomes sufficiently negative to allow the activation of K^+ uptake via inward rectifier K^+ channels. It appears that the effect is highly species-specific. It was shown that a salt-tolerant species, *Thelluingiella halophila*, was able to recuperate the initial very negative potential difference value while its salt-sensitive relative, *Arabidopsis thaliana*, was not (Volkov and Amtmann 2006).

Long-term potentiation of the H^+-ATPase activity may be achieved via facilitating of 14-3-3 protein binding by spermine, accumulated in salinized tissues (Garufi et al. 2007). 14-3-3 proteins may also directly regulate K^+ channel activity; they are required for KIR activity and could differentially modulate KORs (Bunney et al. 2002; Wijngaard et al. 2005). Thus, it appears that 14-3-3 proteins may control intracellular K^+ homeostasis both directly, by regulating K^+ permeable channels, and indirectly, by modulating H^+-ATPase activity. This is consistent with the idea that 14-3-3 proteins might mediate cross-talk between the salt stress and potassium signaling pathways in plant roots (Xu and Shi 2006).

3.4 Maintaining the Optimal Cytosolic K^+/Na^+ Ratio

Being an overall result of Na^+/K^+ exchange across the plasma membrane, regulation of K^+ efflux is a more complex trait than control of either Na^+ uptake or exclusion. The extent of NaCl-induced K^+ efflux depends on both the magnitude of salt-induced membrane depolarization (H^+ pump activity *vs* Na^+ entrance through NSCC) and on the activity of all K^+-release channels (e.g., KOR and NSCC). Also, the initial rapid loss of cytosolic K^+ is rapidly reversed through repletion by

K^+ from the vacuole (Shabala et al. 2006). Thus, regulation of K^+ transport across membranes of other organelles has to be considered. However, the above repletion could only postpone but not abolish the need to reabsorb K^+ from the salinized soil. As a result, plants with initially better designed PM Na^+ and K^+ transport systems (e.g., with a reduced Na^+ influx and K^+ efflux) will have a longer lasting advantage. Comparative analysis of ion currents in *Thellungiella* and *Arabidopsis* may serve as an interesting extension of this principle. Both KOR and NSCC in *T. halophila* root plasma membranes display a higher degree of the K^+/Na^+ selectivity (Volkov et al. 2004). Consequently, Na^+ influx in *T. halophila* was reduced as compared to *A. thaliana*. Moreover, despite a higher (and more energy consuming) Na^+ export from roots, *A. thaliana* accumulated more Na^+ (Wang et al. 2006).

In addition, leaf epidermal cells of *T. halophila* also displayed a higher ratio of Na^+ to K^+ currents compared with mesophyll cells, which could underlie the preferential accumulation of Na^+ in the leaf epidermis as compared to the tissues with higher metabolic workloads. Leaf epidermis and mesophyll could possess contrasting concentrations of cytosolic Na^+ and K^+ (Karley et al. 2000), which argues for a mainly apoplastic way of communication between these cell types. A redistribution of Na^+ and K^+ between these cell types in salinized barley leaves seems to be an important strategy for maintaining constant K^+ activity and a high K^+/Na^+ ratio in the cytosol of mesophyll cells (Cuin et al. 2003).

3.5 *Long-term Salinity Exposure and Regulation of K^+ Transport*

Long-term salinity exposure adds another dimension to the regulation of K^+ transport, causing tissue- and genotype- specific changes in the expression levels of K^+ channels. In addition to GORK channels, several more Shaker-type channels are involved in plant adaptive responses to salinity. These are briefly summarized in Table 1 and are commented on below.

AKT1 channels are most abundant in root epidermis and responsible for K^+ uptake by roots. Not being perfectly K^+-selective, AKT1 may mediate some Na^+ influx when the Na^+/K^+ ratio in the external medium is high (Amtmann and Sanders 1999). Therefore, it comes as little surprise that in halophytes (e.g., ice plant) and relatively tolerant rice cultivars, expression levels of this channel are down-regulated (Table 1) whereas this did not occur in salt-sensitive rice and *Arabidopsis* (Golldack et al. 2003). Interestingly, salt tolerant rice cultivars differed from the salt sensitive ones by a slower accumulation of Na^+, whereas K^+ content remained relatively constant. This ability for Na^+ exclusion was lost, however, at very low external K^+ (Golldack et al. 2003). This not only emphasizes the importance of maintaining a high K^+/Na^+ ratio, but also implies the participation of additional K^+ import systems. On the other hand, *Atakt1* mutants displayed a higher salt-sensitivity (Qi and Spalding 2004). It appears, therefore, that the expression of this

Table 1 Expression patterns of plasma membrane Shaker-type potassium channels in salt-stressed plants

Name (Species)	Type	Location	Expression under salt stress	References
AKT1 (*Arabidopsis*)	IR	Root cortex, epidermis, endodermis & hairs; leaf mesophyll	Mostly unchanged in roots and shoots	Pilot et al. 2003
AKT1 (rice)	IR	Root (epidermis and endodermis mostly); leaves xylem parenchyma; phloem mesophyll	Mostly unchanged in sensitive varieties; down-regulated in roots and coleoptiles of tolerant genotypes	Golldack et al. 2003; Fuchs et al. 2005
MKT1 (ice plant)	IR	Roots	Strongly downregulated	Su et al. 2001
AKT2/3 (*Arabidopsis*)	WIR	Phloem, xylem, leaf mesophyll, guard cell	Down-regulated in shoots Up-regulated in leaves	Pilot et al. 2003
MKT2 (ice plant)		Leaves, stems, flowers seed capsules	No significant changes	Su et al. 2001
KCT2 (*Brassica rapa*)			Upregulated	Zhang et al. 2006
KAT1 (*Arabidopsis*)	IR	Guard cell		Szyroki et al. 2001
KAT1 (rice)	IR	Internodes, rachides	Improves K/Na ratio	Obata et al. 2007
KMT1 (ice plant)		Leaves, stems, seed capsules	Strongly upregulated	Su et al. 2001
SKOR (*Arabidopsis*)	OR	Root pericycle; stelar parenchyma cells	No significant changes	Pilot et al. 2003; Qi and Spalding 2004
KC1 (*Arabidopsis*)		Roots, shoots, leaves	Strong up-regulation in shoots and leaves; no changes in roots	Reintanz et al. 2002; Pilot et al. 2003

IR inward rectifier, *OR* outward rectifier, *WIR* weakly inward rectifying channel

inward rectifier has to be fine tuned to match the activity of high-affinity K^+ transporters to support K^+ absorption, whereas at the same time toxic Na^+ influx is avoided. A modification of the inward rectifier properties may be achieved by the formation of heteromeric complexes with subunits of a different K^+ channel. AtKC1, which does not form functional channels by itself (Dreyer et al. 1997), could form functional heteromeric channels with AKT1 in roots, and with both AKT1 and AKT2 in leaf mesophyll (Dennison et al. 2001; Pilot et al. 2003). An increase of AtKC1 transcript in leaves and shoots would increase the formation of heteromeric channels which require a higher activation voltage compared to AKT1 homomers and thus would be less active.

3.6 Tonoplast (Vacuolar) Channels

3.6.1 Properties of K^+-Permeable Vacuolar Channels

Three major types of K^+-permeable channels are known to be present at tonoplast membranes. These include the following:

- slow vacuolar (SV) channels;
- fast vacuolar (FV) channels;
- vacuolar K^+ (VK) channel.

In *Arabidopsis* the SV channel is a product of a single gene, *ATPC1*, encoding a unique double-pore Ca^{2+} channel (Peiter et al. 2005). This channel is activated by cytosol-positive voltages and elevated cytosolic Ca^{2+} (Hedrich and Neher 1987; Pottosin et al. 2001) and, when open, conducts K^+, Na^+, Ca^{2+}, and Mg^{2+} almost indiscriminately. The activation threshold of the SV channel is shifted to positive voltages by vacuolar Ca^{2+}, thus strongly limiting the channel activity at physiological transtonoplast potentials (Pottosin et al. 1997, 2004). Recent work on beet vacuoles has shown that, although the SV channels dominate the vacuolar Ca^{2+} release at diverse signaling conditions, this release is strongly restricted (Pérez et al. 2008; Pottosin et al. 2009). Consistent with these observations is a report of Ranf et al. (2008) that SV-mediated vacuolar Ca^{2+} release does *not* contribute significantly to early Ca^{2+} responses to a variety of abiotic and biotic stresses.

The FV forms a non-selective monovalent cation channel which is inhibited by micromolar Ca^{2+} and Mg^{2+} at either membrane side (Tikhonova et al. 1997; Brüggemann et al. 1999a, 1999b; Dobrovinskaya et al. 1999a, 1999b). At present, the gene(s) encoding the FV channels are not identified. At physiological conditions, the FV-mediated currents are delimited by the intrinsic voltage dependence, Mg^{2+}, and polyamines (see Pottosin and Muñiz 2002 for a review).

The VK channels were originally found in guard cells (Ward and Schroeder 1994) but reported later in other plant tissues (Pottosin et al. 2003; Gobert et al. 2007). In *Arabidopsis* mesophyll and guard cell vacuoles VK channels are encoded by TPK1, a tandem pore K^+ channel. These channels mediate vacuolar

K^+ release during stomatal closure, seed germination, and K^+ accumulation during seedlings growth (Gobert et al. 2007). AtTPK2, AtTPK3, AtTPK5, and AtKCO3 also encode tonoplast proteins, but their channel function is not yet demonstrated. There is also evidence that TPK family members do not form heteromeric proteins and their expression patterns poorly overlap, implying tissue-specific functions (Voelker et al. 2006). *Arabidopsis* TPK1/VK channels are voltage-independent and require elevated cytosolic Ca^{2+} for their activation (Bihler et al. 2005; Gobert et al. 2007).

3.6.2 Vacuolar Channels and Cytosolic K^+ Homeostasis

Two vacuolar pumps, a V-type H^+-ATPase and a pyrophosphatase, generate the electrochemical potential difference for H^+ across the tonoplast, which fuels different secondary transports (see for a review Barkla and Pantoja 1996; Maeshima 2001; Gaxiola et al. 2002). Under salt stress, the import of Na^+ into the vacuole via Na^+/H^+ antiport is central to plant survival, as it leads to osmotic adjustment and Na^+ detoxification. Therefore, both over-expression of H^+-pumps and Na^+/H^+ antiporters corroborated the increased salt tolerance in plants (see Gaxiola et al. 2002; Apse and Blumwald 2007 for a review).

Under salt stress conditions, leaf vacuoles can accumulate between 200 and 400 mM Na^+ in crop species (Cuin et al. 2003; James et al. 2006) and up to 1 M in some halophytes (Barkla and Pantoja 1996). In salinized plant tissues, the tonoplast electric potential difference is close to zero (Cuin et al. 2003), whereas the difference in Na^+ concentration between the vacuole and cytosol could reach 10-fold (Apse and Blumwald 2007). Thus, any passive tonoplast conductance for Na^+ implies a Na^+ leak from the vacuole. Proper Na^+ sequestration could be achieved only when the Na^+ leak from the vacuole is abolished or at least greatly reduced.

Both SV and FV channels are highly Na^+ permeable, with $P_{Na} \approx P_K$ (Amodeo et al. 1994; Brüggemann et al. 1999a; Pottosin et al. 2003). Thus, they have to be shut down under salt stress conditions. On the other hand, the function of the tonoplast K^+-selective channel, VK, could be beneficiary under salt stress. This benefit is dual: (1) providing a shunt conductance for H^+-pumping, and (2) exporting K^+ from the vacuole to improve the cytosolic K^+/Na^+ ratio (Pottosin et al. 2003).

3.6.3 Regulation of Vacuolar Channel Activity Under Saline Conditions

Cytosolic K^+ can not be eternally maintained at the expense of the vacuolar K^+ pool, so the role of VK channels may decrease with the progression of salt stress. However, non-selective FV and SV channels should be down-regulated at most times under saline conditions. Indeed, salinity treatment resulted in a dramatic decrease of the SV currents in root vacuoles in *Plantago* species (Maathuis and

Prins 1990); the effect was more pronounced in a salt-tolerant species. Recent studies did not demonstrate significant salt-induced changes in *AtPC1* (SV) transcripts in *Arabidopsis* and TPK (VK) transcription was only moderately regulated by external stresses (Maathuis 2006; Voelker et al. 2006; Hamamoto et al. 2008). Thus, changes in the expression of vacuolar channels seem to have a low impact on vacuolar function under salt stress conditions.

Ivashikina and Hedrich (2006) reported that vacuolar Na^+ increased a threshold voltage for the SV channel activation. Thus, accumulation of Na^+ in the vacuole *per se* could reduce the channel-mediated Na^+ leak. However, such effects were only observed at zero vacuolar Ca^{2+}; at physiological luminal Ca^{2+}, the SV channel activity is *potentiated* by high monovalent cation concentrations, especially by Na^+ which ameliorates the inhibitory effect of vacuolar Ca^{2+} (Pottosin et al. 2005a; Pérez et al. 2008). On the other hand, salt-induced vacuolar Ca^{2+} accumulation could suppress SV channel opening. Although such accumulation has not been directly demonstrated yet, the expression of the vacuolar Ca^{2+}/H^+ antiporter *CAX3* is strongly induced by salt and is important for salt tolerance (Maathuis 2006; Zhao et al. 2008), whereas another antiporter, *CAX1,* is activated by *SOS2,* a serine/threonine kinase, whose function is essential for salt tolerance (Cheng et al. 2004).

Both SV and VK channels are directly activated by cytosolic Ca^{2+}, but SV channels may be also regulated via Ca^{2+} dependent protein phosphorylation. Cytosolic Ca^{2+} signaling under salinity triggers a variety of downstream targets such as calcium- and calmodulin-binding proteins, calmodulin, and calcineurin B-like proteins (see Luan et al. 2002; Mahajan and Tuteja 2005 for a review). SV channels are calmodulin-dependent and possess at least two phosphorylation sites (Bethke and Jones 1997) allowing both stimulation and inhibition of their activity, depending on calcineurin B concentration (Allen and Sanders 1995). Thus, under salt stress the SV channel activity might be indirectly regulated by cytosolic Ca^{2+} via CBL-CIPK signaling pathways.

SV activity under saline conditions may also be regulated by stress-induced ROS production. SV channel activity from several plant species is stimulated by reducing agents and it is also conceivable that ROS, because of their oxidizing effects on both calmodulin and calcineurin, may impact on SV channel activity *in vivo* (Carpaneto et al. 1999; Scholz-Starke et al. 2005). A strong inhibitory effect of H_2O_2 on the SV channel was recently demonstrated (Pottosin et al. 2009).

Last but not least, both FV and SV channels show the highest sensitivity to polyamines among plant channels, with K_d values for inhibition by spermine of ~5 and ~50 μM, respectively (Brüggemann et al. 1998, Dobrovinskaya et al. 1999a, 1999b). Given the fact that free polyamine concentrations in plant tissues may rise above these levels under saline conditions (Alcázar et al. 2006), polyamine blockage of SV and FV channels may be an efficient adaptive mechanism to prevent Na^+ efflux into the cytosol. Interestingly, VK channels are almost insensitive to polyamines (Hamamoto et al. 2008). Thus, selective inhibition of FV and SV channels by polyamines will make the overall passive transport of cations across the tonoplast much more K^+-selective under salinity, promoting the efficient sequestration of Na^+ in the vacuole.

3.7 Chloroplasts and Mitochondria

3.7.1 Salinity and Photosynthesis

The drop in photosynthetic activity is one of the principal factors determining plant sensitivity to salt stress. At low and moderate salinities, this decline is usually attributed to a decreased CO_2 conductance (the so-called "stomatal limitation" component; Munns 2002), as well as by a decrease in leaf size. Photosynthetic activity of isolated chloroplasts decreases only slightly despite a large decrease of K^+ and increase of Na^+ content under salinity (Robinson et al. 1983; Flexas et al. 2004). More severe salinity treatments result in non-stomatal inhibition of photosynthesis, where an impact on leaf photochemistry becomes more important. There is a large body of evidence that an extreme rise of salt in chloroplasts provokes a disorganization of their ultrastructure, lipid peroxidation, and separation of membrane components (Navarro et al. 2006; Barhoumi et al. 2007), as well as increased photoinhibition and reduced PSII repair (Takahashi and Murata 2008). A direct consequence of a lower photosynthesis rate is an increased generation of ROS which is partly compensated by an increased activity of the ROS-scavenging enzymes. Indeed, plants expressing high levels of ROS detoxifying enzymes naturally or transgenic overexpressors display a relatively higher salt tolerance (Mittova et al. 2002; Tseng et al. 2007).

3.7.2 Photosynthetic Activity, Stromal pH, and Membrane Transport in Chloroplasts

CO_2 fixation is optimal at stromal pH ~ 8 and is strongly suppressed at pH < 7.3. Protons could be expelled from the stromal compartment by light-driven H^+ pumping into the thylakoid lumen (Junge and Jackson 1982) and/or by ATP-dependent export across the chloroplast envelope (Wu and Berkowitz 1992b; Berkowitz and Peters 1993; Shingles and McCarty 1994). The latter mechanism of H^+ extrusion is important, bearing in mind that envelope membranes are permeable to protons (Thaler et al. 1991). To electrically counterbalance H^+ pumping across the envelope, Na^+ or K^+ uptake is required, which has been shown to reverse stroma acidification and photosynthesis inhibition (Demmig and Gimmler 1983; Heiber et al. 1995). The resulting trans-envelope $K^+(Na^+)/H^+$ exchange is electroneutral and, as a very minimum, involves a functionally coupled H^+-ATPase and putative cation channel(s) of the inner envelope membrane (Wu and Berkowitz, 1992a, 1992b; Berkowitz and Peters 1993; see Neuhaus and Wagner 2000 for a review). A search of suitable channels was mainly performed by reconstitution of the inner envelope membrane fractions into the artificial bilayers and revealed a variety of high-conductance, poorly regulated and low selective cation channels (Mi et al. 1994; Heiber et al. 1995). In contrast, a tightly voltage-regulated cation channel, FACC, has been described upon direct patch-clamping of intact pea

chloroplasts (Pottosin et al. 2005b). Taking into the account the K^+ transport rate across the envelope (Demmig and Gimmler 1983), the corresponding K^+ current would be 0.25–0.8 pA per single chloroplast. Low conductance (~30 pS) and low-activity (open probability ~2% at physiological pH, Ca^{2+}, and voltage) make FACC a suitable candidate to mediate the trans-envelope K^+ fluxes. The relative Na^+ to K^+ permeability of FACC is ~0.5 (Pottosin et al. 2005b), which matches the relative Na^+ to K^+ envelope conductance (Wang et al. 1993). The FACC partial down-regulation by a physiologically relevant stromal alkalinization (from pH 7.3 to 8.0) may explain the experimentally observed light-induced increase of the transenvelope electric potential difference (Demmig and Gimmler 1983). Therefore, FACC seems to also play a role in controlling the transenvelope potential.

3.7.3 Role of the Envelope $K^+(Na^+)/H^+$ Antiport in Salt Tolerance

The molecular identity of the putative envelope H^+-translocating ATPase and cation (e.g., FACC) channels is unknown. Recent studies have shown the existence of additional components (AtCHX23 protein) that could mediate electroneutral $K^+(Na^+)/H^+$ exchange across the envelope membranes of *A. thaliana* chloroplasts (Song et al. 2004). The *chx23* loss-of-function mutants had yellowish leaves (lower chlorophyll content), altered chloroplast ultrastructure (lack of grana thylakoid stacks), an increased cytosolic pH (leaves), and decreased growth at higher medium pH (7.0) as well as increased K^+ and salt sensitivity. On the basis of physiological cytosol to stroma $K^+(Na^+)$ and H^+ gradients, a secondary $K^+(Na^+)/H^+$ antiport across the envelope could function only in the direction of the H^+ uptake by chloroplasts paralleled by a $K^+(Na^+)$ release. Thus, some effects, like an increase in cytosolic pH in *chx23* mutants, could not be explained in a straightforward manner. However, together with the results of other authors, these data point out that proper functioning of the chloroplast pH-stat depends on the $K^+(Na^+)/H^+$ antiport across the envelope, and is important for salt tolerance.

3.7.4 Mitochondrial Channels

In contrast to chloroplast membranes (where so far only weakly selective cation channels were reported), the inner membrane of plant mitochondria contains highly K^+-selective ATP-sensitive channels (PmitoK_{ATP}; Pastore et al. 1999; Petrussa et al. 2001). This channel forms a part of the mitochondrial energy-dissipating system. In the energized mitochondria, a large matrix-negative voltage difference exists across the inner mitochondrial membrane, which decreases due to a K^+ influx upon the activation of PmitoK_{ATP}. Depending on the function of anion channels (Laus et al. 2008) and additional inner membrane K^+ transport systems (K^+/H^+ antiporter), a swelling of mitochondria may be observed, with a rupture of the outer membrane and cytochrome c release, which in turn could promote programmed cell death (Vianello et al. 2007). Together with chloroplasts, mitochondria are a major source of ROS production in plant cells, in particular during salt- and drought-induced

responses (Foyer and Noctor 2005; Pastore et al. 2007). PmitoK$_{ATP}$ is rapidly (within seconds) stimulated by superoxide anions, and its activation in a feedback manner decreases the ROS production by mitochondria (Pastore et al. 1999). In durum wheat, salt and drought stresses increased the activity of the PmitoK$_{ATP}$ by several-fold, which could be reversed by addition of superoxide dismutase and catalase. A twofold increase in the $^{\bullet}O_2^-$ production by mitochondria was measured, which could in turn be reduced by 60% by PmitoK$_{ATP}$ activity (Trono et al. 2004). Thus, PmitoK$_{ATP}$ could be part of a mechanism which protects the mitochondria and the cell from excessive ROS production under stress. Alternative/additional potent dissipative pathways via K^+ uniport may exist in the inner membrane of plant mitochondria, which are independent of the metabolic status and ATP level, but equally useful in the prevention of the ROS generation (Ruy et al. 2004). An important aspect of mitochondrial operation under stress is their collaboration with chloroplasts in the regulation of cell redox homeostasis. Mitochondria can discharge reducing equivalents produced by chloroplasts without a large increase in ROS generation, thus decreasing photoinhibition and over-reduction of chloroplasts/cytosol under conditions of delimited CO_2 supply, e.g., under salt and drought stresses (Pastore et al. 2007).

4 Concluding Remarks and Future Prospects

As summarized above, K^+ channels are instrumental to nearly all aspects of salinity stress signaling and tolerance, and the plant's ability to control intracellular K^+ homeostasis appears to be central to salinity tolerance (e.g., Chen et al. 2005, 2007a, 2007b). Given the fact that NaCl-induced K^+ efflux is mediated predominantly by GORK channels, it would be very tempting to suggest that knocking out GORK genes would increase salt tolerance. Such a statement may be a bit naïve, in the light of the multiple roles these channels play in plants, and any benefits of such modification may be outweighed by the potentially numerous physiological disturbances caused by such a mutation (see Shabala and Cuin 2008). Instead, the major focus of plant physiologists and breeders should be on revealing the specificity of K^+ channel regulation under saline conditions and a "fine tuning" of all mechanisms involved in the regulation of K^+ homeostasis in plants. Such "tuning" should be not restricted to just the plasma membrane, but also has to include tonoplast, mitochondrial and chloroplast K^+ channels, and transporters. Special attention should be paid to control of voltage gating and ROS scavenging/production, two major factors affecting activities of GORK and NSCC K^+-permeable channels. Importantly, the function of these channels under saline conditions, as well as modes of their regulation, should be studied *in planta* and particularly in crop species. Finally, special attention should be given to the tissue-specific aspects of the function and regulation of K^+-permeable channels under saline conditions. There is no doubt we have enough unanswered questions to keep us busy for many years!

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Regulation of Ion Channels by the Calcium Signaling Network in Plant Cells

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Abstract Free calcium (Ca^{2+}) has been considered as a second messenger in all eukaryotes. In response to many extracellular signals, plants often alter cellular Ca^{2+} status, and such changes in many cases are required and sufficient for downstream responses. The specific Ca^{2+} changes triggered by different signals are reflected by not only the concentration but also the temporal and spatial patterns, forming the so-called "Ca^{2+} signature" for each of the different signals. In decoding such Ca^{2+} signatures, plants cells express and organize a large number of sensors that recognize the Ca^{2+} signals and transmit the signals into downstream cellular responses. One of the cellular targets for such Ca^{2+} sensors is the ion channels that are involved in a variety of cellular processes. Such ion channels can be regulated by Ca^{2+} signaling in many ways including both transcriptional and posttranslational modifications. Here, we review the recent studies and conclusions on the ion channel regulation by various signaling pathways involving calcium sensors and their targets.

1 Introduction

Despite the lack of a specific nervous system, plants are capable of perceiving external stimuli, processing the signals, generating specific responses, and sometimes "remembering" the stimulus-response process. This process, often referred to as "signal transduction" or "acclimation," is reminiscent of the "learning" process in animals. In between the signal (input) and response (output), there exists a complicated molecular network for processing the information regardless of the specific organism in question. Within the molecular network for plant signaling, calcium serves as a critical component and plays a role in the signaling of many extracellular

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stimuli including biotic and abiotic stress factors and developmental cues (Trewavas and Knight 1994; Bush 1995; Braam et al. 1997; Felle and Hepler 1997; Holdaway-Clarke et al. 1997; McAinsh et al. 1997; Wymer et al. 1997; Sanders et al. 1999; Rudd and Franklin-Tong 2001). One important question in calcium signaling concerns the specificity of signal-response coupling as different signals elicit distinct and specific cellular responses. Recent studies in both animal and plant cells suggest that a Ca^{2+} signal is characterized not only by the concentration of Ca^{2+} but also by its spatial and temporal information (Franklin-Tong et al. 1996; Holdaway-Clarke et al. 1997; Dolmetsch et al. 1998; Li et al. 1998; Trewavas 1999; Allen et al. 2001; Hetherington and Brownlee 2004). A combination of changes in all Ca^{2+} parameters produced by a particular signal is always different from that produced by any other signal and therefore referred to as a "Ca^{2+} signature." If the specificity of the calcium signals is encoded by these signatures, a particular plant cell must be equipped with the mechanisms for decoding various signatures leading to specific responses. Although the decoding process is not well understood, studies indicate that this process starts with calcium sensors, often calcium-binding proteins that bind calcium with high-affinity and alter their own structural properties. Such structural changes result in functional changes in the sensor proteins (with effector domains) or trigger interaction with the target proteins of the sensors (without effector domains). The sensors or their targets are often regulatory proteins that modulate the function of others and elicit changes in cellular processes.

Several families of Ca^{2+} sensors have been identified in higher plants. Perhaps, the best known is calmodulin (CaM) and CaM-related proteins, which typically contain four EF-hand domains for Ca^{2+}-binding (Zielinski 1998; Snedden and Fromm 2001; Luan et al. 2002). Another class is the Ca^{2+}-dependent protein kinases (CDPKs), which contain CaM-like Ca^{2+}-binding domains and a kinase domain in a single protein (Roberts and Harmon 1992; Harmon et al. 2000). CDPK proteins function both as Ca^{2+} sensors and as effectors of their Ca^{2+}-sensing activity. A more recent addition of Ca^{2+} sensors are proteins similar to both the regulatory B-subunit of calcineurin and the neuronal Ca^{2+} sensor in animals (Luan et al. 2002). These plant Ca^{2+} sensors are referred to as calcineurin B-like (CBL) proteins (Kudla et al. 1999).

CaM and CBL are small proteins that contain multiple Ca^{2+}-binding domains but lack other effector domains like the kinase domain in CDPKs. To transmit the Ca^{2+} signal, CaMs and CBLs interact with target proteins and regulate their activity. CaM target proteins have been identified in higher plants and include protein kinases, metabolic enzymes, cytoskeleton-associated proteins, and others (Reddy et al. 1996; Snedden et al. 1996; Zielinski 1998; Snedden and Fromm 2001; Luan et al. 2002; Reddy et al. 2002). A family of SNF1-like protein kinases called CIPKs has been identified as targets for CBL proteins (Shi et al. 1999). The target proteins of these small Ca^{2+} sensors then regulate activities that constitute cellular responses triggered by an external signal. The CDPKs bind calcium and regulate the kinase activity present in the same molecule, more tightly linking calcium sensing and effector activity. Both small (CaM- and CBL-type) and large (CDPK-type) Ca^{2+} sensors are therefore part of a complex signaling network of interconnected pathways. A prime goal of many plant biologists is to understand how this network is established and

how it functions to link discrete external signals to specific cellular and physiological responses. In this chapter, we focus on CDPKs and the small Ca^{2+} sensors (CaMs and CBLs) that regulate ion channel activities in plant cells.

Many ion channels and ion transporters are important candidates for transducing Ca^{2+} signals. Plant ion channels are transmembrane proteins possessing hydrophilic pore structures that locate in the plasma membrane (PM) or intracellular membranes (e.g., tonoplast, plastid, and mitochondrial membranes) of living cells. Solute ions move through the open pore of ion channels driven by a transmenbrane electrochemical potential at extremely high rates (10^6–10^8 ions per second through one channel protein) (Maathuis et al. 1997). According to the mechanism that controls channel opening and closing (gating), plant ion channels can be classified into four categories: (a) voltage gated channel; (b) exogenous ligands/regulators gated channel; (c) endogenous ligands/regulators gated channel; and (d) mechanical action (stretch, pressure, shear, or displacement) gated channel (Krol and Trebacz 2000). Alternatively, ion channels can be categorized according to ion selectivity. Plant ion channels are expressed in various tissues, located at various cellular and subcellular membranes, and differ in terms of gating mechanisms, ion selectivity, activation kinetics, as well as regulatory mechansims by modulatory factors (Barbier-Brygoo et al. 2000; White et al. 2000; Lebaudy et al. 2007). These ion channels, together with their regulatory components as well as other ion transporters, establish various complex transmembrane transport systems and play essential roles in plant cell nutrient uptake, membrane potential controlling, ion homeostasis, and signal transductions (Barbier-Brygoo et al. 2000; Krol and Trebacz 2000; White et al. 2000; Lebaudy et al. 2007).

A number of studies have revealed that direct or indirect regulation of ion channel activity by Ca^{2+} is important for plant responses to various stimuli. For example, stomatal movement is controlled by changes in guard cell turgor, which are modulated via Ca^{2+}-regulated ion fluxes (Assmann 1993; Ward et al. 1995; MacRobbie 1998). The elevation of $[Ca^{2+}]_{cyt}$ inhibits inward K^+ channel-mediated K^+ influx across the PM of guard cells (Schroeder and Hagiwara 1989) and activates K^+ efflux from guard cell vacuoles to cytoplasm mediated by K^+ channels on the tonoplast (Ward and Schroeder 1994). The anion channels in the PM of stomatal guard cells are also activated by the elevated cytosolic Ca^{2+} (Schroeder and Hagiwara 1989; Hedrich et al. 1990; Allen et al. 1999) and in concert the Ca^{2+}-regulated ion fluxes result in stomatal closure. The following sections briefly describe Ca^{2+} sensors (CDPKs, CaMs, and CBLs) and how they mediate regulation of ion channel activities in different types of plant cells.

2 CDPKs, Plant Calcium "Sensor-Responders" that Regulate Ion Channels

Calcium's role as a second messenger has been identified in all eukaryotes. Before the finding of calmodulin-domain protein kinases (CDPKs), the general paradigm for the mode of action of calcium was its binding to a sensor protein (e.g., CaM),

altering protein conformation. Conformational changes in the sensor protein trigger interaction with downstream effectors (often enzymes) leading to the modification of target enzymes. Identification of CDPK in plants represented a new paradigm for calcium sensing because the sensor protein itself contains a kinase domain that serves as an effector (Harper et al. 1991). Therefore, CDPKs are also referred to as "sensor-responders" as they contain both a sensing and a response domain for the calcium signaling process (Harper and Harmon 2005).

2.1 Structural Diversity and Regulation of CDPK Superfamily

The first CDPK to be cloned represented a typical CDPK that contains calmodulin-like calcium-binding domains (Harper et al. 1991). Subsequent studies identified several sub-types of protein kinases that were highly related to CDPKs and may reflect evolutionary diversification of the same type of protein kinases. These include the CDPK-Related Kinases (CRKs) and calcium and calmodulin-dependent kinases (CCaMKs). The CRKs have high sequence homology to CDPKs and retain a general similarity in their structural domains. For example, the kinase domains in the CRKs are followed by a long C-terminal domain corresponding to the CaM-like domains in CDPKs, although the calcium-binding EF hand motifs are not conserved in CRKs. The structural features of CCaMK are rather unique in that they contain both calcium-binding and CaM-binding domains in the same protein. Instead of four EF-hands, as found in CDPKs, CCaMKs usually have three EF hands, a feature also described for the animal calcium binding protein visinin (Patil et al. 1995).

Biochemical studies on CDPKs have identified several regulatory features that represent important models for the regulation of calcium-regulated protein kinases in plants (reviewed by Harper and Harmon 2005). The structural domains carry straightforward features related to their function and regulation. For example, the kinase domain contains the catalytic site of the enzyme; the EF-hand motifs are calcium-binding domains; the autoinhibitory domain is located between the kinase domain and EF hands and represses kinase activity when the calcium signal is absent. Presumably, the autoinhibitory domain serves as a pseudosubstrate that binds to the kinase active site and blocks the access of substrates. Upon calcium binding, the conformational change results in the release of the inhibitory domain from the active site thereby making the kinase site available for substrate access. In addition to calcium-dependent regulation, some CDPKs have been shown to be modified by myristoylation and palmitoylation (Martin and Busconi 2000). By attaching a lipid module to the N-terminus of the protein, these modifications can effectively target the protein to the cell membranes. For the regulation of CCaMKs, calcium-binding to the visinin-like domain enhances autophosphorylation that in turn increases calmodulin-binding affinity, leading to maximal activation of the kinase (Takezawa et al. 1996). The identification of plant CDPKs and CCaMKs significantly expands the repertoire of CDPKs in eukaryotes.

2.2 Functional Diversity of CDPKs and CCaMKs

Where and when a gene is expressed, its subcellular localization often determines the function of the gene (product). A number of studies address the temporal and spatial expression patterns of CDPK genes. Recent transcriptional profiling studies further enriched the information at the genome scale on gene expression patterns. Although a comprehensive study of all CDPKs is lacking, some CDPK isoforms have been shown to be ubiquitously expressed, whereas others are expressed with tissue-specificity, regulated by various signals such as stress conditions, light, hormones, and pathogens (reviewed by Cheng et al. 2002; Hrabak et al. 2003; Harper et al. 2004). Concerning the subcellular localization, studies have shown that CDPKs can be either soluble or associated with cell membranes (Harmon et al. 2000). Some isoforms are found to be located throughout the cytoplasm and the nucleus. The subcellular compartments that contain CDPKs include the PM, peroxisomes, endoplasmic reticulum, seed oil bodies, and mitochondria (Harper and Harmon 2005). Interestingly, most of the CDPKs contain both myristoylation and palmitoylation sites at their N-termini, which could be responsible for their recruitment to cell membranes. It is yet to be determined how the subcellular locations of these CDPKs are related to their functions.

Toward the understanding of CDPK function in plant physiology, several approaches have been taken and a number of results are revealing. Using biochemical approaches, a growing list of substrates for CDPKs has been identified, that are involved in a number of cellular processes. Substrates include enzymes involved in carbon, nitrogen, and sulfur metabolism (Tang et al. 2003; Hardin et al. 2004; Liu et al. 2006), enzymes for secondary metabolism (Cheng et al. 2001), and proteins for ion and water transport (Hwang et al. 2000; Guenther et al. 2003). The phosphorylation of substrates by CDPKs can alter enzyme/transporter activity against the substrates (in the case of aquaporin and phenylalanine ammonia lyase) and change the regulatory properties of substrates (Liu et al. 2006) or protein stability (Tang et al. 2003). However, these biochemical studies have yet to be connected to the physiological functions of the relevant CDPKs in plants.

Although significant effort has been dedicated to the functional analysis of CDPKs, it has been challenging to assign function to specific CDPKs using either forward or reverse genetics approaches. Available data so far suggest that significant redundancy among CDPK isoforms may account for difficulty in genetic analysis. For example, a recent report (Mori et al. 2006) showed that two CDPKs (CPK3 and CPK6) are involved in the regulation of stomatal response to ABA. The plant hormone ABA is a well-known chemical messenger that is produced upon stress exposure especially under drought conditions. An important response of plants to drought is the closing of their stomata to preserve water, a process that involves a number of signaling components including ABA and calcium. Furthermore, calcium has been shown to serve as a downstream second messenger for ABA in stomatal closing response. However, little is known regarding the

mechanism of calcium action in guard cells except that ion channels responsible for turgor regulation are potential targets for ABA-induced calcium fluctuation (Allen et al. 2000; MacRobbie 2000; Schroeder et al. 2001; Luan 2002). As CDPKs are important sensor-responders in plants, it is speculated that they may play a role in calcium-regulated stomatal closure. The work by Mori et al. (2006) showed that disruption of CPK3 and CPK6 resulted in rather subtle phenotypic changes at the whole plant level despite changes in ion channel activities in the guard cells. This study therefore indicates that, in addition to functional redundancy, genetic analyses to identify whole plant phenotypes may not be successful because of cellular specificity of particular CDPKs.

Calcium signaling is crucial for many aspects of reproductive biology. The earliest evidence for such a conclusion was obtained by the finding of a calcium "wave" during the fertilization process in sea urchins. In plants, pollen tube growth has been used as a single-cell model for the study of calcium signaling for decades. Directional pollen tube elongation critically depends on calcium oscillations (Franklin-Tong et al. 1996). Although it is not known how the calcium waves are decoded by sensors and effectors in the male gametophyte, some studies indicate the involvement of CDPKs. This includes findings that a large number of CDPK isoforms are expressed in pollen grains, the effect of CDPK antisense RNA interference on pollen tube growth (Estruch et al. 1994; Yoon et al. 2006), and recent reverse genetics analyses in *Arabidopsis* (J. Harper, personal communication). It is possible that a high degree of functional redundancy may also be found in pollen CDPKs.

Although CCaMKs are not found in the model plant *Arabidopsis*, studies have demonstrated a critical role of such calcium-regulated protein kinases in plant-microbe symbiosis. One example is the legume−*Rhizobium* symbiosis important in nitrogen fixation. An early signaling event in plant recognition of the bacterial partner is a calcium oscillation in root hairs (Ehrhardt et al. 1996) and similar calcium signaling has been observed during the establishment of plant-fungus symbioses. A genetic screen identified a mutant defective in nodule formation in the legume *M. truncatula* and the gene affected in the mutant encodes a CCaMK containing typical visinin-like EF hands in the calcium sensing domain. In addition, several other genes that encode receptor-like kinases and a cation transporter are predicted to generate and decode calcium signals during the legume−*Rhizobium* interaction reviewed in (Oldroyd and Downie 2004). The CCaMK-type kinases are clearly candidates for decoding calcium changes during legume−microbe interaction. More recent studies using CCaMK mutants lacking the autoinhibitory domain (making the kinase constitutively active) demonstrate that a CCaMK (DMI3) is required and sufficient for the nodulation-related plant cell morphogenesis (Gleason et al. 2006; Tirichine et al. 2006), highlighting the possibility of transferring nitrogen fixation to nonlegume plants by manipulation of CCaMKs and other molecular components in the plant−*Rhizobium* interaction pathway. Because Arabidopsis does not seem to have any type of symbiotic relationship with microbes, it is speculated that CCaMKs may be specifically involved in such symbiotic processes.

2.3 Ion Channel Regulation by CDPKs

It was spectulated for more than a decade that CDPKs might have potential functions in regulation of ion channels in plant cells. So far, there have been several studies showing evidence supporting this notion. Pei et al. (1996) demonstrated that a recombinant *Arabidopsis* CDPK activates Cl^- channels located in the *Vicia faba* tonoplast. Later, a soybean CDPK was demonstrated to inhibit KAT1-mediated inward K^+ currents (Berkowitz et al. 2000). Recently, *in vivo* experiments in *Arabidopsis* guard cells directly confirmed that CPK6 and CPK3 are involved in ABA and Ca^{2+} dependent activation of S-type anion channels as well as ABA activation of Ca^{2+}-permeable channels (Mori et al. 2006). However, lack of direct evidence for the identification and characterization of ion channel phosphorylation by specific CDPKs has become a challenging issue in this field. Future studies should therefore give attention to the following related questions: (a) identification of CDPKs and ion channels specifically expressed in the given cell types (such as guard cells, pollen cells, root cells, and so on), (b) redundancy analyses of specifically expressed CDPKs in one cell type, (c) identification of interaction and phosphorylation between CDPKs and ion channels, and (d) an analysis of the physiological function CDPK-regulated ion channel activity.

3 Calmodulins : Small Calcium Sensors that Target a Family of Ion Channels (CNGCs)

3.1 Plant Genomes Encode a Large Number of CaMs and CaM-Related Proteins

Perhaps the best known calcium-binding proteins are CaMs, highly conserved proteins in all eukaryotic systems. Compared to animals and fungi, which contain only a few CaM isoforms, plants contain an extended superfamily of CaMs and CaM-related proteins with a diverse number of Ca^{2+}-binding EF hands and additional domains (Snedden and Fromm 1998; Zielinski 1998; Snedden and Fromm 2001; Luan et al. 2002). In addition, a large number of CaM-like and CaM-related proteins have been identified in plant species. In Arabidopsis, typical CaM isoforms include CaM1-7 that are highly similar to animal CaMs and to each other (>95% similar on amino acid sequence). Other proteins (CaM8-14) share 50–75% amino acid identity to the typical CaM2, and some of them have been shown to have CaM activity. They are referred to as CaM-like (CaM8, 9, 13, and 14) or, when they have additional non-CaM domains, CaM-related proteins (CaM10−12). For example, Arabidopsis CaM8 is a CaM-like protein because of its more

divergent sequence. This protein can function as a CaM in Ca^{2+}-binding and yeast complementation experiments, but it appears to interact with a more limited set of target proteins as compared to typical CaM isoforms (Zielinski 2002). A good example of a CaM-related protein is petunia CaM53, which has been demonstrated to have CaM activity but it contains a polybasic C-terminal domain that is not found in a typical CaM. As discussed later, this extra domain in CaM53 regulates its cellular localization (Rodriguez-Concepcion et al. 1999). It is also interesting that the genes encoding CaM10, CaM12, and CaM2 are organized in a tandem array in this order on chromosome 2. This could result from gene duplication and incorporation of additional domains in a sequence of events from CaM2 to CaM10 to CaM12.

The EF hands in CaM proteins are organized into two distinct globular domains, each of which contains one pair of EF hands. Each pair of EF hands is considered to be the basic functional unit. Pairing of EF hands is thought to stabilize the protein and increase its affinity toward Ca^{2+} (Seamon and Kreetsinger 1983). Although each globular domain binds Ca^{2+} and undergoes conformational changes independently, the two domains act in concert to bind target proteins (Nelson and Chazin 1998). Upon increase of Ca^{2+} to sub-micromolar or low micromolar levels, all CaM molecules will be activated. Cooperative binding is required for this "on-off" mechanism to function efficiently. Cooperativity of Ca^{2+} binding ensures that full activation of the CaM occurs in a narrow region of calcium concentrations during a signaling event.

Selectivity of CaM toward Ca^{2+} is also an important factor in effective transduction of the Ca^{2+} signal. CaMs bind Ca^{2+} selectively in the presence of high concentrations of Mg^{2+} and monovalent cations in the cell. This selectivity is achieved by optimizations in the structure folds of the binding loop. For example, the discrimination between Ca^{2+} and Mg^{2+} is accomplished through the reduction in the size of the binding loop. Binding of Mg^{2+} ions would collapse the EF-hand loop, thereby reducing the distance between negatively charged side chains and destabilizing the CaM-Mg^{2+} complex (Falke et al. 1994). Even small changes in the chemical properties of the Ca^{2+} binding loop (e.g., Glu12→Gln) can drastically reduce the binding affinity for Ca^{2+} (Beckingham 1991; Haiech et al. 1991). The Glu12→Gln mutation changes the carboxylate side chain into carboxylamide, which removes the oxygen ligand for Ca^{2+} (Nelson and Chazin 1998). Structural analyses in combination with site-directed mutagenesis established that CaMs (and other EF-hand containing proteins) have evolved as highly specific Ca^{2+} sensors.

Structural analysis of the Ca^{2+}-free and Ca^{2+}-bound states of CaM proteins reveals the conformational changes induced by Ca^{2+} binding. In the Ca^{2+}-free state, CaM adopts a closed conformation. Ca^{2+} binding triggers a conformational change and the protein now adopts an open conformation with near perpendicular inter-helical angles between the globular domains. This open conformation exposes a hydrophobic surface within each globular domain and permits binding of protein targets (Babu et al. 1988; Kuboniwa et al. 1995; Zhang et al. 1995).

3.2 *Calmodulin Targets a Large Array of Proteins Including Ion Channels*

The diversity of gene expression and protein localization patterns is important for generating functional diversity and specificity. The temporal and spatial expression patterns of CaMs, like those for CDPKs or any gene family in plants, are diverse. Some CaMs are ubiquitously expressed, whereas expression of others is regulated by various factors including light, mechanical stress, heat/cold shock, wounding, osmotic stress, pathogens, and plant hormones. Certain CaM genes are also developmentally regulated and show tissue- and cell-specific expression patterns. Despite extensive analysis of expression patterns, relevant physiological functions are not known. Some touch-induced genes (*TCH*) encode CaM-related proteins, which are rapidly induced by mechanical manipulation, cold- and heat-shock, phytohormones, and Ca^{2+} itself (Braam et al. 1997). The magnitude and kinetics of mRNA induction differ between the different *TCH* genes (Braam et al. 1997). Extensive work with *TCH3* established that the gene is expressed in the shoot apical meristem, vascular tissue, and root pericycle cells during vegetative growth in Arabidopsis. Following wind stimuli, *TCH3* becomes abundant in branch points of leaf primordia and stipules, pith parenchyma, and vascular tissues, although the functional consequences of this induction are not understood.

As plants can establish specific cellular Ca^{2+} signatures by restricting Ca^{2+} to a specific compartment of the cell (reviewed in (Rudd and Franklin-Tong 2001), the subcellular location of CaMs and other calcium sensors plays a role in decoding "local" calcium signals and is not fixed. A good example for this type of regulation is petunia CaM53 (Rodriguez-Concepcion et al. 1999): Similar to rice OsCaM61, CaM53 contains a polybasic 34-residue C-terminal extension ending with a CaaX-box motif for prenylation. CaM53 prenylation (Caldelari et al. 2001) and processing (Rodriguez-Concepcion et al. 2000) lead to targeting the PM. However, when prenylation is blocked, the polybasic domain targets the protein to the nucleus. A similar prenylation-dependent membrane vs. nuclear localization has been recently reported for OsCaM61 (Dong et al. 2002). Prenylation and PM targeting of CaM53, however, do not depend on calcium binding. The prenylation status of CaM53 is likely an important aspect of its function, as the set of proteins with which CaM53 could potentially interact upon calcium binding is expected to be very different depending on the subcellular localization of the protein.

An important clue for the function of intracellular calcium sensors is the identity of their target proteins. The Ca^{2+}-bound CaM binds and regulates the activity of a wide range of proteins that are not necessarily related in structure. How can Ca^{2+}-CaMs bind to so many different proteins? More specifically, the plasticity of the Ca^{2+}-CaM structure must accommodate the variation in both the molecular size and composition of the target proteins. This issue has been addressed by structural analyses of Ca^{2+}-CaMs and target-bound Ca^{2+}-CaMs. Studies show that the two globular domains of Ca^{2+}-CaM are interconnected by a flexible tether that

can accommodate peptides of varying sizes (Nelson and Chazin 1998). Upon binding a peptide, the two globular domains fold toward each other to form a hydrophobic channel rich in methionine residues that have flexible hydrophobic side chains. In this channel, Ca^{2+}-CaM interacts with peptides mostly through nonspecific van der Waals interactions that form between the exposed hydrophobic domains of Ca^{2+}-CaM and the target peptides, which explains why Ca^{2+}-CaM can bind many target proteins (O'Neil and DeGrado 1990; Osawa et al. 1998; Zhang and Yuan 1998). Together, the structures of CaM illustrate how this class of proteins can function as extremely efficient Ca^{2+} sensors and on/off switches, allowing them to transduce Ca^{2+} signals with high efficiency and accuracy. Different affinities for Ca^{2+}-CaM interactions with specific target proteins may be sufficient for the differential transduction of the Ca^{2+} signal.

The interaction between CaM and CaM-dependent protein kinases in animal cells provides a good model that illustrates how Ca^{2+}-CaM regulates the activity of the target. For example, CaMKII contains an autoinhibitory domain, which occludes the active site in the resting state. Ca^{2+}-CaM binds to a site near or overlapping with the autoinhibitory domain, thereby releasing it from the active site and activating the enzyme (reviewed by Hook and Means 2001). On the basis of the available results, this model also appears to be applicable to interactions between CaMs and their target proteins in plant cells. CaM targets in plants have been extensively reviewed (Snedden and Fromm 1998; Zielinski 1998; Snedden and Fromm 2001; Reddy et al. 2002), and therefore we will only introduce the conceptual framework, using several examples, to explain how CaMs regulate protein target activity in plants.

CaM target proteins can be identified using labeled CaMs to screen expression cDNA libraries (Fromm and Chua 1992). A large number of CaM-binding proteins have been identified from plants. Glutamate decarboxylase (GAD) is one of the best studied (Baum et al. 1993; Baum et al. 1996; Snedden et al. 1996; Zik et al. 1998). The enzyme catalyzes conversion of L-glutamate into gamma-aminobutyric acid (GABA) and is rapidly activated during several stress responses (Snedden and Fromm 1998, 2001). GAD is activated by binding either to a CaM or to a monoclonal antibody that recognizes the CaM-binding domain of GAD. In analogy to Ca^{2+}-CaM−CaMK interaction, binding of Ca^{2+}-CaM to GAD probably relieves the autoinhibitory effect of the CaM-binding domain, as GAD mutants that lack the CaM-binding domain (GAD-C) are constitutively active. Overexpression of GAD-C in transgenic tobacco induced developmental abnormalities associated with increased GABA levels, concomitant with reduced levels of glutamate (Baum et al. 1996). The activation of GAD by environmental stimuli via the Ca^{2+}-CaM signaling system is very rapid, exemplifying the highly cooperative on/off switch of the CaM response (Snedden and Fromm 1998).

Ca^{2+}-ATPases are localized in the endo-membranes or PM and play a key role in removing Ca^{2+} from the cytoplasm to terminate a signaling event, which is critical for Ca^{2+} homeostasis in all eukaryotic cells (reviewed by Sze et al. 2000). Among the Ca^{2+}-ATPases in higher plants, type IIB Ca^{2+}-ATPases are major targets of Ca^{2+}-CaM regulation. Unlike homologs in animal cells, plant type IIB ATPases are

located in both endo-membranes (ER and tonoplast) and the PM (Sze et al. 2000). Ca^{2+}-CaM interacts with type IIB ATPases to activate the pump by releasing an autoinhibitory domain from the active site, similar to the Ca^{2+}-CaM−CaMKII interaction in animals. It is noteworthy that plant Ca^{2+}-ATPases are subject to regulation by CDPKs, as briefly described earlier. Interestingly, while Ca^{2+}-CaM activates the pump, CDPK phosphorylation inhibits the pump, demonstrating the complexity in the regulation of Ca^{2+} signal termination by feedback from two different types of Ca^{2+} sensors (Hwang et al. 2000). Several plant nucleotide-gated ion channels may also be regulated by Ca^{2+}-CaM (Schuurink et al. 1998; Arazi et al. 1999; Kohler et al. 1999; Leng et al. 1999; Arazi et al. 2000). These channel proteins contain six transmembrane domains and a high-affinity CaM-binding site overlapping with a cyclic nucleotide-binding (CNB) domain (Arazi et al. 2000).

Ca^{2+} signaling and the role of CaM in the nucleus are drawing increased interest (Rudd and Franklin-Tong 2001; Snedden and Fromm 2001). CaMs participate in transcriptional regulation either directly by binding to transcription factors (Szymanski et al. 1996) or indirectly by activating kinases or phosphatases that control transcription factor activity (Marechal et al. 1999). Studies in animal cells demonstrated that CaM localization to the nucleus could be facilitated by differential Ca^{2+} oscillations (Craske et al. 1999; Teruel et al. 2000; Teruel and Meyer 2000), suggesting additional and complex levels of transcriptional regulation. As discussed earlier, changing the metabolic status of plant cells induced translocation of CaM53 to the nucleus where it appears to activate specific signaling (Rodriguez-Concepcion et al. 1999). Selective Ca^{2+} signals were measured in the cytoplasm and the nucleus of transgenic plants expressing either cytoplasmic or nuclear forms of the Ca^{2+} reporter protein aequorin (van Der Luit et al. 1999; Pauly et al. 2000). Such Ca^{2+} signals may be required for the expression of specific genes. For example, expression of tobacco *NpCaM1* (but not *NpCaM2*, which encodes an identical CaM protein) in response to wind was stimulated by nuclear Ca^{2+} transients, whereas cold-responsive expression was primarily induced by a cytoplasmic Ca^{2+} transient (van Der Luit et al. 1999). Thus, spatially separated Ca^{2+} signals can also control the function of closely related CaM proteins through the regulation of their genes.

Although many target proteins have been identified for CaMs, relatively little is known about the specific physiological function of each CaM member. Like the situation with CDPKs, functional redundancy may have hindered the genetic analysis of CaM members in model plants such as Arabidopsis.

3.3 Regulation of Cyclic Nucleotide-Gated Channels by CaMs

Although CaMs in plants have been identified for more than two decades, their target ion channels appear to be limited to cyclic nucleotide-gated channels (CNGC). The first plant CNGC channel HvCBT1 (*Hordeum vulgare* CaM-binding

transporter) was cloned when screening for the CaM-binding proteins in barley (Schuurink et al. 1998). Afterward, more CNGC proteins similar to HvCBT1 from several species including *Arabidopsis* (Köhler and Neuhaus 1998; Kohler et al. 1999; Maser et al. 2001), tobacco (Arazi et al. 1999), and rice (Maser et al. 2001) were identified. The protein sequences of CNGCs show similarity to Shaker-like K^+ channels with six transmembrane domains (S1–S6), a pore domain between S5 and S6, and a CNB domain (Demidchik et al. 2002; Talke et al. 2003). In contrast to animal CNGCs, plant CNGCs possess a CaM-binding domain at the C-terminal that overlaps with the CNB domain (Kohler et al. 1999). The CaM binding activities of CNGCs have been confirmed by different research groups (Schuurink et al. 1998; Arazi et al. 2000; Kohler and Neuhaus 2000). The protein structure and functional analyses indicate that CNGC activity is enhanced by binding cyclic nucleotides and inhibited by binding CaM (Arazi et al. 2000; Kohler and Neuhaus 2000; Leng et al. 2002; Balague et al. 2003; Ali et al. 2007). Activities of AtCNGC2 (Hua et al. 2003) and AtCNGC10 (Li et al. 2005) have been demonstrated to be inhibited by CaMs. Several studies showed that plant CNGCs are permeable to monovalent (K^+, Na^+) and/or divalent (Ca^{2+}) cations. For example, AtCNGC2 could conduct K^+ and Ca^{2+} but not Na^+ (Leng et al. 1999; Leng et al. 2002; Ali et al. 2007) and AtCNGC4 is permeable to both K^+ and Na^+ equally (Balague et al. 2003). In addition to their permeabilities to K^+ and/or Na^+, some CNGCs can probably mediate Ca^{2+} influx, and the consequent cytosolic Ca^{2+} elevation may bind and activate CaMs to subsequently regulate CNGC activity. Several CNGCs, such as AtCNGC2, AtCNGC4, AtCNGC11, and AtCNGC12, have been demonstrated to have important roles in plant responses to pathogen infection (Clough et al. 2000; Balague et al. 2003; Yoshioka et al. 2006; Ali et al. 2007). A recent report showed that AtCNGC18 may function as a cation channel and is involved in the polarized tip growth of pollen tubes (Frietsch et al. 2007). Further investigation on physiological functions of Ca^{2+}/CaM modulation of CNGCs as well as other candidates of ion channels *in planta* is expected.

4 The CBL-CIPK Network

4.1 Plant CBLs are Related to Calcineurin B but have Significantly Diverged into a Group of Proteins with New Functions

Earlier studies on calcium signaling implicate a calcineurin-like protein in the signaling processes of ion channel regulation and salt tolerance (Luan et al. 1993; Allen and Sanders 1995; Pardo et al. 1998). Calcineurin is a calcium/calmodulin-dependent protein phosphatase highly conserved in eukaryotes from yeast to mammals (Klee et al. 1998). Like CaM-dependent protein kinase, calcineurin contains a CaM-binding domain in the catalytic subunit (calcineurin A). In addition, another

regulatory subunit (calcineurin B) binds to the catalytic subunit and is required for the activation of the phosphatase. Calcineurin B, like CaM, contains four EF-hand domains, although the overall sequence is not related to CaM. Because calcineurin serves as a critical molecular switch for many cellular processes in eukaryotes from yeast to mammals, it was speculated that similar molecules might also exist in plants. Extensive effort focused on the isolation of calcineurin-like proteins and genes from plants and a family of genes encoding CBLs was eventually identified in Arabidopsis (Kudla et al. 1999). Independently, a genetic analysis of salt mutants identified a gene related to calcineurin B, called SOS3, (Liu and Zhu 1998) and it is a member of the CBL family (also referred to as CBL4). CBLs are encoded by a multigene family of at least ten members in Arabidopsis, which have similar structural domains with small variations in the length of the coding regions (Kudla et al. 1999; Kim et al. 2000; Albrecht et al. 2001; Guo et al. 2001a, b). Their amino acid sequence identity, which ranges from 20–90%, would be sufficient for functional redundancy among the closely related members, while allowing for functional specificity among more diverged members. Unlike CaMs, CBLs as yet have been identified only in higher plants, suggesting that CBLs may function in plant-specific signaling processes. Comparing CaM with CBL proteins, the two families do not show significant similarity in their primary amino acid sequences except for the conserved positions in the EF-hand motifs. In addition to a general sequence difference, CaMs and CBLs also differ in the number of typical EF-hand motifs in their basic structure. Typically, CaMs contain four EF-hands and CBLs contain three canonical EF-hands. Recent studies have solved the 3D structure of two members in the CBL family and in both cases, the fourth "EF hand" appears to diverge into a Mn-binding domain (Nagae et al. 2003; Sanchez-Barrena et al. 2005).

4.2 The CBL-Type Calcium Sensors Target a Family of Protein Kinases–a Shift-of-Paradigm from Calcineurin in Yeast and Animals

As discussed earlier, small calcium sensors function by targeting downstream effectors. Unlike CaMs that interact with a large variety of target proteins, CBLs appear to interact with a single family of protein kinases (Shi et al. 1999). These kinases, referred to as CBL-interacting protein kinases (CIPKs), are most similar to sucrose non-fermenting (SNF) protein kinase from yeast and animals in the kinase domain but retain unique C-terminal regulatory domains. The CBL−CIPK interaction represents a major paradigm shift in calcium signaling as compared to yeast and animals where calcineurin B protein interacts and regulates a protein phosphatase. The CBLs interact with CIPKs through the C-terminal nonkinase domain that contains a conserved region among different CIPK members (Shi et al. 1999; Kim et al. 2000; Albrecht et al. 2001; Guo et al. 2001a, b). Interestingly, interaction between CBL1 and CIPK1 requires micromolar levels of Ca^{2+}. This

Ca^{2+}-dependent interaction is consistent with the general paradigm established for Ca^{2+}-sensor interactions with target proteins in animals (e.g., Ca^{2+}-CaM−CaMKII interaction). Another study (Halfter et al. 2000) using SOS3 (also referred to as CBL4) as a "bait" also identified several interacting protein kinases that belong to the CIPK family. In particular, SOS3 interaction with SOS2 (also called CIPK24) stimulates kinase activity against a peptide substrate, suggesting that SOS3 serves as a regulatory subunit of SOS2. SOS2 and SOS3 were initially identified by a genetic screen for Arabidopsis mutants that are salt-overly-sensitive (reviewed by Zhu 2003).

Regarding the biochemical properties of CIPKs, studies showed that CIPKs have strong substrate specificity with very low activity against generic substrates (Shi et al. 1999). In addition, the CIPK kinase activity prefers Mn^{2+} as a cofactor over Mg^{2+} (Shi et al. 1999). Interaction with CBLs activates the kinase activity of CIPKs. One study suggests that the CBL-interacting domain may serve as an autoinhibitory domain that blocks the kinase active site (like the situation with CDPK or CaMK) (Guo et al. 2001a, b). The CBLs interact with the autoinhibitory domain in CIPKs and by doing so may release the kinase domain for substrate access.

The Arabidopsis genome contains a large number of genes for putative CIPK proteins. At least 25 CIPK genes have been confirmed by cDNA cloning and sequencing (Luan et al. 2002). Further experiments have extended the analysis of CBL−CIPK interactions to the entire family of CBLs and a large fraction of the CIPK family in an effort to determine the functional pairs of CBLs and CIPKs. These studies revealed that each CBL interacts with a subset of CIPKs and each CIPK interacts with one or more CBLs. Some CBLs share common CIPK targets and some CIPKs share common CBL regulatory subunits. Such interaction specificity and overlap among various members in the CBL and CIPK family may well reflect functional specificity and redundancy (Kim et al. 2000; Albrecht et al. 2001; Guo et al. 2001a, b). It must be noted, however, that these interaction studies were performed using mostly the yeast two-hybrid system and therefore may not necessarily represent the physiological situations in plants. In addition to matching the CBLs with their target kinases, the interaction studies further defined the functional domains of CBLs and CIPKs. For example, the CBL-interacting domain in the C-terminal region of CIPKs was localized to a small region of approximately 20 amino acids (Kim et al. 2000; Albrecht et al. 2001; Guo et al. 2001a, b). This domain may be important in kinase regulation by releasing the autoinhibitory domain (Guo et al. 2001a, b).

Besides regulating the activity of the CIPK kinases, certain structural features of CBLs also suggest these Ca^{2+}-sensors can change cellular localization of the CBL-CIPK complexes. Several CBLs have a conserved myristoylation site in their N-terminal region (Liu and Zhu 1998; Kudla et al. 1999; Kim et al. 2000; Albrecht et al. 2001). It would be expected that these CBLs are localized to cell membranes, which could serve as a regulatory mechanism for establishing a local signal cascade similar to the model discussed for CaM53 above. For example, a significant amount of SOS3/CBL4 is always found associated with the membrane fraction and the

myristoylation site is required for the function of the protein (Ishitani et al. 2000). CBL1 and CBL9 are also associated with the membrane (D'Angelo et al. 2006; Xu et al. 2006), and target CIPK1 and CIPK23 to the PM, thereby enabling CIPK phosphorylation of membrane associated protein substrate(s) (see details in later sections). Together, the view emerges that in plants certain calcium sensors (including CDPK, CBL, and CaM) have acquired protein domains that restrict their localization, serving as a mechanism to establish local signal transduction pathways that initiate specific cellular responses.

4.3 Physiological Pathways Involving CBL-CIPK Signaling Modules that Regulate Ion Channels and Transporters

So far, there have been several CBL-CIPK signaling pathways whose physiological functions have been well investigated, and most of these pathways have been identified to regulate the activities of ion channels or transporters.

The interaction pairs CBL4 (SOS3)-CIPK24 (SOS2) and CBL10-CIPK24 (SOS2) have been both confirmed to participate in salt tolerance in *Arabidopsis* (Liu and Zhu 1998; Chinnusamy et al. 2004; Kim et al. 2007; Quan et al. 2007). By genetic screening, the *Arabidopsis* salt-overly-sensitive (SOS) mutants were identified and a "SOS" pathway of plant responses to salt stress was established in *Arabidopsis* roots. CBL4 (SOS3), a PM-located Ca^{2+} sensor, may sense the salt stress induced Ca^{2+} signal in *Arabidopsis* roots (Liu and Zhu 1998; Ishitani et al. 2000), and transduce this signal to its downstream target CIPK24 (SOS2). By interacting with CIPK24 (SOS2), CBL4 (SOS3) recruits CIPK24 (SOS2) to the PM (Quintero et al. 2002) and activates the kinase activities of CIPK24 (SOS2) (Halfter et al. 2000; Guo et al. 2001a, b). The PM-located Na^+/H^+ exchanger SOS1, as the downstream target of the SOS3-SOS2 complex, is phosphorylated by SOS2 and activated (Quintero et al. 2002). As a result, cytosolic Na^+ is transported out of the cell by SOS1 contributing to salt tolerance. In addition, the SOS3-SOS2 complex seems also to prevent Na^+ entry into root cells by inactivating or down-regulating another PM-located Na^+ transporter AtHKT1 (Rus et al. 2001; Zhu 2002).

Two recent independent studies (Kim et al. 2007; Quan et al. 2007) showed that CIPK24 (SOS2) can also interact with CBL10 and regulate *Arabidopsis* responses to salt stress by regulating the activities of Na^+ transporters. Interestingly, the CBL10-CIPK24 (SOS2) complex may target the tonoplast and transport cytoplasmic Na^+ into the vacuole by activating an unknown tonoplast-located Na^+ transporter (Kim et al. 2007). These studies demonstrated that two Ca^{2+} sensors (CBL4 (SOS3) and CBL10) can interact with CIPK24 (SOS2) and regulate activities of Na^+ transporters at PM or tonoplast, respectively. Both these pathways would decrease the cytoplasmic Na^+ load.

The CBL-CIPK signaling pathway also has been demonstrated to be involved in regulation of K^+ acquisition and/or translocation. Using a reverse genetics approach, CIPK23 was identified to regulate K^+-uptake in *Arabidopsis* roots,

particularly under K^+-deficient conditions (Xu et al. 2006). Low-K^+ stress signals may trigger the cytosolic Ca^{2+} elevation and lead to activation of PM-located calcium sensors CBL1 and/or CBL9. The CBL1 and/or CBL9 proteins interact with CIPK23 and recruit CIPK23 to the PM where the K^+ channel AKT1 is phosphorylated (Li et al. 2006; Xu et al. 2006; Cheong et al. 2007). As a result, AKT1 is activated in *Arabidopsis* roots to augment K^+ uptake under low-K^+ conditions. It is noteworthy that the results of K^+ content measurements indicated that, in addition to AKT1, CIPK23 might also regulate other K^+ transporters involved in *Arabidopsis* K^+ uptake (Xu et al. 2006).

As discussed above, each designated CBL-CIPK pair may possess specific physiological functions in plant cells, depending on its subcellular location and the specificity of its downstream target. It appears that CBLs play crucial roles in determining the specific subcellular location of CBL-CIPK complexes by interacting with CIPKs and recruiting CIPKs to the PM or tonoplast (Quintero et al. 2002; Xu et al. 2006; Cheong et al. 2007; Kim et al. 2007; Quan et al. 2007). So far, most identified targets of CBL-CIPK complexes are ion transporters or channels (Rus et al. 2001; Quintero et al. 2002; Zhu 2002; Xu et al. 2006), which suggests particular importance of CBL-CIPK signaling pathways in regulation of ion (particularly K^+ and Na^+) transport and cellular ion homeostasis as well as in plant responses to environmental stimuli.

5 Plant Calcium Signaling Network in Response to Abiotic Stresses

Calcium signaling has been considered as the most important regulatory system in plant cells, particularly for plant responses to various environmental stresses, such as salt, high and low temperatures, drought, and low K^+ (LK) (Ng and McAinsh 2003; White and Broadley 2003). The initial generation and subsequent transduction of cytoplasmic Ca^{2+} signals in response to various environmental stress conditions require numerous molecular components including Ca^{2+}-permeable ion channels, reactive oxygen species (ROS) as well as various Ca^{2+} sensors, which constitute a complex Ca^{2+} signaling network in plant cells (Fig. 1).

The model entails the following processes. When the plants are subjected to a stress signal, the PM-located nonselective cation channels (NSCCs), such as CNGCs, are activated and mediate the Ca^{2+} influx into plant cells (Talke et al. 2003; Donaldson et al. 2004). The elevation of cytoplasmic Ca^{2+} could stimulate NADPH oxidase-mediated production of ROS by means of CaM-regulated NAD kinase (Yang and Poovaiah 2003). Consequently, the increased ROS (such as H_2O_2 and HO^-) may directly activate some NSCCs or other Ca^{2+}–permeable channels, leading to further Ca^{2+} influx (Pei et al. 2000; Demidchik and Maathuis 2007). ROS has been well documented as a signal molecule involved in plant stress responses (Apel and Hirt 2004; Mori and Schroeder 2004; Pitzschke et al. 2006) and is tightly

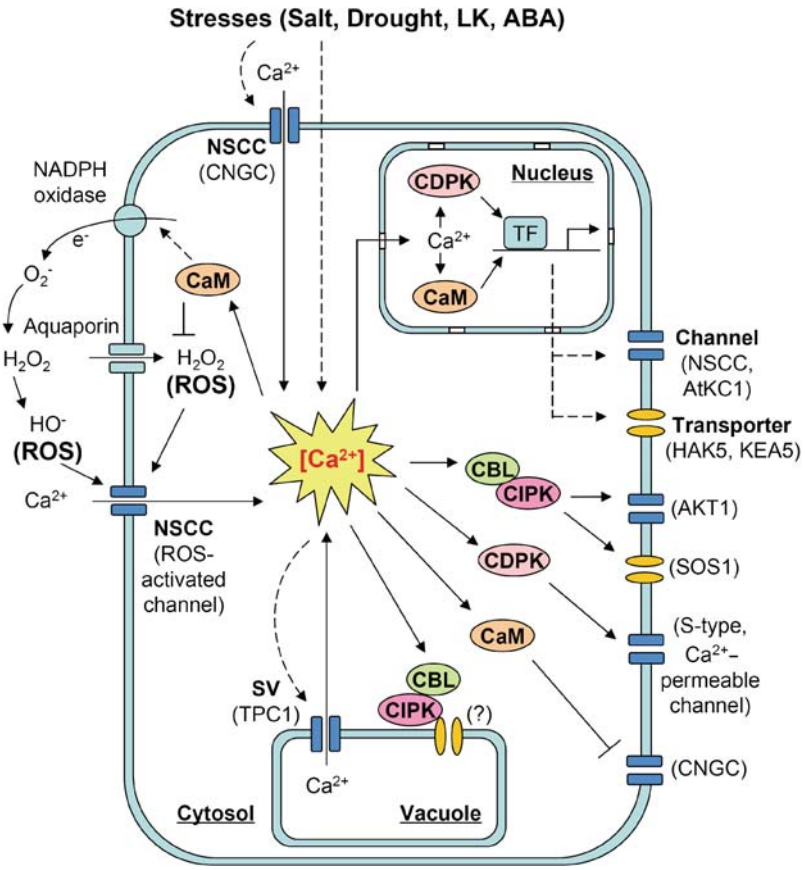

Fig. 1 The schematic model of plant calcium signaling network in response to abiotic stresses (After Demidchik and Maathuis 2007; Kim et al. 2009 with modifications). This model illustrates the generation and transduction of cytoplasmic Ca^{2+} signals integrating with ion channels or transporters in plant cells in responses to various abiotic stresses (salt, drought, nutrient-deficiency, etc.). The details of this working model are described in the text.

linked to the Ca^{2+} signaling pathway by activating Ca^{2+}-permeable ion channels (Pitzschke et al. 2006; Demidchik and Maathuis 2007).

In addition, the increased cytoplasmic Ca^{2+} could also activate the endomembrane NSCCs, such as AtTPC1 (a vacuole-located SV-type channel) that may mediate Ca^{2+} release from vacuole (Peiter et al. 2005). The Ca^{2+} signals generated by the combination of transporters through fluxes from both extracellular and intracellular stores will be patterned with spatio-temporal variations under the different stress conditions (Ng and McAinsh 2003), leading to specific "signatures" that are then recognized and transduced downstream by specific Ca^{2+} sensors (CaMs, CDPKs and CBLs) (Luan et al. 2002; White and Broadley 2003; Luan 2009; Luan et al. 2009).

The growing data show that the Ca^{2+} sensors could interact and regulate downstream target proteins in plant cells via transcriptional and/or posttranslational modulations (White and Broadley 2003; Kim et al. 2009). Here, we summarize the regulations of ion channels or transporters by Ca^{2+} sensors under various abiotic stresses (Fig. 1). In one pathway, the cytoplasmic Ca^{2+} signals are transduced into nucleus, where the nucleus-localized CaMs or CDPKs may sense the signals and regulate transcription factors (Kim et al. 2009). Consequently, the genes encoding ion channels (*CNGCs*, *AtKC1*, *OsAKT1*, *TaAKT1*) or transporters (*AtHAK5*, *AtKEA5*) are transcriptionally regulated (Buschmann et al. 2000; Shin and Schachtman 2004; Fuchs et al. 2005; Ashley et al. 2006; Maathuis 2006). Alternatively, the cytosol-located Ca^{2+} sensors (CaMs, CDPKs and CBLs) are activated by Ca^{2+} signals and regulate the ion channel activities (AKT1, S-type anion channel, Ca^{2+}-permeable channel) or transporter activities (SOS1) at the PM or vacuolar membrane leading to changes in the ion fluxes across these membranes. The overall regulations of ion channels or transporters by Ca^{2+} sensors could affect the ion and osmotic homeostasis in plant cells, which may enhance the tolerance of plants to various abiotic stresses.

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The Role of Cyclic Nucleotide-Gated Channels in Cation Nutrition and Abiotic Stress

Christen Y.L. Yuen and David A. Christopher

Abstract The plant cyclic nucleotide-gated channels (CNGCs) are a large family of ion channels that are regulated by both cyclic nucleotides (CNs) and calmodulin (CaM). CNGCs are generally permeable to a wide range of cations, including the essential macronutrients K^+ and Ca^{2+}, as well as potentially toxic cations such as Na^+ or Pb^{2+}. Several members of the CNGC family have been implicated in the uptake of cations and/or their subsequent distribution across plant organs. Others may participate in plant responses to salinity and abiotic stress by mediating Ca^{2+} signaling. Some CNGCs localize to the plasma membrane (PM) whereas others localize to intracellular membranes such as the tonoplast, and may therefore regulate the sequestration and release of cations among intracellular stores. It thus appears that plants have adapted certain CNGCs for specialized roles in maintaining cellular cation homeostasis.

Abbreviations

AKT	*Arabidopsis* K^+ transporter
AtKC1	*Arabidopsis thaliana* K^+ channel 1
AtNHX7	*A. thaliana* Na^+/H^+ exchanger 7
AtSOS1	*A. thaliana* salt overly sensitive 1
CaM	Calmodulin
CaMBD	Calmodulin-binding domain
CAMP	3′,5′-Cyclic adenyl monophosphate
CGMP	3′,5′-Cyclic guanyl monophosphate
CML	CaM-like

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CN	Cyclic nucleotide
CNBD	Cyclic nucleotide-binding domain
CNGA	Cyclic nucleotide-gated channel subunit type A
CNGB	Cyclic nucleotide-gated channel subunit type B
CNGC	Cyclic nucleotide-gated channel
CNTE	Cyclic nucleotide-dependent thioesterase
DEPC	Diethyl pyrocarbonate
EAG	Ether-a-go-go
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	Endoplasmic reticulum
GLR	Glutamate receptor
GORK	Gated outwardly-rectifying K^+ channel
GUS	β-Glucuronidase
HACC	Hyperpolarization-activated Ca^{2+} channel
HCN	Hyperpolarization-activated cyclic nucleotide-gated
HEK	Human embryonic kidney
HvCBT1	*Hordeum vulgare* calmodulin-binding transporter 1
KAT	K^+ transporter of *Arabidopsis thaliana*
KUP	K^+ uptake transporter
MPSS	Massively parallel signature sequencing
NSCC	Nonselective cation channel
NtCBP4	*Nicotiana tabacum* calmodulin-binding protein 4
PM	Plasma membrane
ROS	Reactive oxygen species
SKOR	Stelar K^+ outward rectifier
TPM	Transcripts per million
VI-NSCC	Voltage-insensitive nonselective cation channel

1 Introduction

Inorganic cations are essential macro- and micronutrients in plants, playing crucial roles in many cellular processes, such as signal transduction, the stabilization of cell walls and membranes, osmoregulation, and the activation of numerous enzymes (Maathuis 2009). Plants have evolved several distinct classes of transporters to facilitate the movement of cations across cellular membranes. Electrophysiological studies have shown that channels permeable to a wide range of cations are present at the plasma membrane (PM) and other organellar membranes of all major plant organs (Demidchik et al. 2002b). These channels are collectively referred to as nonselective cation channels (NSCCs). NSCCs are proposed to facilitate the passive uptake of essential nutrient cations (e.g. K^+ and Ca^{2+}), to deliver cations across tissues or between the cytosol and intracellular compartments such as the vacuole, and to participate in Ca^{2+} signaling pathways critical for stress responses and development (Demidchik and Maathuis 2007).

Many NSCCs discriminate poorly between different monovalent cations, and thus root-expressed NSCCs may serve as a major pathway for the uptake of Na^+ (Davenport and Tester 2000; Maathuis and Sanders 2001; Demidchik and Tester 2002). Although most plants do not require Na^+, it can be beneficial to the growth of many plant species, particularly under conditions of K^+-deficiency (Flowers and Läuchli 1983). Because of their physicochemical similarity, Na^+ can substitute for K^+ as an osmoticum within the vacuole, and may also be able to take the place of K^+ as a counter-ion in long-distance transport in certain plants (Subbarao et al. 2003). However, an excess of Na^+ within the cytosol is toxic to plants, as Na^+ can compete for K^+ binding sites of various proteins that require K^+ for proper activity (Maathuis and Amtmann 1999). Root-expressed NSCCs can also mediate the influx of Cs^+ (Demidchik and Tester 2002), another cation that is deleterious to plant growth because of its ability to interact with K^+ binding sites (Hampton et al. 2004).

Plants possess at least two large gene families encoding for known or putative NSCCs: the glutamate receptor-like (*GLR*) and cyclic nucleotide-gated channels (*CNGC*) (Demidchik et al. 2002b). The genome of *Arabidopsis thaliana* contains twenty *CNGC* genes, which can be divided into five subfamilies, designated by Mäser et al. (2001) as groups I, II, III, IV-A, and IV-B. Each subfamily is represented by multiple CNGC homologs in rice, indicating that their divergence predates the split between monocots and dicots (Bridges et al. 2005). Plant CNGCs are evolutionarily related to (but phylogenetically distinct from) the CNGCs found in animals (Pilot et al. 2003b), and are predicted by modeling studies to assemble into tetrameric channels (Hua et al. 2003a). Each CNGC subunit possesses a centrally-located hydrophobic core region, common to all members of the Shaker ion channel superfamily, as well as overlapping cyclic nucleotide (CN)- and calmodulin (CaM)-binding sites located near the C-terminus (Fig. 1; for additional information on the structure of CNGCs, please refer to Sect. 2 in Chapter "The Function of Cyclic Nucleotide Gated Channels in Biotic Stress").

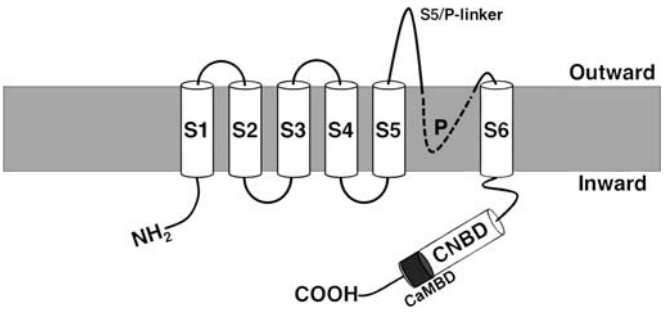


Fig. 1 *Structural features of plant CNGC subunits*. Each CNGC subunit possesses a centrally-located hydrophobic core composed of six membrane-spanning α-helices (S1–S6), with the channel's pore-forming segment (P) situated between S5 and S6. The S5 and P segments are separated by a relatively large linker region. The cytoplasmic C-terminal region harbors partially overlapping cyclic nucleotide-binding and calmodulin-binding domains (CNBD and CaMBD, respectively)

In animals, CNGCs mediate sensory signal transduction in olfactory cells and photoreceptors, and are present in several other neuronal and non-excitable cell types (Kaupp and Seifert 2002). Thus far, eight of the 20 CNGCs in *Arabidopsis* have been ascribed physiological functions. As in the case of animal *CNGC*s, some plant *CNGC*s encode Ca^{2+}-permeable cation channels that participate in signaling pathways (Talke et al. 2003). These include AtCNGC2 and AtCNGC4, which are essential for activation of the hypersensitive response in plants (Clough et al. 2000; Balagué et al. 2003), AtCNGC11 and AtCNGC12, which stimulate the pathogen defense response signaling pathway (Yoshioka et al. 2006), and AtCNGC18, which is required for the proper elongation of pollen tubes (Chang et al. 2007; Frietsch et al. 2007). However, there is a growing body of evidence that some CNGCs directly mediate cation uptake and contribute to maintain homeostasis by regulating the passive exchange of cations between the cell and its extracellular environment (Sunkar et al. 2000; Li et al. 2005; Gobert et al. 2006; Ma et al. 2006; Guo et al. 2008). In this chapter, we discuss the role of CNGCs in plant nutrition and abiotic stress in relation to their ability to transport both essential and toxic cations, and as components of Ca^{2+} signaling.

2 Molecular Characteristics of Plant CNGCs

2.1 Transport of Monovalent and Divalent Cations

Shaker-like K^+ channels contain a characteristic motif, GYGD, which is situated at the narrowest part of the ion-conducting pore and functions as an ion selectivity filter, enabling the selective permeation of K^+ over Na^+ (Doyle et al. 1998; Long et al. 2005). The pore domains of plant CNGCs are structurally similar to that of K^+-selective channels, but do not share the GYGD selectivity filter sequence. Instead, the most common motif found at the analogous position of *Arabidopsis* CNGCs is GQNL, which is present in all members belonging to subfamily I, as well as several members of subfamily III (Table 1). Two paralogs harboring this motif, AtCNGC1 and AtCNGC3, are capable of transporting both K^+ and Na^+ in heterologous systems (Hua et al. 2003a; Gobert et al. 2006). In addition, *AtCNGC10* antisense lines exhibit alterations in the K^+ and Na^+ content within roots and shoots (Li et al. 2005; Guo et al. 2008), suggesting that AtCNGC10 channels also mediate K^+ and Na^+ transport. Thus, channels sharing this selectivity filter sequence generally are not K^+-selective. AtCNGC4, which possesses the unique pore motif GN–L, also is permeable to both K^+ and Na^+ (Balagué et al. 2003). By contrast, AtCNGC2 preferentially conducts K^+ over other alkali metal cations (Cs^+, Li^+, and Rb^+), and does not transport Na^+ (Leng et al. 2002). Its novel selectivity filter sequence, ANDL, has been demonstrated experimentally to be responsible for AtCNGC2's ability to discriminate between K^+ and Na^+ (Hua et al. 2003a).

Table 1 Molecular Properties of *Arabidopsis* CNGCs

Name	Pore	Permeability	Voltage	cNMPs	CaM	References
Group I						
AtCNGC1	GQNL	Ca^{2+}, K^+, Na^+	Inward-rectifying	Activated by cAMP	Interacts with CaM2 and CaM4; Inactivated by yeast CaM	Köhler and Neuhaus 2000, Leng et al. 2002, Hua et al. 2003a, Ali et al. 2006
AtCNGC3	GQNL	K^+, Na^+ (not Ca^{2+})				Gobert et al. 2006
AtCNGC10	GQNL	K^+	Possibly non-rectifying	Activated by cGMP	Partially inactivation by AtCaM2	Li et al. 2005, Christopher et al. 2007
AtCNGC11	GQNL	Ca^{2+}, K^+		Activated by cAMP (not cGMP)		Yoshioka et al. 2006, Urquhart et al. 2007
AtCNGC12	GQNL	Ca^{2+}, K^+		Activated by cAMP (not cGMP)		Yoshioka et al. 2006, Urquhart et al. 2007
AtCNGC13	GQNL					
Group II						
AtCNGC5,6,7,8,9	GQGL					
Group III						
AtCNGC14,15,17	GQNL					
AtCNGC16	GQSL					
AtCNGC18	GQNI	Ca^{2+}				Frietsch et al. 2007
Group IV-A						
AtCNGC19	AGNL					
AtCNGC20	AGNQ					
Group IV-B						
AtCNGC2	ANDL	$K^+ > Cs^+ \approx Li^+ \approx Rb^+$; also Ca^{2+}, but not Na^+. External Ca^{2+} blocks K^+ permeation	Inward-rectifying	Activated by cAMP and cGMP	Inactivated by CaM4; also interacts with CaM2	Leng et al. 1999, Köhler and Neuhaus 2000, Leng et al. 2002, Hua et al. 2003a, b
AtCNGC4	GN–L	K^+, Na^+. Blocked by Cs^+	Weakly outward-rectifying	Activation by cGMP > cAMP		Balagué et al. 2003

The CNGCs are sorted on the basis of their phylogenetic subgroup, according to the convention used by Mäser et al. (2001). The pore motif sequences provided in the 2nd column coincide with the position of the GYGD motif of AtAKT1, based on CLUSTAL W amino acid sequence alignment

Many, but not all, plant CNGCs are capable of efficiently transporting Ca^{2+}, as indicated by the ability of AtCNGC1, AtCNGC11, and AtCNGC12 to rescue Ca^{2+}-uptake deficient yeast mutants (*cch1 mid1*), whereas AtCNGC3 cannot (Ali et al. 2006; Gobert et al. 2006; Urquhart et al. 2007). As the pore regions of these four CNGCs all share the same selectivity filter motif (GQNL), other sequences must account for their differences in Ca^{2+} permeability. In the case of animal CNGCs, the S5 and S6 transmembrane segments and the S5/P-linker region all influence Ca^{2+} permeation (Seifert et al. 1999). Some animal CNGCs have a high affinity for Ca^{2+}; the longer retention of Ca^{2+} ions within their pores (relative to low Ca^{2+}-affinity CNGCs) blocks the permeation of monovalent cations (Frings 1999). Electrophysiological studies indicate that Ca^{2+} blocks the permeation of K^+ through the pore of AtCNGC2, perhaps via a similar mechanism (Leng et al. 2002). Although AtCNGC2 does not transport Na^+, it is conceivable that Ca^{2+} may impede other (Na^+-permeable) CNGCs. In this regard, it is interesting to note that external Ca^{2+} partially inhibits the influx of Na^+ via NSCCs in root protoplasts, which may provide an explanation for the ameliorative effect of Ca^{2+} on salt stress (Demidchik and Tester 2002).

Unlike their animal homologs, which display very little voltage dependence (Kaupp and Seifert 2002), certain plant CNGCs are reportedly voltage-sensitive. AtCNGC1, its tobacco ortholog NtCBP4, and AtCNGC2 all appear to be strictly inward-rectifying channels (Leng et al. 2002). *AtCNGC2* probably encodes for the CN-dependent hyperpolarization-activated Ca^{2+} channel (HACC) detected in *Arabidopsis* guard cells (Ali et al. 2007). HACCs are have also been detected in both trichoblast and atrichoblast cells of the *Arabidopsis* root epidermis (Véry and Davies 2000; Demidchik et al. 2002a), though it remains to be determined if these are also related to inward-rectifying plant CNGCs. Two other members of the CNGC family, AtCNGC4 (Balagué et al. 2003) and AtCNGC10 (Christopher et al. 2007), conduct both inward and outward currents, with the former being weakly outward-rectifying. These could be related to voltage-insensitive, or weakly voltage-sensitive, NSCCs (VI-NSCCs) present at the PM of *Arabidopsis* root and leaf cells (Maathuis and Sanders 2001; Shabala et al. 2006). VI-NSCCs are universally blocked by Gd^{3+} and La^{3+}, but vary in their sensitivities to low pH, high external Ca^{2+}, CNs (cAMP or cGMP), quinine, and the amino acid modifier DEPC, indicating that VI-NSCC currents in roots are mediated by more than one type of channel (Maathuis and Sanders 2001; Demidchik and Tester 2002). In addition to mediating K^+ and Na^+ influx, VI-NSCCs are implicated in the efflux of K^+ from root and leaf cells in response to salt stress (Shabala et al. 2006). Some VI-NSCCs are also permeable to Ca^{2+} and are inferred to be essential for Ca^{2+} uptake, as treatment of intact *Arabidopsis* roots with the potent VI-NSCC and HACC blocker, Gd^{3+}, strongly inhibits Ca^{2+} accumulation, whereas the HACC-specific blocker verapamil does not (Demidchik et al. 2002a).

Both reactive oxygen species (ROS) and nitric oxide (NO) are produced during abiotic and biotic stress (Lamattina et al. 2003; Apel and Hirt 2004). Interestingly, a subpopulation of root VI-NSCCs are activated by hydroxyl radicals ($OH^{\bullet}$), leading to increased Ca^{2+} influx into the cytosol (Demidchik et al. 2003). The

pharmacological profiles of these channels suggest that they are distinct from the VI-NSCCs involved in toxic Na^+ influx. An increase in cytosolic Ca^{2+} levels can also be induced in guard cells by NO; this occurs via the release of Ca^{2+} from intracellular stores, and is blocked by agonists of guanylate cyclase, indicating that the process is cGMP-dependent (Garcia-Mata et al. 2003). It is unclear if NO directly activates Ca^{2+}-permeable channels, or influences their activity through a cGMP-dependent cascade. NO may also induce Ca^{2+} release through a cGMP-independent mechanism (Durner et al. 1998). To our knowledge, no data have yet been reported concerning the effects of ROS and NO on plant CNGC activity; however, NO has been shown to directly activate the olfactory CNGC of rats (Broillet 2000).

2.2 Regulation by CN Monophosphates

CN second messengers (cAMP and cGMP) have been implicated in a wide range of physiological processes in plants, including phytochrome-mediated gene repression, cell cycle progression, adventitious root formation, pollen tube growth, production of the plant antibiotic phytoalexin, and regulation of ion transport (Newton and Smith 2004). Most of the putative direct targets for CNs in *Arabidopsis* are cation transporters harboring an evolutionarily conserved CN-binding domain (CNBD). These include the twenty CNGCs, the nine Shaker-like K^+ channels, and the putative Na^+/H^+ antiporter AtNHX7/SOS1 (Maathuis 2006). Two putative thioesterases, AtCNTE1 and AtCNTE2, also possess a CNBD (Bridges et al. 2005). Molecular characterization of plant CNGCs has revealed that some CNGCs are preferentially activated by cAMP over cGMP (Yoshioka et al. 2006), and vice versa (Balagué et al. 2003). Thus, different sets of CNGCs may be regulated by separate CN signaling pathways.

Several lines of evidence indicate that CNs are involved in plant responses to abiotic stresses. The cGMP content of *Arabidopsis* seedlings rapidly rises ($\leq$ 5 s) after the onset of salt or osmotic stress (Donaldson et al. 2004), and salt-stressed seedlings supplied with exogenous membrane-permeable analogs of cAMP or cGMP exhibit improved growth characteristics and accumulate less Na^+ (Maathuis and Sanders 2001). One mechanism through which CNs may mediate plant responses to salt stress is through the activation or deactivation of cation transporters. Indeed, it has been demonstrated that a subset of root-expressed Na^+-permeable VI-NSCCs are deactivated when cAMPs or cGMPs are supplied to the cytoplasmic side of the PM (Maathuis and Sanders 2001). It is presently unclear if these CN-deactivated VI-NSCCs are related to CNGCs, as all CNGCs analyzed to date are CN-activated (Table 1). However, the regulatory properties of many *Arabidopsis* CNGCs are still unknown, and it remains possible that some are repressed by CNs.

As many CNGCs are permeable to Ca^{2+}, they may also influence abiotic stress by mediating Ca^{2+} signaling. An influx of Ca^{2+} to the cytosol is elicited by cAMP treatment in cultured carrot cells (Kurosaki 1997). Similarly, treatment of tobacco

protoplasts with cAMP or cGMP triggers an elevation in cytosolic Ca^{2+} levels that can be inhibited by verapamil, a known blocker of HACCs (Volotovski et al. 1998). It is interesting to note that the transient increase in cytosolic Ca^{2+} that occurs in response to salt stress is partially suppressed by an inhibitor of guanylyl cyclases, suggesting the possible involvement of CN-regulated Ca^{2+} channels, such as CNGCs (Donaldson et al. 2004).

2.3 Regulation by Calmodulin

Developmental and environmental cues, including biotic and abiotic stresses, elicit Ca^{2+} signals. A key transducer of these signals is the Ca^{2+}-binding protein, calmodulin (CaM). Though lacking inherent enzymatic activity, CaM binds to and modulates the activities of a diverse range of proteins (Snedden and Fromm 1998). The ability of CaM to interact with target proteins is dependent upon conformational changes that occur when it binds Ca^{2+} (White and Broadley 2003). The CaM-binding domain of plant CNGCs is delimited to a stretch of 23–24 amino acids located at the C-terminus, coinciding with the terminal α-helix (αC) of the CNBD (Arazi et al. 2000; Hua et al. 2003b). It is hypothesized that the overlapping nature of the two domains allows CaM to influence CNGC activity by weakening or impairing its interaction with CNs (Köhler et al. 1999; Arazi et al. 2000). Indeed, an antagonistic relationship between CaM and CNs in modulating CNGC activity has been described in two instances. Using the HEK cell expression system, Hua et al. (2003b) demonstrated that recombinant AtCaM4 reversed the ability of cAMP to activate AtCNGC2 currents in a time-dependent manner. Similarly, our lab has observed that co-expression of AtCaM2 with AtCNGC10 partially suppresses the ability of AtCNGC10 to complement the K^+ uptake-deficient *E. coli* strain LB650 (*trkG trkH*); this effect was reversed by the addition of the membrane-permeable CN analog, 8-Br-cGMP (Li et al. 2005). In both studies, the CNGC channels appeared to interact specifically with the Ca^{2+}-bound conformation of CaM, as the effects of CaM were abolished in the presence of the Ca^{2+}-chelating agent, EGTA. A similar requirement of Ca^{2+} for interaction with CaM has been reported for AtCNGC1, 3–6, and 9 (Reddy et al. 2002).

The *Arabidopsis* genome contains seven highly-conserved calmodulin (*CAM*) genes, encoding four distinct isoforms (CaM1/4, CaM2/3/5, CaM6, and CaM7), and 50 less-conserved *CAM*-like (*CML*) genes (McCormack and Braam 2003). Although several CaM/CML proteins interacted with multiple binding partners in protein microarray experiments, the majority of target proteins interacted specifically with only one or a few CaM/CMLs *in vivo*, indicating that many CaM/CMLs operate through distinct sets of target proteins (Popescu et al. 2007). Several *CAM* and *CML* genes are upregulated in response to external stimuli, such as mechanical stimulation (*CAM2*, *CML12*, and *CML24*: Braam and Davis 1990) or salt stress (*CML9*: Magnan et al. 2008; *CML37* and *CML39*: McCormack et al. 2005). Increased *CAM* transcript levels are also elicited in response to salt stress in tomato

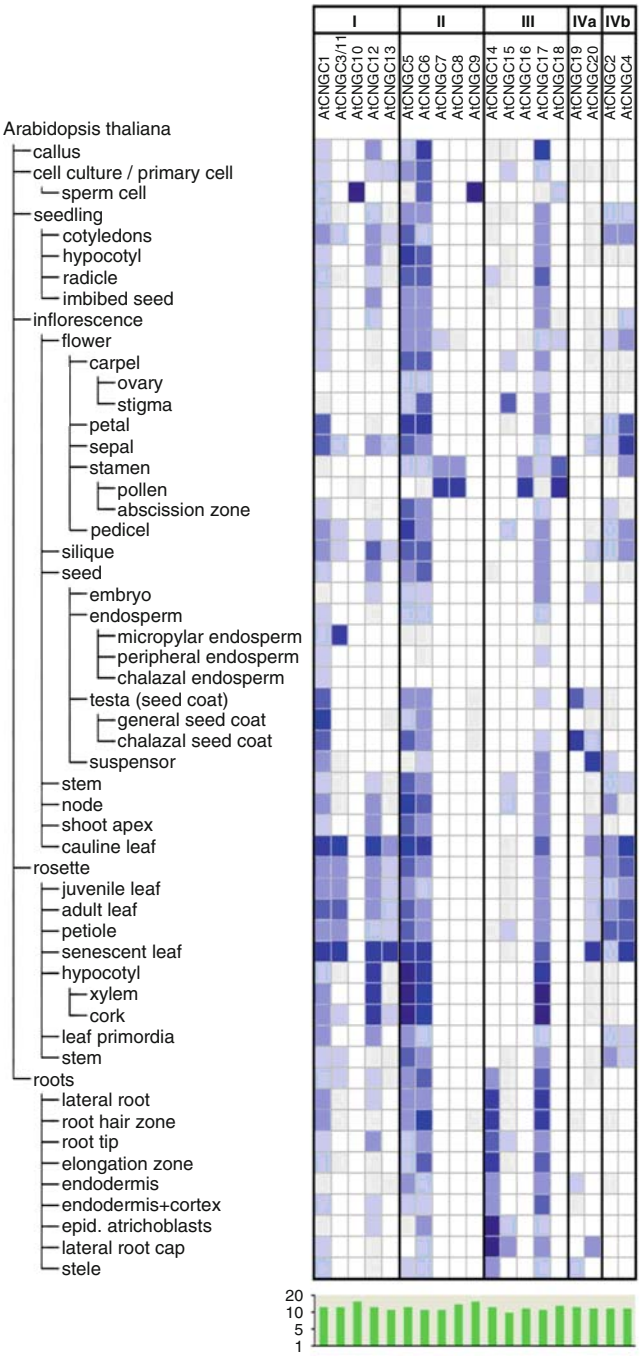
(Delumeau et al. 2002). There is currently very limited information concerning the relative affinities of plant CNGCs for different CaM/CML proteins (Table 1). It would be interesting to see if any of the stress-induced CaM or CML proteins interact with CNGCs to generate physiological responses to environmental stresses.

3 CNGC Expression and Subcellular Localization

3.1 Tissue-Specific Expression Patterns

Characterization of the expression patterns of *CNGC* genes may provide insight into their possible functions. For example, *CNGC* genes active in root hairs may contribute to the acquisition of nutrient cations from the soil, while those expressed in vascular tissues may mediate long-distance cation transport. The expression profiles of *Arabidopsis CNGC* genes, derived from Genevestigator analysis of publicly available microarray datasets, are shown in Fig. 2. The results indicate that multiple paralogs are transcriptionally active in nearly all plant tissues, and are generally consistent with massively parallel signature sequencing (MPSS) data reported earlier by Talke et al. (2003). Note that although both MPSS and microarray data indicate that *AtCNGC9* and *AtCNGC10* are significantly expressed in roots and shoots, this is not reflected in the heat maps of Fig. 2 because the signal values for their expression in pollen sperm cells are an order of magnitude higher than in other tissues. The relative transcriptional activity in sperm cells may be overestimated, as the normalization methods for datasets in the Genevestigator database may be inadequate for pollen (Becker and Feijó 2007).

On the basis of MPSS data, the most highly expressed paralog in roots is *AtCNGC10*, which has a normalized transcript abundance value of 122 TPM (transcripts per million); by comparison, *AtAKT1*, which encodes the principle inward-rectifying K^+-selective channel found in roots (Hirsch et al. 1998), has an abundance value of 174 TPM. Immunoelectron microscopy has shown that AtCNGC10 is present within multiple cells types of the root apex, including meristematic, and peripheral and columella root cap cells (Christopher et al. 2007). Labeling by AtCNGC10 antibodies was also observed in stele cells, albeit at much lower frequency. *AtCNGC1*, *AtCNGC2*, *AtCNGC3*, and *AtCNGC6* are moderately expressed in roots (20–50 TPM; Talke et al. 2003). Promoter-GUS transcriptional fusion studies have shown that *AtCNGC3* is primarily expressed in the cortical and epidermal cells of the root mature zone, including root hairs (a primary site of ion uptake), but does not appear to be expressed within endodermal or stele cells, or at the root tip (Gobert et al. 2006). The expression patterns of *AtCNGC1*, *AtCNGC2*, and *AtCNGC6* in roots have not been characterized in detail, but available microarray data suggest that *AtCNGC1* and *AtCNGC6* are active at the root tip, elongation, and mature (root hair) zones, while *AtCNGC2* expression is restricted to the mature zone (Fig. 2). Notably, of the five most highly-expressed *CNGC* genes in roots, three (*AtCNGC1*,


I
II
III
IVa
IVb
AtCNGC1
AtCNGC3/11
AtCNGC10
AtCNGC12
AtCNGC13
AtCNGC5
AtCNGC6
AtCNGC7
AtCNGC8
AtCNGC9
AtCNGC14
AtCNGC15
AtCNGC16
AtCNGC17
AtCNGC18
AtCNGC19
AtCNGC20
AtCNGC2
AtCNGC4
Arabidopsis thaliana
callus
cell culture / primary cell
sperm cell
seedling
cotyledons
hypocotyl
radicle
imbibed seed
inflorescence
flower
carpel
ovary
stigma
petal
sepal
stamen
pollen
abscission zone
pedicel
silique
seed
embryo
endosperm
micropylar endosperm
peripheral endosperm
chalazal endosperm
testa (seed coat)
general seed coat
chalazal seed coat
suspensor
stem
node
shoot apex
cauline leaf
rosette
juvenile leaf
adult leaf
petiole
senescent leaf
hypocotyl
xylem
cork
leaf primordia
stem
roots
lateral root
root hair zone
root tip
elongation zone
endodermis
endodermis+cortex
epid. atrichoblasts
lateral root cap
stele
20
10
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1

AtCNGC3, and *AtCNGC10*) have loss-of-function phenotypes associated with altered cation accumulation, and one (*AtCNGC2*) is hypersensitive to growth on media containing high concentrations of Ca^{2+} (see Sect. 4 in Chapter "New Approaches to Study the Role of Ion Channels in Stress Induced Signaling; Measuring Calcium Permeation in Plant Cells and Organelles Using Optical and Electrophysiological Techniques"). It is not known whether *AtCNGC6* and other, less prominently-expressed CNGCs also play roles in cation nutrition and homeostasis, or instead regulate other aspects of root biology. Several paralogs are transcribed in the root stele, perhaps indicating a role in long-distance transport.

AtCNGC3 and *AtCNGC10* are also significantly expressed in shoots. In mature rosettes, *AtCNGC3* is predominantly expressed in leaf veins, and may therefore be involved in the distribution and/or unloading of cations transported through the xylem (Gobert et al. 2006). *AtCNGC3* is also transcriptionally active in the cotyledons of mature embryos from ungerminated seeds. In contrast to the vasculature-predominant expression of *AtCNGC3* in leaves, immunolabeling studies have detected AtCNGC10 at the PM of spongy mesophyll, palisade parenchyma, and xylem cells (Christopher et al. 2007). The mesophyll and parenchyma cells of *AtCNGC10* antisense plants are smaller (Borsics et al. 2007), demonstrating the importance of AtCNGC10 in these cell types. As noted earlier, *AtCNGC10* is also strongly active in sperm cells. Its role in sperm physiology has not yet been defined, but the relative scarcity of T-DNA insertions within *AtCNGC10* (Borsics et al. 2007) may indicate that disruption of this gene causes gametophyte lethality. Interestingly, although microarray and MPSS data indicate that *AtCNGC1* mRNA is abundant in both roots and shoots, AtCNGC1 protein was only detected in microsomal membranes of roots, but not leaves (Ma et al. 2006).

3.2 Responses to Abiotic Stress

Plants use several strategies to cope with inadequate nutrient availability or excessive concentrations of toxic ions within their environment. One strategy is to alter the expression of genes relevant to ion transport. Using an *Arabidopsis* transporter-specific gene array, Maathuis et al. (2003) analyzed the root expression patterns of multiple transporter families in response to nutrient (K^+ and Ca^{2+}) deprivation and salt (Na^+) stress. The effects of salt stress on the expression of transporter genes were further characterized subsequently by Maathuis (2006). These studies

Fig. 2 *Tissue-specific expression patterns of plant CNGC*s. The Genevestigator software suite was used to mine publicly-released *Arabidopsis* ATH1 microarray datasets. The results are depicted as a heat map, where dark blue indicates a relatively high expression level within a particular tissue type, and progressively lighter shades of blue indicate lower levels of expression for a given gene. The green bars below the chart indicate the log2 of the expression potential for each gene (i.e. the maximum expression intensity for each gene observed across all data sets). *AtCNGC3* and *AtCNGC11* are recognized by the same non-unique probe set, and have thus been combined as AtCNGC3/11

revealed that Ca^{2+} starvation and Na^+ stress affected a large number of genes, including several *CNGC*s. However, K^+ deficiency modulated relatively few genes in roots. In the experimental conditions used by Maathuis et al. (2003), the abundance of K^+ in roots was not substantially affected after 4 days starvation, whereas shoot K^+ levels decreased by ~30%. By contrast, Pilot et al. (2003a) observed that the K^+ contents of roots were ~50% lower after K^+ deprivation over a similar period. Differences in media composition (e.g. the presence of NH_4^+) may be responsible for the variance in root K^+ levels (Maathuis et al. 2003).

To supplement earlier transcriptomics analysis, we utilized Genevestigator to characterize *CNGC* expression in response to several additional types of abiotic stress (Fig. 3). *AtCNGC9* was the only CNGC regulated by K^+ starvation in roots; mRNA levels decreased 5-fold 24–96 h after the onset of deprivation (supplemental data in Maathuis et al. 2003). However, no difference in *AtCNGC9* root expression was observed in a separate experiment (Fig. 3). These differences could be due to experimental methodology or a high level of variability inherent to transcriptomics analyzes (Maathuis 2006). Prolonged K^+ starvation deceased *AtCNGC8*, *AtCNGC17*, and *AtCNGC19* mRNA levels by at least four-fold in shoots (Fig. 3). Cs^+ also strongly represses the expression of these three genes, which is consistent with Cs^+ toxicity due to interfering with the transport, accumulation and biological activity of K^+ (Hampton et al. 2004). The expression of several *CNGC* genes was altered two-fold or more after Ca^{2+} starvation, with *AtCNGC8*, *AtCNGC9*, and *AtCNGC17* being downregulated, and *AtCNGC12*, *AtCNGC19*, and *AtCNGC20* upregulated (supplemental data in Maathuis et al. 2003). With the exception of *AtCNGC12*, which plays a role in disease resistance (Yoshioka et al. 2006), the physiological functions of the genes affected by K^+ and/or Ca^{2+} stress have not been determined. Interestingly, *AtCNGC8* expression, which appears to be pollen-specific (Fig. 2), is downregulated in response to Na^+ stress (Maathuis 2006), and upregulated in response to nitrate or phosphate deficiency (Fig. 3). The availability of phosphate has been shown to influence pollen production and morphology in some plants (Lau and Stephenson 1994).

Putative NSCCs, such as CNGCs, are speculated to transport toxic levels of Na^+ into the root. Surprisingly, however, Na^+ stress only affects transcription of a few *CNGC* genes. The mRNA levels for *AtCNGC1*, *AtCNGC19*, and *AtCNGC20* increased in roots in response to salinity, while *AtCNGC3*, *AtCNGC8*, and *AtCNGC19* transcript levels were reduced in shoots (Maathuis 2006). As *AtCNGC3* is mainly expressed in the leaf vasculature, reduced activity of this gene may serve to limit the accumulation of Na^+ in leaves. The significance of *AtCNGC19* and *AtCNGC20* upregulation in response to Na^+ treatment is currently unclear, as *Atcngc19* and *Atcngc20* single mutants do not exhibit altered sensitivity to salt stress (Maathuis 2006). The two genes belong to their own *CNGC* subgroup (IV-A) in *Arabidopsis*, and encode for highly similar products (~75% sequence identity); thus, the lack of an apparent phenotype may be due to functional redundancy. *AtCNGC19* also exhibits increased expression in response to several pathogens and osmotic/drought stress (Fig. 3), and may therefore have a common function in both biotic and abiotic stress pathways.

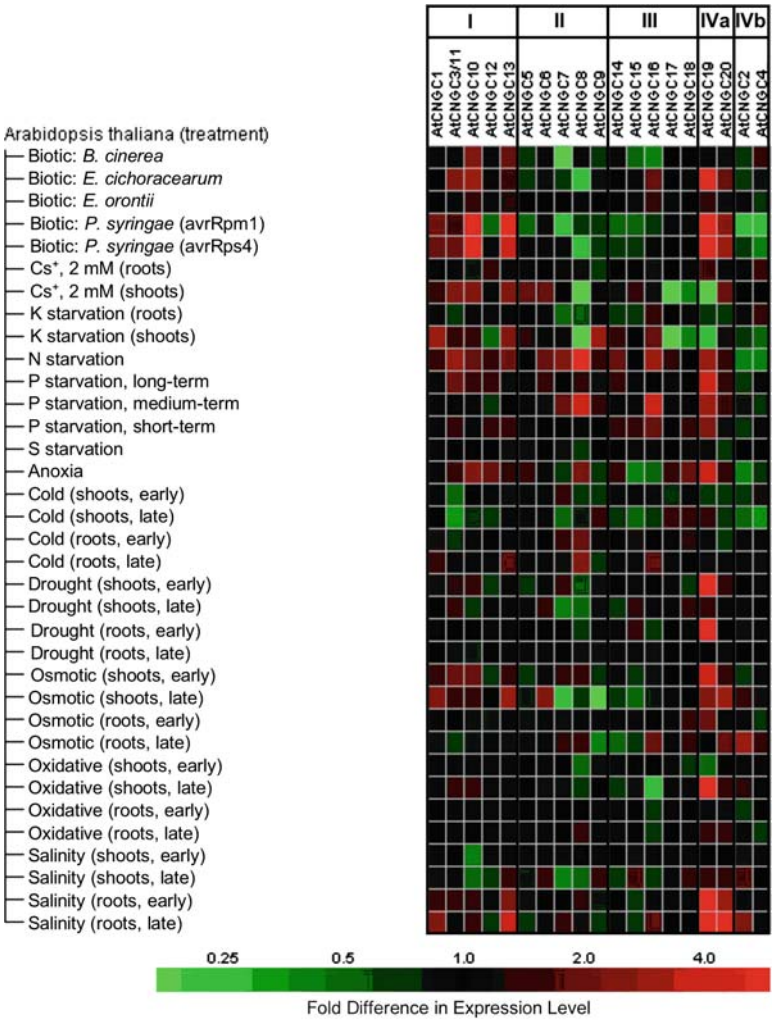

Fig. 3 *Effects of biotic and abiotic stresses on CNGC expression*. Genevestigator was used to analyze the effects of different stresses on *CNGC* expression levels. The results are depicted as a heat map. Red indicates increased expression relative to control samples, while green indicates decreased expression

3.3 Subcellular Localization

Translational reporter fusions have been utilized in several instances to define the subcellular localization patterns of plant CNGCs. For example, translational fusions to GFP were used to demonstrate that the barley homolog HvCBT1 localizes to the PM in aleurone protoplasts (Schuurink et al. 1998), and that AtCNGC3,

AtCNGC10, AtCNGC11, and AtCNGC12 localize to the PM when expressed in *Arabidopsis* leaf protoplasts (Gobert et al. 2006; Christopher et al. 2007; Baxter et al. 2008). In most cases, the chimeric CNGC proteins were evenly distributed throughout the PM of transfected protoplasts, although HvCBT1-GFP aggregated into numerous discrete patches, which may coincide with specialized functional domains in the PM (Schuurink et al. 1998). The tobacco homolog, NtCBP4, was also shown by immunoblotting to associate with the PM fraction (Arazi et al. 1999). All of these CNGCs belong to subgroup I.

There are four pollen-specific *Arabidopsis* CNGCs: AtCNGC7 and 8 (group II), and AtCNGC16 and 18 (group III) (Fig. 2). YFP-tagged versions of AtCNGC7 and 8 localize to the tonoplast, while AtCNGC16-YFP appears to be targeted to an intracellular membrane different from that labeled by AtCNGC7-YFP (Chang et al. 2007). Therefore, some CNGCs may mediate the exchange of cations between the cytosol and intracellular compartments such as the vacuole and the ER, which is consistent with experiments by Volotovski et al. (1998) demonstrating that a significant portion of the Ca^{2+} released into the cytosol in response to CN treatment originates from intracellular stores. The only pollen-specific CNGC localizing to the PM is AtCNGC18, which is targeted specifically to the apex of pollen tubes (Chang et al. 2007; Frietsch et al. 2007). The essential role of AtCNGC18 in pollen tube elongation raises the possibility that CNGCs may similarly regulate the expansion of root hairs, which also elongate via tip growth. It will be interesting to determine if other CNGCs exhibit asymmetrical subcellular localization patterns, leading to polar ion fluxes within cells, and perhaps across plant tissues.

An alternative method that has recently been utilized to define the subcellular distribution patterns of CNGCs is immunoelectron microscopy (Christopher et al. 2007). This approach enables the analysis of protein localization at a much higher resolution than currently possible by light or confocal microscopy. For example, immunoelectron microscopy has revealed that AtCNGC10 is present in the PM (which confirmed the localization of the AtCNGC10-GFP fusion), and is also detected within the ER, Golgi, trans-Golgi vesicles, and cell plate membranes (Christopher et al. 2007). These localizations likely define steps in the trafficking pathway of AtCNGC10 from initial synthesis to final integration in the PM and to the expanding cell plate by way of Golgi-derived vesicles (Nebenführ et al. 2000).

4 Physiological Roles in Plant Nutrition

4.1 Cation Uptake and Homeostasis

Classical studies on the absorption of cations by intact barley roots have shown that plants possess at least two distinct mechanisms of K^+ uptake: a high-affinity system (K_M = 0.021 mM) that preferentially transports K^+ over Na^+ and a low-affinity system (K_M = 11.4 mM) that is capable of transporting both monovalent cations

(Epstein et al. 1963). On the basis of loss-of-function studies, at least three CNGCs contribute to the nonselective uptake of monovalent cations in *Arabidopsis*: AtCNGC1, AtCNGC3, and AtCNGC10. The *Atcngc1* T-DNA insertion mutant accumulates less Ca^{2+} and K^+ in shoot tissues than wild type, and is less sensitive to toxic concentrations of Na^+ (Hampton et al. 2004; Ma et al. 2006; Maathuis 2006). Given that AtCNGC1 protein is predominantly located in roots (Ma et al. 2006), and that heterologously-expressed AtCNGC1 channels are inward-rectifying for K^+, Na^+, and Ca^{2+} (Leng et al. 2002; Ali et al. 2006), these phenotypes can be explained by the *Atcngc1* being deficient in the uptake of all three cations. The relatively modest decrease in Ca^{2+} content within *Atcngc1* shoots (6–22% lower), is suggestive of additional Ca^{2+} uptake pathways in *Arabidopsis* (Ma et al. 2006). Consistent with evidence that entry of toxic Pb^{2+} into root cells occurs at least in part via Ca^{2+}-permeable channels (Huang and Cunningham 1996), *Atcngc1* mutants have a higher tolerance to Pb^{2+}, and accumulate lower amounts of Pb^{2+} than wild-type plants (Sunkar et al. 2000).

As for *Atcngc1*, T-DNA insertion mutants of *AtCNGC3* are more resistant to Na^+ stress, albeit at a restricted concentration range (40–80 mM; Gobert et al. 2006). The similar sensitivities of *Atcngc3* and wild-type plants at higher Na^+ concentrations may be due to the downregulation of AtCNGC3 activity by wild-type plants under extreme salt stress. Indeed, a decrease in *AtCNGC3* expression in shoots during salt stress was reported (Maathuis 2006). The Na^+ content of *Atcngc3* shoots (but not roots) is ~30% lower than that of wild type when grown on media containing 80 mM NaCl (Maathuis 2006). The mutants are also less sensitive to growth-inhibiting concentrations of K^+, and accumulate ~20% less K^+ than wild type on high (120 mM) K^+ media (Gobert et al. 2006). As *AtCNGC3* is expressed in the leaf vasculature and the epidermal and cortical cells of the root mature zone, the higher tolerance of *Atcngc3* mutants to K^+ and Na^+ stress may be due to decreased monovalent cation uptake by roots, impaired translocation of these cations to shoots, or both (Gobert et al. 2006). The germination of *Atcngc3* seeds was significantly lower than that of wild type seeds on high concentrations of NaCl, suggesting that mutant embryos are hypersensitive to salt stress. In developing embryos, AtCNGC3 is hypothesized to mitigate ionic toxicity by facilitating the movement of Na^+ from salt-sensitive to salt-tolerant tissues (Gobert et al. 2006). AtCNGC3 channels do not appear to transport Ca^{2+}, as AtCNGC3 cannot rescue a Ca^{2+}-uptake defective yeast strain, and *Atcngc3* mutant seedlings do not exhibit altered sensitivities to Pb^{2+} or high concentrations of Ca^{2+} (Gobert et al. 2006).

Altered expression of *AtCNGC10* also affects nutrient cation transport in *Arabidopsis*. Constitutive overexpression of *AtCNGC10* under the CaMV 35S promoter can partially compensate for *akt1-1*, a knockout mutation of a *Shaker*-type gene implicated in the high-affinity uptake of K^+ by root hairs (Li et al. 2005). Furthermore, antisense-mediated silencing of *AtCNGC10* causes plants to be hypersensitive to growth on low K^+ media (10–100 μM), as indicated by decreased biomass production relative to wild type (Borsics et al. 2007). The effects of salt stress on antisense lines are complex, with roots exhibiting increased tolerance to NaCl compared to wild type, whereas the biomass production and photosynthetic

activity of mutant shoots were more severely reduced than in control plants (Guo et al. 2008). The effect of antisense suppression of *AtCNGC10* on the accumulation of monovalent cations within tissues is likewise complex, and appears to be dependent upon the developmental states and experimental conditions utilized. Initial studies observed ~40% less potassium in the leaves of mature antisense plants relative to wild type plants grown in soil, suggesting the involvement of *AtCNGC10* in K^+ uptake (Li et al. 2005). However, the roots and shoots of younger *AtCNGC10* antisense plants grown on Hoagland's solution had higher levels of K^+ than those of wild type, and shoots (but not roots) contained more Na^+ than those of wild type when plants were subjected to salt stress, which suggests a defect in monovalent cation efflux under these growth conditions (Guo et al. 2008). Thus, AtCNGC10 appears to mediate cation influx under certain circumstances, and efflux in others, which is consistent with the observation that heterologously-expressed AtCNGC10 can conduct both inward and outward cation currents in HEK cells, as dictated by the electrochemical gradient (Christopher et al. 2007). The efflux of K^+ from antisense lines upon NaCl treatment is significantly reduced relative to wild type (Guo et al. 2008), supporting conclusions by Shabala et al. (2006) that the NaCl-induced K^+ loss from *Arabidopsis* cells is mediated in part by a VI-NSCC. Although the ability of AtCNGC10 to transport Ca^{2+} has not been analyzed at the molecular level, preliminary experiments from our lab indicate that *AtCNGC10* antisense plants accumulate ~20% less Ca^{2+} in mature leaves (Borsics and Christopher, unpublished data). It is interesting to note that AtCNGC10 was detected in root cap cells (Christopher et al. 2007), as removal of the cap from maize roots strongly inhibits the rapid transport of exogenously applied Ca^{2+} across the root tip (Lee et al. 1983).

VI-NSCCs are permeable to Cs^+ (Demidchik and Tester 2002), and theoretical models suggest that the toxic influx of Cs^+ into root cells occurs predominantly through VI-NSCCs, with high-affinity K^+/H^+ symporters of the AtKUP family transporting the remainder (White and Broadley 2000). A survey of the effects of *CNGC* mutations on Cs^+ accumulation in *Arabidopsis* revealed that some mutations reduced Cs^+ content in shoots (*AtCNGC2*, *AtCNGC3*, *AtCNGC16*, *AtCNGC19*, and *AtCNGC20*), whereas others increased Cs^+ content (*AtCNGC1*, *AtCNGC9*, and *AtCNGC12*) (Hampton et al. 2004). The decreased Cs^+ content of *Atcngc3* shoots is consistent with AtCNGC3's putative role in the translocation of monovalent cations to leaves. It is speculated that since K^+ deficiency increases Cs^+ influx, the enhanced accumulation of Cs^+ in *Atcngc1* shoots may result from the mutant's defect in K^+ uptake leading to upregulation of an alternative K^+ and Cs^+-permeable pathway, such as AtKUPs (Hampton et al. 2004).

4.2 Ca^{2+} Signaling

Some of the effects of CNGC on plant growth may be indirect, by way of Ca^{2+} signaling pathways. As previously mentioned, there is circumstantial evidence

supporting the involvement of CNGCs in mediating Na^+-induced transient increases of cytosolic Ca^{2+} (see Sect. 2.2 in Chapter "New Approaches to Study the Role of Ion Channels in Stress Induced Signaling; Measuring Calcium Permeation in Plant Cells and Organelles Using Optical and Electrophysiological Techniques"). CNGCs may also serve a similar function in the response to Ca^{2+} stress, as the growth of *Atcngc2* is strongly impaired under moderately calcified (10–30 mM Ca^{2+}) conditions (Chan et al. 2003), but mutant plants do not exhibit altered Ca^{2+} accumulation (unpublished data in Chan et al. 2003; Hampton et al. 2004). In addition, although AtCNGC1 and AtCNGC10 participate in cation uptake and distribution, their possible involvement in Ca^{2+} signaling cannot be excluded. Indeed, both mutants exhibit alterations in gravitropism, a process which involves polar Ca^{2+} movement from the ER (Plieth 2005) and mechanosensitive ion channels (Leitz et al. 2009). The *Atcngc1* roots 'overbend' upon gravistimulation (Ma et al. 2006), while *AtCNGC10* antisense roots display slower reorientation kinetics (Borsics et al. 2007). As CNs can induce the release of Ca^{2+} from intracellular stores (Volotovski et al. 1998), CNGCs that localize to the vacuole or ER membranes are also potentially involved in Ca^{2+} signaling.

5 Conclusions and Future Perspectives

Plant CNGCs have diverse temporal and spatial distributions, voltage-dependence, cation permeabilities, and regulation by CaM and CNs. Loss-of-function studies indicate that at least three CNGCs contribute to the nonselective uptake and transport of cations in *Arabidopsis*: AtCNGC1, AtCNGC3, and AtCNGC10. It is presently unclear if these CNGCs exist natively as homomeric channels, or interact with each other and/or other paralogs to form heterotetramers, as has been demonstrated for animal CNGCs found in olfactory cells and photoreceptors (Kaupp and Seifert 2002). Interestingly, all three are group I CNGCs, perhaps indicating that nutrient transport represents the ancestral function of this phylogenetic subgroup. There are several reasons why plants may have adapted CNGCs for the purpose of nutrient absorption. Plants do not appear to possess canonical voltage-gated Ca^{2+} channels (Demidchik et al. 2002b), and thus one of the main functions of this channel family may be to serve as a pathway for Ca^{2+} uptake. In addition, low-affinity transport systems, such as NSCCs, have a greater influx capacity than highly selective, high-affinity transporters (Britto and Kronzucker 2006). This property may be advantageous in natural environments, where plants must compete for limited resources, by permitting faster rates of cation uptake at the expense of decreased selectivity against potentially toxic cations. Plants may also utilize CNGCs as a means of acquiring Na^+, for although excessive concentrations of Na^+ within the cytosol are detrimental to plant survival, moderate levels of Na^+ can be beneficial to the growth of many plant species, especially when the availability of K^+ is limited (Subbarao et al. 2003).

In addition to their role in regulating cation homeostasis, there is considerable evidence that CNGCs mediate Ca^{2+} signaling in response to both biotic (see Chapter "The Function of Cyclic Nucleotide-Gated Channels in Biotic Stress") and abiotic stresses. Such channels may be located at the PM, or the membranes of intracellular Ca^{2+} stores such as the vacuole and ER. CNGCs targeted to these intracellular compartments may also be involved in regulating the sequestration of monovalent and divalent cations. The cation permeabilities and subcellular locations of the various CNGCs must therefore be determined to complete their functional characterization.

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The Function of Cyclic Nucleotide-Gated Channels in Biotic Stress

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Abstract Plant cyclic nucleotide-gated ion channels conduct Ca^{2+} across the plasma membrane (PM) and facilitate cytosolic Ca^{2+} elevation during pathogen response signaling cascades. Until recently, not much was known about the specific ion channels involved in Ca^{2+} influx into plant cells, or how Ca^{2+} signals are generated and impact on downstream events during pathogen resistance responses. Recent studies, involving the cyclic nucleotide gated ion channel (CNGC) family of proteins, have provided new information relevant to these two areas of plant biology and will be reviewed in this chapter. Current evidence points to specific proteins that synthesize cyclic nucleotides and that function as ligands to activate CNGCs. The role of these channels in Ca^{2+} conduction appears critical to the generation of the hypersensitive response to pathogens, an important defense mechanism that limits disease in plants. Signaling downstream from Ca^{2+} during biotic stress responses involves cytosolic Ca^{2+}-binding proteins such as calmodulin.

Abbreviations

AC	Adenylyl cyclase
avr	Avirulence
CaM	Calmodulin

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CML	CaM-like protein
CDPK	Ca^{2+}-dependent protein kinase
CNBDs	Cyclic nucleotide binding domains
CNGC	Cyclic nucleotide gated channel
GC	Guanylyl cyclase
HR	Hypersensitive response
LPS	Lipopolysaccharide
LRR-RLKs	Leucine-rich-repeat receptor-like kinases
NC	Nucleotidyl cyclase
NO	Nitric oxide
NOS	Nitric oxide synthase
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PM	Plasma membrane
ROS	Reactive oxygen species
SA	Salicylic acid
TM	Transmembrane
VPE	Vacuolar processing enzyme

1 Introduction

Plants exposed to pathogens are capable of invoking a complex, multilayered, programmed cellular-level suite of defense responses that act to limit disease progression (Dangl et al. 1996). Plants lack a defense network of circulating mobile sentry cells equivalent to macrophages of the jawed vertebrate immune system. Consequently, recognition of a pathogen as "non-self" by plants is a critical and little understood feature of system fitness and plant immune defense responses.

Plant immune responses are triggered by the perception of non-self through the recognition of pathogen-associated molecular pattern (PAMP) molecules and elicitor molecules that can be pathogen-derived toxins. Typically, PAMPs are evolutionarily conserved components of microbes (including pathogens) that are not present in the plant cell (i.e., "self"). Examples are lipopolysaccharide (LPS; the glycolipid component of the outer membrane found in Gram negative bacteria), flagellin (the structural protein component of the bacterial motility organ), the bacterial elongation factor Tu, chitin (found in fungal cell walls), and ergosterol (found in fungal membranes) (Zipfel 2008).

One of the earliest components of the pathogen response signal transduction cascade in a plant cell is an increase in cellular Ca^{2+} (Nürnberger et al. 1994) upon perception of non-self (PAMP) presence. Cytosolic Ca^{2+} elevation upon pathogen perception leads to a suite of basal defense responses ("PAMP triggered innate immunity" as described by Jones and Dangl 2006), and the hypersensitive response (HR) to avirulent pathogens. HR, one of the immune responses triggered by specific effector molecules in pathogens, involves reactive oxygen species (ROS) and nitric

oxide (NO) production leading to programmed cell death (PCD) in cells neighboring the infection site, which limits the spread of the disease (Bent and Mackey 2007). HR occurs when a specific avirulence (avr) gene product generated by pathogens interacts (directly or indirectly) with a corresponding resistance (or "*R*" gene encoded) protein present in the plant cell. PCD associated with HR that is evoked by this interaction is distinct from, and augments basal level innate immunity resistance responses. However, in both cases, a range of cytosolic defense systems is initiated in the cells at the infection site upon pathogen perception due to cytosolic Ca^{2+} elevation (Ma and Berkowitz 2007; Lecourieux et al. 2006). Thus, pathogen recognition mechanisms lead to a cascade of defense responses through Ca^{2+} signaling.

Early electrophysiological analysis of plant cell responses to pathogen perception demonstrated that plasma membrane (PM) Ca^{2+}-conducting channels contribute to pathogen-induced cytosolic Ca^{2+} elevation (Gelli and Blumwald 1997). Only recently, however, has genetic evidence linked Ca^{2+} conductance through specific ion channel gene products to plant pathogen defense signal transduction cascades. Several isoforms of the 20-member cyclic nucleotide-gated ion channel (CNGC1-20) family have been found to be involved in plant defense responses to biotic stress. The information in this chapter will focus on linking the molecular properties of CNGC channels to the cytosolic Ca^{2+} elevation that is a critical aspect of this signaling. Details regarding steps upstream from cytosolic Ca^{2+} elevation that link Ca^{2+} conductance to pathogen perception, as well as steps downstream from PM Ca^{2+} conductance during pathogen signaling will be also be discussed.

Excellent reviews have been published recently covering topics related to the focus of this chapter. Readers are referred to the following articles which provide expansive reviews covering CNGCs (Sherman and Fromm 2009; Kaplan et al. 2007; Talke et al. 2003), Ca^{2+} signaling (McAinsh and Pittman 2009; Wheeler and Brownlee 2008; Bouché et al. 2005; Hetherington and Brownlee 2004), Ca^{2+} conducting channels (Ward et al. 2009; Demidchik and Maathuis 2007), Ca^{2+} involvement in plant responses to pathogens (Ma and Berkowitz 2007; Garcia-Brugger et al. 2006; Lecourieux et al. 2006), plant PAMP receptors (Zipfel 2008), plant immune response to pathogens (Bent and Mackey 2007; Bittel and Robatzek 2007; Hofius et al. 2007; Jones and Dangl 2006), NO signaling (Courtois et al. 2008; Delledonne 2005; Lamotte et al. 2005), and cyclic nucleotide signaling (Martinez-Atienza et al. 2007).

2 CNGC Structure and Function

Higher plants contain no canonical genes encoding voltage-gated Ca^{2+} channels; current reviews point to CNGCs as facilitating Ca^{2+}-signaling in land plants (McAinsh and Pittman 2009; Wheeler and Brownlee 2008). Patch clamp studies indicate that the major inward Ca^{2+} current across the plant PM occurs through nonselective weakly voltage gated cation channels (Demidchik and Maathuis 2007; Demidchik et al. 2002). All relevant experimental evidence indicates that plant

CNGCs are specifically localized to the PM, although CNGC20 may be targeted to the chloroplast (Sherman and Fromm 2009). Application of cyclic nucleotides to plant (Arabidopsis) leaf (guard and mesophyll cell) protoplasts activates an inwardly rectified Ca^{2+} current (Lemtiri-Chlieh and Berkowitz 2004); work with isolated tobacco (*Nicotiana plumbaginofolia*) protoplasts has shown that cAMP and cGMP application leads to cytosolic Ca^{2+} elevation as well (Volotovski et al. 1998). The electrophysiological characterization of cyclic nucleotide dependent Ca^{2+} conductances across the PM included studies showing ligand activation of the current in the detached patch configuration (i.e., in the absence of endogenous cytosolic signaling molecules), suggesting that it is due to a direct interaction between ligand and channel (Lemtiri-Chlieh and Berkowitz 2004). Thus, this evidence documents the presence of native CNGC channels in the plant PM.

CNGCs native to animal membranes are relatively well characterized as ligand-gated cation-conducting channels, with varying degrees of selectivity for Ca^{2+} and monovalent cations. Animal CNGCs are activated by cyclic nucleotides (cGMP and/or cAMP to different relative extents) and allosterically inhibited by calmodulin (CaM) binding to a region of the protein distinct from that binding cyclic nucleotides. In animals, native CNGCs are uniformly heterotetramers formed by two and oftentimes three different CNGC subunits. No experimental evidence supports this quaternary structure in plant membranes. However, modeling studies suggest they are tetramers (Hua et al. 2003b). Expression of cDNAs encoding single plant CNGC gene products in heterologous systems such as *Xenopus laevis* oocytes and cultured human embryonic kidney cells, along with patch clamp analysis of currents, indicates that functional channels can be formed as homomeric protein complexes. We cannot assume, however, that this is the case with CNGCs in native plant membranes. Whether or not native plant CNGC channel complexes are formed from more than one subunit has some implications (discussed below) for understanding their role in plant pathogen signaling.

The tetrameric quaternary structure of the plant CNGC ion conduction pathway is similar to the "inverted tee pee" found in members of the superfamily of 6 transmembrane (TM1-6) "Shaker-like" pore-loop ion channels. Each of the four subunits forming the ion conduction pathway through the membrane has a pore region selectivity filter (between TM5 and 6) that determines the specificity of ion permeation. Importantly, the amino acids experimentally verified to form the selectivity filter of plant CNGCs differ from their animal counterparts. Of the 20 plant CNGCs, only isofoms 1, 2, 18, 11, 12, and the chimeric mutant channel CNGC11/12 have been experimentally verified to conduct Ca^{2+}. The native plant CNGC isoforms 2, 4, 11, and 12 are involved in signaling cascades related to plant pathogen defense responses. Some indirect evidence indicates that at least some of the other plant CNGC isoforms (e.g., CNGC3) do not conduct Ca^{2+} (Gobert et al. 2006, also R. Ali and G.A. Berkowitz, unpublished data). It should be noted, however, that all plant CNGCs studied to date conduct K^+ and in some cases Na^+ or Ca^{2+}; thus they are considered "nonselective" cation channels.

CNGCs are the only plant channels that are activated (i.e., showing increased open probability at a given membrane potential) by cyclic nucleotides. They have

cyclic nucleotide binding domains (CNBDs) at the cytosol-localized carboxyl terminus. Several tertiary three-dimensional structural models of the plant CNGC CNBD have been generated (Baxter et al. 2008; Kaplan et al. 2007; Bridges et al. 2005; Hua et al. 2003a) and these studies identified residues that may contribute to ligand binding. Members of the plant "Shaker-like" (i.e., 6 TM) K^+-selective KAT and AKT channel families also have carboxyl terminus CNBD domains; however, in these cases cyclic nucleotide deactivates the channel by shifting the voltage threshold for activation to more negative values (e.g., Hoshi 1995). At present, it is thought that plants lack functional cyclic nucleotide activated protein kinases (Kaplan et al. 2007; Martinez-Atienza et al. 2007; Bridges et al. 2005). Therefore, CNGCs can be considered to be the only (or at least primary) cellular target of cyclic nucleotides that can transduce elevation of these messenger molecules in the cytosol to downstream steps of a plant signaling cascade. It should be noted that most of the functional characterizations of cyclic nucleotide effects on plant CNGCs have shown that cAMP acts to activate the channel. Only in a few instances has cGMP been used as an activating ligand (Leng et al. 1999) and in some cases cGMP was found not to act as an activating ligand (Lemtiri-Chlieh and Berkowitz 2004). Our understanding of cyclic nucleotide signaling in plants that involves CNGCs, therefore, is primarily based on studies using cAMP.

The regulatory carboxyl terminus of plant CNGCs has a CaM binding domain that overlaps the region of the polypeptide that binds cyclic nucleotides (the CNBD). Some experimental evidence has shown that CaM blocks plant CNGC ion conductance (Ali et al. 2007; Ali et al. 2006; Li et al. 2005; Hua et al. 2003a). CaM may compete with cyclic nucleotide for binding to the channel, and therefore prevent cyclic nucleotide activation. This, possibly competitive–interaction between cyclic nucleotide activation and CaM inhibition, may have some ramifications for CNGC-dependent Ca^{2+} signaling with regard to pathogen defense responses in plants.

In the presence of exogenously added activating ligand (cyclic nucleotides), plant CNGC currents are non-inactivating, both in native membranes and in heterologous expression systems. Perhaps in the presence of an exogenous supply of cyclic nucleotide, build up of Ca^{2+}/CaM does not affect the channel. However, native CNGC functioning during pathogen response signaling may be different. In this case, a rise in cytosolic Ca^{2+} could lead to binding of Ca^{2+}/CaM complex to the CaM binding domain of the channel and thus reduce current. Thus, CNGC functioning during a signaling cascade could lead to a transitory increase in Ca^{2+} conductance across the PM and concomitant transitory elevation in cytosolic Ca^{2+}. As cytosolic Ca^{2+} is elevated, further current through the channel could be then blocked. The normal suite of plasma- and endomembrane Ca^{2+} ATPase pumps and transporters could provide a mechanism for rapid return of cytosolic [Ca^{2+}] to homeostatic levels as further inward CNGC current is blocked.

Current reviews of Ca^{2+} signaling in plants (e.g., McAinsh and Pittman 2009; Ward et al. 2009) distinguish CNGCs as distinct from the (unknown) family of genes responsible for hyperpolarization activated (inwardly rectified) Ca^{2+} currents across the PM. However, plant CNGCs may incorrectly be categorized as "solely" ligand gated channels. They do retain the voltage-sensor region of voltage-gated

channels (i.e., evenly spaced positively charged amino acids in the TM4 domain, although the number of lysines and arginines in this region of plant CNGCs is reduced). Addition of exogenous cyclic nucleotide clearly increases the open probability of these channels but plant CNGCs may also be gated by voltage; hyperpolarizing membrane potentials may activate these channels (see Hua et al. 2003b, Fig. 5). In this case, their functional properties may be more related to animal hyperpolarization-activated and cyclic nucleotide gated (HCN) channels than animal CNGCs (Wahl-Schott and Biel 2009). The current:voltage relationship of plant CNGCs expressed in oocytes is quite different from that found for animal CNGCs (compare Leng et al. 1999 and Yao et al. 1995, for example). The much larger range of membrane potentials observed in plant cells and their change in response to various signals could affect the population of open CNGC channels.

A recent study has provided some new insights into what is referred to as the "C-linker" region of the plant CNGC polypeptide (Baxter et al. 2008). In this work, a point mutation of a residue (E527K in CNGC12) in the CNBD was identified that abolished channel function but did not affect ligand binding. The results suggested that the C-linker region of plant CNGCs plays an important role in channel function, perhaps providing a physical link between the CNBD and the pore that transmits ligand binding to facilitate channel opening.

3 Ca^{2+} Signaling, CNGCs, and Pathogen Defense Responses

Early studies (Grant et al. 2000) have shown that inoculation of leaves with a pathogen (*Pseudomonas syringae*) leads to an elevation of plant cell cytosolic Ca^{2+} (Fig. 1). This Ca^{2+} elevation may be affected by the presence in the pathogen of some (but not all) avr genes. Exposure of leaves to a Ca^{2+} channel blocker prevents HR in wild type plants inoculated with pathogen and this result supports the concept that pathogen/PAMP-associated influx of Ca^{2+} is an early signal initiating plant defense responses which, in the presence of pathogen avr and corresponding plant *R* genes, leads to HR. Mutations in several (Arabidopsis) CNGCs have been associated with altered plant responses to pathogens. Loss-of-function of CNGC2 (the "defense-no-death" or *dnd1* mutant) and CNGC4 (the "HR-like lesion mimic *hlm1* or *dnd2*" mutant) alters plant responses to avirulent pathogens (including *P. syringae*). Arabidopsis plants with these mutations display impaired HR, constitutive expression of salicylic acid (SA), altered expression of pathogen defense-related genes, and (despite the lack of HR) increased resistance to pathogen growth unrelated to the HR.

The *Arabidopsis* mutant "constitutive expresser of *PR* (pathogenesis related) genes 22" (*cpr22*) was identified in a screen for mutations associated with altered activation of pathogen defense responses (Yoshioka et al. 2001). The *cpr22* mutant displays different phenotypes from *dnd1* and *hlm1* (*dnd2*) plants, but does overlap with these CNGC loss-of-function mutants in having constitutively activated defense responses and enhanced resistance to *P. syringae* (*cpr22* also was shown

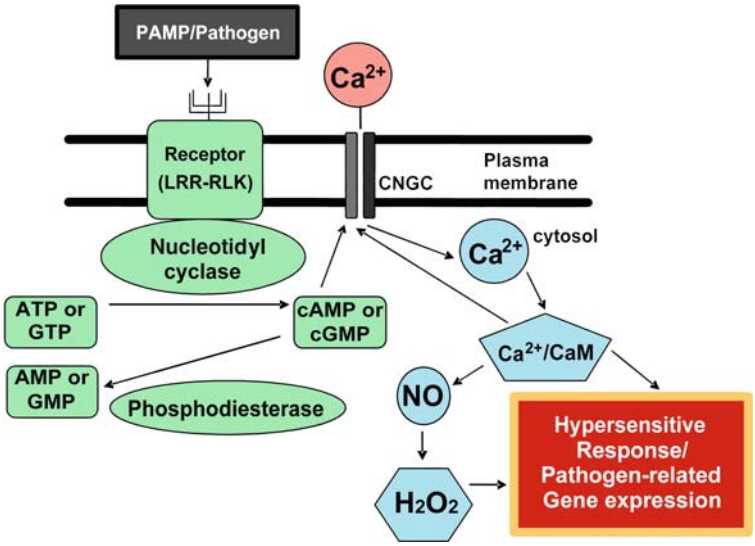

Fig. 1 Model of early steps in the plant immune response signal transduction pathway. Pathogen/PAMP binding to a receptor results in a rise in cytosolic cyclic nucleotides through activation of a nucleotidyl cyclase. The elevation of cyclic nucleotide levels activates a possibly heteromeric CNGC, leading to Ca^{2+} influx. Cytosolic Ca^{2+} elevation leads to a rise in Ca^{2+} complexed with CaM (or CML), which leads to NO and H_2O_2 production, gene expression, and CNGC inactivation. When plants are exposed to an avirulent pathogen, CNGC-mediated Ca^{2+} influx results in HR

to have enhanced resistance to the oomycete pathogen *Hyaloperonospora parasitica*) (Moeder and Yoshioka 2008; Yoshioka et al. 2006; Yoshioka et al. 2001). The *cpr22* mutation was identified as a 3-kb deletion that fuses two CNGC genes, *CNGC11* and *CNGC12*, to generate a novel chimeric gene, *CNGC11/12* (Yoshioka et al. 2006). Based on genetic and molecular analyzes, it is suggested that the phenotype conferred by *cpr22* is attributable to the expression of *CNGC11/12*. Since loss-of-function mutants of *CNGC11* or *12* show a partial breakdown of *R*-gene mediated pathogen resistance but display enhanced resistance to pathogen growth, one function of *CNGC11* and *12* could be the activation of *R*-gene mediated pathogen resistances and *CNGC11/12* may activate their downstream signal constitutively (Yoshioka et al. 2006; Urquhart and Yoshioka unpublished data).

Urquhart et al. (2007) further investigated the nature of HR like cell death induced by expression of *AtCNGC11/12* using a transient expression system. In this study, they found that cell death development depends on Ca^{2+} and the caspase like vacuolar processing enzyme (VPE). Caspases are aspartate-specific cysteine proteases that play an essential role in executing PCD in various organisms (Ho and Hawkins 2005). Caspase-like activity is required for the development of PCD in plants and VPE, a plant cysteine protease, was identified as a potential plant counterpart to animal caspases that is essential for pathogen-induced HR (Hatsugai et al. 2004; Rojo et al. 2004).

The phenotypes displayed by these CNGC mutants (*dnd1*, *hlm1*/*dnd2*, and *cpr22*) suggest that native CNGC channel protein complexes are heteromeric in plants. CNGC2 and 4 have overlapping expression patterns (and functions) while a loss-of-function mutation in either gene alone causes similar alterations in pathogen-related responses. This could be explained by the possibility that these two CNGC polypeptides are part of the same tetrameric channel protein in native plant membranes. A loss of either polypeptide could impair the same channel protein complex. Analysis of the CNGC11/12 mutant suggests that CNGC12 also forms heteromeric channel protein complexes that are positive regulators of plant resistance to avirulent pathogens.

Cytosolic Ca^{2+} elevation during pathogen response signaling could occur through influx into the cytosol of Ca^{2+} from internal stores as well as from the apoplast. For example, electrophysiological studies have identified a tonoplast Ca^{2+} current activated by cytosolic Ca^{2+} (Peiter et al. 2005). However, the expression product of the only known gene encoding an endomembrane-localized plant Ca^{2+} conducting channel (TPC1) apparently does not contribute to pathogen-associated Ca^{2+} signaling (Ranf et al. 2008).

Studies with the *dnd1* mutant provide some insight into specific mechanisms underlying the role that CNGC-mediated cytosolic Ca^{2+} elevation plays in pathogen signaling (Ali et al. 2007). In this work, some differences were noted in wild type and *dnd1* plant responses to avirulent pathogens related to the HR. HR was restored in *dnd1* plants supplied with an exogenous NO donor. NO generation is required for development of HR. PAMP (LPS) application (using guard cells in epidermal peels as a model cell system) led to Ca^{2+}-, and CNGC-dependent NO generation. Thus, it was concluded that CNGC-dependent cytosolic Ca^{2+} elevation mediates pathogen/PAMP-induced NO generation which, in the presence of an appropriate avr gene in the pathogen and a corresponding *R* gene in the plant, leads to HR. Several lines of evidence suggest that the Ca^{2+} binding protein CaM, or a CaM-like protein (CML), mediates the aforementioned NO generation downstream from CNGC-mediated cytosolic Ca^{2+} elevation during pathogen signaling (Ma et al. 2008). It is likely that CaM (or one of the many CMLs in plants) provides a mechanism to transduce CNGC-dependent cytosolic Ca^{2+} elevations occurring upon pathogen perception to a number of downstream responses.

4 CaM and Ca^{2+} Signaling During Pathogen Defense Responses

In addition to mediating some of the downstream signaling from the initial PAMP/pathogen-induced cytosolic Ca^{2+} elevation, an increase in activated CaM via initial Ca^{2+} influx through CNGCs could also shape the Ca^{2+} signal (Fig. 1). The specific "signature" of a Ca^{2+} elevation in many cases provides information for specific downstream responses (McAinsh and Pittman 2009). In the case of cytosolic Ca^{2+} elevation due to Ca^{2+} conductance through CNGCs, a rise in cytosolic Ca^{2+}/CaM could feedback and block further Ca^{2+} current (as mentioned above). Deactivation

of CNGC currents during pathogen response signaling by CaM is supported by the following experimental evidence: Application of a PAMP results in sustained Ca^{2+} current only in the presence of a CaM antagonist (Ali et al. 2007). The cytosolic Ca^{2+} elevation occurring in leaves inoculated with avirulent pathogen was increased and sustained (above background) for a longer duration in the presence of CaM antagonist (Ma et al. 2008). The application of a broad range of PAMPs and elicitors to plant cells results in a transitory "spike" in cytosolic Ca^{2+} typically lasting from seconds to minutes (Errakhi et al. 2008; Lecourieux et al. 2006). A feedback mechanism to down regulate CNGCs by CaM could therefore contribute to the transitory rise and then fall in cytosolic Ca^{2+} during the signaling cascade.

CaM (or CML) probably affects pathogen response signaling downstream from the cytosolic Ca^{2+} elevation by interacting with enzymatic proteins and transcriptional regulators. Overexpression of soybean (*Glycine max*) CaMs in tobacco (*Nicotiana tabacum*) leads to enhanced resistance to a range of pathogens (Heo et al. 1999). Tobacco CaM silencing lines display increased susceptibility to virulent bacterial and fungal pathogens (Takabatake et al. 2007). Silencing expression of the CML *APR134* in tomato (*Solanum lycopersicum*) impaired HR formation while overexpression of the Arabidopsis ortholog of APR134 (*AtCML43*) hastened HR development (Bouché et al. 2005). LPS-induced NO generation in plant cells can be quenched by the addition of CaM antagonist (Ali et al. 2007).

An arginine-dependent nitric oxide synthase (NOS) type enzyme is responsible for NO generation during pathogen response signaling cascades (Delledonne et al. 1998). A gene encoding a NOS-type enzyme has not yet been identified in plants; however a number of studies indicates that NOS-dependent NO generation is Ca^{2+}/CaM dependent (e.g., Ma et al. 2008; Corpas et al. 2004). Thus, CNGC-mediated cytosolic Ca^{2+} elevation upon pathogen perception could lead to NO generation due to Ca^{2+} binding to CaM (or a CML) which then interacts either directly or indirectly with NOS.

During plant-pathogen interactions, Ca^{2+}/CaM can activate NAD kinase, which would result in increased NADPH, the substrate for the oxidative burst responsible for ROS production during the HR (Harding and Roberts 1998). A rise in cytosolic Ca^{2+} could affect ROS production during HR in other ways, such as through Ca^{2+}-dependent protein kinase (CDPK) signaling. Some studies suggest involvement of CDPKs in HR. Two CDPK isoforms in potato (*Solanum tuberosum*) have been shown to phosphorylate NADPH oxidase, which generates the ROS product H_2O_2 during HR (Kobayashi et al. 2007). Other studies have suggested that this ROS-generating enzyme may be affected by direct binding of Ca^{2+} (Torres et al. 2006).

Recent works have begun to provide insights into how CNGC-dependent conductance of Ca^{2+} into the cytosol could impact on expression of defense-related genes during pathogen response signaling. The Arabidopsis *CBP60g* gene product is a CaM-responsive protein involved in pathogen responses; it is required for PAMP-mediated SA generation and other defense responses (Wang et al. 2009). The absence of CaM interaction with this protein results in enhanced disease progression. The expression product of the barley (*Hordeum vulgare*) *MLO*

(mildew resistance locus *o*) gene is another CaM target involved in plant responses to pathogens (Kim et al. 2002). MLO may be a negative regulator in plant defense responses. The Arabidopsis signal responsive protein SR1, a member of the CaM binding transcription activator protein family, is also a negative regulator of plant pathogen signaling (Du et al. 2009; Galon et al. 2008) that could act downstream from cytosolic Ca^{2+} elevation through CaM/CML signaling to regulate expression of defense-related genes (including *PR1* and regulators of SA synthesis).

5 Activation of CNGCs During Immune Signaling Cascades

Not much is known at present about the molecular mechanisms linking PAMP perception at the PM to Ca^{2+} involvement in the early steps of the pathogen response signal transduction cascade occurring in the plant cell cytosol. We suspect that CNGC-dependent cytosolic Ca^{2+} elevation in plant cells occurs during immune signaling due to activation of the channels by a rise in the level of cAMP and/or cGMP upon pathogen perception. Not much evidence supporting this speculation is available in the published literature at present. However we found a coordinated rise of cAMP and Ca^{2+} occurring minutes after inoculating Arabidopsis leaves with avirulent pathogen (*P. syringae*) (Ma et al. 2009), and a rise of cGMP several hours post inoculation with the same pathogen (S. Meier and C. Gehring, unpublished results). An alternative possible mechanism for CNGC activation could be pathogen/PAMP-mediated post-translational modification of the channels, leading to increased open probability and Ca^{2+} conductance. Some indirect evidence suggests that pathogen-dependent phosphorylation or nitrosylation of PM proteins may occur during plant immune signaling (Besson-Bard et al. 2008; Garcia-Brugger et al. 2006). This putative signaling mechanism may impact on Ca^{2+}-conducting channels either directly, or indirectly; however, no evidence links these post-translational modifications to CNGC-mediated Ca^{2+} conductance at present. Here, we focus on the possible role cyclic nucleotides may have as signaling molecules upstream from CNGCs in plant immunity.

6 Cyclic Nucleotide Generation and Its Role in Biotic Stress Responses

Identification of nucleotidyl cyclases (NCs) responsible for cAMP and cGMP generation in higher plants has remained elusive. This is somewhat surprising given that the unicellular green alga *Chlamydomonas reinhardtii* contains >100 annotated NCs (Schaap 2005) with 22 different domain architectures with 13 different partners [http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY (Madera et al. 2004)]. The first *bona fide* NC identified in higher plants was an adenylyl cyclase (AC) from

corn (*Zea mays*) pollen (Moutinho et al. 2001). However, BLAST searches of the *Arabidopsis* genome with this sequence or known NCs from lower and higher eukaryotic species do not identify orthologs. An alternative systematic search for NCs, based on a search motif of functionally assigned and conserved amino acid residues in the catalytic center of annotated guanylyl cyclases (GCs) from both lower and higher eukaryotes, was undertaken by Ludidi and Gehring (2003) and 7 *Arabidopsis* candidate GCs including the soluble AtGC1 (Ludidi and Gehring 2003) and a wall associated kinase-like protein (*AtWAKL10*; At1g79680) were identified. Microarray expression analysis of *AtWAKL10* (Zimmermann et al. 2005) supports a role for this GC in pathogen signaling.

An extended bioinformatic search for potential GCs in higher plants by relaxing the stringency of the originally used motif identified a number of annotated kinases and receptor kinases, in particular leucine-rich-repeat receptor-like kinases (LRR-RLKs)(Kwezi et al. 2007). One of these LRR-RLKs, the brassinosterosid receptor AtBRI1, has since been expressed as a recombinant protein and shown to have GC activity *in vitro* (Kwezi et al. 2007) suggesting that several other receptor kinases that contain the same motif also function as GCs. One of these is AtPepR1 (At1g73080) (Kwezi et al. 2007) that is PM localized and has a role in pathogen defense responses through binding the pathogen response-related *Arabidopsis* peptides of the AtPep family (Ryan et al. 2007). Expression of some members of the AtPep peptide family is induced by application of either PAMPs or pathogens (*P. syringae*) to leaves (Ryan et al. 2007). We have found that recombinantly expressed and affinity-purified AtPepR1 has GC activity, and that the AtPep ligands which bind to this receptor induce AtPepR1- and CNGC2-dependent inward Ca^{2+} currents in *Arabidopsis* mesophyll cells (Z. Qi, R. Verma, G.A. Berkowitz, unpublished results). Thus, it appears that AtPepR1 may generate cyclic nucleotides and activate CNGC-dependent inward Ca^{2+} currents in response to pathogen perception during plant immune signaling.

NC catalytic motif searches can also provide cues to identify putative plant ACs since prior experimental work has shown that the catalytic domains of ACs and GCs are highly similar (Roelofs et al. 2001). Searches identified 16 putative *Arabidopsis* ACs one of which is KUP5 (K^+ uptake permease 5). While the domain combination of an AC with a potassium channel would be entirely novel in plants, such homodimeric AC/ion-channels are known and functional in *Paramecium* and *Plasmodium falciparum* (Weber et al. 2004).

Thus, although AC, GC, and cyclic nucleotide phosphodiesterase activities are clearly present in protein extracts from plants the exact identity of the responsible enzymes has yet to be established. However, we suspect that NCs play a critical role in translating pathogen perception to CNGC activation and downstream pathogen defense responses in the plant immune signaling cascade. Indeed, prior published work with cultured cells challenged with fungal elicitors suggests that cyclic nucleotides may act in plant immune signaling. Application of fungal extracts induced elevation in the endogenous level of cAMP in cultured carrot (*Daucus carota*), alfalfa, and French bean (*Phaseolus vulgaris*) cells (Cooke et al. 1994;

Bolwell 1992; Kurosaki et al. 1987). Other work indicates that application of exogenous cyclic nucleotides can in some cases activate pathogen response signaling in cultured plant cells (Bindschedler et al. (2001). Furthermore, cyclic nucleotides as well as the PAMP LPS induce cytosolic Ca^{2+} elevations within minutes in leaves of wild type but not *dnd1* plants. This rapid Ca^{2+} elevation leads to NO and ROS generation that can be blocked by AC inhibitors. Inhibitors of cAMP generation also block PAMP-induced NO generation, the coordinated pathogen-induced cAMP and cytosolic Ca^{2+} elevation occurring minutes after inoculation with pathogen, and HR in response to inoculation with avirulent pathogen (Ma et al. 2009).

7 Summary and Perspectives for the Future

Early work on plant CNGCs focused on their characterization as ion channel proteins, and on phenotypes related to their conductance of cations into plant organs during growth and development. In addition, some CNGCs were associated with plant responses to pathogens. More recent work, as discussed in this chapter, has provided a fuller and more nuanced picture of (a) CNGC function related to their molecular structure, (b) the role of CNGCs as Ca^{2+} conducting channels in plant pathogen response signaling cascades, (c) several events downstream from CNGC-mediated Ca^{2+} conduction in the pathogen response signaling cascade, (d) how CNGC ligands (cyclic nucleotides) are generated in plants and (e) how generation of cyclic nucleotides may be involved in linking pathogen perception to CNGC activation and cytosolic Ca^{2+} signaling. Outstanding questions related to these points are as follows: How is pathogen perception, through binding of PAMPs to a receptor, linked to generation of cyclic nucleotide and/or other mechanism(s) that activate CNGCs? Is the specific nature of the CNGC-mediated Ca^{2+} conductance that occurs during the signaling cascade different during responses to PAMPs and other elicitor molecules, avirulent and virulent pathogens, and/or sensing of non-pathogenic microbes as non-self by plant cells? How does the molecular architecture of CNGCs impact on their function *in planta*, with specific reference to issues such as their gating properties and regulation by CaM? What specific molecular events occur downstream from CNGC-mediated cytosolic Ca^{2+} elevation that act to initiate a range of defense responses to pathogens? Do members of the family of proteins recently identified as having a NC domain and in some cases in vitro NC activity act to generate cyclic nucleotides in the plant during signaling cascades such as the immune response to pathogenic biotic stress? We suspect that in the near future, some aspects of these questions will be answered in the relevant literature.

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New Approaches to Study the Role of Ion Channels in Stress-Induced Signalling: Measuring Calcium Permeation in Plant Cells and Organelles Using Optical and Electrophysiological Techniques

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Abstract Calcium is one of the most important second messengers in plant cells; an increase in intracellular calcium is believed to be a major pathway in the plant stress response. Several techniques were developed to measure cytoplasmic calcium changes and there is an increasing effort to unravel spatial and dynamic properties of calcium signals. Calcium influx in plant cells is typically mediated by non-selective cation channels. Patch-clamp still plays a fundamental role in studying calcium permeation; however, recent advances in microscopy are very promising to characterize calcium fluxes even at the level of the single channel protein. In this chapter, we focus on techniques that combine electrophysiological and optical approaches to study the local and temporal characteristics of calcium signalling in plants.

Abbreviations

ABA	Abscisic acid
ATP	Adenosine-5′-triphosphate
AtTPC1	*Arabidopsis thaliana* two pore channel 1
CITC	Cold-induced transient conductance
$DiBAC_4(3)$	Bis-(1,3-dibutylbarbituric acid)-trimethine oxonol
DSTORM	Direct stochastic optical reconstruction microscopy
FLEP	Fluorescence combined with excised patch
FPALM	Fluorescence photo-activated localization microscopy
GFP	Green fluorescent protein
GSD	Ground-state depletion

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GSDIM	Ground-state depletion and single-molecule return
PAINT	Points accumulation for imaging in nanoscale topography
PALM	Photoactivatable localization microscopy
PALMIRA	PALM with independently running acquisition
PyMPO maleimide	1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl) pyridinium methanesulfonate
RESOLFT	Reversible saturable optical fluorescence transitions
SCAM	Scanning cysteine accessibility mutagenesis
SCCaFTs	Single-channel calcium fluorescent transitions
SPEM	Scanning photoemission microscope
SSIM	Saturated structured illumination microscopy
STED	Stimulated emission depletion microscopy
STORM	Stochastic optical reconstruction microscopy
TEVC	Two-electrode voltage-clamp
TIRF	Total internal reflection microscopy
TL-PALM	Time-lapse photoactivatable localization microscopy

1 Introduction

Pollack (1928) was probably the first author reporting calcium changes in the cytoplasm of a living cell. He injected *Amoeba dubia* and *Amoeba proteus* with a saturated solution of alizarin which precipitated with calcium ions; the resulting compound, calcium alizarinate, could be seen under the microscope as "purplish red crystals". Pollack could demonstrate an "appreciable amount of calcium ions in the living ameba" and that amebae should have a calcium reserve used to recover from sublethal doses of alizarin. Moreover, he could observe a "shower of these purplish red granules" at the places in which the ameba was attempting to put forth a pseudopod.

Nowadays, there is emerging evidence of the importance of calcium in plant cells (Sanders et al. 2002; McAinsh and Pittman 2009; for a critical view see Plieth 2005), in particular, in stress responses for example, mediated by abscisic acid (ABA), mechanical stimulation, osmotic, salt and drought signals, oxidative stresses, temperature changes, light, pathogens (Hetherington and Brownlee 2004; Israelsson et al. 2006; Lecourieux et al. 2006; Oldroyd and Downie 2008), and in plant development (Hepler 2005; Michard et al. 2008).

Methods to measure Ca^{2+} concentration in living cells can be divided into 4 groups (Blinks et al. 1982), namely those based on bioluminescent indicators, metallochromic indicators, fluorescent indicators, and Ca^{2+}-selective microelectrodes.

This chapter summarizes the state of the art of optical calcium detection combined with electrophysiological techniques in plant cells. This emerging approach is essential to investigate the spatio-temporal properties of calcium signalling in plants.

2 Plant Cell Impalement

Membrane voltage in plant cells can be monitored over time using microelectrode impalement. The impalement of a plant cell loaded with a calcium reporter, such as aequorin or fura-2 (see below), allows the investigation of how changes in cytosolic calcium and electrical signals are synchronized. Because guard cells are electrically isolated, it is possible to perform voltage-clamp measurements with single or double barreled electrodes (see for comparison Roelfsema et al. 2001) and thus record current flowing through guard cell electrogenic channels/transporters; in these cases, simultaneous calcium detection can give information on Ca^{2+} permeability of the active channels.

2.1 Aequorin

Aequorin belongs to a class of photoproteins found in coelenterates that emit light upon binding of calcium ions (Shimomura 2005; Knight and Knight 1995; Brini 2008). Functional aequorin is synthesized by the jellyfish *Aequorea victoria* and consists of an apoprotein (a single polypeptide chain of 189 aminoacids and molecular weight of 21.4 kDa, Inouye et al. 1985) and a prosthetic group (a hydrophobic luminophore called coelenterazine) in a peroxidized form (Shimomura and Johnson 1978). From the structural point of view, aequorin is a globular molecule containing a hydrophobic core cavity that accommodates coelenterazine-2-hydroperoxide (Head et al. 2000). It has four helix-loop-helix EF-hand domains, of which only three are able to bind calcium. Upon calcium binding, only two calcium ions are sufficient to trigger an irreversible reaction in which the prosthetic group and CO_2 are released and a photon is emitted ($\lambda_{max} = 470$ nm). Aequorin can be loaded into cells by a variety of techniques. The most used approach in the plant field is based on genetic transformation of plants with the apoaequorin gene and subsequent incubation in coelenterazine to obtain reconstituted active aequorin (Knight and Knight 1995). In transgenic *Nicotiana plumbaginifolia* plants, a variety of stress stimuli such as mechanical (touch, wind) stress, cold-shock, and elicitors, induces an increase of intracellular calcium characterized by stimulus specific kinetic patterns (Knight et al. 1991; Knight et al. 1992).

An important advance provided by aequorin is the possibility to target apoaequorin towards intracellular organelles by adding sorting sequences to the primary sequence of the protein (Brini 2008). In plant cells, one remarkable application, besides targeting aequorin to the nucleus and to the ER, was to fuse apoaequorin with a tonoplast proton-pyrophosphatase in a way that active aequorin is adjacent to the cytoplasmic face of the vacuolar membrane (Knight et al. 1996). The authors were able to prove that the cytosolic calcium increase due to rapid cooling (a temperature drop of several degrees in a few seconds described as "cold-shock" Minorsky 1989; for a detailed study see Plieth et al. 1999) resulted

from influx of calcium from both the external medium and from the vacuole (Knight et al. 1996).

As the vacuole is a very important calcium store (Martinoia et al. 2007), efforts were made to characterize calcium-permeable tonoplast channels (Pottosin and Schonknecht 2007). To study the involvement of Slow Vacuolar (AtTPC1) channels from *Arabidopsis thaliana* (Hedrich and Neher 1987; Peiter et al. 2005) in calcium signalling, aequorin was expressed in wild-type, tpc1-2 knockout, and AtTPC1-overexpressing plants. No significant differences were found among these plants when they were challenged with abiotic (cold, hyperosmotic, salt, and oxidative) stresses, elevation in extracellular Ca^{2+} concentration, and elicitors such as elf18 and flagellin (Ranf et al. 2008).

Recently the Cold-Induced-Transient Conductance (CITC), localized on the plasma membrane of mesophyll cells, was characterized by patch-clamp as an outward rectifying calcium-permeable channel. The authors tested several *Arabidopsis* mutants but it was not possible to identify CITC genes (Carpaneto et al. 2007). To gain insights into the timing of cold-induced voltage- and Ca^{2+}-changes, current-clamp recordings on apoaequorin-expressing *Arabidopsis* leaves were performed (Fig. 1). Leaf sections were impaled with microelectrodes and correspondent Ca^{2+} changes were followed. Upon perfusion of leaf sections with cold solution (indicated by the downward arrow in the figure) the membrane potential depolarized and aequorin bioluminescence, recorded by a high resolution CCD camera, was emitted. When the cold stimulus was removed (at the time indicated by the upward arrow) by perfusion with a solution kept at room temperature the membrane potential repolarized and luminescence decreased. These data confirm previous findings showing that the initial phase of the depolarization is synchronous with Ca^{2+} influx (see Knight 2002).

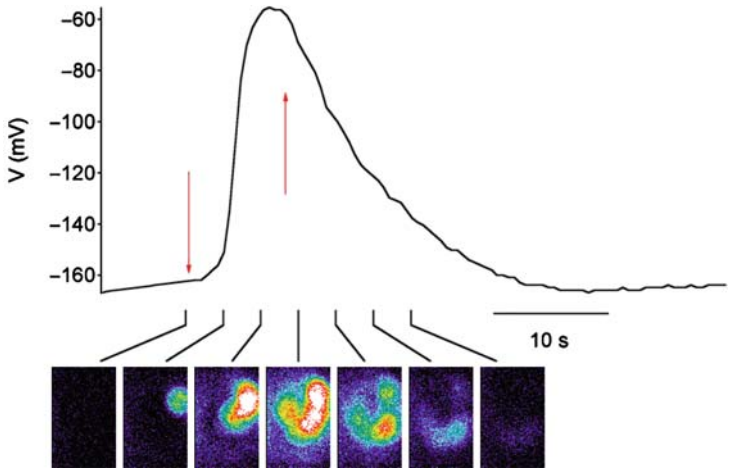

Fig. 1 Cold-induced changes in membrane potential and cytosolic Ca^{2+}. From Carpaneto et al. (2007, copyrighted by the American Society of Plant Biologists and reprinted with permission)

2.2 *Fura-2*

The major disadvantage in the use of aequorin for calcium detection is that the photon intensity is too low to allow single cell imaging. Indeed, only one photon per molecule and only a small fraction of the protein pool emits light (Brini 2008). Therefore other fluorescent dyes such as indo-1 and fura-2 were used. However, unlike many animal cells, plant cells do not readily take up the esterified form of these molecules dyes (McAinsh et al. 1990; Gilroy et al. 1991; Gilroy 1997) and a "low-pH" loading method was successfully developed for plant protoplasts (Bush and Jones 1987). Alternatively, the dye can be micro-injected into the cytoplasm by iontophoresis (McAinsh et al. 1990; Gilroy et al. 1991). The latter approach showed that in guard cells the increase in calcium is induced by ABA, a phytohormone known to report the water status of the plant, and that this signal preceded stomatal closure.

To simultaneously control the membrane voltage and record calcium concentration, multi-barrelled microelectrodes are necessary. *Vicia faba* guard cells were impaled using four-barrelled microelectrodes (Grabov and Blatt 1997): two barrels (one for membrane voltage measurement and the second for current injection) were used for voltage-clamping the cell while the others were connected to an iontophoresis module for fura-2 loading. Extreme acid loads (applying 30 mM Na^+-butyrate in the external solution) evoked cytosolic calcium increase and a parallel negative shift in the activation threshold of inward rectifying potassium channels. Using the same technique in a subsequent paper, the authors showed that hyperpolarization triggered a calcium influx (Grabov and Blatt 1998).

Microinjection of fura-2 in guard cells by iontophoresis can also be performed by three barreled microelectrodes. Levchenko et al. (2005) loaded *V. faba* guard cells through current injection with up to 500 pA from the third barrel whereas cells were kept at a holding voltage of −100 mV. In this case the injection current from the third barrel was automatically compensated by a current coming from the second barrel. Application of ABA in the external solution transiently activated anion channels after a lag phase of about 2 min, without a parallel rise in cytosolic free-calcium concentration.

Similar experiments were performed in *Nicotiana tabacum* guard cells (Marten et al. 2007): ABA triggered a transient rise in cytoplasmic calcium in 14 out of 19 cells under investigation while in the remaining five cells ABA stimulated anion channels without a change in cytoplasmic Ca^{2+}. The authors concluded that guard cells have evolved both calcium-dependent and -independent ABA signaling pathways and that the use of these pathways is species-dependent. In guard cells from *N. tabacum* anion channels could also be activated by CO_2 and darkness in a Ca^{2+}-independent manner but the anion channel activity was enhanced by parallel increases in the cytosolic Ca^{2+} concentration (Marten et al. 2008). Both the activation of anion channels induced by CO_2/darkness and Ca^{2+} signals were repressed in NtMPK4-encoded MAP kinase-silenced guard cells (Marten et al. 2008).

The three barreled microelectrode technique was applied to both intact leaves and epidermal strips from *V. faba* in order to compare calcium homeostasis and stimulus-induced calcium signals (Levchenko et al. 2008). At hyperpolarizing potentials of −100 mV, intact guard cells were able to maintain much lower cytoplasmic calcium concentration than epidermal strips. Further hyperpolarization opened hyperpolarized-activated calcium permeable channels (McAinsh and Pittman 2009) with a consistent rise in Ca^{2+} concentration that returned to the pre-stimulus level in intact plants but not in epidermal strips (Levchenko et al. 2008). Epidermal strips are thus less efficient than intact cells in buffering free cytosolic calcium concentration.

2.3 Green-Fluorescent-Protein-Based Calcium Indicators

Green fluorescent protein (GFP, Shimomura 2005) represents one of the most investigated proteins, especially as a marker of gene expression and protein targeting in intact cells and organisms (Tsien 1998; Fricker et al. 2006). Indicators based on GFP, specific for calcium detection, were constructed such as cameleon (Miyawaki et al. 1997), camgaroo (Baird et al. 1999), Flash- and Inverse Pericam (Nagai et al. 2001), and the troponin C-based sensor TN-L15 (Heim and Griesbeck 2004).

In plant cells the pH-independent, GFP-based calcium indicator cameleon 2.1 was constitutively expressed in *A. thaliana* and allowed time-dependent measurements of cytoplasmic calcium at the level of single guard cells (Allen et al. 1999). Until now there were no attempts to combine electrophysiological approaches and calcium detection with cameleon in plant cells.

3 Patch-Clamp

Patch-clamp is the most powerful technique to investigate the functional properties of ion channels at the single cell level (Sakmann and Neher 1995; Hille 1992). Fluorescent substances such as Lucifer Yellow were used in guard cell protoplasts of *V. faba* to monitor the equilibration time between the pipette solution and the cytoplasm after reaching the whole-cell configuration (Marten et al. 1992).

The compound fura-2 (Grynkiewicz et al. 1985) is generally known as an indicator dye for measuring the free calcium concentration inside living cells. As pointed out by Neher (1995) fura-2 is actually a divalent metal ion chelator and can influence calcium signals. The chelating property of the dye can be used to estimate the actions of endogenous buffers or, when fura-2 is present at high concentration, to outcompete them. In the latter case, calcium fluxes across the membrane can be measured in order to estimate fractional calcium current in non-selective cation channels (see, as an example, Burnashev et al. 1995).

3.1 Whole-Cell Measurements

In plant cells there are a few interesting examples where fura-2 was used in combination with patch-clamp. Schroeder and Hagiwara (1990) sealed a patch-clamp pipette containing 100 μM fura-2 to the plasma membrane of a *V. faba* guard cell; after reaching the whole-cell configuration and waiting 15 min for equilibration of the pipette solution with the cytoplasm, exposure of the cells to ABA produced (in responsive guard cells) a transient increase in calcium concentration with a parallel increase of an inward ion current. Reversal voltage measurements proved that these currents were mediated by non-selective cation channels. These data provided direct evidence of ABA-activated calcium-permeable channels. Using a similar approach applied to *A. thaliana* guard cells, Pei et al. (2000) discovered activation of plasma membrane calcium permeable channels by hydrogen peroxide.

Romano et al. (2000), using patch pipettes filled with calcium green-1 or (for ratiometric images) indo-1, showed that increases in cytosolic calcium were not necessary for ABA-inhibition of inward rectifying potassium channels in *V. faba* guard cells, pointing to a calcium-independent mechanism in impairment of stomatal opening by ABA. Finally, Levchenko et al. (2008) applied negative voltages through a patch pipette in whole-cell configuration to protoplasts from *V. faba* guard cells. This stimulus elicited negative currents and parallel calcium increases that could be only reverted by depolarization or by blocking the calcium entry. This is different to what was observed in intact cells where similar hyperpolarizing voltages induced transient calcium rise (see Sect. 2 in Chapter "The Role of Ion Channels in Plant Salt Tolerance").

It is worth noting that in both *A. thaliana* and *V. faba* mesophyll protoplasts the fura-2 concentration in the cytoplasm never reached a steady state level and continuously rose after attaining the whole cell configuration. Possibly, the central vacuole and the chloroplasts prevent effective dye loading or alternatively, the large mesophyll cells need more loading time. In addition, chloroplasts partially absorb the fluorescence, resulting in low fluorescence intensities. All these reasons make simultaneous patch-clamp and optical measurements in mesophyll cells very demanding (P. Dietrich, University of Erlangen, Germany, personal communication). Recently the redox properties of fura-2 were investigated by cyclic voltammetry and it was shown that fura-2 could be reversibly oxidized (Gulaboski et al. 2008). These results suggest that the redox transformations of the fura-2 forms do not affect its calcium binding ability and thus, indipendently of which redox form of fura-2 is present inside the cell, no change in calcium detection by fura-2 is expected.

3.2 Fluorescence Combined with Excised Patch (FLEP)

The same approach as used for guard cells, i.e. loading fura-2 through the patch pipette in a whole-cell configuration, has also been applied in plant vacuoles. However, under the experimental conditions tested by the authors, loading was

not efficient in the whole vacuole configuration (Gradogna et al. 2009). This could be due to either low diffusion rates in the highly viscose vacuolar sap or to the presence inside the vacuole of substances able to quench fura-2 fluorescence. To overcome this problem the authors excised the membrane patch and focused the photomultiplier on the tip of the recording pipette where fura-2 was present. Obvious advantages of this recording mode were the absence of any loading time and no interference by luminal calcium buffers. Moreover photobleaching was not a problem because calcium/fura-2 complexes near the tip were continuously substituted by free fura-2 molecules contained in the upper volume of the patch pipette (representing a quasi-infinite reservoir of the dye). FLEP recordings are presented in Fig. 2; the patch-clamp technique was applied to carrot root vacuoles, as described in Gradogna et al. (2009), in the cytosolic-side out configuration in the presence of 100 μM fura-2 inside the patch pipette. Fluorescence signals induced by excitation light at 380 and 340 nm (Fig. 2a, upper panel) were recorded simultaneously with vacuolar currents (Fig. 2a, lower panel) elicited by the voltage protocol (Fig. 2a, middle panel). Upon voltage depolarization, SV channels opened and fluorescence signals at 380 and 340 nm respectively decreased and increased, thus indicating a calcium flux from the cytosol to the vacuolar side. For the sake of clarity, the same SV currents for all clamping voltages are shown in Fig. 2a and b. In these experimental conditions, the outward rectifying SV currents were active from −10 mV, as shown in Fig. 2c. Indeed −10 mV elicited a negative potassium current and simultaneously an opposite flux of calcium was recorded, see panel A and Gradogna et al. (2009). From a mechanistic perspective at the protein level, it is clear that the opposite passage of potassium and calcium is not contemporaneous but each of the two ions enters into the pore according to its accessibility and electrochemical gradient. It is worth noting that the opposite unidirectional fluxes cannot be distinguished with conventional patch-clamp recordings and this underlines the potential benefits of the FLEP technique in investigating divalent ion channels/transporters.

Several lines of evidence indicated that calcium was permeating through the SV channel: SV channel blockers such as nickel, zinc, lanthanum, and chloramine-T abolished both currents and fluorescence signals. Moreover, *A. thaliana tpc1-2* mutants which lack SV channels, did not show any variation in current or fluorescence signals in the presence of these modulators. If it is assumed that (1) in symmetrical potassium conditions (105 mM) with 2 mM/5 nM calcium in the cytosol/vacuole, the outward current at 0 mV is only mediated by calcium, and (2) a linear relationship holds between calcium current and fluorescence (see appendix in Gradogna et al. 2009), then the fractional calcium current (P_f) of the SV channel can be estimated. The authors evaluated that P_f is voltage dependent and that it approximately corresponds to 10% of the total SV currents at strongly depolarized potentials (Gradogna et al. 2009).

Interestingly, besides calcium, fura-2 can also detect other divalent ions (i.e. cadmium, zinc) whereas metal ions such as manganese, nickel, and cobalt act as quenchers of fura-2 fluorescence. This adds to the possibility of using the FLEP technique to study the transport of these ions through different channels/transporters.

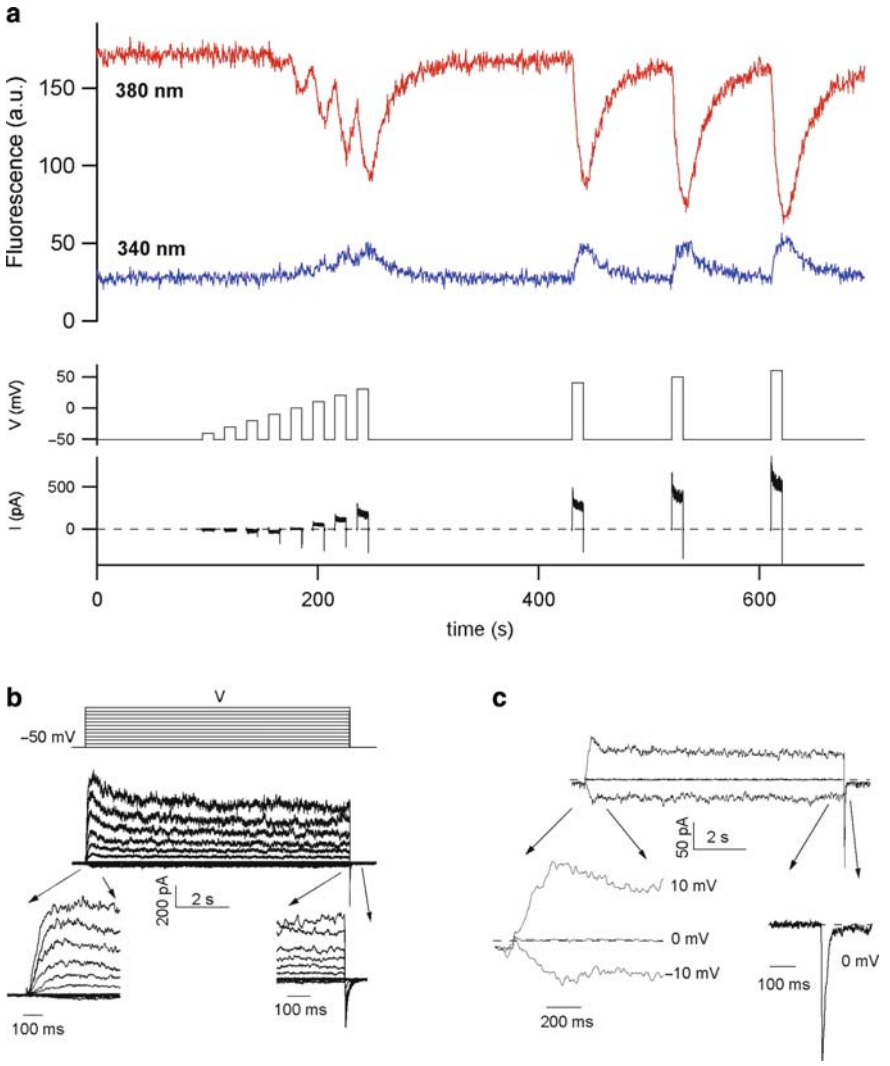

Fig. 2 Simultaneous recordings of ionic currents using the patch-clamp technique and calcium flux measured with the fluorescent calcium-sensitive dye fura-2 in isolated *Daucus carota* vacuoles. (**a**) *Upper panel*: fura-2 fluorescence excited at 380 and 340 nm. Middle panel: applied voltage versus time. *Lower panel*: SV currents recorded in cytosolic side-out configuration versus time. (**b**) and (**c**) The same Slow Vacuolar currents as shown in A are superimposed for clarity. Cytosolic bath solution was (in mM) 100 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 20 Hepes/Tris, 2 DTT, pH = 7. Pipette (luminal) solution was (in mM) 100 KCl, 0.1 fura-2, 2 $MgCl_2$, 20 Hepes/Tris, pH = 7. Sorbitol was added in both solutions to obtain a final osmotic pressure of 450 mOsM

From a certain point of view the FLEP technique resembles radiotracer flux measurements: focusing on a relatively small volume of a few picoliters inside the pipette, fluorescence signals can be amplified by increasing the duration of voltage

stimulus. Calcium tends to accumulate inside the pipette due to relatively slow diffusion (Gradogna et al. 2009). As plant ion channels rarely show inactivation, this technique seems thus very promising in addressing important challenges in divalent ion transport through plant cell membranes.

4 New Prospects in Investigating Calcium Permeable Channels

Several new optical/electrophysiological approaches were recently developed to investigate ion channel functions. Among many attempts to reveal single channel openings using only optical detection, the combination of voltage-clamp with fluorescence calcium imaging using Total Internal Reflection Microscopy (TIRF) is the most successful (Demuro and Parker 2005). The use of voltage sensitive dyes is promising to study the changes in membrane potential of a large number of cells or organelles like plant vacuoles (Konrad and Hedrich 2008). Voltage-clamp fluorometry is very useful to observe real-time conformational changes associated with ion channel gating (Gandhi and Olcese 2008). Finally, in light microscopy, "diffraction-unlimited" resolution has become most evident: the first example was represented by STED (Stimulated Emission Depletion Microscopy) that displayed a resolution down to 28 nm (Hell 2003). These topics are briefly discussed below.

4.1 Voltage-Clamp and TIRF

After the introduction of highly-sensitive fluorescent calcium-indicator dyes it became possible to visualize the diffusional spread of calcium induced by the openings of calcium-permeable channels. The concerted openings of calcium channels generated puffs and sparks, or smaller events called blips and quarks (for review see Demuro and Parker 2006). Combining electrophysiological and optical approaches Zou et al. (2002) and Wang et al. (2001) could measure Single-Channel Calcium Fluorescent Transitions (SCCaFTs) for the first time. However the best recordings of the so called optical patch-clamping was performed by Demuro and Parker (2005); different from FLEP, the activity of single calcium channels can be monitored by the excitation of the fluorophore close to channel mouth in a volume of approximately 0.1 fl (Shuai and Parker 2005). This was accomplished using the evanescent wave generated by TIRF to slightly (100 nm) penetrate inside a *Xenopus* oocyte expressing muscle nicotinic acetylcholine receptor channels and previously loaded with fluo-4 dextran (Demuro and Parker 2005). The voltage of the oocytes was controlled by the Two Electrode Voltage-Clamp (TEVC) technique. Fluorescence changes provided information on the simultaneous activity of more than 400 channels in the imaging field, namely on open and closed durations, open probability vs acetylcholine concentration, and relative single channel amplitude vs voltage. The authors also studied the spatial

distribution of the channels that were randomly distributed without evidence of clustering. Moreover, the positions of the channels did not change over tens of seconds. Such information regarding the spatial distribution and lateral diffusion of ion channels cannot be obtained with conventional electrophysiological techniques. Unfortunately, the thickness of the cell wall (usually > 100 nm) is one of the major problem for the application of similar TIRF methods to plant cells (Shaw 2006).

4.2 Voltage-Sensitive Dyes

As seen before, the membrane voltage of plant cells can be accurately measured using microelectrodes. Impalement is, however, not suited for large sample screening or to monitor the membrane potential changes in intracellular organelles. Recently, Konrad and Hedrich (2008) established a method for quantification of the membrane potential in guard cell protoplasts and in vacuoles based on the fluorescence properties of bis-(1,3-dibutylbarbituric acid)-trimethine oxonol ($DiBAC_4(3)$). They were able to follow ABA-induced depolarization in guard cells as well as changes in tonoplast voltage upon ATP exposure. Simultaneous use of voltage and calcium sensitive dyes will be an interesting, non-invasive, approach to study the interconnection between voltage and calcium release in plant cells.

4.3 Voltage-Clamp Fluorometry

Voltage-clamp fluorometry (see for review Gandhi and Olcese 2008) was originally developed in Isacoff's lab (Mannuzzu et al. 1996) and is derived from two different techniques namely TEVC (Stuhmer 1998) and SCAM (scanning cysteine accessibility mutagenesis Akabas et al. 1992). The channel of interest is modified inserting a single cysteine accessible from the external side. The cysteine is then labeled with a thiol-reactive fluorophore such as PyMPO maleimide or tetramethylrhodamine maleimide. These fluorophores change their fluorescence emission depending on their exposure to solvent/lipid environment or to quenching groups. Voltage-clamp fluorometry was successfully used to report conformational changes in voltage and ligand-gated channels and to follow the dynamic properties of channel opening. This approach could be applied to plant calcium-permeable channels, such as AtTPC1, providing useful information about gating processes.

4.4 Far-Field Fluorescence Nanoscopy

Lens-based (far-field) optical microscopes were believed to have resolution limits (d) of $d = \lambda/(2n \sin\alpha)$ where λ is the wavelength of light, $n \sin\alpha$ the numerical

aperture of the objective (n is the refractive index of the media and α one-half the angular aperture of the objective). This limit is imposed by the diffraction properties of light. However, recently, diffraction-unlimited resolution has become evident (see, as an excellent review, Hell 2009). The basic idea of techniques such as STED (Hell and Wichmann 1994; Klar et al. 2000), GSD (Hell and Krough 1995), SPEM (Heintzmann et al. 2002), SSIM (Gustafsson 2005), RESOLFT (Hofmann et al. 2005), PALM (Betzig et al. 2006), PALMIRA (Egner et al. 2007), STORM (Rust et al. 2006), FPALM (Hess et al. 2006), PAINT (Sharonov and Hochstrasser 2006), dSTORM (Heilemann et al. 2008), TL-PALM (Biteen et al. 2008), and GSDIM (Folling et al. 2008) is to switch off or on signals from fluorescent molecules so that single molecules can be seen consecutively (for a brief summary of all these techniques see Hell 2009). Remarkably, resolution of less than 20 nm was obtained (Betzig et al. 2006). Microscopy is thus being replaced by nanoscopy and the investigation of calcium microdomains is being substituted with the study of calcium at the nanometer level.

5 Conclusion

In this chapter, we underlined the power of combined electrophysiological approaches and optical calcium detection in order to investigate calcium permeable ion channels in plant cells. The recent developments in microscopy offer new advanced tools to characterize calcium permeation in plant systems entering the nanometer scale. In turn, this will help to elucidate the molecular and physiological role of calcium and other divalent ions in plant stress responses.

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Vacuolar Ion Channels: Roles as Signalling Mechanisms and in Plant Nutrition

Frans J.M. Maathuis

Abstract Vacuoles play various roles in many physiologically relevant processes in plants. Some of the more prominent are turgor provision, the storage of minerals and nutrients and cellular signalling. To fulfil these functions a complement of membrane transporters is present at the tonoplast. Prolific patch clamp studies have shown that amongst these, both selective and non selective cation channels (NSCCs) control key vacuolar functions: The non-selective SV channel is Ca^{2+} permeable and has been proposed to have signalling roles during germination, stomatal opening and in response to pathogens. The K^+ selective VK channel impacts on K^+ nutrition and stomatal closure. Ligand-gated channels form possible pathways for vacuolar Ca^{2+} release whereas the FV channel may be important in overall K^+ homeostasis. This chapter will summarise and review the main functions of vacuolar ion channels with particular emphasis on their roles in abiotic and biotic stress.

Abbreviations

cADPR	Cyclic ADP-ribose
FV	Fast vacuolar channel
IP_3	Myo-inositol 1,4,5-triphosphate
LV	Lytic vacuole
NSCC	Non selective cation channel
PMF	Proton motive force
PSV	Protein storage vacuole
SV	Slow vacuolar channel
VK	Vacuolar K^+ channel
TMD	Transmembrane domain

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1 Introduction

Plant cells contain different types of vacuole, the most prominent being the central lytic vacuole (LV) which can occupy as much as 95% of the cellular volume (Leigh and Sanders 1997). LVs play many essential roles both at the cellular and tissue level. They constitute the main site of turgor generation through their role as depository for minerals and water, thereby providing structure to plants. LVs also are the compartment where xenobiotic and toxic compounds are sequestered to minimise potential toxicity in the cytoplasm which contains the bulk of the biochemical machinery. Containment of secondary metabolites and proteins involved in plant defence such as alkaloids and proteases forms a further function whereas the low pH of LVs is believed to be important in degradation of both exogenous and endogenous compounds such as proteins. Since the LV also forms a large store of cellular Ca^{2+} it is important for cellular signalling.

A second type of vacuole is present, predominantly in storage tissues such as seeds, but it is also found in vegetative cells. In seeds, these protein storage vacuoles (PSVs, previously also known as protein bodies) are deposits for minerals and proteins that are essential for the development of the embryo both pre- and post-germination. PSVs contain internal compartments, crystalloids and globoids, which respectively contain protein and complex salts such as phytate (Leigh and Sanders 1997; Bethke et al. 1998).

1.1 The Role of Vacuoles in Plant Nutrition

The general mechanisms regarding the role of vacuoles in plant nutrition have been amply documented: for example, plants grown on K^+-rich media will deposit large quantities of this nutrient in the LVs of vegetative tissues (Maathuis and Sanders 1993). This "luxury" consumption of K^+ allows plants to survive subsequent exposure to K^+ deficient conditions by mobilising the vacuolar store in order to maintain cytoplasmic K^+ homeostasis (Walker et al. 1996). This scenario not only pertains to K^+ but has also been observed for other (macro)nutrients such as P, N and S (Marschner 1995). Indeed, the solute composition in vacuoles is highly dynamic with constant adaptations to changing environmental and developmental conditions and vacuolar contents reflect changes in external nutrient level not only for macronutrients but also for important micronutrients like Fe and Zn (Marschner 1995). This mineral storage function of LVs is to some extent tissue dependent: techniques such as X-ray analysis and single cell sampling have revealed that, in barley leaves, Ca^{2+} and Cl^- are mainly detected in vacuoles of epidermal cells but not mesophyll cells. In contrast, P is more abundant in mesophyll vacuoles compared to epidermal vacuoles (Leigh and Storey 1993).

PSVs are essential for delivery of nutrients to the germinating seed (Otegui et al. 2002). Inorganic minerals such as K^+ and Ca^{2+} are released from phytate and

distributed. Phosphorous is released via break down of phytate whereas reduced carbon and nitrogen derive from metabolisation of starch and storage proteins. The subsequent early stages of seedling growth and development are also often dependent on seed storage although this varies greatly from species to species.

The storage function of both LVs and PSVs not only presents a nutritional buffer essential for plant growth and development but also provides a main source in human and animal diets of sugars, minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and P, and it is a main source of protein.

1.2 Vacuoles and Signalling

LVs provide a major intracellular Ca^{2+} store with concentrations typically in the millimolar range. Both ATP driven Ca^{2+} pumps and H^+-coupled Ca^{2+} antiporters contribute to vacuolar Ca^{2+} accumulation and to maintaining a resting cytoplasmic Ca^{2+} concentration of around 100 nM. LVs are believed to be important contributors to stimulus-invoked changes in cytoplasmic Ca^{2+} (Sanders et al. 2002). Vacuolar Ca^{2+} release during signal transduction occurs through tonoplast located ion channels. Some of these are voltage sensitive whereas others are believed to be under control of ligands (Pottosin and Schoenknecht 2007). Whether PSVs play a similar role to LVs in terms of Ca^{2+} signalling has yet to be established.

1.3 The Role of Vacuoles in Detoxification

Nutrient and non-nutrient minerals are often deficient in natural environments. Yet there are many regions where local concentrations of particular minerals are extremely high. This is particularly evident where salinisation is concerned and levels of Na^+ and Cl^- can easily exceed 100 mM. Other minerals that frequently occur in excess are heavy metals such as Fe^{2+}, Pb^{2+} and Cd^{2+} and metalloids such as arsenic. In all cases, plant uptake of these potentially toxic elements can be significant because they tend to mimic other, often beneficial, minerals. For example, the chemical properties of Na^+ are very similar to K^+ (an essential macronutrient) and Na^+ therefore interferes with K^+ transport. Similarly, Pb^{2+} and Cd^{2+} can be taken up through transport systems that normally move Ca^{2+} while arsenic in the form of arsenate (AsO_4^{3-}) enters plants through phosphate transporters (Ali et al. 2009).

Uptake of toxic minerals induces various detoxification responses which often culminate in vacuolar deposition. In the case of elements like Na^+ and Cl^-, the ions are directly moved across the tonoplast by channel and carrier type transporters. In contrast, heavy metals and metalloids such as arsenic are believed to be chelated to non-protein thiols such as glutathione, phytochelatins or amino acids like histidine.

The entire complex may then be loaded into the vacuole via ABC-type transporters (Martinoia 2007).

Similar mechanisms are in place to sequester more complex xenobiotics in the vacuole. Important examples are chemical herbicides that are conjugated to glutathione and then stored in the vacuolar lumen. The overall effect of these strategies is the removal of potentially harmful substances from the cytoplasm and thus minimising inhibitory impact on enzyme activity.

1.4 Tonoplast Membrane Transporters

The dynamic and spatial aspects of vacuolar nutrient contents and vacuolar signalling events critically depend on the concerted action and regulation of tonoplast transporters. Luminal acidification of LVs is achieved through the activity of primary H^+ pumps, the V-ATPase and the PPase. The resultant Proton Motive Force (PMF) consists of a steep pH gradient (acidic in the vacuolar lumen) and a rather low tonoplast potential which is generally believed to be in the range of -10 to -30 mV (Walker et al. 1996; Pottosin and Schoenknecht 2007). The PMF is used to energise secondary transporters for transtonoplast fluxes of organics such as sugars and amino acids, and minerals such K^+, Na^+, Ca^{2+} and NO_3^-. Ion channels form a third class of tonoplast transporter that mediates fluxes down the electrochemical potential of the permeating ions.

Many primary and secondary transport gene families that localise to vacuolar membranes have been identified (Martinoia 2007). For the tonoplast some of the transporter genes have been cloned and characterised. Molecular identification has often been achieved either through homology with mammalian systems, e.g. Ca^{2+} ATPases and Na^+:H^+ antiporters, or because proteins are highly abundant, e.g. V-ATPases and PPases, and therefore co-purify with tonoplast fractions. Gene families that contain secondary tonoplast transporters involved in vacuolar nutrition and signalling include the cation:H^+ exchangers (CHX family), Ca^+:H^+ exchangers (CAX family), heavy metal transporters (CDF family), Ca^{2+} pumps (ACA family), aquaporins (TIP family), glutathione S-conjugate transporting ABC pumps (MRP and other subfamilies) and possibly ATP energised heavy metal pumps from the CPx-ATPase family. In addition, there is evidence for tonoplast localisation of isoforms from the HAK/KUP family and the CLC family.

The amenability of vacuoles to patch clamp methodology ensured that tonoplast channels were amongst the first and best characterised plant ion channels (Leigh and Sanders 1997). The accumulative data show the presence of several cation and anion channels (Fig. 1). The ubiquitous slow vacuolar (SV) channel has K^+/Na^+ and K^+/Ca^{2+}selectivity ratios of around 1 and 4 respectively, is activated by tonoplast depolarisation, has slow kinetics and requires elevated cytoplasmic Ca^{2+} concentrations. The fast vacuolar (FV) channel has a similar low K^+/Na^+ selectivity ratio as the SV channel but is inhibited by elevated $[Ca^{2+}]_{cyt}$. Vacuolar K^+ (VK) cation channels have been recorded in many plant vacuoles where they are

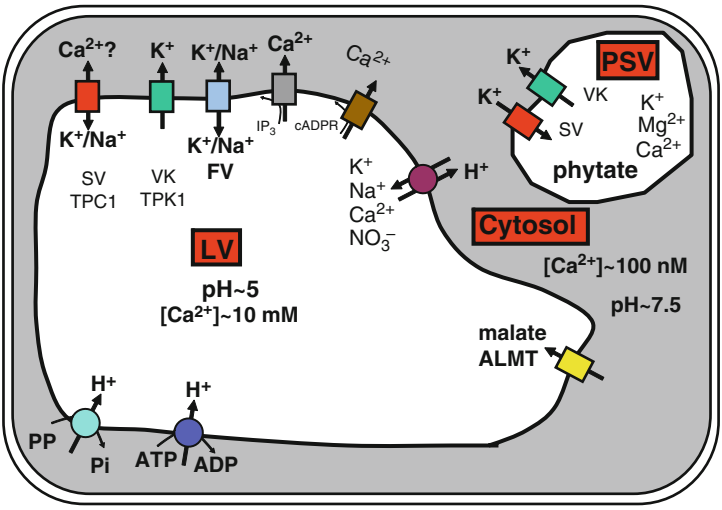

Fig. 1 Overview of the main primary and secondary transport systems in the vacuolar membrane. Plants have at least two different vacuoles, the large lytic vacuole (LV) and the smaller protein storage vacuole (PSV) which is particularly prevalent in seed and storage tissues. The LV membrane, the tonoplast, contains two primary H^+ pumps, the V-ATPase and the PPase which establish a tonoplast PMF by moving protons into the vacuole. The PMF is subsequently used to drive secondary transport through carriers and ion channels. Different antiport systems have been identified that are involved in the transtonoplast movement of cations such as K^+, Na^+ and Ca^{2+} and anions such as NO_3^-. Anion channels are present to conduct Cl^- and malate and non selective cation channels (NSCCs) include the slow vacuolar (SV) and fast vacuolar (FV) conductances whereas the vacuolar K^+ (VK) channel is selective for K^+. In addition, Ca^{2+} selective channels have been described that are gated through binding of cytosolic ligands like IP_3 and cADPR. The SV and VK channels have been shown to be encoded by TPC1 (two pore channel) and TPK1 (two pore K channel) respectively. Far less is known about the PSV tonoplast but both SV and VK type conductances have been recorded from this organelle

involved in turgor regulation and K^+ nutrition. Anion channels permeable to malate, Cl^- and NO_3^- are present at the tonoplast and in addition to the SV channel, two or three further types of Ca^{2+} permeable channel have been described which are either voltage dependent or ligand gated.

Until recently, none of the encoding genes for these channels was known. However, the identification of the *Arabidopsis* SV channel as AtTPC1 (Peiter et al. 2005) and the VK channel as AtTPK1 (Gobert et al. 2007) has led the way to in depth studies regarding their regulation and physiological function. The presence of CLC isoforms on the tonoplast has also been shown although some of these may function as H^+-coupled antiporters to drive vacuolar NO_3^- accumulation (De Angeli et al. 2006). For other types of tonoplast ion channel data mainly derive from electrophysiology. In the subsequent sections I will discuss the data currently available regarding roles of each type of vacuolar cation channel in nutritional and signalling aspects.

1.5 The Slow Vacuolar Channel

The SV channel (TPC1) is now well established as an ubiquitous voltage dependent non-selective cation channel (NSCC) which has been found in many different plant species and all plant tissues. It shows characteristically slow activation at depolarising tonoplast potentials and its current is therefore predominantly outward (i.e. directed out of the cytoplasm). Apart from voltage dependence, the SV channel is sensitive to both cytoplasmic and luminal Ca^{2+} levels (Pottosin and Schoenknecht 2007). Elevation of luminal Ca^{2+} has a strong inhibitory effect on channel opening by shifting the activation potential further positive. The mechanistic details of this process are unknown and so is the location of the binding site. In contrast, two clearly identifiable EF-hand motifs between transmembrane domain 6 and 7 are present. Binding of Ca^{2+} to these domains is believed to be responsible for the steep increase in SV channel open probability. SV channel activity is further regulated by a host of mechanisms including phosphorylation (Bethke and Jones 1997), 14-3-3 proteins (van den Wijngaard et al. 2001), organic cations and redox potential (Scholz-Starke et al. 2004).

Early reports suggested the SV channel may be involved in maintaining turgor and tonoplast potential (Hedrich et al. 1988) and during salt stress by preventing Na^+ leakage from the vacuole (Maathuis and Prins 1990). Much later, the *Arabidopsis* SV channel was shown to be encoded by AtTPC1 (two pore channel; Peiter et al. 2005), a protein with a secondary structure that consists of two times 6 transmembrane domains (TMDs), 2 Ca^{2+} binding EF domains and at least one putative 14-3-3 binding site (Fig. 2). Its pore structure does not resemble that of classical K^+, Ca^{2+} or Na^+ channels. The molecular identification of the SV channel enabled studies into the physiological role of this transporter by manipulating its expression levels. Growth and cation contents during exposure to deficiency and stress levels of a number of minerals were not significantly affected by TPC1 expression (Peiter, Sanders and Maathuis, unpublished data). This indicates that the SV channel is unlikely to be particularly important in plant nutrition. However, in an *Attpc1* loss of function (knockout) mutant, ABA-induced delay of

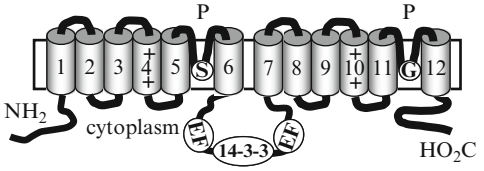

Fig. 2 The SV channel is encoded by TPC1, a protein with a secondary structure that consists of two times 6 transmembrane domains (TMDs). Two 12 TMD subunits form a functional channel. Between TMD 6 and 7, two Ca^{2+} binding EF domains are present that are believed to be responsible for the steep Ca^{2+} dependence of channel opening. In between the EF motifs a putative 14-3-3 binding site is present. The pore regions (P) of TPC1 contain serine (S) and glycine (G) residues resulting in an "SGSG" selectivity filter in the holoenzyme. Ca^{2+} and Na^+ selective channels have "EEEE" and "DEKA" selectivity filters

seed germination was significantly less whereas the opposite was observed in overexpressing lines (Peiter et al. 2005). In *Arabidopsis* guard cells, ABA dependent closure was not affected by AtTPC1 but high external Ca^{2+}, another well documented closing stimulus, largely failed to evoke stomatal closure in the knockout mutant. In rice, OsTPC1 loss of function led to a reduced response to the fungal elicitor xylanase and to fewer cells showing the hypersensitive response, an important mechanism to combat pathogenic infection (Kurusu et al. 2005). A similar phenotype was recorded in tobacco BY-2 suspension cells. In this case the fungal elicitor cryptogein produced a smaller Ca^{2+} signal, less cell death and less induction of defence gene transcription in an *NtTPC1* co-suppression line (Kadota et al. 2004).

Heterologous expression of TPC1 restores Ca^{2+} uptake in *cch*/*mid1* yeast, a strain that lacks its plasma membrane high affinity Ca^{2+} uptake mechanism (Furuichi et al. 2001). This suggests TPC1 can mediate Ca^{2+} flux. Also, many of the TPC1 related phenotypes point to a Ca^{2+} signalling function of this protein: The stomatal phenotype in *Attpc1* mutants is reminiscent of *det1-3* mutants which show a similar lack of stomatal closure in response to elevated external Ca^{2+} (Allen et al. 2000). More detailed studies with the *det1-3* mutant showed that the Ca^{2+} signalling in the guard cell cytoplasm is altered. A similar alteration in *tpc1* Ca^{2+} signalling could therefore explain the observed stomatal phenotype. In tobacco, direct measurements of cytoplasmic Ca^{2+} were made using the Ca^{2+} reporter aequorin. Tobacco suspension cells of the TPC1 co-suppression line showed far smaller Ca^{2+} signals than wildtype cells in response to cryptogein (Kadota et al. 2004). In rice the lack of H_2O_2 production and programmed cell death in the KO mutant also imply a possible role of TPC1 in Ca^{2+} signalling (Kurusu et al. 2005) since this is a well established intermediate in the hypersensitive response. Other pointers towards a potential role of TPC1 in reactive oxygen intermediate and Ca^{2+} signalling comes from studies comparing aequorin signals in wildtype and tpc1 plants in response to H_2O_2. At low concentration (0.1 mM) H_2O_2-induced Ca^{2+} signals are attenuated and delayed in *tpc1* KO mutants (Peiter, Sanders and Maathuis, unpubl. data), however, this effect was not detected at very high (10 mM) H_2O_2 (Ranf et al. 2008) suggesting the presence of multiple systems with varying reactive oxygen species affinities.

Indeed, interpretation of the myriad of TPC1 related data is not always straight forward and the idea that the vacuolar TPC1 channel participates in vacuolar Ca^{2+} release and cellular Ca^{2+} signalling has been challenged for several reasons. Heterologous expression can alter transport properties and membrane targeting which complicates extrapolation of yeast data to plant systems. The work in tobacco and rice was carried out under the mistaken belief that NtTPC1 and OsTPC1 were localised in the plasma membrane and functioned in influx of apoplastic Ca^{2+}. However subsequent work clearly showed that OsTPC1 and NtTPC1, like their *Arabidopsis* counterpart, are vacuolar channels (Ranf et al. 2008).

More importantly, there are many strong electrophysiological data that show the SV channel open probability is exceedingly small in the presence of Ca^{2+} gradients and tonoplast potentials that could produce vacuolar Ca^{2+} release. Although there is

a large inward Ca^{2+} gradient, the presence of millimolar Ca^{2+} in the vacuolar lumen reduces an already low open probability at physiological tonoplast potentials. This results in an effective channel open probability of near zero, even when tonoplasts are significantly depolarised (Pottosin and Schoenknecht 2007).

The role of the SV channel in Ca^{2+} signalling was also studied using a direct Ca^{2+} measuring approach with the Ca^{2+} reporter aequorin: in response to a range of stimuli, aequorin reported Ca^{2+} in the *Attpc1* knockout background was not significantly different from that observed in wildtype plants (Ranf et al. 2008). This work showed that there was no difference in the amplitude and kinetics of Ca^{2+} signals in mutant and wildtype in response to cold shock, osmotic shock, salt stress, oxidative stress or elevation of external Ca^{2+}. In addition, exposure to elicitors such as flagellin or salicylic acid also invoked comparable Ca^{2+} signals.

In combination, these findings appear to argue against a role of SV channels in Ca^{2+} signalling and also plant nutrition and therefore begs the question of what could be the function of this ubiquitous channel which dominates the tonoplast conductance. Several scenarios are possible: there may be unknown factors that affect the SV channel voltage dependence in such a way that open probability is shifted to more physiological tonoplast potentials. For example, in animal cells it has been shown that local cytoplasmic Ca^{2+} concentrations can easily reach 0.1 mM in the vicinity of ER or plasma membrane Ca^{2+} channels (Demuro and Parker 2006). If SV channels were positioned at close range to such systems, their open probability could be sufficient to evoke SV channel mediated Ca^{2+} influx (Pottosin and Schoenknecht 2007). Alternatively, SV channels may participate in overall Ca^{2+} nutrition, rather than Ca^{2+} signalling. Although no evidence was found for this in *Arabidopsis*, overexpression of OsTPC1 in rice led to reduced growth in the presence of excess Ca^{2+} and improved growth rates when Ca^{2+} was deficient (Kurusu et al. 2004). SV channels may also be involved in regulation of cation fluxes other than Ca^{2+} and these may impact on less obvious processes such as tonoplast potential maintenance or osmotic adjustments.

1.6 The Vacuolar K^+ Channel

Vacuolar K^+ (VK) channel currents are typified by a lack of voltage dependence, intrinsic rectification and a much lower requirement for cytoplasmic Ca^{2+} than the SV channel. Due to its high selectivity for K^+ and its presence in guard cells it was hypothesised that the VK conductance might be involved in stomatal functioning.

Electrophysiological and reverse genetics approaches showed that the *Arabidopsis* VK channel is encoded by *AtTPK1* (Gobert et al. 2007). TPK1 is a member of a small gene family of two pore K^+ channels characterised by a four transmembrane/two pore structure with GYGD K^+ selectivity motifs in each pore and a varying number of putative C-terminal EF hands (Fig 3). The *Arabidopsis* genome contains 5 TPK isoforms and similarly sized TPK families have been found in genomes of other species such as rice, tobacco and *Physcomitrella* (Dunkel et al. 2008).

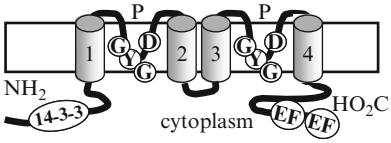

Fig. 3 TPK proteins such as the VK channel contain 4 TMDs and 2 pore regions per subunit with two subunits forming a functional channel. Each pore region (P) contains a GYGD sequence which is characteristic of K^+ selectivity, although some tobacco TPKs may have slightly different motifs. In the N terminal region, TPKs have 14-3-3 binding domains and the presence of 14-3-3 leads to channel activation. Amongst TPK isoforms the occurrence of C-terminal Ca^{2+} binding EF motifs varies from 0 to 2

Like the SV channel, the VK channel activity needs the presence of cytoplasmic Ca^{2+}. The *Arabidopsis* TPK1 is steeply Ca^{2+} dependent and requires a minimum Ca^{2+} concentration of around 1 μM, which is considerably lower than that for the SV channel. However, the Ca^{2+} dependence appears to vary: in tobacco the NtTPK1 was active in the virtual absence of any Ca^{2+} although some residual Ca^{2+} stimulation was recorded (Hamamoto et al. 2008). In rice too, the Ca^{2+} dependence of at least two isoforms (OsTPKa and OsTPKb) is considerably less than that observed for AtTPK1 (Maathuis et al. unpubl results). This divergence is likely to derive from the difference in putative Ca^{2+} binding sites which consist of two well defined EF hands in AtTPK1 to one in NtTPK1 and none in OsTPKb.

VK activity in *Arabidopsis* is also modulated by 14-3-3 binding but in contrast to the SV channel which is strongly inhibited by 14-3-3, VK channel activity is considerably promoted after binding 14-3-3 to its N-terminal 14-3-3 motif (Latz et al. 2007). However, the sequence domain encoding the 14-3-3 binding motif in AtTPK1 varies considerably amongst TPKs so whether 14-3-3 regulation is a generic mechanism for these channels remains to be seen.

No obvious morphological phenotypes are present in *tpk1* mutants when plants are grown in normal conditions. However, comparisons of *tpk1* KO mutant, wildtype and TPK1 overexpressing plants point to multiple functions for TPK1, a gene expressed in all tissues and cell types. In agreement with earlier suggestions (Ward and Schroeder 1994) TPK1 impacts on K^+ release during stomatal closure: in *Attpk1*, stomatal closure in response to ABA ultimately reached similar apertures compared to wildtype plants but this occurred with much slower kinetics. Stomatal opening, in response to the fungal toxin fusicoccin, was not affected. Thus, these results indicate that TPK1 is a major pathway for K^+ release during stomatal closure but that auxiliary mechanisms are present.

TPK1 expression influences seed germination: in knockout mutants the second phase of germination, i.e. emergence of the radicle through the endosperm layer, was considerably slower, especially in the presence of ABA. In contrast, germination occurred quicker in TPK1 overexpressors. Detailed mechanistic insights into seed germination are rare which hampers interpretation of these findings. However,

germination requires redistribution of inorganic minerals to create turgor that drives cell expansion. This is particularly important during the second phase of germination that is hallmarked by rapid radicle extension. A large fraction of the turgor generating K^+ of seeds is sequestered in PSV globoids as insoluble K-phytate (Herman and Larkins 1999). Release of this K^+ fraction can only take place after break down of phytate by the enzyme phytase and subsequent transport of K^+ out of the globoid and PSV. The latter may very well depend on K^+ selective cation channels such as TPK1. TPK1 also impacts on overall K^+ homeostasis. Both in the presence of excess K^+ (80 mM) and K^+ deficiency (0.01 mM) there was a limited growth advantage in TPK1 overexpressors compared to wildtype plants. However, there was no significant difference in tissue K^+ levels.

Like AtTPK1, the *Arabidopsis* isoforms TPK2, TPK3 and TPK5 express at the tonoplast (Voelker et al. 2006) but functional expression of these genes has so far not been successful. Interestingly, expression of these isoforms in K^+-uptake deficient *E. coli* cells does complement the *E. coli* mutation, pointing to functional expression in this heterologous system (Isayenkov and Maathuis, unpubl. data). TPK homologues in other species have been studied to some extent: the tobacco NtTPK1 comes in several variants and some of these have diverging K^+ selectivity filters in the sense that the GYGD motif is replaced by a GHGD or VHGD motif (Hamamoto et al. 2008). These authors also showed a moderate blocking effect of polyamines on NtTPK1. Although the physiological relevance of this is not clear, polyamine levels are often increased in response to abiotic stresses such as drought and salinity.

In rice, there are two close homologues of AtTPK1, OsTPKa and OsTPKb. Interestingly, the rice isoforms express to different types of vacuole with TPKa being expressed predominantly in the central LV and TPKb primarily found in smaller PSV-type compartments (Fig.4). OsTPKa and TPKb have highly similar protein sequences and how this leads to different membrane targeting is the subject of ongoing studies. Similarly, it remains to be elucidated whether OsTPKa and OsTPKb fulfil different functions in these compartments.

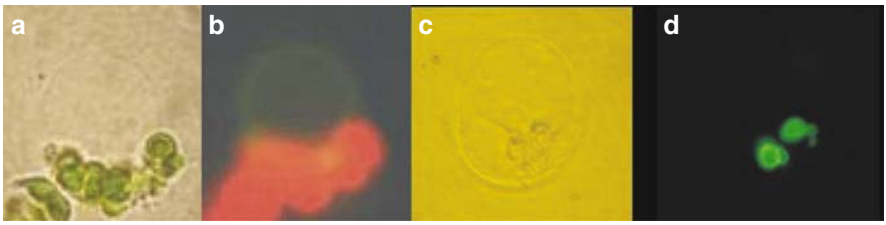

Fig. 4 Rice TPKa and TPKb localise to different types of vacuole. TPKa (**a**, **b**) is predominantly expressed in the main lytic vacuole whereas TPKb mostly resides in protein storage vacuoles (**c** and **d**). Bright light images of rice mesophyll protoplasts are shown in (**a**) and (**c**) with the protoplast in (**a**) osmotically ruptured to release the main vacuole. TPKa (**b**) and TPKb (**d**) were C terminally fused to GFP to visualise expression by fluorescence

1.7 The Fast Vacuolar Channel

Like the SV channel the FV channel has very low selectivity (Bruggemann et al. 1999) is K/Na selectivity is around unity and other monovalent cations can also permeate this protein. Whether it can transport divalent cations remains to be established. Originally described in red beet storage tissue (Hedrich and Neher 1987), few further studies have been published. FV channels become increasingly inactive whenever the cytoplasmic Ca^{2+} concentration exceeds around 200 nM and FV open probability has been reported to be largely insensitive to tonoplast potential. Subsequent publications reported on the presence of FV channels in other tissues such as barley mesophyll vacuoles (Tikhonova et al. 1997) where it shows moderate outward rectification and biphasic voltage dependence. The gene(s) encoding the FV channel is not known and this frustrates in depth studies regarding its characteristics and *in planta* role. Since both luminal and cytoplasmic K^+ levels impact on FV channel open probability, one of the physiological roles of this transporter may be in maintaining cellular K^+ homeostasis (Pottosin and Martinez-Estevez 2003). Other putative roles for the FV channel include providing a shunt conductance for the V-ATPase, osmoregulation, and regulation of the tonoplast potential (Allen and Sanders 1997).

1.8 Ligand-Gated Vacuolar Cation Channels

Several signalling pathways in animal cells are mediated by phosphoinositol compounds. Briefly, these pathways include activation of a G-protein leading to stimulation of phospholipase-C which hydrolyses the membrane lipid phophatidylinositol 4,5-bisphosphate releasing the second messenger myo-inositol 1,4,5-triphosphate (IP_3). IP_3 subsequently causes Ca^{2+} release from intracellular stores (Weernink et al. 2007).

In plants, stress such as rapid changes in osmotic pressure, produces IP_3 within minutes. Longer osmotic stress can also produce IP_3 in an ABA-dependent manner (Burnette et al. 2003). In addition, various reports showed that in plants too, IP_3 could release Ca^{2+} from isolated vacuoles or vacuolar vesicles (Alexandre et al. 1990; Allen et al. 1995) and thus forms one of the major triggers for Ca^{2+} release from internal stores. The main target of IP_3 is IP_3-gated ion channels found in endomembranes. These are capable of conducting $Ca^{2+}$and patch clamp experiments showed the presence of such channels in beet vacuoles (Alexandre et al. 1990). Single channel recordings showed a unitary slope conductance of around 30 pS and, as expected for a true ligand-gated channel, both whole vacuole and single channel currents were strictly dependent on the presence of IP_3. Channel affinity for IP_3 showed a K_m of around 200 nM which agrees well with IP_3 levels determined in tissue (Burnette et al. 2003). Current magnitude was sensitive to IP_3 concentration in a first order fashion (i.e. a Hill coefficient of 1), showing no

evidence of cooperative ligand binding, as is the case for many animal IP_3 receptors. Therefore, plant IP_3 receptors are likely to possess only one ligand binding site per channel protein.

Although this initial work was followed by a few further reports (Allen et al. 1995; Allen and Sanders 1997), surprisingly little progress has been made in the past 15 years regarding plant IP_3 channels. For example, it is not clear whether model systems such as *Arabidopsis* or rice contain IP_3-gated conductances in their vacuole. From a bioinformatics perspective, it is clear that plant genomes and proteomes (at least those that have been sequenced so far) do not contain sequences that resemble animal IP_3 receptors. Identification of genes that encode the observed plant IP_3 channels is therefore likely to be exceedingly difficult.

There is some evidence that a second type of ligand-gated cation channel is localised in plant tonoplasts. Pharmacologically, these channels resemble the mammalian ryanodine receptor, a Ca^{2+} selective channel named after its propensity to bind the plant alkaloid ryanodine. Mammalian ryanodine receptors are most prevalent in muscle sarcoplasmic reticulum where they participate in Ca^{2+} release that is necessary for muscle contraction. Although Ca^{2+} is believed to be the main physiological agonist, there are endogenous and exogenous factors that influence gating such as cyclic ADP-ribose (cADPR). cADPR is capable of releasing Ca^{2+} from intracellular stores in many cell types and this release is often potentiated by Ca^{2+} and ryanodine (Mandi and Bak 2008).

In plants, some studies showed that cADPR was able release Ca^{2+} from microsomal vesicles and also from intact red beet vacuoles (Allen et al. 1995). Nanomolar amounts of cADPR gave rise to Ca^{2+} release which can be inhibited by ruthenium red. Patch clamp experiments showed cADPR dependent currents that are mainly carried by Ca^{2+} (P_K:P_{Ca} between 0.04 and 0.1). Like vacuolar IP_3 induced currents, cADPR dependent currents are prevalent at physiological tonoplast potentials (-10 to -40 mV) and largely absent at positive potentials. Patch clamp data from intact vacuoles further showed that IP_3 and cADPR produce additive Ca^{2+} currents, suggesting that both types of receptor are present in the same membrane.

A recent study (Pottosin et al. 2009) did not find evidence of cADPR-induced Ca^{2+} release in intact vacuoles from red beet. As with plant IP_3-gated channels, little further work has been carried out regarding the exact nature of the putative cADPR dependent channel. Particularly single channel data are needed, preferably from more than one species, to firmly establish that such transporters are common in plant membranes.

With the SV channel as a potential Ca^{2+} release pathway, IP_3-gated Ca^{2+} permeable channels and cADPR-gated Ca^{2+} channels, possibly all co-residing in the tonoplast of a single cell, an obvious question is why cells need such a plethora of different vacuolar Ca^{2+} release pathways. The relevance of multiple pathways may lie in providing specificity for Ca^{2+} signalling. At least 20 stimuli have been described where a rise in Ca^{2+} forms part of the response. Nevertheless, both stimuli and responses are usually highly specific and therefore cannot be mediated by a uniform Ca^{2+} signal. More recent research has shown that Ca^{2+} signals subsume complicated amplitude and frequency modulations, and spatial variations that all contribute to the "Ca^{2+} signature" (Sanders et al. 2002). The presence of multiple mechanisms through

which Ca^{2+} can be released with different kinetics, at different locations and in response to a multitude of factors that impact on channel activity, ensures that cells have an almost inexhaustible repertoire of potential Ca^{2+} signatures.

1.9 Vacuolar Anion Channels

Ion channels in the tonoplast that are sensitive to typical anion channel inhibitors such as DIDS (4,4′-diisothicyanatostilbene-2,2′-disulfonic acid) and A9C (anthracene-9-carboxylic acid) were recorded in early patch clamp studies. These channels broadly fall into two categories: those that showed selectivity for inorganic anions like Cl^- and NO_3^- and those that conduct organic acids such as malate and to a lesser extent fumarate and succinate (Barkla and Pantoja 1996). The roles of these tonoplast anion channels have been hypothesised to be legion and important in both biotic and abiotic stress responses but unfortunately, characterisation of vacuolar anion channels has been less forthcoming than that for cation channels and their exact physiological roles are often ill-defined.

Early reports noted the presence of an anion permeable tonoplast conductance that potentially played a role in vacuolar Cl^- loading during salt stress and malate accumulation (Pantoja et al. 1989). The malate selective channels are inward rectifying which equates to a malate flux into the vacuole. Channel open probability is generally voltage dependent with opening at positive tonoplast potentials. More recently, some of these were genetically identified and this work indicated that one of the malate permeable vacuolar channels is ALMT9 (Kovermann et al. 2007). Loss of function in ALMT9 did reduce vacuolar malate currents but it did not significantly affect plant growth.

Another major group of vacuolar anion channels is encoded by the CLC family. The functions of these are still largely obscure but clearly include nitrogen homeostasis. CLC-c was identified as a regulator of tissue nitrate level and mutations in *CLC-c* led to reduced nitrate levels. The concentrations of chloride, malate, and citrate were also affected in the *clc-c* mutant (Harada et al. 2004). CLC-a also has an important role in nitrate homeostasis. When this transporter was non-functional, the mutants had a greatly reduced capacity to cope with excessive nitrate stress and were more sensitive to the herbicide chlorate (de Angeli et al. 2006). However, in analogy to several animal CLCs, CLC-a does not appear to be an anion channel but functions as an H^+ coupled antiporter to drive vacuolar nitrate accumulation (de Angeli et al. 2006).

2 Concluding Remarks

The amenability of plant vacuoles to patch clamp technology has resulted in the characterisation of many tonoplast ion channels, particularly those conducting cations. At least 5 different cation conductances have been recorded all with a

proposed function in nutrition, stress and signalling. In only two cases has the molecular identity been revealed (Fig. 1) and this has led to more detailed insights into the physiological function of the SV and VK channels. However, for other vacuolar cation channels, even electrophysiological data are scarce. This is particularly pertinent where ligand-gated Ca^{2+} channels are concerned and further data are urgently required. Identification at the molecular level also remains a big priority but it is unclear how this can be achieved: extensive sequence homology between putative plant Ca^{2+} channels and other organisms is lacking and alternative approaches such as tonoplast proteomics methods (Shimaoka et al. 2004; Whiteman et al. 2008) do not detect less abundant proteins such as ion channels. Yeast complementation strategies which have proved very successful in the isolation of plasma membrane plant channels are similarly unsuitable since disruption of yeast vacuolar cation channels does not yield clear phenotypes.

Another complicating factor for analysing physiological roles is the seemingly high level of functional redundancy in vacuolar cation transport: disruption of either the SV or the VK channel does not affect plant growth in most conditions. Even in plants where both these conductances are absent no or little phenotype is observed (F.J.M. Maathuis unpublished data). The role of the SV channel in Ca^{2+} signalling could conceivably be carried out by other, presumably ligand-gated Ca^{2+} permeable channels whereas VK-mediated K^+ release can take place through other cation channels such as the SV or FV conductance.

However, since patch clamping plant vacuoles is relatively straight forward, it should be feasible to optimise conditions and establish protocols to routinely record specific cation conductances such as the FV channel or ligand-gated Ca^{2+} channels. In combination with a reverse genetics approach this should allow testing of large numbers of loss of function mutants to identify encoding genes and hence provide a clearer picture regarding the role of such channels in plant signalling and stress responses.

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Reactive Oxygen Species, Oxidative Stress and Plant Ion Channels

Vadim Demidchik

Abstract Reactive oxygen species (ROS) are important toxic and regulatory agents in plants. They are produced in response to a number of stimuli, including major biotic and abiotic stresses. Disruption of respiratory and photosynthetic electron transport chains, as well as activation of NADPH oxidases (NOXs) and peroxidases, is a major reason for ROS generation and accumulation during stress conditions. ROS production results in an additional challenge for plants that is called oxidative stress. The latter can not only damage plant cells but can also signal prime stresses to gene expression through activation of Ca^{2+} influx and K^+ efflux ion channels. This chapter reviews the mechanisms of stress-induced ROS generation in plants and discusses properties, regulation and possible structure of plant ROS-activated ion channels.

Abbreviations

NSCC	Non-selective cation channel
KOR	K^+ outward rectifier
KIR	K^+ inward rectifier
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
HACC	Hyperpolarisation-activated Ca^{2+} channel
PCD	Programmed cell death
GORK	Guard cell outwardly rectifying K^+ channel
CNGC	Cyclic nucleotide gated channel
NOX	NADPH oxidase
RBOH	Respiratory burst oxidase homologues

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1 Introduction

Stresses are a major problem for agriculture. Understanding their mechanisms is a way to improve crop yield. Stresses also contribute to desertification, deforestation, death of corals and damage of phytoplankton. They have dramatic ecological consequences and directly affect human life through climate change and food shortage.

Over the last decade, induction of oxidative stress has been shown to be a central phenomenon for many stresses (Fig. 1). Examples of plant stresses that are accompanied by ROS accumulation (oxidative stress) include drought (McAinsh et al. 1996), cold (Okane et al. 1996), high light (Karpinski et al. 1998), pathogens (Schwacke and Hager 1992), salinity (Hernandez et al. 1993) and many others. It is widely accepted now that oxidative stress has a broader role than simply being a side effect due to an imbalance between production and removal of radicals. It was shown that plant cells can produce some ROS by special enzymes for regulatory needs. For example, the normal cell response to pathogen attack requires the activation of complex signalling gene and protein networks by oxidative stress (Breusegem et al. 2008). Control of cell functions by ROS and mechanisms of oxidative signal encoding are probably the oldest, and most evolutionary "developed", among all other stress reactions (Dowling and Simmons 2009). This is because the evolution of aerobic organisms (from anaerobes) has been driven by adaptation to constantly rising dioxygen (O_2) levels. Therefore, plants could employ oxidative stress or, in other words, ROS accumulation or radical imbalance,

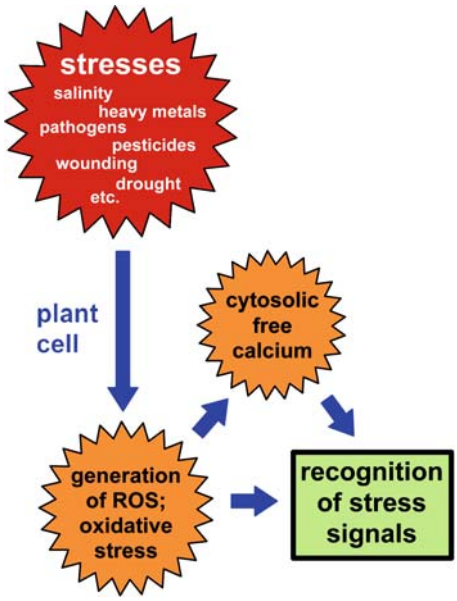

Fig. 1 The recognition of stress stimuli in plant cells. Major plant stresses induce production of reactive oxygen species (ROS) and lead to an oxidative stress which is recognised by plant signalling systems and regulates gene expression

for recognition and encoding of diverse stress factors, including those that did not exist before, such as herbicides, nanoparticles and other xenobiotics.

As soon as ROS are produced they are recognised by cellular sensors. Their nature is still unclear. Although several such sensors (two-component histidine kinases, mitogen-activated protein kinases, some protein phosphatases and transcription factors) have been proposed, involvement of none of these has been proven experimentally (Apel and Hirt 2004). Apart from these systems, plasma membrane and endomembrane ion channels could be involved in ROS sensing in plants. In animal cells, such ion channel-"based" ROS sensors play critical roles in cell physiology (Lahiri et al. 2006). Evidence is now accumulating that ROS and free radicals can activate Ca^{2+}-permeable channels very rapidly in the plant plasma membranes causing Ca^{2+} elevation in the cytosol in seconds (Pei et al. 2000; Demidchik et al. 2003, 2007; Foreman et al. 2003). This resembles (in speed) the receptor-like reaction mediated by ionotropic receptors in animals (Fig. 2). Whether this is due to a direct interaction or not is still questionable. However, it is clear that ion channels link oxidative stress and Ca^{2+} signalling. Ca^{2+} is a central second messenger in plants contributing to a plethora of signalling responses (Hetherington and Brownlee 2004). It is believed that Ca^{2+} transients (Fig. 2) can encode the signal specificity that evokes signal-specific gene expression. Another quick oxidative stress-induced reaction is the activation of K^+-permeable channels by which the cell releases K^+, a process that is often called K^+/electrolyte leakage (Demidchik et al. 2003). The physiological role of this phenomenon has been unclear until recently. It was shown that K^+ loss during plant stress can induce programmed cell death (PCD), a "marginal" type of plant stress response playing critical roles in whole-plant adaptation (Demidchik et al. submitted).

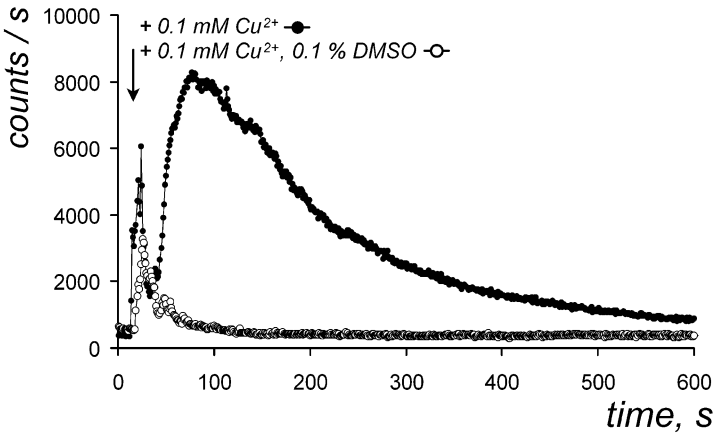

Fig. 2 Cu^{2+}-induced elevation of cytosolic free Ca^{2+} in intact *Arabidopsis thaliana* roots. The signal was inhibited by free radical scavenger, dimethyl sulfoxide. Plants constitutively expressing aequorin in the cytosol were used. Standard procedures and chemiluminometry techniques were used as described elsewhere (Demidchik et al. 2003)

Here, available experimental data on the synthesis of ROS in plants and the interaction between ROS and ion channels in plants are critically reviewed. Major upstream and downstream components of this interaction are also discussed.

2 Synthesis of ROS and Free Radicals and Their Effect on Ion Channels

The main questions that will be answered here are as follows: What are reactive oxygen species? How are they produced? Which channels do they activate? The importance of this section is to emphasise ROS heterogeneity in physiological conditions. "ROS" have often been considered, particularly in plant pathology, as a "single agent". However new data show specificity in the action of different ROS on plant systems, particularly on ion channels (Demidchik et al. 2003, 2007; Foreman et al. 2003). Therefore, the exact chemistry of individual ROS needs to be considered to understand their effect on channels as well as on any other target in plants.

2.1 Oxygen and Radicals

Oxygen (O) is the main oxidiser in aerobic organisms and the second strongest oxidiser in chemistry after fluorine. It is the most prevalent element in the Earth's crust (atomic abundance more than 50%) and the second most abundant gas in the atmosphere (Guido 2001). Release of O_2 to the atmosphere started approximately 2.5 billion years ago when the first blue-green algae appeared. This started an increase of O_2 level and specific O_2-"driven" evolution of species (Dowling and Simmons 2009). As a result, modern plants and animals have evolved sophisticated systems for the use of O_2 in metabolic reduction/oxidation (redox) cascades (85–90% of consumed oxygen is utilised by mitochondria), defence against O_2-mediated oxidation (antioxidants), for regulatory needs (signalling) and sometimes as a "weapon" against parasites, xenobiotics and endogenous "waste" products.

Although some direct inhibitory effects of O_2 on enzymes (for example on nitrogenase) have been reported (Belantine 1982), it is widely believed that O_2 is not particularly toxic or chemically active (Haugaard 1968). Low reactivity is related to the so-called spin restriction phenomenon. Briefly, O_2 is a free radical since it has two unpaired electrons ($\bullet O_2 \bullet$) and it can exist as a free molecule, but both its electrons have the same spin numbers (parallel spins) which limit (restrict) the number of O_2 targets to those that have two electrons with antiparallel spins (Gilbert 1981). To achieve more reactivity O_2 requires an input of energy to remove the spin restriction that can come from ionising irradiation, chemical reactions and heat.

Chemistry studies all varieties of reactive oxygen species (ROS) but, for biology, only a few of these have been proven interesting. These include ubiquitous species in plants such as singlet O_2, hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), superoxide radical ($•O_2^-$) and nitric oxide (NO•). Although NO• is clearly an ROS, this substance, and its derivates, are often called reactive nitrogen species (RNS). RNS will not be discussed here since they have not yet been shown to induce strong oxidative stress in plants (Palavan-Unsal and Arisan 2009), although their regulatory effects on ion channels have been recently delineated (Garcia-Mata et al. 2003) and they may affect other ROS. Yet physiologically important ROS include peroxyl, alkoxyl and hydroperoxyl radicals, ozone and hypochlorous acid.

The terms "ROS" and "free radicals" are repeatedly confused in biological literature. ROS always include activated atom(s) of oxygen but are not necessarily radicals, for example H_2O_2. Free radicals are any species capable of independent existence and containing one or more unpaired electrons (Halliwell and Gutteridge 1999). They may entirely lack oxygen atoms; for example, transition metals are free radicals without oxygen. Both ROS and free radicals promote oxidative stress and, for example, induce well-known lipid peroxidation. The characteristics of key ROS and free radicals in relation to ion channel activation are described below. In many cases, the stresses that induce detectable ROS/free radical generation also cause an increase of cytosolic Ca^{2+} or electrolyte (K^+) leakage, which are both manifestations of ion channel activation.

2.2 Singlet Oxygen

The input of energy to O_2, for example through excess light quanta in photosynthesis, causes the formation of two types of very reactive O_2: non-radical $^1\Delta_gO_2$ (22.4 kcal) and the more reactive free radical $^1\Sigma g^+O_2$ (37.5 kcal) (Schweitzer and Schmidt 2003). The one word "singlet O_2" often covers both of these since it is believed that $^1\Sigma g^+O_2$ can decay into $^1\Delta_gO_2$, although the significance of this reaction for biological systems is not proven. Singlet O_2 is abundantly synthesised in mitochondria and chloroplasts where, if over-produced (for example during photo-oxidative stress or pathogen attack), it causes oxidative damage and contributes to PCD reactions (Møller et al. 2007). In animals, singlet oxygen production inhibits the mitochondrial inner membrane K^+ influx channel which regulates mitochondrial volume, cytochrome c release and transport of the superoxide anion radical to the cytosol (Duprat et al. 1995; Fornazari et al. 2008). Whether similar reactions take place in plants is unknown. The involvement of singlet O_2 in ion channel activation could be through H_2O_2, which can accumulate after singlet oxygen detoxification in organelles and can diffuse to the cytosol where it activates Ca^{2+} channels and triggers signalling cascades, for example sending ROS/Ca^{2+} "messages" to the nucleus. The latter is called retrograde signalling and might play an essential role in the regulation of organelle protein biosynthesis under high light and probably other environmental stresses (Fernándeza and Stranda 2008).

Accurate measurements in intact plants of singlet O_2 have now become possible due to a new commercially available reagent (singlet oxygen sensor green, SOSG), which is highly selective for this ROS (Flors et al. 2006; Driever et al. 2009). It has been shown that singlet O_2 is much more stable in plant systems than previously thought and can diffuse outside the chloroplast and even reach the apoplastic space (Flors et al. 2006). This finding suggests that a much broader spectrum of targets of singlet O_2 in plant cells exists, including plasma membrane and tonoplast ion channels.

2.3 Superoxide Radical

If O_2 (which is actually $\bullet O_2\bullet$) accepts a single electron, for example from NADPH oxidase (NOX) or the electron transport chains of mitochondria and chloroplasts, it becomes more reactive and negatively charged. The resultant $\bullet O_2^-$ harbouring one unpaired electron is called "superoxide anion radical" (also often called by its shorter names, "superoxide radical" or "superoxide"). Note that there is no evidence of direct plant ion channel activation or inhibition by superoxide, although the generation of this ROS, as a precursor of more reactive H_2O_2 and hydroxyl radicals, is certainly crucial for the modulation of ion channel activities in plants (Demidchik et al. 2003, 2009; Foreman et al. 2003). Recent finding by Demidchik et al. (2009) have shown that superoxide produced by NOX in response to purines, such as ATP and ADP (a common signal released by wounded, collapsed or stressed cells), is responsible for the activation of Ca^{2+}-permeable cation channels in the root cell plasma membrane. Therefore, it can be suggested that any other stresses producing superoxide potentially induce Ca^{2+} channel activation.

2.3.1 The Chemistry of Superoxide

Superoxide is short-lived in aqueous solutions due to the dismutation reaction when, ideally (but unlikely) two $\bullet O_2^-$ react with two H^+ to give H_2O_2 and O_2. More probably $\bullet O_2^-$ reacts with H^+, which yields the more reactive and more stable hydroperoxyl radical $HO_2^-\bullet$, similar to water and hypothetically permeable through cell membranes. Two $HO_2^-\bullet$ form H_2O_2 and O_2. The ratio $\bullet O_2^-/HO_2^-\bullet$ depends on pH and is 1/1 at pH 4.8, 10/1 at pH 5.8 (typical for plant cell wall) and 100/1 at pH 6.8 (Sawyer and Gibian 1979). Providing that $HO_2^-\bullet$ has a longer lifetime and higher reactivity than $\bullet O_2^-$, this species could be particularly important at acidic pHs, for example in the apoplast (pH about 5.5–6.0). Unfortunately, the effects of $HO_2^-\bullet$ on plant ion channels have not been studied.

Superoxide is an extremely inert molecule and does not interact with amino acids, lipids or nucleic acids but is a precursor for more reactive ROS, such hydroxyl radicals, and therefore a prime cause of oxidative stress. This role of $\bullet O_2^-$ is related to its capacity to interact with other radicals such as transition

metals, NO•, phenoxyl radical and iron-sulphur clusters, leading to biosynthesis of more reactive ROS (Sawyer and Gibian 1979; Halliwell and Gutteridge 1999).

Although both $•O_2^-$ and $HO_2^-•$ react with transition metals, the prevalent interaction is the reduction of transition metals by $•O_2^-$. In the case of Fe^{3+} and Cu^{2+}, $•O_2^-$ reduces these to Fe^{2+} and Cu^+. These metals can subsequently interact with H_2O_2, which results in the synthesis of extremely reactive hydroxyl radicals (Halliwell and Gutteridge 1999). The reactions of $•O_2^-/HO_2^-•$ with NO• give $ONOO^-$ (peroxynitrite)/ROONO (alkyl peroxinitrite), both of which are critical cytotoxic species more reactive than the original radicals (Squadrito and Pryor 1998). For example, peroxynitrite decomposes to hydroxyl radicals through the intermediate formation of peroxynitrous acid (Pryor and Squadrito 1995). It is very likely that these reactions occur in plants in stress conditions when both $•O_2$ and NO• concentrations are dramatically increased (del Rio et al. 2004). Therefore the effects of NO• on ion channels in plants (Garcia-Mata et al. 2003; Sokolovski and Blatt 2004) could be regulated by $•O_2^-$.

2.3.2 Superoxide Generation during Stress Conditions

Superoxide is probably the most frequently detected ROS during stress conditions, along with H_2O_2 (Apel and Hirt 2004). In some cases, it is generated a few seconds after the addition of a stress factor or stress hormone (Kawano et al. 1998); but sometimes it takes hours to observe detectable superoxide levels (Schraudner et al. 1998).

However, $•O_2^-$ rapidly forms H_2O_2; therefore the same techniques are often used for superoxide and H_2O_2 detection in plants. Moreover, all "established" superoxide detecting techniques, apart from Electron Paramagnetic Resonance (EPR) spectroscopy, are not really specific to this radical, but sense other radical species as well as sometimes H_2O_2 (Halliwell and Gutteridge 1999). The addition of superoxide dismutase (SOD), which is an enzymatic antioxidant specifically breaking down $•O_2^-$, is crucial for accurate superoxide measurement.

Apart from the electron transport chains of mitochondria and chloroplasts that produce $•O_2^-$ inside these organelles (Apel and Hirt 2004), the major system generating this ROS during stress is the Ca^{2+}-activated enzyme NOX encoded by the Respiratory Burst Oxidase Homologues (*RBOH*) gene family which has ten members in *Arabidopsis* and 9 in rice (Keller et al. 1998; Torres et al. 1998; Torres and Dangl 2005; Wong et al. 2007). This system "works in concert" with ROS-activated Ca^{2+} channels to generate and amplify stress-induced Ca^{2+} and ROS transients (Demidchik and Maathuis 2007) (Fig. 3). The more Ca^{2+} enters the cell the more ROS are generated and, *vice versa* – the more ROS are generated the more Ca^{2+} enters (Takeda et al. 2008). It was suggested that this loop helps to amplify weak signals at the level of the plasma membrane and generates sustained Ca^{2+} elevation encoding signal specificity (Demidchik and Maathuis 2007). Regulation of this $•O_2^-$–Ca^{2+} signal amplification mechanism is not well understood although in most cases, it definitely does not "over-produce" ROS or Ca^{2+}, each of which

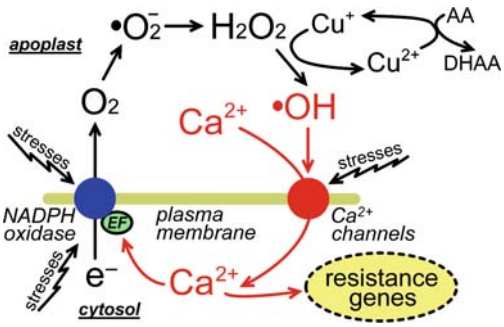

Fig. 3 ROS/Ca^{2+} stress signalling "hub". Stress signals activate NADPH oxidase or Ca^{2+}-permeable channels that induces a self-amplifying regulatory loop, because NADPH oxidase is stimulated by Ca^{2+} binding to its EF-hand domain, while Ca^{2+} channels are activated by ROS. NADPH oxidase transfers electrons from cytosolic NADPH to apoplastic O_2 that causes synthesis of superoxide ($•O_2^-$). The latter rapidly forms hydrogen peroxide (H_2O_2). Cu^+ reduces H_2O_2 that yields the extremely reactive •OH, which activates the Ca^{2+}-permeable channel. Cu^{2+} can be reduced by an apoplastic L-ascorbic acid (AA). Ca^{2+} modifies gene expression responsible for adaptation to stresses

could kill the cell. However, over-production also has physiological "sense" and leads to the induction of plant PCD reactions that are very useful for pathogen defence and in response to some abiotic stresses such as salinity or heavy metals. Physical distribution of the $•O_2^-$–Ca^{2+} system is probably regulated by SCN1/AtrhoGDI1 RhoGTPase GDP dissociation inhibitor that allows focussing AtrbohC-catalysed production of ROS to specific areas of the cell such as root hair tips (Carol et al. 2005). It can be suggested that stimulation of Ca^{2+}-ATPases that removes excess Ca^{2+} from the cytosol to the apoplast, vacuole or organelles, as well as depolarisation of the membrane that decreases Ca^{2+} influx through channels, are critical regulators of the $•O_2^-$–Ca^{2+} loop. Activity of Rop GTPases (small G proteins) that can probably control NOXs is an additional factor for fine regulation of this signalling loop (Baxter-Burrell et al. 2002). In addition, it could be regulated by Ca^{2+}-binding proteins and Ca^{2+}-mediated protein phosphorylation pathways. Inhibition of Ca^{2+} channel conductances will also be a very rapid way to inhibit the $•O_2^-$–Ca^{2+} cycle, though no experimental evidence for this has been found so far.

Recent advances in NOX structural analysis and functional characterisation have shown how this system generates superoxide. The system for electron transfer in NOX includes the C-terminal cytoplasmic region (superdomain) homologous to the enzyme ferredoxin reductase bearing the NADPH-binding and FAD-binding sites that transfer the electron to the N-terminal six transmembrane segments containing the di-heme system (Sumimoto 2008). Hemes react with O_2 and generate $•O_2^-$ outside the cell. An intrinsic property of NOX is its activation by cytosolic Ca^{2+}. This property allows the induction of superoxide production when Ca^{2+} activity increases in the cytosol. The N-terminus of plant NOX contains two helix–loop–helix structural domains binding Ca^{2+} (so-called EF hand) similar to calmodulin and troponin-C. Binding of Ca^{2+} causes a conformational change, which leads to intramolecular interaction of the N-terminal Ca^{2+}-binding domain with the

C-terminal superdomain, culminating in the activation of electron transfer (Bànfi et al. 2004). EC_{50} for Ca^{2+} is about 1 μm, determined in a cell-free activation system for animal NOX5 (Bànfi et al. 2004). Recent findings have also shown that there are additional mechanisms for controlling Ca^{2+} regulation of NOX and potential stress signalling. For example, sensitivity to Ca^{2+} can be stimulated by calmodulin binding to the NADPH-binding domain or by protein kinase C-mediated phosphorylation of specific serine/tryptophan residues in the FAD-binding domain (Jagnandan et al. 2007; Kobayashi et al. 2007; Tirone and Cox 2007).

Despite exciting data on the superoxide role in signalling and the mechanisms of its stress-induced generation by NOX, one cautionary note should be made. Diphenylene iodonium (DPI), which is used as a major tool for the verification of NOX dependent ROS production, is usually dissolved in 0.1–3% dimethyl sulfoxide (DMSO), which is a powerful scavenger of free radicals and significantly inhibits free radical induced Ca^{2+} signals at 0.1% and fully inhibits them at 1% and higher (Fig. 2). Despite this, DMSO control measurements have not been carried out in most studies, particularly in research on plant pathology themes. Clearly, DPI inhibitory effects and NOX involvement in ROS production during stress in plants may require re-evaluating.

2.3.3 Superoxide and Ca^{2+} Channels Form a Stress Signalling "Hub" in Plant Cells

In the last few years, hundreds of reports documenting stimulation of NOX activities in response to almost all known stress factors have been published (Apel and Hirt 2004; Fluhr 2009). These studies have embraced a large number of plant species and preparations, including all organs, the most important tissues, protoplasts and cell cultures. Analysis of the available data shows that NOX activation during stress, which probably in most cases leads to the activation of Ca^{2+}-permeable channels, is mainly required for the following functions: (a) stress recognition (probably through downstream Ca^{2+} signals); (b) stress-induced PCD (response to pathogens and to severe abiotic stresses); (c) stomatal closure (critical for gas exchange and drought response); (d) gravitropic response; and (e) processing stress and growth hormone signals (auxin, ethylene, abscisic acid, gibberellic acid, brassinosteroids, methyl jasmonate etc.). This strongly suggests that NOX and ROS-activated Ca^{2+}-permeable channels function as a regulatory "hub" in plants for processing important internal and external stress stimuli (Fig. 3).

2.4 Hydroxyl Radical

Hydroxyl radicals (•OH) are central to plant ion channel activation during stress conditions and elongation of growth (Demidchik et al. 2003, 2007; Foreman et al. 2003) (Fig. 3). They can be produced by homolytic bond fission of H_2O

(when electrons in covalent bonds are equally distributed to atoms). This requires a large energy input by ultraviolet quanta (for example during UV stress), ionizing radiation, ultrasonication (due to acoustic cavitation), freezing-drying cycles or heat (Halliwell and Gutteridge 1999). Less energy is required to produce •OH from H_2O_2. A significant amount of •OH can be directly generated from H_2O_2 (HOOH) and hydroperoxides (ROOH) by sunlight (Downes and Blunt 1879). Nevertheless, the most important way to produce •OH in cells is through the so-called "Fenton chemistry" reactions known from the 19th century. Although originally Fenton's study was related to the effects of the transition metal Fe^{2+} on tartaric acid (Fenton 1894), now the term "Fenton chemistry" and "Fenton-like reagents" are used to mark reactions that take place in the presence of H_2O_2 and transition metals and lead to the production of •OH, water and superoxide (Goldstein et al. 1993). Although many radicals are formed as intermediates, the net reactions of Fenton-like reagents are as follows: (A) metal reduced + H_2O_2 → metal oxidised + •OH + OH^-; (B) metal oxidised + H_2O_2 → metal reduced + HO_2^{-}• + H^+ (Koppenol 2001). This was originally proposed by Nobel Prize winner Fritz Haber and his student Joseph Weiss in the 1930s and called the Haber–Weiss cycle (Haber and Weiss 1932). Importantly, ascorbic acid is probably a major reductant for iron and copper in the Haber–Weiss cycle in plants, where it can reach millimolar levels in the cytosol and apoplast (Fry et al. 2002). Therefore, ascorbate plays a pro-oxidant role in these oxidative reactions and could be a key component of ion channel activation by ROS and stress signalling.

An effect of •OH on ion channels has been studied by the addition of Fenton-like reagents (a mixture of Cu^{2+}, L-ascorbic acid and H_2O_2) to plant cells (Demidchik et al. 2003, 2007; Foreman et al. 2003). These studies have shown that •OH activates Ca^{2+}-permeable non-selective cation channels and K^+ outwardly rectifying (K^+ efflux) channels in mature root atrichoblasts, root hairs, pericycle, cortex and elongation zone cells. Activation of Ca^{2+} influx and K^+ efflux by •OH has also been found in roots of crop species (clover, pea, wheat, maize and spinach) (Demidchik et al. 2003). Despite this obvious progress, the mechanisms of the major upstream components of the •OH action on ion channels, which are the generation of •OH during stress and •OH interaction with the channel at the structural/molecular level, remain poorly understood. Moreover, genes encoding •OH-activated cation channels have not been identified.

The estimated *in vivo* half-life of •OH is only 1 ns, which allows •OH diffusion only for very short distances (< 1 nm) (Halliwell and Gutteridge 1999). Second-order rate constants for reactions of •OH with organic molecules are so high that these reactions are only limited by •OH diffusion time (so-called "diffusion-controlled rate") (Anbar and Neta 1967). This shows that specific •OH scavengers do not exist in principle and that, in most cases, the effects of mannitol, sorbitol, dimethyl sulfoxide, thiourea or other "established" •OH scavengers on •OH-induced reactions, such as activation of plant cation channels, are not due to •OH scavenging. Realistically, the effects of widely used •OH scavengers are related to

the removal of the •OH precursors, hydroperoxyl, H_2O_2 and superoxide, or to the chelation of transition metals.

Although a number of techniques for •OH detection has been proposed (Halliwell and Gutteridge 1999), only EPR spectroscopy provides a high specificity of •OH measurements (Liszkay et al. 2004). Nevertheless this method is not ideal because •OH spin traps, such as DMPO, DEPMPO or POBN, may decompose at room temperature and react with superoxide (Pou et al. 1989). Yet, it is still far more sensitive than any imaging techniques used for •OH detection. Recent advances in EPR-based techniques allowed the use of a single root for •OH measurements (Renew et al. 2005). These experiments have shown significant production of •OH in intact plant roots (a major cause of Ca^{2+} channel activation) and that NOX (RbohC) produces a precursor of •OH generation, $•O_2^-$. Future work should relate •OH production and changes in ROS-mediated channel activation during stress responses.

2.5 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a relatively stable, non-radical, weak acid synthesised as the end product of many processes involving more reactive ROS and free radicals. It was shown that this ROS activates Ca^{2+}-permeable non-selective cation channels in guard cell protoplasts that probably underlie ABA-induced stomata closure (Pei et al. 2000). H_2O_2 also interacts with the intracellular part of the root Ca^{2+}-permeable cation channels activating them and inhibits KORC in guard cells and root epidermal protoplasts (Demidchik et al. 2003, 2007; Köhler et al. 2003). H_2O_2 does not modulate activity Ca^{2+}, K^+, Cl^- and non-selective ion channels in green algae, or root plasma membrane ion channels when applied in the whole cell configuration (Demidchik et al. 2001, 2003, 2007). Recent data have also shown that H_2O_2 stimulates anion efflux in cultured *Arabidopsis thaliana* cells mimicking ABA (Trouverie et al. 2008). However, this effect was probably related to the activation of Ca^{2+} permeable conductances.

H_2O_2 originates in plants from the dismutation reaction of superoxide (produced in organelles and by NADPH oxidases) and activity in the heme-containing peroxidases (72 members of three major classes in *Arabidopsis*) (Vitch 2004). Functional activity and structure of plant peroxidases are well studied and will not be discussed here (Vitch 2004). Peroxidases were first proposed to play critical roles in ROS production during biotic stress (Bolwell and Wojtaszek 1997; Bolwell et al. 1998; Bindschedler et al. 2006). Now it is widely accepted that some, such as amine oxidases (Cu-containing amino oxidases and polyamine oxidases), gluthatione oxidases and ascorbate oxidases, are stimulated by abiotic stress and probably critically important for ROS production and Ca^{2+} channel activation during long-term salinity, pathogen and photooxidative stress (Rodríguez et al. 2002, 2007; Chang et al. 2009). Oxidases may have negative feedback inhibitory mechanisms since some of them are inhibited by their product H_2O_2 (Kitajima 2008).

H_2O_2 is ubiquitous in plants (Apel and Hirt 2004). It is more stable than other ROS (Halliwell and Gutteridge 1999) and some indirect evidence exists that it can cross the plasma membrane via aquaporins (Dynowski et al. 2008). Therefore, it can accumulate, particularly in the apoplast, because this area is less accessible by cellular antioxidants such as catalase (Rodríguez et al. 2002; Apel and Hirt 2004). In response to almost all known stresses, the cellular level of H_2O_2 rises from 0.1–1 μM to 0.1–10 mM (this phenomenon is often called ROS or H_2O_2 "transient" by the analogy with Ca^{2+} transients) (Apel and Hirt 2004; Demidchik unpublished). Different studies report different time periods required for detectable H_2O_2 accumulation during stress and varies from seconds to several days (Apel and Hirt 2004; Trouverie et al. 2008). The difference is due to several factors: (A) Low [H_2O_2] at early stages of stresses may not be detectable by most fluorescent probes and $TiSO_4$. Moreover, in most cases, the used techniques show the cumulative effect of all H_2O_2 produced. hemiluminescent probes report H_2O_2 faster and they are more sensitive although less specific to H_2O_2. (B) Observations in the first minutes might simply not be carried out, which was typical in many older studies. (C) The experimental physical and chemical conditions may not have been appropriate. For example, many protocols require a high pH (8–9) that affects H_2O_2 generation from superoxide and changes the physiology of plant cells. (D) The nature of the biological object. For example, cuticula and root caps complicate the delivery of probes and delay reaction with H_2O_2 in intact leaves and root tips. (E) The nature and intensity of the imposed stress varies dramatically across studies.

2.6 Transition Metals

Transition metals are crucially important in all organisms. Cu- and Fe-induced activation of non-selective cation channels and inhibition of anion channels were the first described effects of transition metals acting as free radicals in plants (Demidchik et al. 1996, 1997, 2001). According to the IUPAC definition (http://goldbook.iupac.org/), a transition metal is any element with an incomplete d sub-shell, or which can give rise to cations with an incomplete d sub-shell. This means there are 40 chemical elements (21 to 30, 39 to 48, 71 to 80, and 103 to 112) that can act as transition metals (McCleverty 1999). Only a few of these have demonstrated their importance in biological systems (mainly d-elements of the first row) (McCleverty 1999). The most important transition metals for biology are Cu and Fe and to a much lesser extent Mn, Hg, Ni, Cr and Co. In plant cells, Cu and Fe are the most abundant, and more easily change their valence compared to other transition metals (Bergmann 1992; Fry et al. 2002). They function as key electron transport components in most redox enzymes. Mn and Ni show similar properties when coordinated by specific ligands in some redox proteins but they lack electron transfer capacity in free ionic form in biological conditions (Halliwell and Gutteridge 1999).

The toxic and regulatory effects of Cu and Fe on ion channels are mainly related to increased •OH generation. Cu is 60 times more potent as a catalyst of the Haber–Weiss cycle and several million times more soluble than Fe, though Fe is more abundant in the cell (Bergmann 1992; Halliwell and Gutteridge 1999; Fry et al. 2002). Thus, Cu could be a major catalyst of •OH generation, critical for channel activation (Demidchik et al. 2003). Almost all Cu and Fe are bound in organic complexes and their catalytic activity must be considered instead as a concentration of free ionic forms (which is extremely low). It has been established that the catalytic activity of both Fe and Cu increases several times during stress conditions (Becana and Klucas 1992; Moran et al. 1994; Becana et al. 1998). In such conditions transition metal activity can activate Ca^{2+}-permeable non-selective cation channels (NSCCs) and K^+ outwardly rectifying channels (KORC) in the plasma membrane (Demidchik et al. 2003). Increased •OH production leads to oxidative stress and triggers PCD (Becana et al. 1998).

Moran et al. (1997) found that some specific phenolic compounds are synthesised during stress and chelate Fe to increase its catalytic activity. Some promoted DNA and lipid oxidation. However, apart from this finding, mechanisms by which catalytic transition metal activities elevate in response to stress have not been investigated. One possible scenario is that Cu and Fe catalytic activities are modulated in plants cells by polyamines (for example spermine, spermidine and putrescine) that are abundantly synthesised during abiotic and biotic stress (Alcázar et al. 2006; Moschou et al. 2009) and form redox-active complexes with both Cu (Guskos et al. 2007) and Fe (Tadolini 1988). Although this hypothesis has not yet been tested in plants, inhibition by polyamines of KORC has been shown (Shabala et al. 2007). KORCs are known to be activated by •OH and inhibited by H_2O_2 and are involved in salt stress response.

3 Properties of Plant Ion Channels Regulated by ROS and Free Radicals

The properties of any ion channel can be subdivided into two major categories: physiological and molecular. Physiological properties pertain to the number of ions that can be transported through the channel, how transport is regulated, which ions are transported and what the physiological role of the channel is in plant growth and development (Demidchik et al. 2006). Molecular properties describe aspects such as the encoding gene, temporal and spatial variation in the encoding genes and proteins and channel structure.

3.1 Physiological Properties and Involvement in Stress Responses

ROS accumulation and elevation of cytosolic free Ca^{2+} have been reported for almost all major stresses in a number of plant species. Nevertheless, direct

activation of ion channels by ROS or free radicals has been measured only a few times (Demidchik and Maathuis 2007). Here, existing data on ROS/free radical-activated cation channels in plants are critically discussed.

3.1.1 Transition Metal-Activated Cation Channels in Green Algae

Plant free radical-activated ion channels were discovered in the green alga *Nitella flexilis* (Demidchik et al. 1996, 1997, 2001). This microelectrode voltage-clamp study was intended to delineate the mechanisms of heavy metal toxicity in algae at the cellular level. The advantage of this system was the preservation of the cell wall, where H_2O_2 and ascorbate can catalyse •OH generation in the presence of transition metals (see Sect. 2.4 of this Chapter), and minimal cell damage since it is impaled just by one microelectrode. On the other hand, ion channels of *Nitella* have been well characterised and this organism is very sensitive to heavy metals (Sokolik and Yurin 1981, 1986). Exposure of intact *Nitella* cells to 5–100 μM free radicals, Cu^{2+} and Fe^{3+}, activated inwardly directed, voltage-independent conductances with instantaneous kinetics. Cu^{2+}-activated conductances were non-selective for monovalent cations but discriminated against anions and were partially inhibited by H^+, divalent cations (Ca^{2+}, Ba^{2+} and Zn^{2+}) and the Ca^{2+} channel blocker nifedipine. This was the first study of its kind for plant physiology; therefore, it was important to show whether passive or active transporters mediate observed conductance. Temperature coefficient Q_{10} of passive ion diffusion through the channel is between 1.2 and 1.6, while active transporters and pumps relying on chemical reactions have $Q_{10} > 2$ (Hille 2001). Q_{10} of Cu^{2+}-activated conductance was between 1.2 and 1.6 suggesting the involvement of an ion channel based mechanism. Activation of this conductance over time after addition of Cu^{2+} had temperature-dependent rate with Q_{10} of about 3 corresponding to free radical "chain" reactions catalysed by transition metals (Halliwell and Gutteridge 1999). Overall, these data demonstrated that transition metals activate NSCCs in plant cells due to a free radical-mediated process. It was proposed that "excessive" activation of these channels results in ionic imbalance inducing cell death and that this mechanism is a major cause of toxicity of heavy metals in algae. Note that in the mid-1990s, mechanisms of cationic control of PCD through the regulation of caspase-like activities and endonucleases were unknown even in animal cells.

3.1.2 Hydroxyl Radical-Activated Channels in Roots of Higher Plants

The idea that free radicals can stimulate ion channel activities was later tested in higher plants (Demidchik et al. 2003, 2007, 2009, Foreman et al. 2003). Application of Cu^{2+} together with the transition metal-reducing agent ascorbate (Cu/asc) to *A. thaliana* root epidermal cells activated non-selective inwardly-directed cation and K^+-selective outwardly-directed (efflux) conductances. The non-selective conductance showed the following permeability series: K^+ (1.00) ≈ NH_4^+ (0.91) ≈ Na^+

(0.71) ≈ Cs^+ (0.67) > Ba^{2+} (0.32) ≈ Ca^{2+} (0.24) > TEA^+ (0.09). The K^+ efflux conductance demonstrated a much high selectivity to K^+:K^+ (1.00) > Na^+ (0.31) > Ba^{2+} (0.06) » TEA^+ (0.05) that corresponded to so-called "K^+ outward rectifiers" (KOR) previously investigated in *Arabidopsis* (Lebaudy et al. 2007). Activation over time of *Arabidopsis* cation currents after Cu/asc addition was very similar to the time-dependence of NSCC activation by Cu^{2+} in *Nitella*, suggesting the involvement of a free radical-mediated mechanism. Interestingly, Cu^{2+} added without ascorbate did not induce current in *Arabidopsis* protoplasts although it caused elevation of cytosolic free Ca^{2+} in intact roots (Fig. 2) and induced currents in intact *Nitella* cells. This suggests that apoplastic ascorbate, and probably other reductants, can promote •OH generation via the Haber–Weiss cycle in the cell wall. Superoxide/H_2O_2 for this cycle is likely to be produced by NADPH oxidase (see Sects. 2.3 and 2.4) because, when cell wall peroxidases were removed by the protoplast isolation procedure, Cu/asc was still capable of activating currents.

Cells in the root tip elongation zone and root hairs are the first to sense new environments and stresses while the root grows. However, cells of internal tissues, such as the pericycle, do not have contact with the soil and may not be involved in primary stress sensing and signal transduction. This might explain why Ca^{2+} influx through Cu/asc-activated NSCCs in the elongation zone and root hairs is larger than in other tissues and why the pericycle responds poorly to •OH (Demidchik et al. 2003). This hypothesis does not contradict theories of cell expansive and polar growth which are based on localised elevations of ROS-activated Ca^{2+} influx in growing cell parts to stimulate exocytosis and delivery of new cell structural material (Foreman et al. 2003; Coelho et al. 2008; see Chapter "Ion Channels in Plant Development" of this book). It can be suggested that high level of activities of ROS-producing enzymes and Ca^{2+} influx channels in root hairs and elongation zone cells are necessary for both growth and "timely" stress sensing and adaptation to a new environment. For example, our recent findings show that acute salt stress causes NADPH oxidase activation leading to hydroxyl radical generation accompanied by Ca^{2+} influx and K^+ efflux channel activation in intact root cells and this reaction is much larger in the root elongation zone (Demidchik, unpublished).

3.1.3 Hydrogen Peroxide-Activated Channels in Roots and Leaves

In contrast to •OH, H_2O_2 is unable to activate currents in mature *Arabidopsis* root cells when it is applied inside and outside the pipette in a whole-cell patch clamp configuration or added outside in excised outside-out patches (Fig. 4) (Demidchik et al. 2007). H_2O_2 activates Ca^{2+} influx channels in this system only if applied to excised outside-out patches at the cytoplasmic side (Fig. 4). This shows that H_2O_2 should be delivered directly to the channel inside mature epidermal cells. However, H_2O_2 was capable of inducing Ca^{2+} currents in protoplasts from young cells of the root elongation zone (Demidchik et al. 2007) and in *Arabidopsis* guard cells

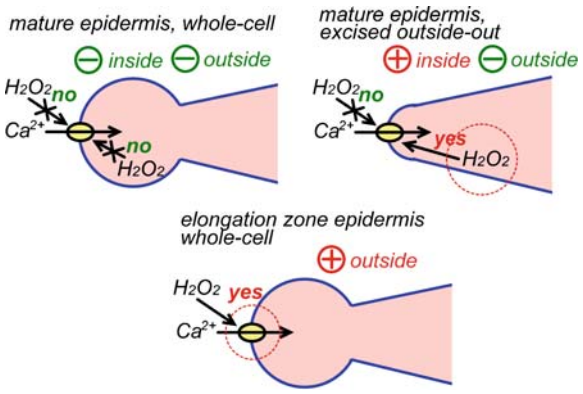

Fig. 4 Hydrogen peroxide effects on Ca^{2+}-permeable channels in *Arabidopsis thaliana* root epidermis according to Demidchik et al. (2007). Both exogenous and endogenous H_2O_2 applications to whole-cell patch-clamped protoplasts isolated from mature epidermis did not activate Ca^{2+} currents. When excised patches of the same protoplasts were used, endogenous application caused activation. Protoplasts isolated from young cells of the elongation zone epidermis showed Ca^{2+} current activation in response to exogenous H_2O_2

(Pei et al. 2000). Several mechanisms could be responsible for these contrasting results: (A) ROS-activated channels in different tissues/cells may be encoded by different genes; (B) young elongating cells and guard cells may have a higher density of H_2O_2-permeable aquaporins (Eisenbarth and Weig 2005; Bienert et al. 2006) which facilitate H_2O_2 delivery to the cytosol (Dynowski et al. 2008); (C) growing root cells and highly specialised guard cells have higher catalytic activities of transition metals than mature root epidermal cells.

Ca^{2+}-permeable channels in guard cells could have a more sophisticated pattern of regulation by ROS than their root counterparts since their reaction to H_2O_2 may be additionally controlled by the cell phosphorylation status (Mori and Schroeder 2004). In guard cell protoplasts, the H_2O_2-mediated activation of Ca^{2+}-permeable NSCCs was impaired in *abi2-1* protein phosphatase mutants that are insensitive to the drought stress hormone ABA (Murata et al. 2001; see also Chapter "Ion Channels and Plant Stress: Past, Present and Future" of this book). Biochemical tests of recombinant ABI2 protein that encodes phosphatase 2C (PP2C) demonstrated that this protein is directly inhibited by H_2O_2 and could be one of the prime targets for H_2O_2 in guard cells (Meinhard et al. 2002). It seems that phosphatase may directly inhibit Ca^{2+}-permeable NSCCs in guard cells because phosphorylation was shown to be crucial for activation of these channels (Köhler and Blatt 2002). Another possible scenario is that PP2C dephosphorylates some intermediate regulators controlling NSCC gating. Another very similar protein phosphatase is also directly inhibited by H_2O_2 (Meinhard and Grill 2001). This phosphatase blocks NADPH oxidase activation and ROS production by ABA that results in the impairment of NSCC activation by ABA (Murata et al. 2001).

3.1.4 ROS-Activated NSCCs Could be Constitutive Hyperpolarisation-Activated Ca^{2+} Channels Involved in Stress Reactions

An important question is whether ROS-activated NSCC and constitutive hyperpolarisation-activated Ca^{2+} channel (HACC; see Chapter "Ion Channels in Plant Development" of this book) are the same. HACCs function in Ca^{2+} uptake at hyperpolarised voltages in a number of cells and tissues, for example in tomato suspension culture cells (Gelli and Blumwald 1997), guard cells (Hamilton et al. 2000; Pei et al. 2000; Köhler and Blatt 2002; Sokolovski et al. 2008), root hair cells (Vèry and Davies 2000; Miedema et al. 2008), pollen tube (Wang et al. 2004; Wu et al. 2007), root mature and elongation zone epidermis (Demidchik et al. 2002; 2007), and algal rhizoids (Coelho et al. 2002). It is believed that they are involved in stress-related Ca^{2+} signalling and Ca^{2+} loading for polar growth stimulation rather than nutritional Ca^{2+} uptake (Demidchik et al. 2002). Actually, these processes are usually accompanied by ROS accumulation leading to the oxidative stress. Recent data have demonstrated that unitary conductances of ROS-activated NSCCs and HACCs are very similar and lie between 15 and 20 pS (Demidchik et al. 2007). Moreover HACCs of the *Arabidopsis* mature root epidermis do not show high selectivity for Ca^{2+}, which additionally points to their possible relationship with ROS-activated NSCCs. Major differences between these systems are that ROS-activated NSCCs do not show steep rectification at hyperpolarised voltages and delayed activation kinetics, typical for HACCs. However, this could be explained by the presence of Cu^{2+} in the solution, which is not only a catalyst of •OH generation but also a divalent cation that can itself permeate and block cation channels. Cu^{2+} could block the rectifying component and change the kinetics of HACCs. Cu^{2+} was shown to modify gating, kinetics and the rectification of animal cation channels, including Ca^{2+}-permeable cation channels (Kiss and Osipenko 1994). Recent data on purine-induced activation of Ca^{2+}-permeable NSCCs through NADPH oxidase-produced ROS have shown that, in the absence of Cu^{2+}, ROS-activated NSCCs in mature root epidermis are similar to HACCs and reveal time-dependence and steep rectification. However, HACCs in the apical part of growing root hairs are probably different from NSCCs/HACCs from root mature epidermis because they are highly selective to Ca^{2+} and Ba^{2+}. It should also be noted that the selectivity of root hair HACCs for monovalent cations has been tested in the presence of Ca^{2+} which probably affected selectivity properties (Vèry and Davies 2000).

3.1.5 ROS-Activated K^+ Efflux Channels and Their Role in Plant Stress Response

Potassium channels were the first ion channels characterised electrophysiologically in plants (Sokolik and Yurin 1981, 1986) and the first to be systematically studied at the genetic level (Sentenac et al. 1992; Gaymard et al. 1998). Owing to their high selectivity for K^+ and abundant expression in the plasma membrane and tonoplast,

they control plant cell K^+ influx (inwardly rectifying K^+ channels, KIRs) and efflux (outwardly rectifying K^+ channels, KORCs or KORs) and thus regulate cell K^+ homeostasis. They are also responsible for maintaining membrane potential since K^+ is the dominant ion in the cytosol.

Demidchik et al. (2003) have found a novel mechanism for regulation of plant K^+ efflux channels: activation by ROS (see selectivity series of ROS-activated KORs in Sect. 3.1.2). This phenomenon has been recently investigated in detail (Demidchik et al. unpublished; Fig. 5). In fact, K^+ (electrolyte) leakage from plant cells is a phenomenon occurring during almost any stress, but virtually unstudied. Salinity causes a severe loss of K^+ which is then replaced by Na^+. This mechanism is believed to be a major reason for Na^+ toxicity at the cellular level (Maathuis and Amtmann 1999). Demidchik et al. (submitted to Plant Journal) have shown that *Arabidopsis* plasma membrane ROS-activated K^+ outwardly rectifying channels activate in response to oxidative stress and salinity and mediate dramatic K^+ loss from the cytoplasm leading to activation of cytoplasmic cell death proteases and endonucleases which cause cell death. These K^+ channels are probably encoded by the *GORK* gene, previously characterised in *Arabidopsis* guard cells and roots (Ivashikina et al. 2001; Hosy et al. 2003). ROS were not able to activate K^+ efflux channels in the GORK KO line. Development of PCD symptoms induced by NaCl and oxidative stress were delayed in *gork1-1* plants confirming the role of K^+ efflux channels in this process.

In animals, specific enzymes (caspases and endonucleases) destroy proteins and DNA in response to the death factor (Remillard and Yuan 2004). They are normally

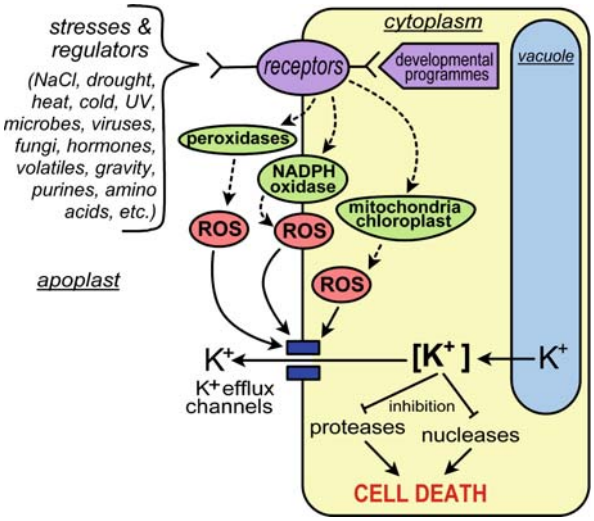

Fig. 5 The mechanism of K^+ channel-mediated cell death in plants based on experimental data obtained from *Arabidopsis thaliana* roots. Stresses and other factors stimulate ROS generation in the apoplast and inside the cell. ROS activate K^+ efflux channels leading to K^+ loss and stimulation of K^+-controlled cell death enzymes

inhibited by the natural blocker, K^+, which is high in the cytosol (70–100 mM) (Yu et al. 1997; Yu 2003; Remillard and Yuan 2004). Death factors can activate K^+ efflux channels causing K^+ loss that leads to stimulation of these enzymes (Yu 2003). A number of animal K^+ channels participate in PCD (Remillard and Yuan 2004). Some, such as human ether-a-go-go (hERG) channels are directly activated by hydroxyl radicals (Yu et al. 1997; Han et al. 2004). Future work should show how individual plant caspase-like activities and endonucleases are regulated by K^+ and whether ROS-activated K^+ efflux channels are involved in other stresses, such as pathogen attack and drought.

3.2 Molecular Properties

In animal cells, ROS-activated ion channels mainly belong to three classes: Shakers, voltage-dependent Ca^{2+} channels and "transient receptor potential" (TRP) channels. Secondary oxidative stress generally causes their activation. Apart from activation by H_2O_2, $\bullet O_2^-$, $HO_2^-\bullet$, some RNS or •OH, these channels are sensitive to a range of regulators, such as ADP-ribose, ATP, cytosolic Ca^{2+}, glutathione and others. Cysteine, histidine and methionine residues are often responsible for ion channel interaction with ROS (Yu et al. 1997; Hoshi and Heinemann 2001; Simon et al. 2004). All three are probably capable of binding transition metals such as copper and iron in complex. The role of transition metal complexes with cysteine and histidine in signalling proteins, such as transcription factors and ion channels, is well-documented (Yu et al. 1997; Yu 2003; Simon et al. 2004; Traore et al. 2009). The reversibility of the ROS effects on channels can be based on incomplete and reversible oxidation of amino acids. For example, ROS-induced cysteine oxidation can lead to a sequential formation of more oxidised derivatives, such as cystine, cysteine sulfenic acid and cysteine sulfinic acid, which are all enzymatically reversible (Biteau et al. 2003). The highest level of Cys oxidation is the cysteic acid, which is believed to be irreversible (Ghezzi 2005). Interaction of His with free radicals leads to the appearance of the histidinyl radical that can produce peroxyl radicals (in reaction with O_2), which, if not quenched, form stable irreversibly oxidised 2-oxo-histidine (Halliwell and Gutteridge 1999). Methionine is oxidised to methionine sulfoxide (MetO, MeSOX, MetSO, or MsX), which can be reduced by methionine sulfoxide reductase to methionine in a thioredoxin-dependent manner (Hoshi and Heinemann 2001).

Plants probably do not have Ca^{2+} and TRP channels; therefore ROS-activated Ca^{2+}-permeable NSCCs and KORs are likely to be encoded by Shaker genes that are abundant in plant genomes (for example plant Shaker K^+ channels and 20 members of CNGCs). Several ROS-sensitive sites as well as putative metal binding centres have been recently identified in plant Shakers and examination of their importance for ROS-mediated ion channel activation is in progress (Demidchik and Maathuis; unpublished).

4 Concluding Remarks

Significant progress has been achieved in understanding the mechanisms of ROS generation and radical imbalance caused in plants by biotic and abiotic stresses and their involvement in stress signal transduction. The major finding of recent years is the role of NADPH oxidase in stress-induced ROS generation. Accumulating evidence also indicates the role of ROS-activated Ca^{2+}- and K^+-permeable cation channels as a downstream target for NADPH oxidase-produced ROS. These channels have been characterised biophysically in *A. thaliana* and their roles in drought stress, PCD and generation stress-induced Ca^{2+} signals have been demonstrated.

Nevertheless, many more questions need to be answered. Despite their extraordinary importance in plant physiology, Ca^{2+}-permeable and other ion channels activated by free radicals and ROS are still poorly studied. Most aspects of their physiology remain obscure. Pilot tests with vibrating ion-selective microelectrodes have shown their existence in some crop species (Demidchik et al. 2003) but patch-clamp analyses in these species have not yet been carried out. Genes encoding ROS-activated channels are yet to be identified. As already mentioned above, hundreds of reports described cation channel activation by ROS leading to elevations in cytosolic free Ca^{2+} and K^+ leakage; however, the molecular identities of corresponding channels are lacking. Attention is often focused on H_2O_2, which is easy to monitor but is probably not a prime cause of many stress-signalling reactions. Studies of hydroxyl radical generation by transition metals and their role in ROS activation of channels are absent. The effects of the important ROS singlet oxygen on plant ion channels are also not studied. Addressing these questions will greatly improve our understanding of the functioning and molecular nature of plant ROS-activated cation channels and will help in the future to control the ROS/Ca^{2+} stress signalling "hub" by genetic manipulation. This could provide us with an opportunity to regulate plant stress responses and stress tolerance.

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