Stress-induced electrolyte leakage: the role of $K^+$-permeable channels and involvement in programmed cell death and metabolic adjustment

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Abstract

Electrolyte leakage accompanies plant response to stresses, such as salinity, pathogen attack, drought, heavy metals, hyperthermia, and hypothermia; however, the mechanism and physiological role of this phenomenon have only recently been clarified. Accumulating evidence shows that electrolyte leakage is mainly related to $K^+$ efflux from plant cells, which is mediated by plasma membrane cation conductances. Recent studies have demonstrated that these conductances include components with different kinetics of activation and cation selectivity. Most probably they are encoded by GORK, SKOR, and annexin genes. Hypothetically, cyclic nucleotide-gated channels and ionotropic glutamate receptors can also be involved. The stress-induced electrolyte leakage is usually accompanied by accumulation of reactive oxygen species (ROS) and often results in programmed cell death (PCD). Recent data strongly suggest that these reactions are linked to each other. ROS have been shown to activate GORK, SKOR, and annexins. ROS-activated $K^+$ efflux through GORK channels results in dramatic $K^+$ loss from plant cells, which stimulates proteases and endonucleases, and promotes PCD. This mechanism is likely to trigger plant PCD under severe stress. However, in moderate stress conditions, $K^+$ efflux could play an essential role as a ‘metabolic switch’ in anabolic reactions, stimulating catabolic processes and saving ‘metabolic’ energy for adaptation and repair needs.

Key words: Electrolyte leakage, ion channels, metabolic adjustment, potassium efflux, programmed cell death, reactive oxygen species, stress response.

Introduction

Electrolyte leakage is a hallmark of stress response in intact plant cells. This phenomenon is widely used as a test for the stress-induced injury of plant tissues and ‘a measure’ of plant stress tolerance (Levitt, 1972; Blum and Ebercon, 1981; Bajji et al., 2002; Lee and Zhu, 2010). The electrolyte leakage is ubiquitous among different species, tissues, and cell types, and can be triggered by all major stress factors, including pathogen attack (Atkinson et al., 1985, 1990, 1996; Ebel and Mithöfer, 1998; Blatt et al., 1999; Maffei et al., 2007), salinity (Nassery, 1975; Maathuis and Amtmann, 1999; Shabala et al., 2006; Demidchik et al., 2010), heavy metals (De Vos et al., 1991; Murphy and Taiz, 1997; Demidchik et al., 2003), oxidative stress (Demidchik et al., 2003, 2010), high soil acidity (pH <4) (Marschner et al., 1966), wounding (Nassery,
The discovery of stress-induced electrolyte (K⁺) leakage

The first measurements of the leakage of electrolytes from freezing- and wounding-treated plants were carried out almost a hundred years ago (Osterhaut, 1922; Dexter et al., 1932). These original studies suggested that the electrolyte leakage is related to membrane damage causing cell death. However, in the 1970s, Palta et al. (1977) demonstrated that freezing does not decrease cell viability and does not change water permeability, although it induces efflux of K⁺ and anions. These authors proposed that freezing increases the passive permeability of cells to K⁺ but does not disturb membrane integrity. Similar reactions have been found in heat- and drought-treated plants, which demonstrated dramatic electrolyte/K⁺ loss (Blum and Ebercon, 1981; Leopold et al., 1981).

Plant physiologists investigating toxic effects of high salinity discovered the K⁺ leakage in the 1960s (Levitt, 1972). Originally, this effect of NaCl was attributed to the non-specific membrane damage and loss of membrane integrity. However, in the 1970s, Nassery (1975, 1979) showed that NaCl (>50 mM) specifically induces efflux of K⁺. Analysing K⁺ efflux from wheat, barley, bean, and chick pea roots, Nassery (1975, 1979) demonstrated that this reaction is sensitive to Ca²⁺ and that it is not induced by osmotic stress. Since then, the problem of NaCl-induced K⁺ efflux has been addressed in a number of reports using different species and techniques (Maathuis and Amtmann, 1999; Demidchik and Tester, 2002; Demidchik et al., 2003, 2010; Shabala et al., 2006; Cuin et al., 2008). Salt-induced K⁺ efflux is a rapidly activating process, which lasts up to 1 h and leads to a significant decrease of K⁺ activity in the cytosol (Shabala et al., 2006). It is currently accepted that maintaining high cytosolic K⁺ activity is crucial for plant tolerance to high NaCl (Maathuis and Amtmann, 1999; Demidchik and Maathuis, 2007; Shabala and Cuin, 2008; Velarde-Buendia et al., 2012).

In the 1980s, Atkinson and co-authors demonstrated that pathogen elicitors cause K⁺ release from tobacco and soybean suspension cultures (Atkinson et al., 1985, 1990, 1996). This reaction activated after a few minutes and lasted ~1 h. Other substances, such as proteins or sugars, were not released. Elicitor-induced K⁺ leakage was accompanied by Ca²⁺ influx and H⁺ efflux. Nowadays, it is believed that K⁺ efflux can play an important role in plant-pathogen interaction (Garcia-Brugger et al., 2006).

Potassium efflux from roots treated by heavy metals was first established in the 1980s (De Vos et al., 1989). This reaction was initially thought to be caused by pores in the membrane that are induced by lipid peroxidation or by activation of ‘heavy metal receptors’ (De Vos et al., 1993; Demidchik et al., 1997, 2001). However, Murphy et al. (1999) have clearly shown that Cu²⁺-induced K⁺ efflux is prevented by blockers of K⁺ channels, such as tetaethylammonium ions (TEA⁺). In the 1990s, an investigation of Cu²⁺-induced reaction of the plasma membrane in algal cells (Nitella flexilis) led to the discovery of oxidative stress-induced cation/K⁺ efflux in root and leaf cells of higher plants (Demidchik et al., 1997, 2001, 2003, 2007, 2010; Shabala et al., 2006).

Potassium leakage is mediated by cation channels

As mentioned above, the idea of an ion channel mechanism of K⁺ efflux (passive permeability of the plasma membrane) was actually proposed by Palta et al. (1977), based on studies of freezing tolerance. Atkinson et al. (1985, 1990, 1996) found the sensitivity of pathogen-induced K⁺ efflux to cation channel antagonists (La³⁺, Gd³⁺, and Co²⁺), and concluded that there was an involvement of Ca²⁺-permeable channels, although polyvalent cations tested in this study can block all cation channel types. Murphy et al. (1999) demonstrated that Cu²⁺-induced K⁺ efflux in Arabidopsis roots is inhibited by TEA⁺ (a specific K⁺ channel blocker). Demidchik et al. (1997, 2001) demonstrated that Cu²⁺ activates non-selective cation channels.
Potassium efflux activated by hydroxyl radicals, salinity, and elicitors in Arabidopsis thaliana root cells has been shown to be sensitive to TEA⁺ and mediated by GORK (guard cell ‘outward-rectifying’ K⁺ channel) (Demidchik et al., 2003, 2010). Purines (markers of wounding stress) have been shown to activate K⁺ efflux through NSCCs (Demidchik et al., 2011). Cation efflux channels with low ionic selectivity, permeable to both cations and anions, but not to organic substances, have been characterized in pea (Zepeda-Jazo et al., 2011). Additionally, Laohavisit et al. (2009, 2012) have recently demonstrated that plant annexins form NSCCs in the plasma membrane in an -OH-dependent manner. Overall, these data clearly show that plant cell electrolyte/K⁺ leakage is mediated by different types of ion channels and that this phenomenon is not related to non-specific membrane damage induced by stress factors.

**Electrophysiology of stress-activated K⁺ efflux channels**

The concentration of K⁺ in the cytosol of plant cells is 70–200 mM, while the external K⁺ level is between 0.01 mM and 1 mM (Leigh and Wyn Jones, 1984; Bergmann, 1992). This creates a huge chemical gradient of K⁺ across the plasma membrane. The cell membrane generates negative electric potential at the cytoplasmic side to hold positively charged K⁺ inside (Nobel, 2009). When depolarization discharges the plasma membrane, K⁺ leaks out through any K⁺-permeable pore. Analysis of available data on stress-induced K⁺ efflux conductances (obtained using various preparations and techniques) shows that they are mediated by two types of ‘pores’. The first type is K⁺-selective channels, which are highly selective for K⁺ (Blatt et al., 1999; Becker et al., 2003; Demidchik et al., 2003, 2010). In most cases, they have time-dependent activation (slowly activating channels) and demonstrate outward rectification (steep voltage dependence). The second type is NSCCs that are almost equally permeable to the range of cations, including K⁺, and usually demonstrate instantaneous (rapid) activation kinetics and weak voltage dependence (Demidchik et al., 2002, 2007, 2011; Laohavisit et al., 2009, 2012; Zepeda-Jazo et al., 2011). However, this classification is not ‘exclusive’. Some K⁺-selective channels can demonstrate rapid (or instantaneous) activation and weak rectification, while NSCCs can have slow activation and outward rectification (reviewed by Demidchik et al., 2002; Demidchik and Maathuis, 2007; Hedrich, 2012). In some cases, activation of both K⁺-selective and non-selective channels can be observed (Demidchik et al., 2003, 2010) (Fig. 1).

The pharmacological profile of K⁺ efflux conductances varies a lot across the preparations. However, K⁺-selective channels are always sensitive to TEA⁺ while NSCCs are not inhibited by this K⁺ channel antagonist. Lanthanides (Gd³⁺ and La³⁺) and divalent cations, such as Ca²⁺, Ba²⁺, Zn²⁺, or Co²⁺, decrease K⁺ currents mediated by both groups of channels, including annexins. Algal and pea K⁺ efflux NSCCs are sensitive to nifedipine and verapamil which are antagonists of animal voltage-gated Ca²⁺-selective channels.
Potassium-permeable non-selective channels in pea plasma membrane are also sensitive to anion channel antagonists (Zepeda-Jazo et al., 2011).

**Molecular origin of K⁺ efflux**

Cation channels are di- or tetrameric structures that are composed of the same or different subunits. Each subunit is encoded by one gene and includes from two to 12 transmembrane domains, one or two pore regions, and a number of regulatory domains (Fig. 2). The *A. thaliana* genome contains 77 genes of hypothetical K⁺-permeable channels localized in the plasma membrane or endomembranes and include the following families: (i) K⁺-selective ‘Shakers’ (nine members); (ii) ‘tandem-pore K⁺’ channels (TPKs; six members); (iii) ionotropic glutamate receptors (GLRs; 20 members); (iv) cyclic nucleotide-gated channels (CNGCs; 20 members); (v) two-pore channel (TPC; one member); (vi) mechanosensitive-like channels (MSLs; 10 members); (vii) mechanosensitive ‘Mid1-complementing activity’ channels (MCAs; two members); (viii) mechanosensitive Piezo channel (one member); and (ix) annexins (eight members) (Demidchik et al., 2002; White et al., 2002; Véry and Sentenac, 2003; Demidchik and Maathuis, 2007; Hedrich, 2012; Jam et al., 2012; Sharma et al., 2013; Iida et al., 2014). The quantity of cation channel genes varies significantly across the plant species. For example, poplar and rice genomes contain 61 and 13 GLRs, respectively. Among these families, K⁺-selective Shakers and TPKs contain a pore region (selectivity filter) which is selective for K⁺ (Dreyer and Uozumi, 2011; Sharma et al., 2013). Shakers have one pore region while TPKs contain two such regions (Fig. 2). Thus, Shakers and TPKs form a group of ‘proper’ K⁺ channels or K⁺-selective channels. Other plant cation channels are likely to be NSCCs that are ‘non-selectively’ permeable to different cations, including K⁺, Na⁺, Cs⁺, and Ca²⁺ (Demidchik et al., 2002).

Each subunit of K⁺-selective Shakers contains six transmembrane domains (‘TM-helices’) and one pore region, while TPKs contain four transmembrane domains forming two pore regions (Fig. 2). The pore region of K⁺-selective channels harbours a conservative TXGYGD/E motif in the pore region (also known as the GYG motif; Gly-Tyr-Gly), which is common for plants, animals, fungi, and bacteria (MacKinnon, 2004; Nimsigean and Allen, 2011). Four Gly-Tyr-Gly regions (from two subunits of TPKs or four Shakers) form a ‘tunnel-like’ structure (pore) ‘mimicking’ the hydration shell of K⁺ with electrostatic interactions of oxygen atoms in amino acids (MacKinnon, 2004). Potassium ions, when entering the pore, lose the hydration shell. Other cations have a much higher energy barrier of hydration shell removal than K⁺ when passing this pore. This ensures the ‘selectivity’ for K⁺. Potassium-selective filters also function as K⁺ sensors and directly regulate K⁺ efflux (Choe, 2002; Poree et al., 2005; Johansson et al., 2006; Li et al., 2008). Maximally four potassium ions can occupy the pore, although computational analysis and lipid bilayer studies suggest that, at physiological K⁺ activities (up to 500 mM), maximally two K ions occupy the pore at a given time (with two water molecules intervening) (Morais-Cabral et al., 2001). In the classical K⁺ influx channel, the maximal occupancy (which is likely to be two K⁺) causes the ‘widening’ of the pore ‘tunnel’ and stabilization of its ‘transport’ properties, increasing the channel’s conductance. However, K⁺ efflux channels respond differently to high extracellular K⁺. Their ‘K⁺-filled’ pore (expanded under high external K⁺) directly interacts with the DM1xG-motif.

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**Fig. 2.** Transmembrane topology and major functional domains of putative K⁺-permeable cation channels in plant membranes (see description in the text).
of the sixth TM helix (‘S6 gating domain’), that promotes the channel’s closing.

Shakers are voltage-gated channels while TPKs are voltage-independent systems (Dreyer and Uozumi, 2011; Sharma et al., 2013). The fourth transmembrane domain (S4) of Shakers takes part in voltage sensing. This domain is enriched by basic amino acids harbouring a total high positive charge. When moving in response to the change of the transmembrane electric field, it causes the rearrangement of the other channel’s subunits, resulting in the channel’s opening or closure. Seven K⁺-selective Shakers activate at voltages more negative than the resting potential (hyperpolarization-activated K⁺ channels or inwardly rectifying K⁺ channels), thus catalysing K⁺ influx to the cytosol. However, two Shakers are activated at voltages more positive than the resting potential (depolarization), including SKOR (stelar K⁺ ‘outward-rectifying’ channel) and GORK. Domain S4, when ‘performing’ voltage-dependent rearrangements, opens SKOR and GORK when the total membrane charge decreases (conditions of depolarization). These two channels are likely to form K⁺ leakage pathways.

The SKOR-encoded channels catalyse the K⁺ ‘leak’ from root stelar cells into xylem (Gaymard et al., 1998). The GORK-encoded channels are highly expressed in root epidermis (both atrichoblasts and trichoblasts) cortex and guard cells (Ache et al., 2000; Ivashikina et al., 2001; Demidchik et al., 2003; 2010). In these tissues, they appear to have the function of ROS-, abscisic acid-, jasmonic acid-, and ethylene-controlled K⁺ efflux systems. The activation of K⁺ release through GORK is critically important for closing stomata, which is regulated by blue light, pH, Ca²⁺, and phytohormones (Hosy et al., 2003; T.H. Kim et al., 2010). Potassium efflux increases H₂O chemical potential in guard cells and promotes the H₂O efflux from guard cells, causing their ‘relaxation’ and shrinkage (closing stomata). Potassium efflux in leaves may also be catalysed by AKT2, which is a K⁺-selective Shaker with an unusual ‘bell-like’ shape of the I–V curve, conducting a large K⁺ efflux current at positive membrane voltages (reviewed by Hedrich, 2012; Sharma et al., 2013).

TPKs are voltage-independent channels lacking a voltage-sensing domain (Dunkel et al., 2008). They seem to be ‘permanently’ active and conducting K⁺-selective current at any membrane voltage. TPKs can potentially be responsible for voltage- and time-independent K⁺ efflux currents which participate in K⁺/electrolyte leakage mechanism (Demidchik and Tester, 2002; Demidchik et al., 2003, 2010; Shabala et al., 2006). Nevertheless, only TPK4 is localized in the plasma membrane (Becker et al., 2004). This channel is expressed in the pollen tube plasma membrane and mediates voltage-independent instantaneously activating cation currents.

CNGCs have a Shaker-like structure sharing principal domains with K⁺-selective Shakers, but not a GYG motif (Demidchik et al., 2002; Demidchik and Maathuis, 2007). Thus, these systems are potentially non-selective for cations. They contain a cyclic nucleotide-binding domain overlapping with a calmodulin-binding moiety. This allows regulation by cyclic nucleotides (cAMP/cGMP) and additional control provided by calmodulin (preventing binding of cyclic nucleotides). Despite the high level of expression in different tissues (Gobert et al., 2006), the K⁺ efflux currents through CNGCs have not been investigated in intact plant cells. On the other hand, animal CNGCs are capable of catalysing Ca²⁺ influx and K⁺ efflux (Kaupp and Seifert, 2002; Cheng and Kodama, 2004). Some plant CNGCs, when expressed in animal cells, probably form K⁺-selective channels (Hua et al., 2003).

Some animal ionotropic glutamate receptors mediate K⁺ efflux currents (Kriedowski and Mieneville, 2001), triggering K⁺-dependent PCD in neurons (Xiao et al., 2001). Hypothetically, plant GLRs can be involved in K⁺ efflux. They share a principal structure with animal ionotropic glutamate receptors, which seem to be homo- or heteromers of four subunits and contain an extracellular glutamate/glycine binding domain (each consisting of subdomains) (Fig. 2). However, they contain completely different amino acid sequences in the pore region (Demidchik et al., 2002; Price et al., 2012). Moreover, plant GLRs have very long N-terminal domains exposed to the extracellular space. The function of this domain is unknown. The addition of exogenous amino acids to the bathing solution activates voltage-independent NSCCs in the plasma membrane (Demidchik et al., 2004). Whether these channels can mediate K⁺ efflux is unclear.

Potassium permeability of plant ‘mechanosensitive-like’ and ‘mechanosensitive’ channels has not yet been studied; however, their bacterial counterparts are permeable to K⁺ (Haswell et al., 2011; Cox et al., 2013). Such channels can potentially be involved in K⁺ release under mechanical stress.

Reactive oxygen species are ‘partners’ of K⁺ leakage in plant stress response

The generation of ROS is the most often reported reaction accompanying K⁺ leakage in plants in stress conditions. In most cases, superoxide (O₂⁻) production via one-electron reduction of triplet oxygen (O₂) is a starting point for ROS biosynthesis, oxidative stress, and redox regulation in plants (Halliwell and Gutteridge, 1990; Demidchik, 2012). Further acceptance of two electrons leads to sequential formation of hydrogen peroxide and hydroxyl radicals, respectively (Halliwell and Gutteridge, 1999). Hydroxyl radicals are the most powerful oxidants in biological systems, which can potentially modify any organic molecule. This short-living oxygen derivative seems to be responsible for the majority of toxic and regulatory effects of ROS in plant cells (Halliwell and Gutteridge, 1999). Transition metals, such as iron or copper, and reducing agents, for example 1-ascorbic acid (ascorbate), supply electrons for ·OH formation while enzymatic and non-enzymatic antioxidants, and metal chelators, interfere with this process, detoxifying O₂⁻, peroxides, and free metals.

Recent data have clarified major sources of ROS in plants, and show that, apart from classical extracellular chloroplast, mitochondrial, and peroxisome sources, ROS are synthesized in the apoplast by plasma membrane NADPH oxidases, class III peroxidases, poly(dI)amine oxidases, and oxalate
oxidases (reviewed by Lane, 1994; Moschou et al., 2008; Demidchik, 2010, 2012; Jiang et al., 2011; Marino et al., 2012). In the case of moderate stress, ROS production predominantly acts as a regulatory mechanism activating defence and immunity reactions. However, when stress is ‘severe’, ROS generation superimposes the oxidative stress on top of a given stress factor, damaging cellular components and causing their dysfunction. A special class of nitrogen-containing ROS (NO, peroxinitrite, and others), called reactive nitrogen species (RNS), has also been shown to be involved in plant stress reactions (Molassiotis and Fotopoulos, 2011). This adds complexity to ROS metabolism and pathophysiology. Combined production of ROS and RNS is currently considered a major stimulus for plant stress adaptation (under moderate stress) and PCD (under severe stress) (Apel and Hirt, 2004; Molassiotis and Fotopoulos, 2011).

Plants produce \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \( \cdot \text{OH} \) in response to salinity (Kawano et al., 2002; Demidchik et al., 2003, 2010), pathogens (Huckelhoven et al., 2000; Demidchik et al., 2003; Giovannini et al., 2006), drought (Menconi et al., 1995; Selotea et al., 2003; Sgherri et al., 1996), hyperthermia (Lee et al., 2002; Dong et al., 2009), hypothermia (Edreva et al., 1998), heavy metals (Wang et al., 2008), herbicides (Song et al., 2007), and other stresses. The kinetics of ROS production are similar to the kinetics of the increase of \( K^+ \) efflux in response to the same stresses. This points to the existence of a link between ROS production and \( K^+ \) efflux.

Pathogen attack is the best studied case of both ROS generation and \( K^+ \) leakage. Production of \( \cdot \text{OH} \) and \( K^+ \) leakage have been observed in plants treated with pathogenic elicitors from \( \text{Cladosporium fulvum} \) (Veraestrella et al., 1992; Blatt et al., 1999), \( \text{Alternaria alternata} \) (Jennings et al., 2002), \( \text{Botrytis cinerea} \) (Govrin et al., 2006), and \( \text{Magnaporthe grisea} \) (Pasechnik et al., 1998). Treatment of protoplasts isolated from tobacco guard cells by \( C. \text{fulvum} \) elicitor activated \( K^+ \) efflux currents (Blatt et al., 1999). \( \text{Trichoderma viride} \) elicitor induced \( \text{TEA}^+\)-sensitive \( K^+ \) efflux in intact \( A. \text{thaliana} \), suggesting that \( K^+ \) channels are activated by this elicitor (Demidchik et al., 2010). ROS scavengers, inhibitors of ROS-producing enzymes, cation channel antagonists, as well as overexpression of antioxidant systems and specific defence proteins can prevent or significantly delay plant responses to pathogens, including \( K^+ \) efflux (Ebel and Mithofer, 1998; De Gara et al., 2003; Apel and Hirt, 2004; Pike et al., 2005; Shetty et al., 2008; Demidchik et al., 2010).

ROS-activated cation channels link ROS production, \( K^+ \) leakage, and PCD

The discovery of hydroxyl radical-activated \( K^+ \) outwardly rectifying channels has linked stress-induced ROS generation and \( K^+ \) release (Demidchik et al., 2003, 2010). In this case, the ROS production is an upstream reaction inducing \( K^+ \) efflux (Fig. 3). Using electron paramagnetic resonance spectroscopy, Demidchik et al. (2010) have shown that salt stress induces the generation of \( \cdot \text{OH} \), which activates \( K^+ \) efflux channels in intact root epidermis of wild-type plants.

![Fig. 3. The scheme of the hypothetical mechanism of \( K^+ \) leakage at the plasma membrane of plant cells.](image-url)
-OH activation was not observed in *gork1-1* plants lacking the gene that encodes the K⁺ efflux channel, GORK. In intact roots, both -OH and NaCl caused dramatic K⁺ efflux that was much smaller in *gork1-1*. Similar effects have been measured for biotic stress (Trichoderma viride elicitor cellulysin). Potassium efflux channels activated by -OH have also been characterized in pea root epidermis (Zepeda-Jazo et al., 2011) and in barley root cells, where their activation correlated with salt sensitivity (Velasco-Buenia et al., 2012).

Generation of ROS (particularly -OH) seems to be a key cause of plant PCD (Apel and Hirt, 2004). Stress-induced PCD begins from the effect of stress factors on as yet unidentified receptors (plasma membrane and/or intracellular), which activate ROS-producing enzymes, such as NADPH oxidases (Ca²⁺-dependent proteins), peroxidases, and chloroplast and mitochondrial redox cascades (Demidchik, 2012) (Fig. 3). It is widely accepted that ROS induce the elevation of cytosolic free Ca²⁺, and this is not only a stress signal, but probably also a trigger of PCD reactions that result in damage to endomembranes and collapse of vacuoles (Apel and Hirt, 2004; Demidchik and Maathuis, 2007). Calcium influx, which is amplified by the Ca²⁺-ROS amplification mechanism (Fig. 3) can promote long-term plasma membrane depolarization which is required for GORK activation.

In animals, PCD-specific hydrolytic enzymes (caspases and endonucleases) are directly inhibited by their natural blocker, K⁺, which is, similarly to the case of plant cells, high in the cytosol (70–100 mM) (Remillard and Yuan, 2004). The cytoplasmic K⁺/Na⁺ ratio is a major parameter regulating animal PCD (Bortner et al., 1997, 2001; Orlov et al., 1999). Na⁺ cannot substitute for K⁺ in its protease inhibition reactions, so the replacement of K⁺ by Na⁺ results in PCD (Orlov et al., 1999). Death factors activate animal K⁺ efflux channels leading to K⁺ loss, and this stimulates protease and endonuclease activity (Yu, 2003). Discovery of ROS-activated K⁺-permeable cation channels in plant cells suggests that a similar mechanism of hydrolase activation exists in plants. Supporting this hypothesis, Demidchik et al. (2010) have shown that both K⁺ channel blockers and the lack of a functional K⁺ efflux channel (GORK) inhibit the NaCl- and oxidative stress-induced activation of PCD proteases and endonucleases.

PCD can help plants to survive under pathogen attack, herbicide treatment, and heavy metal stress (Cutler and Somerville, 2005; Mur et al., 2008). Dead cells provide a ‘shield’ from stress factors and signal stress to surviving cells. However, PCD is harmful in the case of salinity and some other stresses (drought, hypothermia, and hyperthermia). It can be hypothesized that most crop plants ‘over-react’ to salt stress. The salt-induced PCD response seems to have been acquired through evolution long before the development of modern agriculture. In simple terms, plants ‘do not know’ that they will be rescued by a farmer. Hypothetically, the ‘over-reaction’ to pathogen attack, known as the pathogen-induced hypersensitive response, might not always be a useful process. Supporting this hypothesis, the hypersensitive response can be beneficial or detrimental, depending on whether the plant was attacked by a biotrophic or necrotrophic pathogen (Marina et al., 2008).

**Molecular identity of plant ROS-activated cation channels**

Plant ROS-activated cation channels mediating electrolyte leakage have a complex molecular identity. Demidchik et al. (2010) have provided the evidence that these channels are encoded by the K⁺-selective Shaker (GORK), while Laohavisit et al. (2012) have demonstrated the presence of an additional K⁺ efflux pathway that is catalysed by annexins. These mechanisms seem to co-exist and act in concert to amplify K⁺ efflux. Moreover, annexins may lie upstream by catalysing steady-state Ca²⁺ influx, which is required for sustained plasma membrane depolarization, and prolong activation of GORK.

Garcia-Mata et al. (2010) have recently investigated the molecular mechanism of ROS-induced activation of plant K⁺ efflux channels. These authors have shown that the K⁺-selective Shaker, SKOR, heterologously expressed in HEK293 cells, was activated in response to H₂O₂. However, substitution of the Cys168 residue on the S3 α-helix of the voltage sensor complex by another amino acid led to loss of SKOR’s sensitivity to H₂O₂. SKOR serves as a major K⁺ efflux channel in xylem parenchyma cells, but this channel is a close relative of GORK. Analysis of the GORK structure demonstrates the presence of a similar cysteine residue which may possess H₂O₂ sensitivity. Thus, ROS-sensitive residues seem to exist in major K⁺ efflux channels and control ROS-induced K⁺ leakage.

In free radical chemistry, transition metals, such as copper, iron, and manganese, function as electron donors to convert H₂O₂ (weak oxidant) to the more powerful oxidizing species, the hydroxyl radical (Halliwell and Gutteridge, 1999). Metals that lose electrons in this reaction (called the Fenton reaction or Fenton-like reaction) gain them from reduced organic substances, such as ascorbic acid or some phenolic compounds. This means that ascorbate, transition metals, and H₂O₂ can non-enzymatically produce hydroxyl radicals in the cell and apoplast (these chemical reactions are called the Haber-Weiss cycle; Halliwell and Gutteridge, 1999). Hydroxyl radical biosynthesis seems to play a critical role in the activation of K⁺ efflux in intact roots because the addition of H₂O₂ without transition metal induced a much smaller K⁺ efflux (Demidchik et al., 2010; Rodrigo-Moreno et al., 2013). Demidchik et al. (2007) have demonstrated that H₂O₂ can activate cation currents in mature root epidermal cells only when it was added to the cytosolic side of the plasma membrane (inside a patch-clamp pipette). This points to the existence of a transition metal-binding site in the cation channel mediating ROS-activated K⁺ efflux. Supporting this hypothesis, Rodrigo-Moreno et al. (2013) have recently shown that copper acts on K⁺ efflux at the cytosolic side of the plasma membrane.

We have carried out bioinformatics analysis of structures of all *A. thaliana* cation channels using Metal Detector v.2.0 software (Universities of Florence and Trento). This analysis has revealed two candidates with putative Cu/Fe-binding sites, namely CNGC19 and CNGC20 (Fig. 4). Cys102, Cys107, and Cys110 of CNGC19, and Cys133, Cys138, and Cys141...
of CNCG20 coordinate transition metals and form metal-binding sites (the probability is close to 100%). These cysteine metal pockets are situated in the first cytosolic domain of CNGC. Notably, cysteine residues have recently been shown to be responsible for hydroxyl radical-mediated activation of animal Ca^{2+}-permeable channels (Simon et al., 2004) and transcription factors (Dubbs and Mongkolsuk, 2012).

Fig. 4. The scheme of hypothetical cysteine-containing transition metal-binding sites in plant cyclic nucleotide-gated channels. A Cu or Fe ion is thought to reside in the cysteine 'pocket' at the cytosolic face of the plasma membrane [N-terminus]. This 'fixed' metal is catalytically active and requires H_{2}O_{2} for generation of extremely reactive hydroxyl radicals (·OH) (insert). It can be hypothesized that the ·OH reaction with neighbouring amino acids increases the probability of the open state of the channel. 1–6, transmembrane domains on the Shaker structure of the CNGC; P, pore region between the fifth and sixth transmembrane domains; CaM/CNBD, putative calmodulin and cyclic nucleotide-binding domain.

Is stress-induced K^{+} release required for ‘metabolic adjustment’?

In the majority of cases, stress-induced K^{+} efflux lasts at least 30–40 min (Marschner et al., 1966; Demidchik et al., 2003, 2010; Britto et al., 2010) and results in a stable 3- to 5-fold decrease in cytosolic K^{+} activity (Shabala et al., 2006; V. Demidchik et al., unpublished). For example, treatment with 50 mM NaCl decreases cytosolic K^{+} in Arabidopsis root epidermal cells from 70–80 mM to 10–20 mM within 10–15 min after NaCl addition to the bathing solution. The addition of 50 mM NaCl to the growth medium delays root growth by 50–60% but does not cause PCD, which is observed at higher NaCl concentrations (>100 mM). Calculations of total K^{+} loss based on K^{+} efflux curves obtained by the MIFE™ (microelectrode ion flux estimation) technique demonstrate that major stresses induce reduction of cytosolic K^{+} several fold (Demidchik et al., 2003, 2010; Shabala et al., 2006, 2010; Shabala, 2011; Rodrigo-Moreno et al., 2013). This reaction is reversible after the removal of the stress factor or after a period of adaptation (in the case of moderate, non-lethal, stress treatment).

Adequate K^{+} supply stimulates the growth rate and development of plants. However, plants may be losing K^{+} from cells during K^{+} starvation (Bergmann, 1992). For example, Szczерба et al. (2006) have demonstrated that the K^{+} level in barley root cells decreases from 200 mM to 40 mM under K^{+} deficiency conditions. In Arabidopsis roots exposed to low external K^{+}, this value drops to 5–15 mM (Armengaud et al., 2009). Although K^{+}-deficient plants survive, they demonstrate stunted growth and a low level of anabolism (Bergmann, 1992; Marschner, 2012).

Some plants avoid stress-induced loss of K^{+}. A number of stress-tolerant species are capable of maintaining K^{+} at higher levels in stress conditions (Shabala and Cuin, 2008). This can be achieved through increased K^{+} selectivity of total plasma membrane conductance and high H^{+}-ATPase activity preventing long-term depolarization.

Maintaining the K^{+} ‘steady state’ (or K^{+} homeostasis) in living cells is a very old evolutionary phenomenon (Derst and Karschin, 1998). Eukaryotes evolved sophisticated transport and regulatory proteins that control K^{+} accumulation and release. Therefore, K^{+} loss seems to be an acquired evolutionary reaction and, perhaps, beneficial in some conditions. It is an established fact that K^{+} is an allosteric inhibitor of animal proteases and nuclease (Bortner et al., 1997, 2001; Orlov et al., 1999; Yu, 2003; Remillard and Yuan, 2004). This suggests that K^{+} loss may stimulate catabolic processes and release of energy.

Textbooks on plant mineral nutrition indicate that K^{+} is a non-specific activator of cytosolic enzymes (Bergmann, 1992; Marschner, 2012). ‘Targets’ for direct K^{+} action may include pyruvate kinase, protein biosynthesis enzymes, and other systems (Walker et al., 1996, 1998; Armengaud et al., 2009). Some recent data on the metabolite profile and corresponding enzymatic activities in Arabidopsis roots in plants cultivated on media with normal and low K^{+} suggest that enzymatic activities related to anabolic processes decrease under K^{+} starvation (Armengaud et al., 2009). K^{+}-deficient plants accumulate soluble sugars (sucrose, fructose, and glucose) and non-acidic amino acids. However, levels of nitrate, glutamate, aspartate, pyruvate, 2-oxoglutarate, and malate decrease. Most of these modifications of metabolites were reversed within a few hours of K^{+} resupply (Armengaud et al., 2009), which correlates with the time of K^{+} ‘re-fill’ in the cytosol (Szczерба et al., 2006). These results point to the ‘inhibiting’ interaction of K^{+} with enzymes catalysing ‘anabolic’ processes, which build up cell polymers from monomeric sugars and amino acids.

It can be hypothesized that the decrease in cytosolic K^{+} plays the role of a ‘switch’ which inhibits energy-consuming ‘anabolic’ reactions and stimulates ‘energy-releasing’ catabolic processes. Overall, this stops growth and ‘redirects’ the energy flow to adaptation and reparation needs. This could be a critical step in plant cell adaptation to any stress factor. Stressed plants stop growing and use the released energy to fight stress-induced injuries. Supporting this hypothesis, all
stresses that induce K\(^{+}\) efflux and K\(^{+}\) loss by roots eventually lead to reduction in plant growth, while K\(^{+}\) has always been considered a major factor stimulating growth (Beringer and Troldenier, 1980). For example, Ben-Hayyim et al. (1987) have found that the ability to maintain high cytosolic [K\(^{+}\)] is directly linked to the ability of cultured cells to grow. Walker et al. (1998) have demonstrated that cytosolic K\(^{+}\) stimulates both root expansion growth and protein biosynthesis. Future research should therefore concentrate on the detailed investigation of mechanisms of the relationship between K\(^{+}\) efflux, stress tolerance, and growth retardation.

The relationship between K\(^{+}\) metabolism and ROS production can be even more sophisticated because K\(^{+}\) starvation, which results in a decrease of cytosolic K\(^{+}\) (Armengaud et al., 2009), can also stimulate ROS generation through NADPH oxidase- (Shin and Schachtman, 2004) and peroxidase- (M.J. Kim et al., 2010) mediated pathways.

**Conclusions**

Electrolyte leakage is a constituent part of the plant’s response to stress. This reaction is mainly related to the efflux of K\(^{+}\), which is abundant in plant cells. There are many facts which show that K\(^{+}\) efflux is mediated by ion channels consisting of two groups. The first group are the slowly activating outwardly rectifying K\(^{+}\)-selective channels, which are encoded by GORK, SKOR, and annexin genes. The second group are voltage-independent instantaneously activating NSCCs with unknown molecular identity. Most probably, the second group belongs to multigene families of CNGCs or ionotropic glutamate receptors. This has to be investigated in future studies.

The stress-induced electrolyte leakage is always accompanied by ROS generation and often leads to PCD. These phenomena are not independent of each other. Recent data demonstrate that ROS (hydroxyl radicals and H\(_2\)O\(_2\)) are capable of activating GORK, SKOR, and annexins catalysing K\(^{+}\) efflux from plant cells. Moreover, GORK-mediated K\(^{+}\) efflux has been shown to cause PCD under salinity and oxidative stress. Potassium ions seem to block intracellular proteases and endonucleases; therefore, their efflux stimulates these hydrolytic enzymes, leading to PCD. In moderate stress conditions, K\(^{+}\) efflux could also play the role of a ‘metabolic switch’ which decreases the rate of abiotic reactions and stimulates catabolic processes, causing the release of energy for adaptation and repair needs.

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