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REVIEW PAPER

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 Mithofer, 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,

Abbreviations: GLR, glutamate receptor; GORK, guard cell 'outward-rectifying' K^+ channel; I–V curve, current–voltage curve; MIFE™, microelectrode ion flux estimation; MSL, mechanosensitive-like channel; NSCC, non-selective cation channel; ROS, reactive oxygen species; SKOR, stelar K^+ 'outward-rectifying' channel.

1971, 1972), waterlogging (Shabala, 2011), drought (Blum and Ebercon, 1981; Leopold *et al.*, 1981; Shcherbakova and Kacperska, 1983), heat (Liu and Huang, 2000), and others. The electrolyte leakage is detected almost instantaneously after the application of a stress factor and lasts from a few minutes to several hours. It is mainly caused by the efflux of K^+ and so-called counterions (Cl^-, HPO_4^{2-}, NO_3^-, citrate^{3-}, malate^{2-}) that move to balance the efflux of positively charged potassium ions (Palta *et al.*, 1977; Bajji *et al.*, 2002). Some stresses induce a dramatic loss of K^+. The activity of this macronutrient in the cytosol and other cellular compartments can decrease from 70–200 mM to 10–30 mM (for example, as measured by Shabala *et al.*, 2006 in the cytosol using sharp K^+-selective microelectrodes). This is particularly important for roots, where K^+ leakage is a common phenomenon leading to irreversible K^+ loss by plants during stress response.

Despite the high physiological importance and direct correlation with stress tolerance, the mechanisms of electrolyte leakage are far from understood. Pharmacological analyses have demonstrated that this reaction is sensitive to cation channel antagonists and accompanied by Ca^{2+} and H^+ efflux, and reactive oxygen species (ROS) generation. Here we revise existing data on the electrolyte leakage (focusing on K^+) in plants and discuss recent studies which shed light on the mechanism and physiological roles of this phenomenon. We propose that the ROS-activated outwardly rectifying K^+ channels are responsible for stress-induced K^+ release from plant cells (a major component of electrolyte leakage). Ion channel-mediated K^+ efflux can lead to K^+ loss that triggers programmed cell death (PCD) (Demidchik *et al.*, 2010; Zepeda-Jazo *et al.*, 2011; Demidchik, 2012). Hypothetically, this reaction can also be involved in metabolic adjustment, which is essential for adaptation.

We acknowledge that electrolyte leakage may be induced by a range of factors, such as oxidative degradation of the lipid bilayer or mechanical defects; however, the sensitivity of this phenomenon to K^+ channel blockers clearly demonstrates that the activation of K^+-permeable channels is a dominant mechanism. Thus the focus of this article is on K^+ leakage mediated by K^+-permeable channels.

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 Ca^{2+} 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-Buendía *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 Ca^{2+} 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 Cu^{2+}-induced K^+ efflux is prevented by blockers of K^+ channels, such as tetraethylammonium ions (TEA^+). In the 1990s, an investigation of Cu^{2+}-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 (La^{3+}, Gd^{3+}, and Co^{2+}), and concluded that there was an involvement of Ca^{2+}-permeable channels, although polyvalent cations tested in this study can block all cation channel types. Murphy *et al.* (1999) demonstrated that Cu^{2+}-induced K^+ efflux in *Arabidopsis* roots is inhibited by TEA^+ (a specific K^+ channel blocker). Demidchik *et al.* (1997, 2001) demonstrated that Cu^{2+} activates non-selective cation channels

(NSCCs) in the plasma membrane of charophyte algae, which are permeable to a range of cations, including K^+, and inhibited by nifedipin and verapamil (antagonists of animal Ca^{2+} and non-selective channels). In 1999, Blatt *et al*. demonstrated that elicitors from *Cladosporium fulvum* activate outwardly rectifying K^+ channels in the plasma membrane of tomato guard cells. The direct involvement of K^+ efflux channels in NaCl-induced K^+ release has recently been demonstrated in *Arabidopsis* root epidermis and leaf mesophyll (Shabala *et al.*, 2006; Demidchik *et al.*, 2010) as well as barley root cells (Chen *et al.*, 2007; Zepeda-Jazo *et al.*, 2008). Shabala *et al.* (2007) discovered the polyamine-sensitive K^+ efflux channels in pea leaf mesophyll, which can mediate K^+ leakage in response to salinity in leaves.

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 (Gd^{3+} and La^{3+}) and divalent cations, such as Ca^{2+}, Ba^{2+}, Zn^{2+}, or Co^{2+}, 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 Ca^{2+}-selective channels.

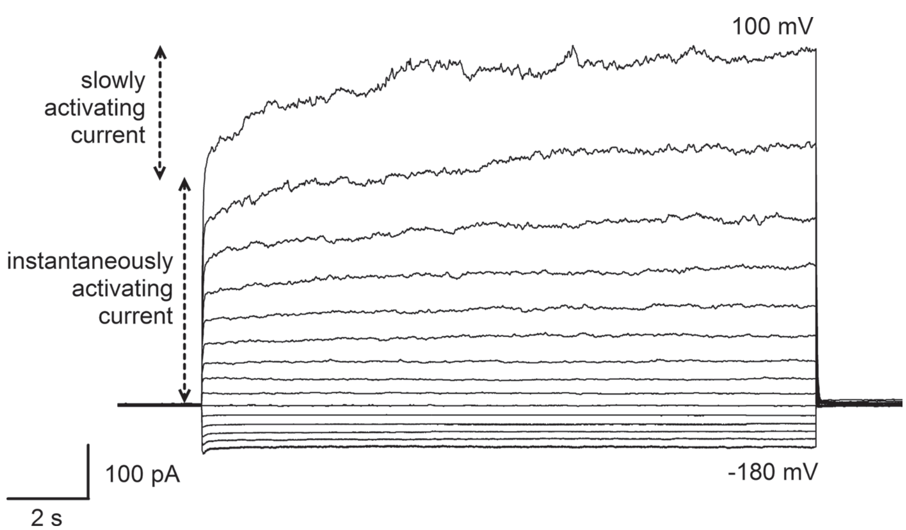

Fig. 1. Typical slowly and instantaneously activating K^+ efflux currents activated by hydroxyl radicals through the plasma membrane of the protoplast (d=21 µm) isolated from *Arabidopsis thaliana* root epidermis. Techniques for protoplast isolation and patch-clamp measurements are described elsewhere (Demidchik and Tester, 2002; Demidchik *et al.*, 2010). Protoplast was exposed for 15 min to a hydroxyl radical-generating mixture comprising 1 mM $CuCl_2$, 1 mM L-ascorbic acid, and 1 mM H_2O_2. The holding potential was –80 mV. The pipette solution contained 100 mM K^+, 10 mM Cl^-, 90 mM gluconate$^-$, and 100 nM Ca^{2+} ($CaCl_2$/BAPTA); pH 7.2 (MES/TRIS).

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; Jami *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 Ca^{2+} (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 DMIxG-motif

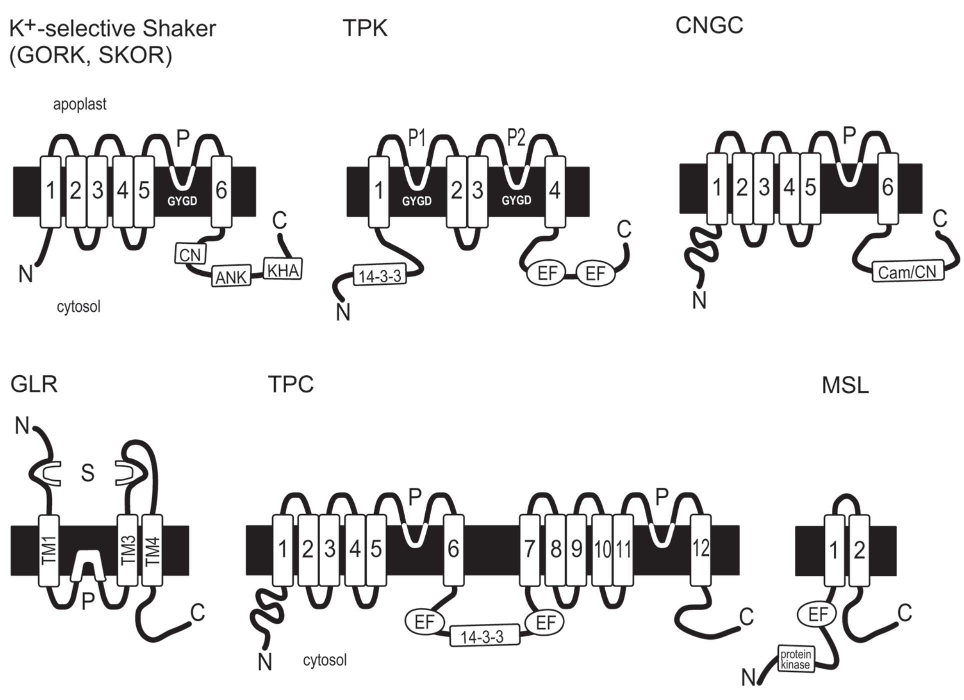

Fig. 2. Transmembrane topology and major functional domains of putative K^+-permeable cation channels in plant membranes (see description in the text).

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, Ca^{2+}, and phytohormones (Hosy *et al.*, 2003; T.H. Kim *et al.*, 2010). Potassium efflux increases H_2O chemical potential in guard cells and promotes the H_2O 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 Ca^{2+} 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 (Kiedrowski and Mienville, 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 ($O_2{\cdot}^-$) production via one-electron reduction of triplet oxygen (O_2) 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 L-ascorbic acid (ascorbate), supply electrons for ·OH formation while enzymatic and non-enzymatic antioxidants, and metal chelators, interfere with this process, detoxifying $O_2{\cdot}^-$, 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 pathopysiology. 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 $O_2^{\cdot-}$, H_2O_2, and ·OH in response to salinity (Kawano *et al.*, 2002; Demidchik *et al.*, 2003, 2010), pathogens (Huckelhoven *et al.*, 2000; Demidchik *et al.*, 2003; Giovanini *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 ·OH and K^+ leakage have been observed in plants treated with pathogenic elicitors from *Cladosporium fulvum* (Veraestrella *et al.*, 1992; Blatt *et al.*, 1999), *Alternaria alternata* (Jennings *et al.*, 2002), *Botrytis cinerea* (Govrin *et al.*, 2006), and *Magnaporthe grisea* (Pasechnik *et al.*, 1998). Treatment of protoplasts isolated from tobacco guard cells by *C. fulvum* elicitor activated K^+ efflux currents (Blatt *et al.*, 1999). *Trichoderma viride* elicitor induced TEA^+-sensitive K^+ efflux in intact *A. 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 ·OH, which activates K^+ efflux channels in intact root epidermis of wild-type plants.

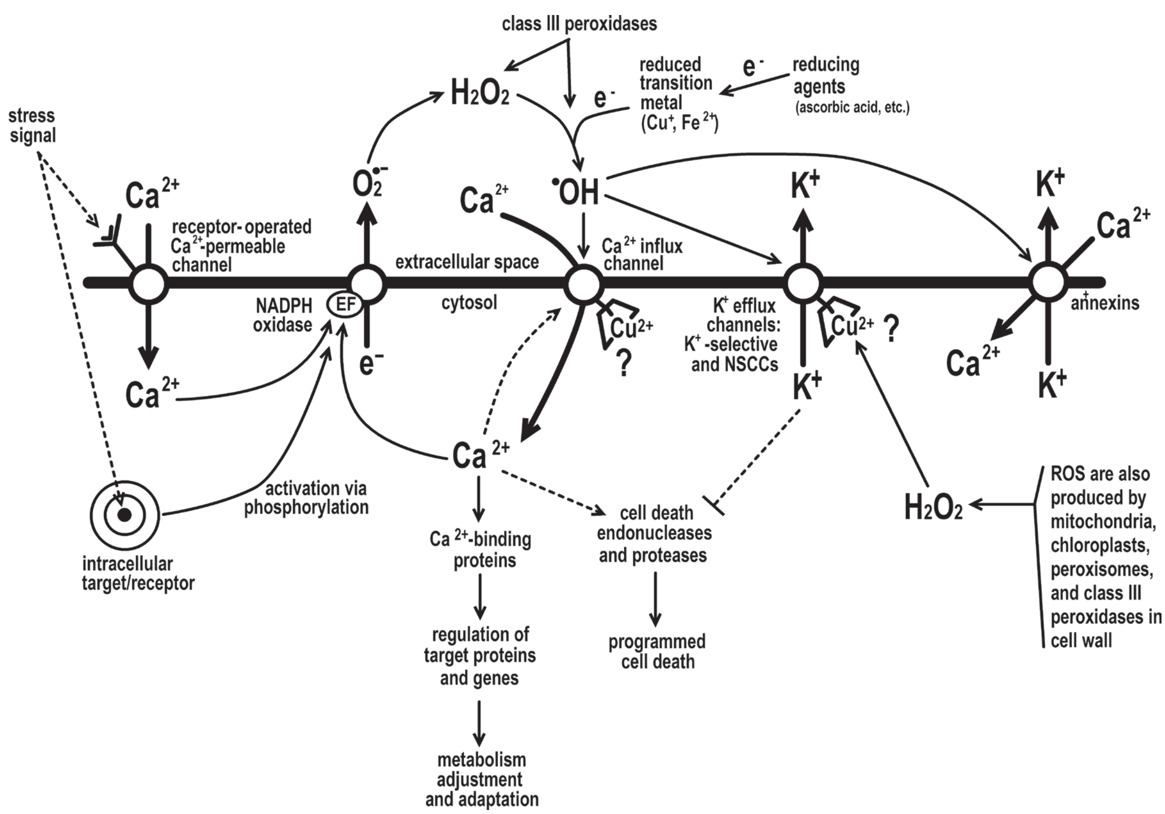

Fig. 3. The scheme of the hypothetical mechanism of K^+ leakage at the plasma membrane of plant cells.

·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 (Velarde-Buendía *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 undetermined receptors (plasma membrane and/or intracellular), which activate ROS-producing enzymes, such as NADPH oxidases (Ca^{2+}-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 Ca^{2+}, 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 Ca^{2+}–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 Ca^{2+} 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 H_2O_2. 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 H_2O_2. 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 H_2O_2 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 H_2O_2 (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 H_2O_2 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 H_2O_2 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 H_2O_2 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

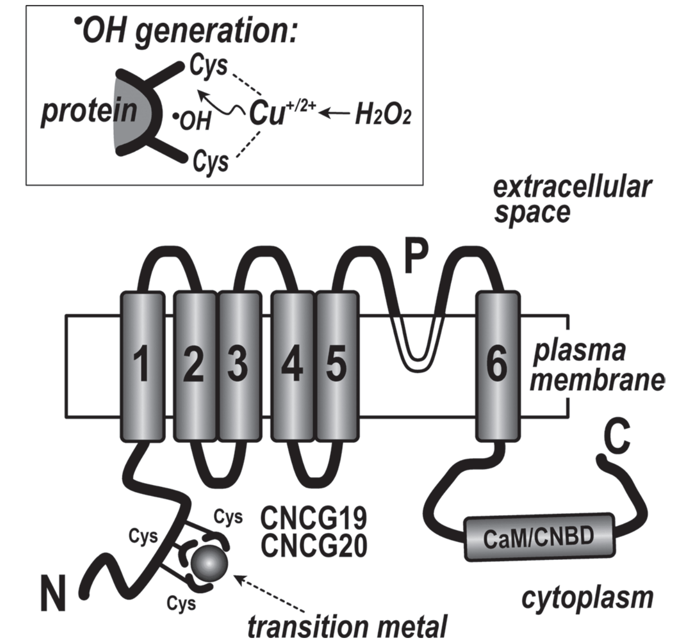

Fig. 4. The scheme of hypothetical cysteine-containing transition metal-binding sites in plant cyclic nucleotide-gated channels. A Cu or Fe ion is thought to reside in the cysteine 'pocket' at the cytosolic face of the plasma membrane (N-terminus). This 'fixed' metal is catalytically active and requires H_2O_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.

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).

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, Szczerba *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 nucleases (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 (Szczerba *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_2O_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 anabolic reactions and stimulates catabolic processes, causing the release of energy for adaptation and repair needs.

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