Cross-talk between reactive oxygen species and polyamines in regulation of ion transport across the plasma membrane: implications for plant adaptive responses

Igor Pottosin1,2,*, Ana María Velarde-Buendía1, Jayakumar Bose2, Isaac Zepeda-Jazo3, Sergey Shabala2 and Oxana Dobrovinskaya1

1 Biomedical Center, University of Colima, Mexico
2 School of Agricultural Science, University of Tasmania, Australia
3 Universidad de la Ciénega del Estado de Michoacán de Ocampo, Mexico

* To whom correspondence should be addressed. E-mail: pottosin@ucol.mx

Received 28 August 2013; Revised 11 November 2013; Accepted 12 November 2013

Abstract

Many stresses are associated with increased accumulation of reactive oxygen species (ROS) and polyamines (PAs). PAs act as ROS scavengers, but export of putrescine and/or PAs to the apoplast and their catabolization by amine oxidases gives rise to H2O2 and other ROS, including hydroxyl radicals (•OH). PA catabolization-based signalling in apoplast is implemented in plant development and programmed cell death and in plant responses to a variety of biotic and abiotic stresses. Central to ROS signalling is the induction of Ca2+ influx across the plasma membrane. Different ion conductances may be activated, depending on ROS, plant species, and tissue. Both H2O2 and •OH can activate hyperpolarization-activated Ca2+-permeable channels. •OH is also able to activate both outward K+ current and weakly voltage-dependent conductance (ROSIC), with a variable cation-to-anion selectivity and sensitive to a variety of cation and anion channel blockers. Unexpectedly, PAs potentiated •OH-induced K+ efflux in vivo, as well as ROSIC in isolated protoplasts. This synergistic effect is restricted to the mature root zone and is more pronounced in salt-sensitive cultivars compared with salt-tolerant ones. ROS and PAs suppress the activity of some constitutively expressed K+ and non-selective cation channels. In addition, both •OH and PAs activate plasma membrane Ca2+-ATPase and affect H+ pumping. Overall, •OH and PAs may provoke a substantial remodelling of cation and anion conductance at the plasma membrane and affect Ca2+ signalling.

Key words: Abiotic stress, amine oxidase, apoplast, calcium, hydroxyl radical, hypersensitive response, ion channel, P-type ATPase, plasma membrane, polyamine, reactive oxygen species, root, stomata.

Introduction

Reactive oxygen species (ROS), partly reduced oxygen forms, are by-products of aerobic metabolism that, being stronger oxidants than molecular oxygen, are potentially damaging to the intracellular machinery. Under stress conditions, an imbalance between ROS generation and scavenging often occurs, so that oxidative stress is a common part of all 'generic' abiotic stresses. Plants can also generate ROS on purpose, which was originally discovered as oxidative burst, during the hypersensitive response of plants to pathogens (Levine et al., 1994). Later, ROS signalling was also documented for plant responses to abiotic stresses (Apel and Hirt, 2004; Foyer and Noctor, 2005; Garg and Manchanda, 2009; Mittler et al., 2004).
Generation of ROS is a downstream step in stomata closure induced by the drought hormone, abscisic acid (ABA) (Wang and Song, 2008). ROS are also involved in the regulation of stomata aperture by ethylene (Desikan et al., 2006), methyl jasmonate (Munemasa et al., 2007), and salicylic acid (Mori et al., 2001). Generation of H₂O₂ during ABA-induced stomata closure causes the activation of inward-rectifying Ca²⁺-influx channel and the transient rise of cytosolic Ca²⁺ (Pei et al., 2000). Intracellular Ca²⁺ increases further affect plasma membrane (PM) transporters, facilitating stomata closure and preventing stomata re-opening. ROS and ROS-induced Ca²⁺ signalling are also important for plant growth and development. Root hair-defective (rhd) mutants in Arabidopsis, impaired in PM-bound NADPH oxidase (NOX) activity, have stunted roots and underdeveloped root hairs. They are incapable of generating intracellular Ca²⁺ signals in the tip, due to the lack of ROS-, 'OH-, or H₂O₂-induced Ca²⁺ influx across the PM (Foreman et al., 2003; Demidchik et al., 2003, 2007). As NOX is activated by cytosolic Ca²⁺, there is a positive feedback relationship between NOX activity and ROS-induced Ca²⁺ influx (Fig. 1; Takeda et al., 2008). The activity of NOX and the resulting tip-focused Ca²⁺ gradient appear to be universal for polarized growth in plants (Cárdenas, 2009; Swanson and Gilroy, 2010). Finally, the level of ROS accumulation acts as important switch between survival and programmed cell death (PCD) scenarios (see below).

Polyamine (PA) levels are frequently increased during stress (Gupta et al., 2013). Rather than being merely a collateral effect of stress-induced metabolic changes, these increases are often beneficiary for plant performance following stress, as evidenced by a variety of studies utilizing externally applied PAs, inhibitors of PA synthesis, or gain- or loss-of-function mutants of PA biosynthesis enzymes (Álcazar et al., 2010). In addition, induction of the different enzymes of PA biosynthesis appears to be stress specific (Álcazar et al., 2010), and transcriptomic analysis of spermine (Spm⁴⁺) and putrescine (Put²⁺) overproducers has revealed marked differences in the gene expression patterns and stress response pathways where these two PAs occur (Marco et al., 2011). The detailed mechanisms of how PAs exert their protective function are not clear. Among various possibilities is that they can act as molecular chaperons, protecting membranes and biomolecules, due to their ability to bind to negatively charged surfaces (Galston and Kaur Sawhney, 1990; Kusano et al., 2008). Alternatively, as PAs may act as ROS and free-radical scavengers (Bors et al., 1989; Ha et al., 1998; Das and Misra, 2004) and activate the antioxidant enzyme machinery, reducing oxidative stress-induced membrane injury and electrolyte leak (Nayyar and Chander, 2004; Tang and Newton 2005a; Kubiš, 2008; Gill and Tuteja, 2010). There are also reports of PAs interacting with some signalling components like 14-3-3 proteins (Athwal and Huber, 2002; Garufi et al., 2007), nitric oxide (NO)-generating systems (Tun et al., 2006; Yamasaki and Cohen, 2006; Arasimowicz-Jelonek et al., 2009), and phospholipase C (Echevarría-Machado et al., 2004). On the other hand, PAs are unique polycationic metabolites and, as such, they are known to cause a direct block of a variety of cation and K⁺-selective channels in animal cells (Drouin and Hermann, 1994; Lopatin et al., 1994; Williams, 1997; Lu and Ding, 1999), as well as fast-acting and slow-activating vacuolar-type channels in plant vacuoles (Brüggemann et al., 1998; Dobrovinskaya et al., 1999a,b) and ammonium channels in the peribacteroid membrane of root nodules (Whitehead et al., 2001). At the PM, PAs inhibit inward-rectifying K⁺ channels (KIRC) and some non-selective cation channels (NSCCs) in leaves and roots, restricting Na⁺ influx and NaCl-induced K⁺ efflux, shoot-to-root K⁺ recirculation, and stomata aperture (Liu et al., 2000; Shabala et al., 2007; Zhao et al., 2007). If PA inhibition of K⁺ and NSCCs at the PM was the dominant effect, this could reduce Na⁺ entrance into the cytosol and membrane depolarization. In turn, this would lead to a reduced salt-induced K⁺ efflux, resulting in improved cellular K⁺/Na⁺ relationships and overall better salt tolerance (Zepeda-Jazo et al., 2008). Direct testing of this hypothesis revealed, however, that the immediate effects of PAs on NaCl-induced K⁺ efflux from intact roots can range from beneficiary (suppression of K⁺ leak) to detrimental (Pandolli et al., 2010), depending on the plant and PA species, root zone, and growing conditions. Acute application of PAs can produce membrane instability, supposedly due to the oxidative damage caused by ROS, generated upon PA catabolization in the apoplast (DiTomasso et al., 1989). The relationship between ROS-induced membrane conductance and PAs is another question. As ROS mainly activate cation-permeable channels, are such channels sensitive to PAs, and how? Apart from channels, are there other PM transporters affected by ROS and PAs? Last but not the least, how are the effects of the two stress-related factors, ROS and PAs, integrated at the level of membrane transport? These and other questions are addressed in more detail below.

ROS generation and interconversion in the apoplast: the role of PA catabolism

The apoplast is an important site for ROS generation and scavenging. Intact Arabidopsis cells demonstrated 100 times higher resistance to H₂O₂ compared with isolated protoplasts (Neill et al., 2002). Yet, under certain conditions, the production of ROS in the apoplast may overweight their scavenging. The main sources of H₂O₂ in the apoplast are PM NOX, cell-wall-associated peroxidase, and oxalate oxidases (Fig. 1; Bolwell and Wojtaszek, 1997; Kawano, 2003). H₂O₂ is also generated upon catabolization of PAs by amine oxidases: copper-containing diamine oxidase (DAO; EC 1.4.3.6) and flavin-containing PA oxidase (PAO; EC 1.5.3.3) (Moschou et al., 2008b). DAO mainly catalyses oxidation of Put²⁺ and cadaverine (Cad⁵⁺) and possesses a lower affinity for higher PAs, whereas PAO oxidizes exclusively higher PAs: spermidine (Spd⁴⁺), Spm⁴⁺, and thermospermine. Thermospermine is a structural isomer of spermine (Fig. 1), recently discovered in bacteria and plants, which is absent in animals; in plants, it controls stem elongation and xylem vessel development (Takahashi and Kakehi, 2010). DAO is loosely bound
Fig. 1. Generation of ROS in the apoplast. Peroxide is generated in different reactions, including oxidation of oxalate by oxalate oxidase, enzymatic or non-enzymatic reduction of oxygen by available organic reductant (XH₂), and spontaneous or superoxide dismutase (SOD)-catalyzed dismutation of the superoxide anion (O₂⁻), generated by membrane-bound NOX. Another source of H₂O₂ involves intracellular synthesis of polyamines (PAs) (ADC and ODC are arginine and ornithine decarboxylase, SPDS and SPMS are spermidine and spermine synthase), their export to the apoplast against an electrochemical gradient via an unknown mechanism, and oxidation by PA oxidase (PAO) and diamine oxidase (DAO). H₂O₂ can be further converted to •OH via the Haber–Weiss mechanism, including reduction of iron by O₂⁻, and further reduction of peroxide by reduced iron, catalyzed by a special state of cell-wall peroxidase (POX), called compound III (POX-Fe²⁺-O-O). The last step, which is called the Fenton reaction, can be catalyzed by other transition metals (e.g. copper), reduced by ascorbate. Additional steps are shown, including H₂O₂ internalization, positive feedback regulation of NOX by ROS-activated Ca²⁺ influx, scavenging of •OH by polyamines, and the differential effects of H₂O₂ and •OH on the cell wall. The structures of plant polyamines are shown below. After Rodríguez et al. (2009), Polyamine oxidase activity contributes to sustain maize leaf elongation under saline stress. *Journal of Experimental Botany* 60, 4249–4262, by permission of the Society for Experimental Biology.
to the cell wall and occurs at high levels in dicots, especially in Fabaceae. PAOs are mainly expressed in monocots, especially in Gramineae. They can be subdivided into two classes: terminal oxidases or enzymes, catalysing backconversion of the PAs Spm\(^{4+}\) to Spd\(^{3+}\), and Spd\(^{3+}\) to Put\(^{2+}\) (Moschou et al., 2008b, 2012). The former PAOs are mainly apoplastic enzymes, associated with cell walls. PAO and DAO activity in the apoplast is an important source of H\(_2\)O\(_2\) (Fig. 1), and PA catabolism is an integral part of ROS signalling and ROS-induced PCD. Plants overexpressing PAO show hypersensitive responses to abiotic stresses, as, due to very high ROS production, the balance is switched from survival to a PCD scenario (Moschou et al., 2008a). Generation of 'OH, the most powerful ROS, in the apoplast occurs via the Haber–Weiss mechanism, involving cell-wall-bound peroxidase (Liszkay et al., 2004). The last step of this mechanism, conversion of H\(_2\)O\(_2\) to 'OH (the Fenton reaction), can be mediated by alternative ways, including iron, copper, or manganese ions, either inorganic or as a part of a reaction centre in metal proteins (e.g. Cu in DAO or Mn in the cell-wall-bound superoxide dismutase; Kukavica et al., 2009), in the presence of natural reducing agents (e.g. ascorbate, phenolics, or NADH). Two ROS species, H\(_2\)O\(_2\) and 'OH, affect cell-wall components in contrasting ways. 'OH promotes polysaccharide degradation, leading to wall relaxation and allowing pressure-driven extension growth (Schoepfer, 2001; Liszkay et al., 2004). In contrast, H\(_2\)O\(_2\) promotes cell-wall stiffening through peroxidase-mediated formation of covalent cross-links between components of cell walls. Externalization of PAO has been observed in the mature root zone compared with mainly cytosolic PAO localization in the root apex, suggesting a role in cell-wall maturation (Cona et al., 2005).

The apoplast does not contain enzymes for PA biosynthesis, so they have to be exported there. Roots can absorb PAs from the rhizosphere, either from decomposed organic matter or by exchanging metabolites with microorganisms at the root–soil interface (Yamasaki and Cohen, 2006; Jones et al., 2009). A more general mechanism, which is not restricted to the roots but is also observed in other plant tissues, is PAs export to the apoplast from the cytosol. PA exodus to the apoplast is not trivial, because these polycations have to be transported against a large and negative electric potential difference across the PM. Thus, PA export has to be either active or via exocytosis, but the underlying mechanism is still unknown for plants (Igarashi and Kashiwagi, 2010). Expression of apoplastic DAO and PAO are stress inducible (Møller et al., 1998; An et al., 2008; Toumi et al., 2010). A signalling pathway segment, which involves PA export, their oxidation by available amine oxidases, and ROS-activation of Ca\(^{2+}\) influx across the PM, is common for a variety of plant responses to abiotic and biotic stresses, hormonal regulation, growth and development, and, ultimately, diverse PCD scenarios (for recent reviews, see Moschou et al., 2012; Tavaldoraki et al., 2012). For a variety of biotic and abiotic stresses, as well as for plant development, extracellular PAs cause also rapid NO generation (Yamasaki and Cohen, 2006), so ROS and NO often operate synergistically (Wimalasekera et al., 2011).

Catabolism of PAs in the apoplast controls, in a tissue-specific manner, different aspects of root development, affecting rooting frequency, increasing or at higher concentration decreasing elongation, altering cell division, and inducing cell-wall maturation and adventitious rooting (Tang and Newton, 2005b; She et al., 2010; Tisi et al., 2011). DAO expression has been shown to be induced upon formation of nematode feeding sites (galls) in roots and is involved in the respective vascular redifferentiation (Møller et al., 1998). Two non-exclusive cellular mechanisms of action are fuelled by released H\(_2\)O\(_2\): stiffening of cell walls and developmental PCD. PAO and DAO are highly expressed in the xylem and xylem parenchyma, and in root caps. Generation of H\(_2\)O\(_2\) by extracellular amine oxidases there probably underlies tissue differentiation due to the coordinated processes of cell-wall maturation and PCD (Møller and McPherson, 1998; Cona et al., 2006; Tisi et al., 2011).

Oxidative burst, generated extracellularly, is an early response of plants to pathogen attack. The sources of ROS may be different: depending on host and pathogen species, it may be mainly due to the activity of NOX (Torres et al., 2002), cell-wall peroxidase (Bindschedler et al., 2006), or apoplastic amine oxidases. Involvement of NOX and amine oxidases may dominate ROS production at immediate and later phases of the response, respectively (Yoda et al., 2006). H\(_2\)O\(_2\), produced in the apoplast, is a part of the hypersensitive response that leads to PCD. Accumulation of PAs in the apoplast may be a local response and does not require an increase in the total cellular PAs (Yamakawa et al., 1998). PCD may be beneficial for plants attacked by biotrophic pathogens (by elimination of infected tissue) or, in contrast, may be detrimental, as in case of nectrotropic pathogens (Yoda et al., 2006; Marina et al., 2008; Moschou et al., 2009). There appears to be a complex interplay between the host and the pathogen, so that some biotrophic pathogens, like rust fungi or certain viruses, implement mechanisms that prevent PCD in infected host tissues (Walters, 2003). In the case of the hypersensitive response to the tobacco mosaic virus, maximal H\(_2\)O\(_2\) generation and a peak of activation of salicylic acid-induced protein kinase and wound-induced protein kinases coincides with a transient minimum of antioxidant activity in the apoplast, whereas PAs export and increases in the activity of PAO may continue far beyond this point (Yoda et al., 2006). Production of H\(_2\)O\(_2\) by PA catabolism is important for cell-wall reinforcement, to handicap pathogen penetration (Cona et al., 2006). Apoplastic PAO and DAO activities support wound healing by providing H\(_2\)O\(_2\) for cell-wall peroxidase, which catalyses ligno-sburgerization along the wound periderm. Expression of these enzymes is induced by wounding, and their role as specific sources for ROS in this case was supported by the use of specific inhibitors of PAO and DAO (Rea et al., 2002; Tisi et al., 2008). Plants may utilize more sophisticated strategies when attacked by a relatively large herbivorous organism. Lima bean leaves, when attacked by herbivorous mites, synthesize and emit volatiles, attracting the mites’ natural enemies. Volatile production depends on ROS-induced Ca\(^{2+}\) influx, due to Spm\(^{4+}\) catabolization. Spm\(^{4+}\) also induces synthesis of jasmonate, and Spm\(^{4+}\) and jasmonate amplify the
PA catabolism plays important role in the control of stomata aperture. Stomatal closure induced by ABA in *Vicia faba* and by ethylene in *Arabidopsis* involves induction of DAO and PAO activity, respectively, as a source of H$_2$O$_2$ (An et al., 2008; Hou et al., 2013). It has also been demonstrated that the activities of DAO and NOX contribute to ABA-induced H$_2$O$_2$ production independently and that both routes converge at the level of H$_2$O$_2$-induced Ca$^{2+}$ influx (An et al., 2008). Intracellular Ca$^{2+}$ activates Ca$^{2+}$-dependent protein kinase, which upregulates the activity of slow anion channels, governing stomatal closure (Schroeder et al., 2001; Brault et al., 2004; Trouverie et al., 2008). Both H$_2$O$_2$ and elevated intracellular Ca$^{2+}$ cause the inhibition of KIRCs (Grabov and Blatt, 1997; Zhang et al., 2001; Köhler et al., 2003), preventing stomata reopening. Peroxide-induced Ca$^{2+}$ influx also provokes partial inhibition of Na$^+$ influx through PM NSCCs under NaCl stress, thus reducing stomatal movements (Zhao et al., 2011). ABA-induced NO synthesis depends on H$_2$O$_2$ generation in guard cells of *Vicia* (Dong et al., 2005) and *Arabidopsis* (Bright et al., 2006). PAs, together with DAO and PAO, cause a rapid NO synthesis; this may be indirectly mediated by H$_2$O$_2$ or via an alternative as-yet-unknown pathway (Wimalasekera et al., 2011). NO, in turn, may affect a variety of PM channels in guard cells, causing the inhibition of outward-rectifying K$^+$ channels (KORCs) via direct nitrosylation (Sokolovski and Blatt, 2004) and inhibition and activation of KIRC and anion channels, respectively, via Ca$^{2+}$ release from intracellular stores (García-Mata et al., 2003).

A switch between survival and PCD scenarios under salt and drought stresses is probably dependent on the relationship between PA catabolism, generating ROS in the apoplast, and intracellular PA concentrations: the higher the PA catabolism and the lower the PA concentration, the more probable the PCD scenario (Moschou et al., 2008a; Toumi et al., 2010). Growth of maize leaves and soybean hypocotyls under saline conditions, when NOX is strongly suppressed, is supported by apoplastic PAO and DAO activities, respectively (Rodriguez et al., 2009; Campestré et al., 2011), reflecting the relative abundance of these enzymes in monocots and dicots.

ROS-induced ion conductance in roots is a multicomponent phenomenon, facilitating transport of cations and anions

Background cation conductance of the PM, including K$^+$-selective currents and voltage-independent NSCCs (VICCs) are inhibited by H$_2$O$_2$ (Köhler et al., 2003; Zhao et al., 2011, although see Garcia-Mata et al., 2010 for a conflicting result). In addition to VICCs and K$^+$ channels (Demidchik et al., 2003; Zepeda-Jazo et al., 2011), ‘OH also inhibits DACCs, depolarization-activated outward-rectifying NSCCs (I. Pottosin, unpublished). Both H$_2$O$_2$ and ‘OH are able to activate membrane conductance, but the magnitude and biophysical properties of the evoked currents depend on ROS and the plant tissue. H$_2$O$_2$-induced current is reminiscent of HACCs, constitutive hyperpolarization-activated Ca$^{2+}$-permeable channels, based on their voltage dependence, unitary channel properties, and pharmacology (Demidchik et al., 2002). In *Arabidopsis* guard cells and epidermis of the root elongation zone, H$_2$O$_2$-activated current conducts Ba$^{2+}$>Ca$^{2+}$>Mg$^{2+}$>TEA$^+$, and Ba$^{2+}$>Ca$^{2+}$ (15–20 pS)>Zn$^{2+}$, respectively (Pei et al., 2000; Demidchik et al., 2007). It is still not clear from which side peroxide controls PM channels, as H$_2$O$_2$ has a relatively long half-life and can permeate the PM via aquaporins (Mori and Schroeder, 2004). Also, with intact systems, conversion of H$_2$O$_2$ to ‘OH in the apoplast needs to be taken into account. Nevertheless, externally applied H$_2$O$_2$ was able to evoke Ca$^{2+}$ influx only in the elongation but not in the mature root zone. Thus, in the mature zone, only ‘OH seems to be efficient in the induction of ion conductance across the PM (Demidchik et al., 2003, 2007; Zepeda-Jazo et al., 2011). ‘OH was inefficient for the induction of any current in inner layers of root cells (Demidchik et al., 2003), arguing against a non-specific effect of ‘OH on membranes that could cause an increased electrolyte leak.

Due to its high reactivity, ‘OH is short-lived (~1 ns; Mori and Schroeder, 2004). Therefore, once generated, ‘OH can hit targets within only ~1 nm distance from the point of its production and may not cross the membrane. In *Arabidopsis* roots (root hairs and epidermis of elongation and mature zone), ‘OH activated NSCCs, with a comparable permeability for Mg$^{2+}$, Ca$^{2+}$, and Ba$^{2+}$. In contrast to the H$_2$O$_2$-activated HACCs, it displayed a relatively high permeability for the non-specific K$^+$ channel blocker tetraethylammonium (TEA$^+$) (Demidchik et al., 2003; Foreman et al., 2003). In the mature zone, ‘OH also induced a large outward-rectifying K$^+$ current, partly instantaneously and partly time dependent with very slow activation kinetics. Instantaneous inward and outward current components were insensitive to TEA$^+$, whereas single channels, underlying voltage- and time-dependent outward K$^+$ current, were inhibited by 60% in the presence of 10 mM TEA$^+$ (Demidchik et al., 2003, 2010). The former two components can be efficiently blocked by lanthanides, known non-specific NSCC blockers. Therefore, as a starting point, it may be concluded that ‘OH induces inward-rectifying NSCCs, similar but slightly different from HACCs, and two outward currents, instantaneous and time dependent, mainly carried by monovalent cations (K$^+$). The latter (time-dependent) current was attributed to GORK, a guard cell-type constitutive outward-rectifying root plasma membrane K$^+$ channel (Demidchik et al., 2010). In a subsequent study, a rather different kinetic pattern for ‘OH-induced current in *Arabidopsis* root epidermis protoplasts was reported, where both inward and outward components were voltage and time dependent, with scarce activity between ~100 and +100 mV (Laohavisit et al., 2012). Root protoplasts from gork loss-of-function mutants did not display any difference from the wild type, arguing against the contribution of GORK to an ‘OH-induced current in this case. Non-invasive measurements of ‘OH-induced K$^+$ efflux on whole roots display further complications. In the elongation zone, loss-of-function annexin *Atann1* mutants show almost zero K$^+$ flux in response to ‘OH, whereas in the mature root zone a significant part of K$^+$ efflux
resides in gork or Atamnl loss-of-function mutants. In both cases, 'OH-induced K⁺ efflux was highly sensitive to TEA⁺, a non-specific blocker of K⁺ channels (Demidchik et al., 2003; Laohavisit et al., 2012). In mature pea root epidermides, 'OH-induced conductance was dominated by weakly voltage-dependent instantaneous current; this current was not inhibited but was stimulated by TEA⁺, implying its significant permeability to this cation (Zepeda-Jazo et al., 2011). As a constitutive TEA⁺-sensitive KORC was inhibited by 'OH, the contribution of KORCs may be ruled out in this case. This weakly voltage-dependent current was mediated by channels with a very low (~1 pS) unitary conductance (I. Pottosin, unpublished) and was termed ROSIC, for ROS-induced conductance (Pottosin et al., 2012). A similar 'OH-induced current was recorded also in epidermal protoplasts from the barley root mature zone (Velarde-Buendía et al., 2012), and multiple evidence for the ability of ROSIC to conduct both cations and anions was provided. The dual conductance for cations and anions could mediate massive electroneutral loss of electrolytes, resulting in a large decrease in intracellular K⁺ and concomitant water loss and cell shrinkage. Such changes, especially those for intracellular K⁺, are common in PCD. In animal cells, an apoptotic volume decrease requires the parallel operation of K⁺ and anion channels. A reduction of any of these conductance handicaps apoptosis (Yu and Choi, 2000). Low K⁺ creates a favourable environment for the activity of caspases and endonucleases, potentiating apoptosis (Hughes and Cidlowski, 1999; Valencia-Cruz et al., 2009). In plants, canonical caspases are absent, being substituted by distantly related metacaspases or cystein proteases. It was hypothesized that a decrease in cytosolic K⁺ may be sensed by these proteases in a similar way, resulting in PCD (Shabala, 2009). Indeed, activation of cell death proteases and endonucleases induced by salt or 'OH exposure, and resulting in PCD, were retarded in Arabidopsis gorkI-I mutants, lacking KORC function. Salt stress induced 'OH production, and 'OH activated a type of KORC in this model (Demidchik et al., 2010). In addition, it was shown that activation of a slow anion channel by intracellular Ca²⁺ and anion (NO₃⁻ and Cl⁻) efflux is a pre-requisite of PCD, induced by agents, provoking oxidative burst such as ozone (Kadono et al., 2010), oxalate (a phytotoxin produced by many necrotrophic fungi; Errakhî et al., 2008), and cryptogen (a toxin secreted by Phytophthora; Wendenenne et al., 2002; Gauthier et al., 2007). K⁺ and anion (Cl⁻) efflux from tobacco cell culture, associated with the volume regulation response to hypotonic stress, depends on oxidative burst and could be suppressed by inhibition of the PM NOX (Cazalè et al., 1998). Therefore, ROS may govern cell shrinkage via modulation of K⁺ and anion efflux through the PM ion channels. This process may be potentiated by activation of the constitutively expressed anion channels by intracellular Ca²⁺ resulting from Ca²⁺ influx through ROS-activated NsCCs, similar to the mechanism implemented in stomatal closure (Munemasa et al., 2007). It remains to be elucidated whether ROSIC contributes significantly to membrane depolarization and cell volume regulation.

K⁺ efflux, associated with ROSIC, can be potentiated by PAs, which by themselves cause no or little K⁺ efflux, nor any direct effect on ROSIC once it has developed (Zepeda-Jazo et al., 2011; Velarde-Buendía et al., 2012). The fact that this potentiation can be observed in intact roots and isolated protoplasts, assayed in a whole-cell configuration (lacking, therefore, the enzymes of PA catabolism), implies that PAs act as co-factors for 'OH in ROSIC activation. In animal cells, PAs were shown to promote binding of an essential functional cofactor, phosphatidylinositol 4,5-bisphosphate, to the KIRCs and activator capsaicin to TRPV1 channels (Xie et al., 2005; Ahern et al., 2006). Thus, it is conceivable that adsorption of PAs may sensitize ROSIC for activation by 'OH. In pea roots, a synergism between ROS and PAs was restricted to the mature zone and was not observed in the elongation zone, further supporting the idea of the diversity of ROS-induced conductance in these zones (Pottosin et al., 2012). In barley, a synergistic effect between ROS and PAs was much more pronounced for a salt-sensitive variety compared with a salt-tolerant one (Velarde-Buendía et al., 2012). These finding supports further the concept that efficient K⁺ retention is primordial for salt tolerance, at least in barley species (Chen et al., 2007). While the detailed mechanism of the above potentiation of the 'OH-induced conductance by PAs is not clear, it appears to deal with PAs but not with their catabolites and to be membrane delimited.

**Effect of ROS and PAs on PM ionic pumps**

The P-type H⁺-transporting ATPase (EC 3.6.3.6) is a key element in controlling the membrane potential, internal pH, and membrane energization for the secondary H⁺-coupled transport (Duby and Boutry, 2009). At the same time, PM Ca³⁺ ATPase (EC 3.6.3.8) is central for intracellular Ca²⁺ homeostasis and signalling (Sze et al., 2000; Kabala and Klobus, 2005; Bose et al., 2011). H⁺-ATPase exports only one or less H⁺ per one ATP hydrolyzed (Duby and Boutry, 2009). Thus, at a moderate pH difference across the PM, the reversal potential for the pump-generated current may be well below ~200 mV (Lew, 1991; Lohse and Hedrich, 1992). Due to a balance between pump and passive currents, the resting membrane potential is normally in the range of ~130 to ~160 mV (Lew, 1991; Chen et al., 2007; Bose et al., 2013). In Arabidopsis, two out of 11 PM H⁺-ATPases, namely AHA1 and AHA2, are expressed in all tissues, with a higher abundance of AHA2 in the roots (Gaxiola et al., 2007). PM Ca³⁺-ATPase in Arabidopsis is represented by multiple isoforms, encoded by ACA8, ACA9, and ACA10 (Kabala and Klobus, 2005; Bose et al., 2011). ACA Ca³⁺-ATPases belong to the P2B type, the distinctive characteristics of which are the presence of an autoinhibitory domain at the N terminus. The autoinhibition is relieved by binding of a Ca²⁺/calmodulin complex, so that the pump activity increases many times by an increase in intracellular Ca²⁺ (Boursiac and Harper, 2007). On the other hand, an intracellular Ca²⁺ increase was shown to inhibit the PM H⁺-pump (Kinoshita et al., 1995; Lino et al., 1998; Brault et al., 2004). Thus, PM Ca³⁺ and H⁺ pumps may communicate via intracellular Ca²⁺ changes (Fig. 2).
There are conflicting reports on the effect of PAs on the PM H⁺ pump. H⁺-ATPase activity in isolated vesicles was enhanced (Reggiani et al., 1992) or suppressed (Whitehead et al., 2001) by PAs indiscriminately. Garufi et al. (2007) found that Spm₄⁺ but not Spd₃⁺ and diamines stimulated the PM H⁺-ATPase activity, by promoting 14-3-3 protein binding to the non-phosphorylated protein. Lee et al. (2004) demonstrated that H₂O₂ (>4 mM) inhibits ATPase activity in PM vesicles; H⁺ pumping was not verified in this work. Under natural conditions H₂O₂-induced H⁺-ATPase inhibition may be mediated by increased cytosolic Ca²⁺, flowing through H₂O₂-activated HACCs (Brault et al., 2004). Blue-light-induced stomata opening was inhibited by 70% with 1 mM H₂O₂. This inhibition decreased the H⁺-ATPase phosphorylation level, also decreasing 14-3-3 protein binding to the non-phosphorylated protein.

Lee et al. (2004) demonstrated that H₂O₂ (>4 mM) inhibits ATPase activity in PM vesicles; H⁺ pumping was not verified in this work. Under natural conditions H₂O₂-induced H⁺-ATPase inhibition may be mediated by increased cytosolic Ca²⁺, flowing through H₂O₂-activated HACCs (Brault et al., 2004). Blue-light-induced stomata opening was inhibited by 70% with 1 mM H₂O₂. This inhibition decreased the H⁺-ATPase phosphorylation level, also decreasing 14-3-3 protein binding to the non-phosphorylated protein.

In some plant models, where H₂O₂ is efficient, it produces Ca²⁺ influx only (Pei et al., 2000; Demichek et al., 2007). In the pea root mature zone, H₂O₂ caused no effect on Ca²⁺ fluxes; ‘OH at low level (0.1 mM Cu ascorbate) caused activation of PM Ca²⁺ pumping, and at a higher level (1 mM Cu ascorbate) both Ca²⁺ pumping and Ca²⁺ influx (Bose et al., 2011; Zepeda-Jazo et al., 2011). Remarkably, using the same concentrations of Cu ascorbate mix (0.1 or 1.0 mM), Hao et al. (2012) observed the opposite effects on stomata aperture, opening or closure, respectively. As stomatal opening requires activation of the PM H⁺ pump, it can be hypothesized that H⁺-ATPase can be modulated by net Ca²⁺ flux through the PM. When it is inward (as in case of H₂O₂), an increase in cytosolic Ca²⁺ will inhibit the pump (Brault et al., 2004). When the net Ca²⁺ flux is outward (as in the case of low ‘OH), the H⁺ pump may be activated, due to a decrease in intracellular Ca²⁺. A decrease in the cytosolic Ca²⁺ will also cause a decrease in NOX activity, reducing the overall ROS production (Fig. 1). Indeed, NOX-generated H₂O₂, in a response to either elicitor or hypo-osmotic treatment in Arabidopsis cell culture, was potentiated and Ca²⁺ efflux was suppressed by a specific Ca²⁺-pump inhibitor, eosin yellow (Romani et al., 2004; Beffagna et al., 2005). ‘OH-induced Ca²⁺ efflux in pea roots was insensitive to the blockers of ROSIC but was strongly inhibited by fluorescein derivatives (eosin yellow, erythrosin B), arguing for the involvement of PM Ca²⁺-ATPase (Zepeda-Jazo et al., 2011). At high ‘OH levels, ROSIC-mediated Ca²⁺ influx dominated over Ca²⁺ pumping at a steady state. The situation changed upon extracellular application of PAs. Net Ca²⁺ influx observed at the steady state was reduced by Put₂⁺, cancelled or reverted by Spd₃⁺, and transformed into a long-lasting Ca²⁺ efflux by Spm₄⁺, both in the elongation and the mature root zones of pea. The PAs Put₂⁺ or Spm₄⁺ alone indiscriminately induced net Ca²⁺ efflux in pea and barley roots (Bose et al., 2011; Velarde-Buendia et al., 2012). Therefore, ‘OH and PAs can activate Ca²⁺ pumping across the PM, but due to the activation of a concurrent Ca²⁺ influx by ‘OH, the net effect on Ca²⁺ flux may be variable and dependent on PAs and plant species. The Ca²⁺-pump activation by ‘OH was characterized by a lower threshold and reached a maximum at lower levels of ‘OH production compared with ROSIC (Zepeda-Jazo et al., 2011). Thus, at lower levels of ‘OH generation, Ca²⁺ efflux would dominate over Ca²⁺ influx across the PM.
Finally, ROS and PAs appear to act upstream of NO generation in plants (Dong et al., 2005; Tun et al., 2006). In addition to important targets of NO in PM, like K\(^+\) and anion channels, it caused a several-fold increase in H\(^+\)-pumping (Zandonadi et al., 2010). Generation of an electrochemical gradient for H\(^+\) across the PM fuels a variety of coupled transports, including Ca\(^{2+}\) /H\(^+\) exchange by PM Ca\(^{2+}\)-ATPase (Fig. 2; Niggl and Sigel, 2008). Therefore, NO generation may be an important step at the crossroads of PA and ROS signalling.

**Outlook**

Figure 2 represents a summary of the reported effects of PAs and ROS on the PM ion transporters. This is an oversimplified scheme, because different ROS, such as \(' OH or H_2O_2\), can provoke different and even adverse effects. In many cases, the effects of ROS and PAs are indirect. They can be mediated by intracellular Ca\(^{2+}\), voltage, as well as by other factors such as 14-3-3 proteins, phosphorylation, and lipid metabolites. In some cases (e.g. P-type pumps), it is also not clear whether PAs themselves or ROS generated by their catabolization are at work. Despite the long period of studies on PAs and the advantages of functional genetic approaches, which have demonstrated the importance of PAs and their metabolism in growth, development, stress responses, and PCD, our knowledge of how PAs actually work on plant-cell components is scarce. While the effects of PAs on vacuolar channels are clearly direct and can be explained mechanistically, this is not true for the PM where the effects on ion channels appear to be indirect, and the immediate targets of PAs are unknown.

On the other hand, cross-talk between PAs and ROS has become a promising theme. The exodus of PAs to the apoplast, where they are normally absent, followed by their oxidation by DAO and/or PAO, is a common mechanism within ROS signalling. It is applicable to a surprisingly broad range of physiological processes in plants. However, nothing is known of how plants export PAs, and knowledge about the mechanisms of their uptake by plant cells is also very limited. Gaining such knowledge will be necessary for a better understanding of PA dynamics and will allow manipulation of the latter process. Another important issue is PA specificity. Plants regulate PAs in such a way that one PA species may become dominant, generating a specific signature, e.g. for a response to a specific stress. There are also many reports suggesting that the ratios of Put\(^{2+}\) to total PAs, or PAs to their catabolic products, may be important for stress signalling, as they correlate with plant performance in a changing environment. Under laboratory conditions, the effects of externally applied PAs on a certain process may be very similar for different PA species. In vivo, however, the dependence of a response on a certain PA may arise. In the case of a signalling pathway involving PA export to the apoplast and catabolization therein, this dependence may be driven by: (i) the relative availability of different PAs in the cytosol, (ii) the specificity and relative expression of their transport systems in the PM, and (iii) the type of amine oxidase, PAO or DAO, dominating in the apoplast. It is also important, whether PA-derived H\(_2\)O\(_2\) is further converted to \(' OH and what the relative expression of different membrane transporters, targets for ROS and PAs action, is. All these aspects are dependent on the plant’s physiological and metabolic status, as well as on tissue and plant specificity. This helps us to understand in general terms why PAs may have adverse effects for different plants, tissues or growing conditions, but complicates concrete predictions and explanations.

When it comes to ROS-induced PM conductance, this appears to be a multicomponent phenomenon, which cannot be laid in a Procrustean bed of the ‘HACC plus KORC’ scheme. A better separation and identification of the multiple current components, induced by different ROS in different tissues, are needed. Newly described ROSIC, and its dual conductance for cations and small anions, is of particular interest. Is it a single transport pathway with a variable cation-to-anion selectivity or a conjunction of different conductances that can be dissected in some way, such as pharmacologically? What is the biophysical mechanism underlying the synergistic effects of ROS and PAs on this conductance? Does ROSIC, due to its dual permeability, play a role in osmotic adjustments/volume regulation, PCD, control of \(' OH membrane voltage, and/or salt (NaCl) uptake? In vivo measurements and pharmacological characterization of the ROS-induced anion fluxes (e.g. by non-invasive MIFE technique) may be a good starting point to address these questions. Modulation of the PM H\(^+\) and Ca\(^{2+}\) pumps by PAs and ROS opens up new avenues and deserves further exploration. To get a better understanding of the underlying mechanisms, measurements of H\(^+\) and Ca\(^{2+}\) fluxes need to be performed on a simpler, cell-wall-free system (protoplasts), where measurements are not confounded by the cell-wall buffering problem and which is lacking the apoplastic machinery for PAs and ROS chemical transformation. Whereas ROS-induced Ca\(^{2+}\) influx and related signalling is established, stimulation of Ca\(^{2+}\) pumping by ROS (or just by \(' OH) is a new phenomenon, potentially important for shaping of the intracellular Ca\(^{2+}\) signal. Further on, \(' OH-induced Ca\(^{2+}\) fluxes can be differentially modulated by different PAs, with Spm\(^{4+}\) provoking a long-lasting net Ca\(^{2+}\) efflux (Pottosin et al., 2012). Direct intracellular Ca\(^{2+}\) measurements are required to unravel the role of Ca\(^{2+}\)-efflux systems in ROS and PA signalling.

**Acknowledgements**

This work was supported by CONACyT, ARC and GRDC grants, and by a University of Tasmania visiting fellowship scheme to Professor I. Pottosin.

**References**


Yoda H, Hiroi Y, Sano H. 2006. Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. Plant Physiology 142, 193–206.


