**Arabidopsis** root K⁺-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death

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Accepted 11 February 2010
Journal of Cell Science 123, 1468-1479
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doi:10.1242/jcs.064352

Summary

Reactive oxygen species (ROS) are central to plant stress response, signalling, development and a multitude of other processes. In this study, the plasma-membrane hydroxyl radical (HR)-activated K⁺ channel responsible for K⁺ efflux from root cells during stress accompanied by ROS generation is characterised. The channel showed 16-pS unitary conductance and was sensitive to Ca²⁺, tetraethylammonium, Ba²⁺, Cs⁺ and free-radical scavengers. The channel was not found in the gork1-1 mutant, which lacks a major plasma-membrane outwardly rectifying K⁺ channel. In intact Arabidopsis roots, both HRs and stress induced a dramatic K⁺ efflux that was much smaller in gork1-1 plants. Tests with electron paramagnetic resonance spectroscopy showed that NaCl can stimulate HR generation in roots and this might lead to K⁺-channel activation. In animals, activation of K⁺-efflux channels by HRs can trigger programmed cell death (PCD). PCD symptoms in Arabidopsis roots developed much more slowly in gork1-1 and wild-type plants treated with K⁺-channel blockers or HR scavengers. Therefore, similar to animal counterparts, plant HR-activated K⁺ channels are also involved in PCD. Overall, this study provides new insight into the regulation of plant cation transport by ROS and demonstrates possible physiological properties of plant HR-activated K⁺ channels.

Key words: Ion channels, Plant, Potassium, Programmed cell death, Reactive oxygen species, Stress

Introduction

Reactive oxygen species (ROS) are important toxic and regulatory agents in plants (Apel and Hirt, 2004; Möller et al., 2007). They are produced in response to a number of stimuli, including biotic and abiotic stresses, hormones, developmental and gravitropic signals, and mineral deficiency (Apel and Hirt, 2004; Kwak et al., 2006). Respiratory and photosynthetic electron-transport chains, as well as NADPH oxidases and peroxidases, are involved in their generation in plants (Apel and Hirt, 2004). Several ‘ROS-sensing’ systems have been proposed, including two-component histidine kinases, mitogen-activated protein kinases, and certain protein phosphatases and transcription factors (Apel and Hirt, 2004; Kwak et al., 2006). In addition, plasma-membrane ion channels are probably also involved in ROS sensing and ROS-mediated regulatory reactions in plants. In animals, ion-channel-based ROS sensors play crucial roles in key physiological functions (Lahiri et al., 2006), but their physiological roles in plants are much less well understood.

There is now ample evidence that ROS can activate plasma-membrane cation influx channels in plant cells, resulting in the elevation of cytosolic Ca²⁺ activity (Pei et al., 2000; Köhler et al., 2003; Demidchik et al., 2003; Foreman et al., 2003). The biophysical properties and physiological roles of such ROS-activated Ca²⁺-permeable channels have been investigated in a number of plant preparations (Pei et al., 2000; Coelho et al., 2002; Demidchik et al., 2003; Demidchik et al., 2007; Demidchik et al., 2009; Foreman et al., 2003; Köhler et al., 2003; Takeda et al., 2008). In contrast to cation influx, the effect of ROS on cation efflux remains poorly studied. Hydroxyl radical (HR)-activated K⁺-efflux conductance has been observed alongside the activation of Ca²⁺ channels (Demidchik et al., 2003), but has not been investigated in detail; the single-channel characteristics, genetic basis and physiological roles of HR-activated K⁺ conductance remain unknown.

HR-activated K⁺-efflux channels can potentially play very important roles in plants. Potassium is an essential nutrient, a major osmoticum and regulator of enzymes, so decreasing the K⁺ activity will result in significant changes in metabolic reactions and physiological processes (Marschner, 1995). Moreover, retaining high cytosolic K⁺ concentration is crucial for plant salinity tolerance, although the mechanisms by which this protects cells from Na⁺ is still unclear (Maathuis and Amtmann, 1999; Munns and Tester, 2008). We have recently demonstrated that K⁺ channels are responsible for K⁺ loss from Arabidopsis roots and leaves during salt stress, and that this triggers an oxidative burst in Arabidopsis roots (Demidchik et al., 2003; Shabala et al., 2006). However, it was not shown whether the ROS produced under salt stress include HRs (capable of activating K⁺-efflux channels). As well as salinity, both K⁺ efflux and ROS generation are induced by pathogen attack, cold and osmotic shock, heavy metals, UV light, paraquat and ozone (Murphy and Taiz, 1997; Apel and Hirt, 2004; Shabala and Cuin, 2008). This suggests that K⁺ efflux and ROS generation are a general plant-cell response to stress.
The main aim of this study was to investigate in detail the activation of plant K+-efflux channels by HRs and to test the possible physiological roles of this phenomenon. The model plant *Arabidopsis thaliana* was used, focusing on the root epidermis as it is the most likely first target of stress. This study reports, for the first time, the single-channel properties of a plant HR-activated K+ channel, reveals the gene encoding this channel and shows its possible role in plant programmed cell death (PCD).

**Results**

**HRs activate single K+ channels in *A. thaliana* root protoplasts**

The exposure of excised outside-out plasma-membrane patches exhibiting no channel activity to a HR-generating mixture containing 1 mM CuCl2 and 1 mM L-ascorbic acid (Cu/a) activated a single-channel outwardly directed conducance (Fig. 1A), with the open probability (Popen) increasing with depolarisation (Fig. 1B). The maximal activation was observed 15-20 minutes after the addition of Cu/a (Fig. 1C; mean ± s.e. Popen=0.55±0.09; n=6; holding potential (HP)=80 mV; 20 minutes exposure to 1 mM Cu/a). This activation was found in 36% of protoplasts (n=136). Removal of Cu/a from the bathing solution decreased the HR-activated currents when washing out started after 20-30 minutes of treatment (Fig. 1D). Longer-term treatment (30-45 minutes) with Cu/a caused irreversible activation of the K+ current that, over time, slowly 'ran down' (Fig. 1D).

To demonstrate that the observed activation of an outwardly directed channel was caused by HRs, the HR scavenger thiourea (10 mM) was applied together with Cu/a (Fig. 1C,E). This treatment inhibited the Cu/a-induced activation of K+ conductance, causing a tenfold decrease in its Popen (mean ± s.e. Popen=0.051±0.019; n=5; HP=80 mV; 20 minutes; 1 mM Cu/a) (Fig. 1C,1E). Dimethyl sulfoxide (DMSO), another free-radical scavenger, resulted in a similar effect (0.3% DMSO; mean ± s.e. Popen=0.021±0.004; n=5; HP=80 mV; 20 minutes; 1 mM Cu/a) (Fig. 1F). In contrast to HRs, exogenous H2O2 (10 mM) did not activate currents (n=14; 20-30 minutes; data not shown).

Exogenous application of the K+-channel blockers Ba2+, Ca2+, Cs+, tetraethylammonium+ (TEA+) and Na+ significantly decreased the Popen of the HR-activated conductance channel (Fig. 1F). The Ca2+ channel antagonists verapamil and nifedipine did not cause significant changes in HR-activated single-channel conductance (Fig. 1F; P=0.624 for verapamil and P=0.552 for nifedipine, ANOVA test). Overall, the pharmacological analysis showed that HR-activated currents were mediated by K+ channels, not by any other group of cation channel.

The extrapolated reversal potential of the single-channel HR-activated conducance was -75.2±10.4 mV (n=6), close to the equilibrium potential of K+ (~107.6 mV) (Fig. 1G). With 80 mM pipette K+ solution, the average unitary conductance derived from the first open state was 16.1±1.9 pS (mean ± s.e.; n=6; Fig. 1G). Substitution of K+ in the pipette solution with NH4+ (Fig. 2A), which has similar physical properties to K+ (Hille, 2001), did not cause any significant change in the open probability (mean ± s.e. Popen=0.49±0.08; n=4; HP=80 mV; 20 minutes; 1 mM Cu/a; Popen derived from the first open state; P=0.643, ANOVA test, comparison with K+) and only slightly decreased the unitary conducance (mean ± s.e. 13.3±1.2 pS; n=4; same conditions; P=0.303, ANOVA test, comparison with K+). Substitution of K+ for the K+-channel blocker Cs+ (Fig. 2A) dramatically decreased the Popen (mean ± s.e. Popen=0.051±0.013; n=5; same conditions; P=0.001, ANOVA test, comparison with K+) and single-channel conducance (mean ± s.e. 8.1±1.3 pS; n=5; same conditions; P=0.009, ANOVA test, comparison with K+). These results show that, typical of K+...
channels, HR-activated channels are highly permeable to K⁺ and NH₄⁺, and poorly permeable to Cs⁺.

**Physiological concentrations of Cu²⁺ and ascorbate activate K⁺-efflux channels**

Ascorbate reaches submillimolar levels in plant cell walls (Pignocchi and Foyer, 2003), and plant cell catalytic activities of transition metals (Cu²⁺ and Fe³⁺) are in the range 0.3 to 15 μM (Becana and Klucsa, 1992). We tested several Cu²⁺ concentrations, added together with 0.1 mM ascorbate (Fig. 2B), and found that the minimal Cu²⁺ concentration that causes K⁺ current activation (mean ± s.e. P_open=0.043±0.009; HP=80 mV; n=6) was 5 μM. Maximal K⁺ efflux was achieved after the addition of 0.3 mM Cu²⁺. These data show that physiological levels of Cu/a are capable of activating K⁺ channels.

**Gork1-1 lacks HR-activated K⁺-efflux channels**

The outwardly directed K⁺ channels in Arabidopsis root epidermis are likely to be GORK1 and/or AKT2/3 (Véry and Sentenac, 2002). However, AKT2/3 has been recently shown to conduct a steeply inwardly rectifying current, when expressed in plants (Latz et al., 2007). Some cyclic-nucleotide-gated channels could also be involved, but this has not yet been proven experimentally (Demidchik and Maathuis, 2007). Therefore, the main focus of this study is GORK, which is known to conduct large outwardly rectifying K⁺ currents (Ache et al., 2000; Ivashikina et al., 2001; Hosy et al., 2003). We tested HR-activated K⁺-efflux currents in root epidermal protoplasts isolated from gork1-1 plants lacking functional GORK (Hosy et al., 2003) (Fig. 2D). The 16 pS channel was not observed in gork1-1 plants, indicating that the HR-activated K⁺ channel is encoded by GORK. Outwardly directed single channels with smaller unitary conductance (mean ± s.e. 5.9±1.6 pS; n=3; data not shown) were activated in 3 out of 18 gork1-1 protoplasts in response to 1 mM Cu/a (note that these channels were not observed in the wild type). Because the probability of observing these ‘smaller’ channels was very low, a detailed investigation was not possible. Nonetheless, it might be speculated that these channels were expressed ‘to fill a gap’ in K⁺-efflux function in the absence of GORK and could be encoded by some other channel gene.

**Whole-cell HR-activated K⁺-efflux conductance in wild-type and gork1-1 plants**

To confirm that the HR-activated K⁺-efflux channel is encoded by GORK at the cellular level, we compared whole-cell currents in wild-type (WT) and gork1-1 plants (Fig. 3A). HRs activated a steeply rectifying, slowly activating (time-dependent), outward current in WT protoplasts (Fig. 3A). After 20 minutes exposure to HRs, the outward current recorded in response to an 18 second depolarising pulse from −90 to 120 mV increased from 87.9±20.2 to 467.7±91.3 mA m⁻² (mean ± s.e.; n=5; P=0.004, ANOVA test).
The current did not increase in response to HRs when the K⁺-channel blocker TEA⁺ replaced K⁺ in the pipette (Fig. 3A). No activation of time-dependent K⁺ currents was found in response to HRs in gork1-1 plants (n=16; Fig. 3A), although the rapidly activating (instantaneous) conductance increased by about 30-40%. This current could be due to a channel with a ‘smaller’ unitary conductance (see above).

In our previous study (Demidchik et al., 2003), the time course of the voltage-activated current was measured over 2.4 seconds. Here, long voltage pulses (18 seconds) were applied, allowing the recording of a more ‘pronounced’ time-dependent current (Fig. 3A). Pulses longer than 18 seconds were not applied because they destabilised the gigaseal contact. The HR-activated whole-cell K⁺ conductance revealed two component currents: instantaneous (time independent) and slowly activating (time dependent). The time-dependent component showed greater activation than the instantaneous (Fig. 3A).

**HR-activated K⁺ channels can mediate K⁺ efflux during stress-induced depolarisation**

An important question is whether HR-activated K⁺ channels can conduct current (efflux) upon depolarisation caused by stress. To explore this, we measured membrane potential changes induced by salinity and oxidative stress (Cu/a) in mature root epidermis (Fig. 3B,C). Measurements were carried out in a bath solution containing 0.1 mM K⁺, a typical soil solution K⁺ concentration (Marschner, 1995; Cui et al., 2003). Both stresses cause depolarisation (Fig. 3B,C). Maximal salt-induced depolarisation (from –127.5±12.7 mV to 7.5±17.1 mV; mean ± s.e.; n=5; P<0.001, ANOVA test) was found after 20 minutes root exposure to 250 mM NaCl (Fig. 3B). Similar to the patch-clamp experiments described in Fig. 2B,C, we recorded changes in the membrane potential resulting from the addition of different Cu²⁺ concentrations, together with 0.1 mM ascorbate (Fig. 3C). The addition of 1 μM Cu²⁺ and 0.1 mM ascorbate did not modify the membrane potential, but 10 μM Cu²⁺ and 0.1 mM ascorbate caused significant depolarisation (Fig. 3C). At 1 mM Cu²⁺ and 0.1 mM ascorbate, strong depolarisation was measured (from –123.2±15.5 mV to –46.2±12.3 mV; mean ± s.e.; n=5; P=0.005, ANOVA test) (Fig. 3C). The mixture that was used in most patch-clamp experiments, 1 mM CuCl₂ and 1 mM ascorbate, depolarised the plasma membrane from –119.9±14.1 mV to –41.9±14.4 mV (mean ± s.e.; n=5; P=0.005, ANOVA test).

Whole-cell HR-activated K⁺-efflux currents were measured with 0.1 mM K⁺ in the bathing medium (Fig. 3D). Application of 0.1 mM K⁺ (as compared to 1 mM K⁺) shifted the reversal potential to more negative voltages. This resulted in the activation of a significant K⁺-efflux current at depolarised voltages (Fig. 3D); for example, at –40 mV, the outward current was 96.4±33.2 mA m⁻² (mean ± s.e.; n=4). Overall, these results reveal that HR-activated K⁺ channels are capable of mediating significant K⁺ efflux from root cells during depolarisation caused by stress.

**HR-activated K⁺ efflux from intact roots in wild-type and gork1-1 plants**

To relate the results obtained in protoplasts with K⁺ flux in intact roots, we used non-invasive vibrating K⁺ electrodes [so-called microelectrode ion flux estimation (MIFE)] (Shabala et al., 1997) to record the time course of K⁺ efflux from mature root cells (Fig. 4). Before HR application (control), the K⁺ efflux was –47.7±24.7 nmol m⁻²s⁻¹ (net K⁺ flux; WT, mean ± s.e.; n=6) (Fig. 4A). Upon application of HR, dramatic K⁺ efflux occurred, reaching a peak of –234.4±29.4 nmol m⁻²s⁻¹ (net K⁺ flux; mean ± s.e.; n=6; P=0.002, ANOVA test, comparison with control) after 2-4 minutes, before slowly decreasing over time. This effect of HRs was significantly inhibited by TEA⁺ (10 mM) (Fig. 4A; P=0.003, ANOVA test, comparison of peaks) and was smaller in gork1-1 plants (Fig. 4B; P=0.025, ANOVA test, comparison of peaks), in agreement with the patch-clamp data (Fig. 2D). HR-activated K⁺ efflux in gork1-1 plants could be related to K⁺ efflux from cortex cells that might have different HR-activated channels. Hypothetically, it might also be due to the ‘smaller’ K⁺ channel observed in the single-channel analyses. Nonetheless, K⁺ efflux in gork1-1 plants is sensitive to TEA⁺ (Fig. 4B), suggesting that efflux is the result of K⁺-channel activity.

**Stress-activated K⁺ efflux from intact roots**

A crucial step of our research was to establish a link between the activity of the HR-activated channel GORK and the K⁺ efflux induced by stress. The generation of ROS in response to NaCl and oxidative stress (Cu/a) in mature root epidermis (Fig. 4A,B) has already been demonstrated for Arabidopsis roots (Demidchik et al., 2003). Here, we tested the possible involvement of GORK in NaCl- and cellulysin-induced K⁺ efflux. The application of 100 mM NaCl to mature WT root cells activated K⁺ efflux (peak net K⁺ flux: –200.9±45.3 nmol m⁻²s⁻¹, mean ± s.e.; n=6) (Fig. 4C). In gork1-1, the NaCl effect was significantly smaller (peak net K⁺ flux: –41.9±6.4 nmol m⁻²s⁻¹, mean ± s.e.; n=6; P=0.006, ANOVA test) (Fig. 4C). Applying 1% cellulysin resulted in dramatic K⁺ efflux in the WT, with a peak net K⁺ flux of –295.9±13.5 nmol m⁻²s⁻¹ (mean ± s.e.; n=6), but caused a much smaller effect in gork1-1 (peak net K⁺ flux: –80.8±32.9 nmol m⁻²s⁻¹; mean ± s.e.;
HR-activated K⁺ efflux in the root elongation zone

The growing root tip, which interacts first with a new soil environment, could potentially have greater sensitivity to signalling molecules such as ROS than other parts of the root. Our attempts to investigate single K⁺ channels in outside-out patches from elongation zone protoplasts were not successful because the gigaseal contact was not stable in this preparation when depolarising pulses were applied (n=32). Therefore, we investigated HR-activated K⁺ efflux in the elongation zone using MIFE (Fig. 5A). HR-activated K⁺ efflux from the WT elongation zone was about eight times greater than that from the mature epidermis. This shows that the elongation zone has an elevated sensitivity to ROS and points towards their possible involvement in plant stress perception and response. In gork1-1 plants, HR-induced K⁺ efflux was again much smaller than in the WT, demonstrating that GORK encodes HR-activated K⁺ channels in the elongation zone as well (Fig. 5A).

The effect of H₂O₂ on K⁺ efflux from root cells

Although some stresses, such as pathogen elicitors, drought and cadmium, have been shown to cause HR generation in plants (Becana and Klucas, 1992; Moran et al., 1994; Shen et al., 1997; Van Doorslaeder et al., 1999), the effect of salinity has not been studied (Chung et al., 2008). Electron paramagnetic resonance (EPR) spectroscopy is one of the most accurate and sensitive techniques for the detection of radicals in living systems (Halliwell and Gutteridge, 1999). addition of substances is indicated by an arrow.

Salt-induced generation of HRs in intact roots

The most abundant and stable ROS in plants is H₂O₂, which accumulates in response to stress (Apel and Hirt, 2004). In root protoplasts, H₂O₂ does not activate K⁺-efflux currents (Demidchik et al., 2003). However, the application of H₂O₂ to intact roots could potentially activate K⁺ efflux, because cell-wall transition metals and ascorbate could convert H₂O₂ to HRs (Fry et al., 2002; Fry, 2004). We tested this hypothesis by applying 10 mM H₂O₂ to intact roots (mature cells) and measuring the K⁺-flux responses using MIFE (Fig. 5B). We found a TEA⁺-sensitive increase in K⁺ efflux that was smaller in gork1-1 (Fig. 5C). The affect of H₂O₂ developed more slowly and was smaller than that caused by HRs, NaCl and cellulysin (Fig. 4). The difference in response between WT and gork1-1 (Fig. 5B, C) was also not as pronounced as with HR-, NaCl- and cellulysin-induced K⁺ efflux (Fig. 4B-D).

Salt-induced generation of HRs in intact roots

Although some stresses, such as pathogen elicitors, drought and cadmium, have been shown to cause HR generation in plants (Becana and Klucas, 1992; Moran et al., 1994; Shen et al., 1997; Van Doorslaeder et al., 1999), the effect of salinity has not been studied (Chung et al., 2008). Electron paramagnetic resonance (EPR) spectroscopy is one of the most accurate and sensitive techniques for the detection of radicals in living systems (Halliwell and Gutteridge, 1999). We added the classical spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) to intact roots. Spectra with four characteristic peaks, indicative of the formation of HR-DMPO adducts (Yamazaki et al., 1990; Halliwell and Gutteridge, 1999; Ikeda et al., 2002), were detected (Fig. 6). Intact roots demonstrated a small basal level of HR production (Fig. 6A), consistent with the literature on ‘constitutive’ HR generation in Arabidopsis roots (Renew et al., 2005). The effect of two NaCl concentrations (100 and 250 mM) was examined (note that neither NaCl concentration, when added to DMPO without plants, changed the EPR signal; data not shown). Although it is not a lethal concentration, 100 mM NaCl does affect germination and growth of Arabidopsis; 250 mM NaCl does cause cell death (Demidchik and Tester, 2002; Munns and Tester, 2008; Shabala and Cuin, 2008). The shape of the EPR spectrum did not change after treatment with NaCl, whereas the intensity significantly increased (Fig. 6B). A 2.5- to 3-fold increase in HR generation was caused by the addition of 100 mM NaCl, whereas 250 mM NaCl triggered a 4- to 4.5-fold increase compared to the basal level of HR generation (Fig. 6B,F; P<0.01, ANOVA test).

The mixture of Cu²⁺ and ascorbate can produce HRs in the presence of H₂O₂ or O₂ (Shtamm et al., 1977). Because some O₂ is always dissolved in water, we used deoxygenated water; all solutions were sealed with parafilm (Halliwell and Gutteridge, 1999). Solution containing 1 mM Cu/a prepared in this way caused HR generation was caused by the addition of 100 mM NaCl, whereas 250 mM NaCl triggered a 4- to 4.5-fold increase compared to the basal level of HR generation (Fig. 6B,F; P<0.01, ANOVA test).

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reaction media caused the disappearance of the four-peak signal, confirming that NaCl induced HR generation (Fig. 6E).

Involvement of HR-activated K+ channels in programmed cell death

One important affect of HR-activated K+ loss in animal cells is to trigger PCD, a ‘self-destruction’ mechanism associated with the induction of specific K+-inhibited proteases and endonucleases (Hughes et al., 1997; Yu, 2003; Ajiro et al., 2008). Here, 250 mM NaCl and oxidative stress (1 mM Cu/a) were applied to initiate cell death. We found that practically all root cells (in both WT and gork1-1) showed PCD symptoms (Lam, 2004) during 2-3 days treatment with these stresses (Fig. 7). Treatment with HR (Cu/a) caused membrane damage and protoplasm shrinkage (Fig. 7B), whereas NaCl mainly induced the appearance of condensed dark areas in place of nuclei (Fig. 7C). However, the observed morphological changes varied significantly and the extent of damage was not possible to determine quantitatively. Therefore, we used biophysical and biochemical approaches to assess whether HR-activated K+ channels are involved in root PCD.

Sustained depolarisation is perhaps the best marker of cell damage and death (Bortner et al., 2001); consequently, we measured changes in the plasma-membrane potential. Membrane potential tests do not differentiate between PCD and necrosis, but distinguish, very accurately compared with other techniques, dead cells from those that are alive. Measurements were taken from mature root epidermal cells in both WT and gork1-1 plants, and the stresses (250 mM NaCl and 1 mM Cu/a) were washed out for 2 hours before measurement. For WT cells, 36% and 24% demonstrated sustained depolarisation (above –40 mV) after 15 hours treatment with Cu/a and NaCl, respectively (Fig. 8). Forty hours exposure to 250 mM NaCl resulted in the collapse of the membrane potential in all tested cells, whereas the same exposure to a HR-generating mixture induced sustained depolarisation in 85% of cells. gork1-1 had about three times fewer cells with a collapsed membrane potential than WT cells after 15 hours treatment with both stresses (Fig. 8). The difference between gork1-1 and WT cells was also evident after 40 hours treatment with stresses; however, it was not observed for exposure times over 3 days (Fig. 8). Overall, these measurements show that the absence of functional GORK delays stress-induced cell death.

Fluorescently labelled protease inhibitor zV AD-fmk (FITC-V AD-fmk) is a widely used tool for the observation of PCD protease activation in intact tissues, because, at micromolar concentrations, this substance does not significantly inhibit proteases, although it binds to and fluorescently labels them (Elbaz et al., 2002; Bonneau et al., 2008). Increased intracellular fluorescence indicates a higher protease activity and tests on
Arabidopsis roots showed only a little background staining under control conditions (Fig. 9). However, fluorescence increased under stress treatment, indicating protease activation. Fluorescence was about three to four times lower in gork1-1 compared with the WT under either HR or NaCl treatments. DMSO, TEA$^+$ and Ba$^{2+}$ inhibited PCD protease activation, as reported by FITC-VAD-fmk (Fig. 9A,B,D), demonstrating that the HR-mediated K$^+$-efflux channel is essential for stress-induced stimulation of proteases. Longer-term treatment of gork1-1 (>40 hours NaCl) caused a strong increase in protease activity, indicating that K$^+$-efflux channels are mainly involved in the early PCD phase (Fig. 9B). A total of 10 mM Cu/a and H$_2$O$_2$ (used as a positive control) induced a very strong signal in both WT (fluorescence intensity 494±19.7; n=16; mean ± s.e.) and gork1-1 (fluorescence intensity 468±14.1; n=15; mean ± s.e.) cells (Fig. 9C).

TdT-mediated dUTP nick end labelling (TUNEL) is a standard technique for visualising DNA cleavage in PCD (Elbaz et al., +2002; Reape et al., 2008). Gork1-1 plants revealed about four times less TUNEL staining after 15 hours exposure to stress (Fig. 10A,B), implying that HR-activated K$^+$-efflux channels are likely to be involved in endonuclease activation caused by oxidative stress and salinity. DNase controls confirmed that the measured fluorescence was caused by DNA degradation (Fig. 10).

**Discussion**

The data presented here demonstrate that HRs activate an outwardly directed K$^+$-selective channel with unitary conductance of 16 pS that is encoded by the Shaker channel GORK (Figs 1-3). Activation can be induced by physiological concentrations of Cu/a and thus can probably take place in nature (Fig. 2B,C). The HR-activated K$^+$ channel mediates K$^+$ efflux caused by both biotic and abiotic stress – stresses that are able to stimulate HR generation (Figs 4-6) and are likely to contribute to stress-induced PCD (Figs 7-10). Overall, this study shows the key functional properties of HR-activated K$^+$ channels and provides evidence for their role in linking stress-induced ROS, K$^+$ leakage and PCD (Fig. 11).

A newly observed mechanism of plant K$^+$-channel regulation

The regulation of K$^+$ channels is an important issue because these proteins control membrane potential, ion homeostasis and the activity of signalling systems (Hille, 2001). Potassium channels are the best-studied group of plant ion channels (Ward et al., 2009). They were initially discovered in the late 1970s in algae (Sokolik and Yurin, 1981), and a detailed analysis of their voltage control, selectivity, single-channel properties, pharmacology and regulation by exogenous and endogenous factors, G proteins and phosphorylation network then followed (Tester, 1990; Maathuis and Amtmann, 1999; Dreyer and Blatt, 2009; Ward et al., 2009). The data presented here suggest a new mechanism of regulation of plant K$^+$ channels – activation by ROS. This mechanism has not been characterised in plants before, although, in animals, it has been shown to be important for regulation of blood pressure, pacemaker activities in the heart and brain, phagocytosis, and maintaining ionic and osmotic homeostasis (Lahiri et al., 2006).

The genes and structure of plant K$^+$ channels (nine genes in Arabidopsis) have been extensively investigated (Véry and Sentenac, 2002; Véry and Sentenac, 2003). Here, we demonstrate that one of these genes, GORK, encodes a root HR-activated K$^+$ channel. To our knowledge, this is the first identification of the gene encoding the ROS-activated ion channel in plants. BLAST analyses (NCBI) show that genes similar to GORK exist in other plants (such as Populus trichocarpa, Nicotiana tabacum, Samanea...
saman, Solanum tuberosum), suggesting that HR-activated K+ channels are widespread in the plant kingdom.

The 'well-defined' physiological functions of plant outwardly directed K+ channels include K+ efflux from guard cells leading to stomata closure (mediated by GORK) and K+ loading to the xylem (catalysed by SKOR) (Gaymard et al., 1998; De Boer, 1999; Hosy et al., 2003). K+-efflux channels probably also repolarise the plasma membrane after depolarisation caused by activation of Ca2+ and Cl– channels (Tester, 1990; Ward et al., 2009). Our results suggest that these functions might be regulated by ROS (through HR activation of GORK).

The presence of two components in the whole-cell HR-activated K+-efflux current (Fig. 3A) can be explained by either the activation of two channels encoded by different genes or the coexistence of two populations of the same channel with different activation kinetics (Hille, 2001). The latter is more likely for HR-activated K+-efflux channels because only a 16 pS single channel has been found in excised patches. Nonetheless, this requires further investigation.

HRs caused an approximately fivefold increase in the outward K+ current (Fig. 3A). This is two to three times greater than the cytosolic alkalinisation-induced outward K+ current previously measured (Miedema and Assmann, 1996). Locking G proteins in which are weaker oxidants than HRs (Halliwell and Gutteridge, 1999), have been shown to inhibit K+ conductance in roots (Demidchik et al., 2003) and leaves (Torsøenhaugen et al., 1999; Köhler et al., 2003; Sokolovski and Blatt, 2004).

Properties of plant HR-activated K+ channels and plant response to stress

HR-activated K+-efflux channels demonstrate electrophysiological properties (selectivity, voltage dependence, kinetics of activation, pharmacological profile and single-channel characteristics) very similar to those of 'constitutive' outwardly rectifying K+ channels that were previously identified in Arabidopsis (Demidchik et al., 2006). A classical study carried out by Maathuis and Sanders showed that Arabidopsis root K+-efflux channels have unitary conductance of about 15 pS (Maathuis and Sanders, 1995). This is similar to results reported here (16 pS; Fig. 1G). The unitary conductance of heterologously expressed GORK is 13.5 pS (Ache et al., 2000), also very close to the values measured for the HR-activated K+-efflux channel in this study. Taking into account the absence of HR-induced activation in gork1-1, it can be concluded that HRs activate a 'constitutively' expressed outwardly directed K+ channel encoded by the GORK gene in Arabidopsis roots.

The stimulatory effect of HRs on plant K+ channels appears to be HR specific; other oxygen-derived species (H2O2, O3 and NO),
an inactive state using the G-protein blocker guanosine 5′-β-thio-diphosphate and decreasing the cytosolic Ca\(^{2+}\) concentration (Li and Assmann, 1993) also causes smaller effects than HRs. However, the *Cladosporium fulvum* elicitor resulted in very similar activation of outward K\(^+\) currents in *Nicotiana tabacum* guard cells (3-4-fold increase) (Blatt et al., 1999). Interestingly, this elicitor is known to induce the generation of HR (Veraestrella et al., 1992).

Notably, both HR production and K\(^+\) leakage have been observed in plants treated with other pathogenic elicitors, originating, for example, from *Botrytis cinerea* (Govrin et al., 2006), *Alternaria alternate* (Jennings et al., 2002) and *Magnaporthe grisea* (Pasechnik et al., 1998). In these cases, however, K\(^+\)-channel activation was not characterised. The *T. viride* elicitor cellulysin, which previously was shown to induce ROS production in *Arabidopsis* roots (Demidchik et al., 2003), activated K\(^+\) efflux and activation was smaller in *gork1-1* (Fig. 4D). This shows that the HR-activated channel GORK can probably mediate elicitor-induced K\(^+\) efflux, which could potentially be involved in pathogen-induced PCD.

### Possible mechanisms of K\(^+\)-channel activation by HRs

H\(_2\)O\(_2\), which accumulates abundantly in plant cells during stress (Apel and Hirt, 2004), probably requires conversion (catalysed by transition metals) to the more reactive HR species to activate K\(^+\) channels and efflux. Intriguingly, transition metal binding can be bound directly within the Shaker K\(^+\) channel. For example, animal hERG channels, which are closely related to plant GORK channels (BLAST analysis; NCBI), have extracellular transition-metal-binding centres that generate HRs in the presence of H\(_2\)O\(_2\) and are responsible for channel opening (Yu et al., 1997; Yu, 2003). The search for and examination of similar metal-binding centres that can convert H\(_2\)O\(_2\) to HR in GORK (and other plant ion channels) is currently in progress in our laboratories.

We should not exclude other possible modes of action of H\(_2\)O\(_2\) and HRs on channel activity. Oxidation of membrane lipids and/or generation of other signalling molecules, such as NO, could also target the channel; such mechanisms can coexist in animal cells (Yu, 2003).

The oxidising effect of HRs on plant cells is widely believed to be irreversible and harmful, although experimental evidence for this is patchy (Møller et al., 2007). Moderate HR production is essential for cell growth and shaping, cell-wall loosening and probably other reactions (Liszkay et al., 2004; Fry, 2004). Although the role of catalytic copper and iron in plant signalling remains virtually unexplored, some facts point towards their possible involvement in plant stress responses. For example, the catalytic activity of transition metals (Cu\(^{2+}\)/Fe\(^{2+}/3+\)) increases many times in plant cells during senescence, pathogen attack and drought conditions. This leads to an increase in HR generation and cell death (Becana and Klucas, 1992; Moran et al., 1994; Liu et al., 2007). Thus, it can be hypothesised that the regulation of catalytic activities of transition metals could play a crucial role in plant ROS-mediated regulatory reactions.

Our results demonstrate that the effect of HRs on K\(^+\) conductance is reversible after 20 minutes exposure to a HR-generating mixture. This ‘regulatory’ phase is relatively short and is followed by irreversible activation (after 30-45 minutes exposure), very similar to the effect of HR on animal cation channels (Simon et al., 2004; Tang et al., 2004). The reversibility of the HR effect could derive from incomplete and reversible oxidation of amino acids, particularly His, Cys and Met (Tang et al., 2004). For example, HR-induced Cys oxidation leads to the sequential formation of more oxidised derivatives, such as cystine, cysteine sulfenic acid and cysteine sulfenic acid, all of which are enzymatically reversible (Biteau et al., 2003).

### Cation channels and plant PCD

PCD helps plants to survive under stress (Apel and Hirt, 2004; Mur et al., 2008; Cutler and Somerville, 2005). Dead cells probably provide a ‘shield’ from stress factors and signal stress to surviving cells. Generation of ROS seems to be a key mechanism in plant PCD (Apel and Hirt, 2004). Stress-induced PCD begins from the effect of stress factors on as yet undetermined receptors, which activate ROS-producing enzymes, such as NADPH oxidases, peroxidases, and chloroplast and mitochondrial redox cascades (Foyer and Noctor, 2009). It is widely accepted that ROS induces the elevation of cytosolic free Ca\(^{2+}\); 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). The data reported here demonstrate that, as well as Ca\(^{2+}\) elevation, ROS also activate K\(^+\) efflux, thereby inducing PCD hydrolase activation.

Our results show that K\(^+\)-channel blockers and the lack of a functional GORK both inhibit the stress-induced activation of PCD proteases and endonucleases (Figs 9 and 10). In animals, these hydrolytic enzymes are directly inhibited by their natural blocker, K\(^+\), which is at high concentration in the cytosol (70-100 mM) (Yu et al., 1997; Lam, 2004; Remillard and Yuan, 2004). Death factors activate animal K\(^+\)-efflux channels, leading to K\(^+\) loss, and this stimulates protease and endonuclease activity (Yu, 2003; Remillard and Yuan, 2004). Our data strongly suggest that a similar mechanism of hydrolase activation exists in plants. HR-activated K\(^+\)-efflux channels cannot activate without depolarisation, which, in plant cells, relies on Ca\(^{2+}\) influx and anion efflux or can be caused by toxic Na\(^+\) influx (Tester, 1990; Demidchik and Maathuis, 2007). Notably, the involvement of Ca\(^{2+}\) and Cl\(^{-}\) channels in plant PCD has already been reported (Wendehenne et al., 2002). Here, we show that NaCl and oxidative stress cause depolarisation that is sufficient for K\(^+\)-channel activation (Fig. 3B,C).

Other examples of PCD involvement in the plant lifecycle include self-incompatibility of pollen tubes (Bosch and Franklin-Tong, 2007), senescence and death of floral organs (Rogers, 2006), xylogenetesis (Fukuda, 2000) and embryogenesis (Reape et al., 2008). Future experiments should explore a possible role for HR-activated K\(^+\)-efflux channels in these physiological processes.

### The role of HR-activated K\(^+\) efflux in NaCl-induced cell death

Ion disequilibrium is a prime reason for plant damage under salt stress, causing huge crop yield losses worldwide (Munns and Tester, 2008). The ability of plants to retain a high K\(^+\)/Na\(^+\) ratio is crucial for salt tolerance (Chen et al., 2005; Volkov and Amtmann, 2006; Amtmann, 2009). Although some explanations for this phenomenon exist, they are clearly speculative (Shabala and Cuin, 2008). It is still not established experimentally how K\(^+\) loss causes cell death. It could be hypothesised that K\(^+\) efflux from root cells, mediated by K\(^+\)-efflux channels activated by salt-induced HR (Fig. 6), stimulates PCD hydrolases, inducing cell death (Fig. 11). In animal cells, the cytoplasmic and extracellular K\(^+\)/Na\(^+\) ratio is a major parameter regulating PCD (Bortner et al., 1997; Orlov et al.,...
K⁺ cannot substitute for Na⁺ in its protease inhibition reactions, so the replacement of K⁺ by Na⁺ results in PCD (Orlov et al., 1999). It is obvious, then, that K⁺ nutrition and K⁺ fertilisation are crucially important for preventing plant cell damage and death mediated by hydrolases. Future work should show how individual plant caspase-like activities and endonucleases are regulated by K⁺.

Conclusions

In conclusion, HR-activated K⁺-efflux channels have been characterised in detail. They are similar to constitutive plant K⁺-conductance channels and are probably encoded by the GORK gene. Although their functions might be very broad, they are clearly involved in stress-induced K⁺ loss and stimulation of PCD proteases and endonucleases.

Materials and Methods

Plant material

*Arabidopsis thaliana* Wassilevskija (WS 0) ecotype was from the European Arabidopsis Stock Centre (Nottingham). *gork1-1* seeds were a generous gift from Hervé Sentenac (ENSAM, Montpellier, France). Plants were grown vertically, in sterile conditions, on the surface of 0.4% Phytagel with full-strength Murashige and Skoog media (Duchefa, Netherlands), 1% sucrose (w/v), pH 6.0 adjusted with KOH (Demidchik et al., 2003). Four- to nine-day-old root seedlings were used.

Electron paramagnetic resonance spectroscopy

To measure HR generation in intact roots, standard EPR techniques were used (Halliwel and Gutteridge, 1999; Lizziness et al., 2004). Twenty intact seedlings were removed from the medium and washed several times in a buffer solution containing 0.1 mM KCl and 0.1 mM CaCl₂ (pH 6.0; KH₂PO₄/K₂HPO₄). Their 1.5 cm root-tip parts were immersed in the buffer solution containing trap and stress treatments. The HR-specific spin trap DMPO (Sigma; 40 mM) was purified using activated charcoal to remove transition metals and decrease the background. It was applied together with 20 minute stress treatments in a 0.4 ml vial; the vial was sealed from the air to avoid DMPO oxidation (all solutions were first deoxygenated with N₂). Root cells remained viable (did not lose membrane potential or turgor) upon 20 minutes exposure to DMPO.

After treatment, the eluate from the vial was collected in a flat Bruker EPR cuvette. The EPR spectra were recorded in the liquid phase at room temperature using a Bruker EMX (X-band) spectrometer and analysed using the standard Bruker software. The HR-specific radical scavenger thiourea (Lizziness et al., 2004) was used to validate HR production. The line-shape of the EPR spectra of the DMPO adducts (Fig. 6D) was consistent with trapping the HR, because the HR-DMPO and superoxide-DMPO adduct spectra can be clearly distinguished by specific features in the line-shape (Yamazaki et al., 1990; Ikeda et al., 2002). It cannot be excluded that superoxide radicals were generated as precursors of HR, but their activity was not detected using a DMPO-based system. The superoxide-adduct-specific spectrum (Yamazaki et al., 1990) was also not observed after either NaCl or Cu²⁺ treatment.

Patch-clamp experiments and membrane potential measurements

Conventional patch-clamp and protoplast-isolation techniques were used (Demidchik and Tester, 2002; Demidchik et al., 2006; Demidchik et al., 2007, Demidchik et al., 2009). The standard bathing solution contained (in mM): 1 KCl, 0.3 CaCl₂, 2 Tris, adjusted to pH 5.8 with MES and to 290-300 mOs with sorbitol. A freshly prepared mixture of the bathing solution with 1 mM CuCl₂ and 1 mM L-ascorbic acid was applied to generate HRs (Halliwel and Gutteridge, 1999). The standard pipette solution contained 80 mM K⁺, 70 mM gluconate –, 10 mM Cl –, 100 nM Ca²⁺ and 2 mM Tris/MES), pH 7.2 (2 mM Tris/MES). TEA⁺, Cs⁺, Na⁺, Ca²⁺ and Ba²⁺ were added as chlorides. A voltage step protocol was used to activate K⁺-efflux channels as described (Yamazaki et al., 1990) was also not observed after either NaCl or Cu/a treatment. A primary role for K⁺ and pectolytic enzymes (1.5% cellulase Onozuka RS, 1% cellulysin, 0.1% pectolyase Y-23, 0.1 mM KCl, 0.1 mM CaCl₂, pH 6.0 adjusted by 4 mM MES/2 mM Tris).

Screens for salt tolerance by measuring K⁺ flux: a case study for barley


Conclusions

In conclusion, HR-activated K⁺-efflux channels have been characterised in detail. They are similar to constitutive plant K⁺-conductance channels and are probably encoded by the GORK gene. Although their functions might be very broad, they are clearly involved in stress-induced K⁺ loss and stimulation of PCD proteases and endonucleases.

Screening plants for salt tolerance by measuring K⁺ flux: a case study for barley


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