Free oxygen radicals regulate plasma membrane Ca^{2+}- and K^+-permeable channels in plant root cells

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Summary

Free oxygen radicals are an irrefutable component of life, underlying important biochemical and physiological phenomena in animals. Here it is shown that free oxygen radicals activate plasma membrane Ca^{2+}- and K^+-permeable conductances in *Arabidopsis* root cell protoplasts, mediating Ca^{2+} influx and K^+ efflux, respectively. Free oxygen radicals generate increases in cytosolic Ca^{2+} mediated by a novel population of nonselective cation channels that differ in selectivity and pharmacology from those involved in toxic Na^+ influx. Analysis of the free oxygen radical-activated K^+ conductance showed its similarity to the *Arabidopsis* root K^+ outward rectifier. Significantly larger channel activation was found in cells responsible for perceiving environmental signals and undergoing elongation. Quenching root free oxygen radicals inhibited root elongation, confirming the role of radical-activated Ca^{2+} influx in cell growth. Net free oxygen radical-stimulated Ca^{2+} influx and K^+ efflux were observed in root cells of monocots, dicots, C3 and C4 plants, suggesting conserved mechanisms and functions. In conclusion, two functions for free oxygen radical cation channel activation are proposed: initialization/amplification of stress signals and control of cell elongation in root growth.

Key words: *Arabidopsis*, Calcium, Channel, Free oxygen radical, Plasma membrane, Potassium

Introduction

Many fundamental cellular phenomena (Sohal and Weindruch, 1996; Halliwell and Gutteridge, 1999) are mediated by free radical and oxidative mechanisms. Free oxygen radicals (FORs) target ion channel and receptor complexes, which initiate signalling cascades to regulate cellular metabolism (Kourie, 1998; Thannickal and Fanburg, 2000). In the last decade, irrefutable evidence has appeared that in plants, as in animals, elevated production and accumulation of FORs accompanies various processes, for example development, hormone action, gravitropism and stress responses (Alvarez et al., 1998; Karpinski et al., 1999; Bowler and Fluhr, 2000; Pei et al., 2000; Schopfer et al., 2002). Significantly, transient increases in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) as a second messenger also accompany such processes, suggesting FOR/$[Ca^{2+}]_{cyt}$ signalling pathways (Bowler and Fluhr, 2000). Supporting this suggestion, Ca^{2+}-permeable channel modulation by the FOR precursor H_2O_2 was recently demonstrated in *Vicia faba* guard cells (Pei et al., 2000).

This study tests the hypothesis that, in plant cells as in animals, FORs regulate channel activity. The study has focused on root epidermal cells as sites of pathogen interaction, stress perception and gravitropism and on hydroxyl radicals ($OH^\bullet$) as the most potent, biologically relevant FOR that can be produced in various plant systems, including *Arabidopsis* roots (Becana and Klucas, 1992; Moran et al., 1994; Shen et al., 1997; Van Doorslaeder et al., 1999; Smirnoff and Wheeler, 2000; Joo et al., 2001). Moreover, weakly reactive oxygen species (such as hydrogen peroxide and superoxide anion radicals) do not interact directly with most target biomolecules, and thus need to be converted to $OH^\bullet$, which are capable of modifying targets (Halliwell and Gutteridge, 1999). Here, $OH^\bullet$ have been generated at the external face of the epidermal plasma membrane using a Cu^{2+}/ascorbate mixture (Halliwell and Gutteridge, 1999). *In planta*, apoplastic FOR production could occur by several mechanisms including the interaction of transition metals (such as Cu^{2+}) with ascorbate or H_2O_2 (Fry, 1998). It is shown here that FORs activate plasma membrane Ca^{2+}- and K^+-permeable channels in the root epidermis. Activation by FORs forms a novel pathway for the regulation of K^+ channel activity in plant cells and delineates a new family of plant ion channels – FOR-activated Ca^{2+}-permeable nonselective cation channels. This activation of cation conductances is involved in two crucial phenomena in root cells: response to stress and elongation growth.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (Heyn) Columbia and C24 were from our laboratory stock. The *A. thaliana akt1* mutant was obtained from the National *Arabidopsis* Stock Centre (Nottingham, UK). *Arabidopsis* plants constitutively expressing cytosolic aequorin [driven by the *CaMV 35S* promoter (Price et al., 1994)] and plants expressing green fluorescent protein (GFP) in root epidermis [line J0481 (Kiegle et al., 2000a)] and pericycle [line J2661 (Kiegle et al., 2000a)] were kindly provided by M. Knight (University of Oxford, UK) and J. Haseloff (University of Cambridge, UK), respectively. Seedlings were grown aseptically at 22°C for 10-15 days (16 hours day length; 100 μmol

m^{-2} $second^{-1}$ irradiance) on standard medium comprising 0.3% Phytagel (Sigma), full-strength Murashige-Skoog medium (Duchefa, Haarlem, Netherlands) and 1% sucrose. Growth experiments were performed using Columbia. Filter-sterilised test substances were incorporated into the standard medium and main root length was measured after 12 days of growth.

For moving vibrating ion-selective microelectrode (MIFE™) experiments, Columbia seedlings were grown for 3 to 6 days on standard medium or paper rolls (Shabala et al., 1997; Shabala, 2000). All other plants used in MIFE™ experiments (*Triticum aestivum* L., *Zea mays* L., *Vicia faba* L., *Spinacia oleracea* L., *Trifolium pratense* L.) were from commercial sources and were grown for 3 to 6 days on paper rolls.

Isolation of protoplasts from mature root epidermis and pericycle

The method was adapted from Demidchik and Tester (Demidchik and Tester, 2002). Roots from 50 seedlings were cut into approximately 1 mm-long pieces in 4 ml of enzyme solution. This comprised 1.5% Cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), 1% cellulysin (CalBiochem, Nottingham, UK), 0.1% pectolyase Y-23 (Yakult Honsha, Tokyo, Japan), 0.1% bovine serum albumin (Sigma), 10 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM MES, pH 5.6 with Tris; 290 to 300 mOsM adjusted with 330 mM sorbitol. After shaking (60 rpm) in the enzyme solution for 30 to 50 minutes at 28°C, protoplasts were filtered (50 μm pore) and rinsed with holding solution (HS: 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose, 10 mM glucose, 2 mM MES, pH 5.7 with Tris; 290 to 300 mOsM). Protoplasts were collected by 5 minutes centrifugation at 200 ***g*** and diluted with HS. Most viable protoplasts isolated by this procedure were derived from mature epidermis as judged by direct observation and comparison with protoplast release from roots expressing GFP in the epidermis or pericycle. In patch clamp experiments, pericycle protoplasts were identified by GFP expression, while mature epidermal protoplasts were identified by direct observation (light microscopy), larger diameter (20±1.5 μm diameter) and uniform grey colour.

Isolation of protoplasts from root elongation zone epidermis

Root tips (3-4 mm) were isolated directly in the patch-clamp chamber and exposed to the enzyme solution (no shaking) for 1-2 hours (22°C, dark). Direct observation confirmed protoplast release solely from elongation zone epidermis. Protoplasts (20±1.5 μm diameter) were washed with HS for 5 minutes prior to use.

Electrophysiology

Electrophysiological protocols were adapted from Demidchik and Tester (Demidchik and Tester, 2002). Osmolarity of all solutions was adjusted to 300 mOsM with D-sorbitol. The pipette solution contained (mM): 50 K^+, 10 Cl^-, 40 $gluconate^-$, 4 EGTA, 5 HEPES, pH 7.2 (KOH). For selectivity estimates, 50 mM TEA^+ or 25 mM Ba^{2+} were added as chlorides. In preliminary assays, $OH^\bullet$-induced currents (whole cell configuration) were found to be independent of pipette solution Ca^{2+} [1-200 nM: activities calculated using GEOCHEM (Parker et al., 1995)]. To avoid uncertainties arising from ATP-dependent Cl^- currents, ATP was omitted from the pipette solution (Demidchik and Tester, 2002). Control assay (bathing) solution contained (mM): 20 $CaCl_2$, 2 MES, pH 5.7 (Tris). To generate $OH^\bullet$, 1 mM $CuCl_2$ and 1 mM ascorbic acid (Cu/a) (Halliwell and Gutteridge, 1999) were incorporated (pH 5.7) unless stated otherwise. Selectivity estimates were made as described in Demidchik and Tester (Demidchik and Tester, 2002). In pharmacological assays, conductances before ($G_{control}$) and after addition of inhibitors (mM), 20 TEA^+, Ca^{2+} or Ba^{2+}, 0.05 La^{3+} or Gd^{3+}, 0.02 verapamil (G_{block}), were calculated from currents between –80 mV and –160 mV (for the inward Ca^{2+} current) or between 0 mV and +80 mV (for the outward K^+ current). A standard patch-clamp amplifier (IM/PCA, List, Darmstadt, Germany), Digidata 1200 digitiser and pClamp software, version 6.0.2 (Axon Instruments, Foster City, CA) and 8-pole Bessel filter (Frequency Devices, Haverhill, MA) were used. Current was sampled at the end of the 2 second voltage pulse. Liquid junction potentials were measured and corrected as described elsewhere (Ward and Schroeder, 1994). Curve fitting was performed using Statistica (StatSoft, Tulsa, OK) or Sigma Plot (SPSS Science, Chicago).

K^+ photometry

Excised *Arabidopsis* roots (0.1 g) were rinsed for 60 minutes in deionised water and placed in 5 ml of assay solution (10 mM $CaCl_2$, 5 mM MES to pH 5.7 with Tris). Hydroxyl radicals were generated by the addition of 1 mM Cu/a. Samples (0.5 ml) were assayed for K^+ by flame photometry.

Luminometry

Standard luminometry procedures (Price et al., 1994) were used for recording luminescence from about 50 excised roots. Lucigenin-based luminescence assays were adapted from Papadakis and Roubelakis-Angelakis (Papadakis and Roubelakis-Angelakis, 1999). Six to 7 cm-long (from the apex) pieces were placed in the recording cuvette with 2 ml of control assay solution (mM: 0.5 $CaCl_2$, 1 EDTA, 10 Tris, pH 8.7). Stresses were applied as 150 mM NaCl or 1% cellulase (Onozuka) or cooling (2°C). After 30 minutes, the cuvette was placed in the luminometer and 2 mM lucigenin (Sigma) was incorporated. Counting at 1 Hz was performed for 100 seconds. The assay for aequorin luminescence was adapted from Kiegle et al. (Kiegle et al., 2000b) Excised roots were immersed in recording solution (10 mM $CaCl_2$, 2 mM MES, pH 5.7 (Tris), 4 μg ml^{-1} coelenterazine [free base, NanoLight Technology, Prolume, Pittsburgh, PA]) for 20-30 hours at 28°C (dark, 20 rpm). Excised root samples were exposed to inhibitors (incorporated into recording solution) for 30 minutes prior to experimentation. Reactions were initiated by the addition of Cu/a or $CuCl_2$ and luminescence changes were recorded at 1 Hz.

Moving vibrating ion-selective microelectrode technique (MIFE™)

This technique is described in detail elsewhere (Shabala et al., 1997; Shabala, 2000). Each plant was mounted in a Perspex holder by an agar drop and roots were immersed in assay solution comprising (in mM) 0.1 KCl, 0.1 NaCl, 0.1 $MgCl_2$ and 0.05 $CaCl_2$, pH 5.6 with 1 Tris/MES. The microelectrode was placed 20 μm above the root surface and net K^+ and Ca^{2+} fluxes were measured concurrently. Hydroxyl radicals were generated by the addition of 1 mM Cu/a.

Results

Free oxygen radical activation of cation conductances

Under control conditions, protoplasts derived from mature root epidermal cells of *Arabidopsis thaliana* (ecotype Columbia) showed negligible inwardly directed current and a moderate outwardly rectifying current (Fig. 1A,C). $OH^\bullet$ generated at the external face of the plasma membrane by 1 mM Cu^{2+}/ascorbate (Cu/a) (Halliwell and Gutteridge, 1999) activated cation conductances – an inward Ca^{2+} current (Ca_{in}) and an outward K^+ current (K_{out}; Fig. 1B,C). FOR-activated Ca_{in} and K_{out} rectified at voltages <–100 mV and >0 mV, respectively, and both revealed slowly- and rapidly-activating components (Fig. 1B). Whole cell currents transiently decreased after Cu/a

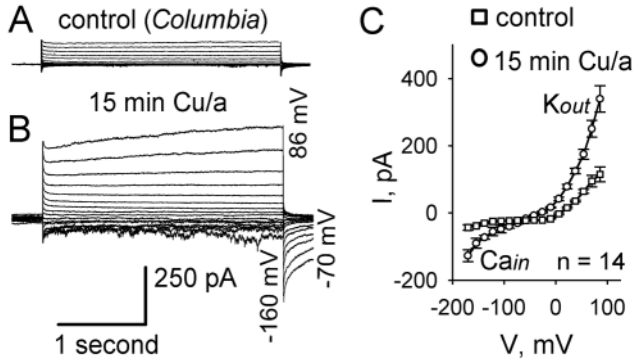

Fig. 1. Hydroxyl-radical-induced whole cell outward K^+ (K_{out}) and inward Ca^{2+} (Ca_{in}) currents in protoplasts from *Arabidopsis* root mature epidermis. (A) Whole cell control currents. (B) Whole cell currents from the same protoplast after 15 minutes exposure to 1 mM Cu^{2+}/ascorbate (Cu/a). (c) Mean (±s.e.m.) current-voltage curves.

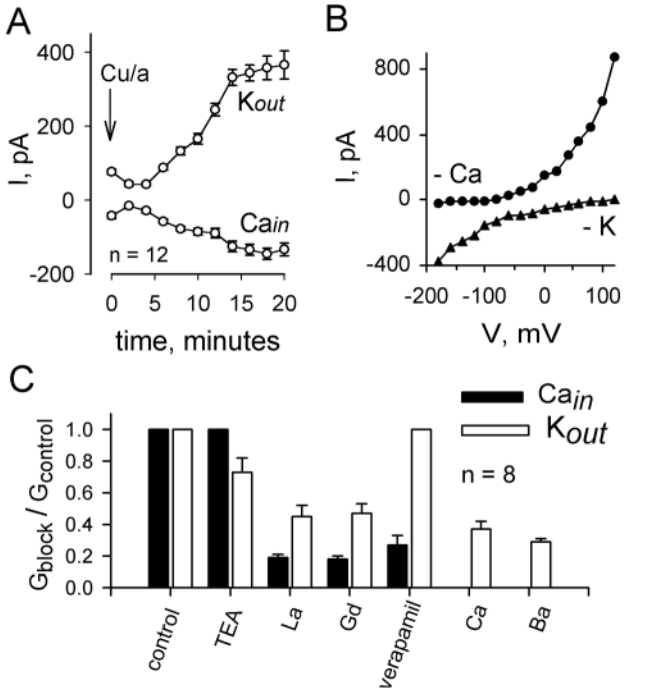

Fig. 2. Observations on the $OH^{\bullet}$-induced K_{out} and Ca_{in} in protoplasts from *Arabidopsis* root mature epidermis. (A) Mean (±s.e.m.) time-courses of $OH^{\bullet}$-induced K_{out} and Ca_{in} measured at +80 and –160 mV, respectively; 1 mM Cu/a added at 0 minutes. (B) The effect of omitting K^+ from the pipette solution (–K) or reducing bathing solution Ca^{2+} to 0.2 mM (–Ca) on currents measured 15 minutes after addition of 1 mM Cu/a (representative current-voltage curves). (C) Pharmacology of $OH^{\bullet}$-induced K_{out} and Ca_{in} (20 mM TEA^+, 50 μM La^{3+}, 50 μM Gd^{3+}, 20 μM verapamil, 20 mM Ca^{2+}, 20 mM Ba^{2+}).

application (for 2-4 minutes) but then dramatically increased over a 15-20 minute period, exceeding control levels several-fold (Fig. 2A). The Cu/a effect was reversible on return to control bathing solution. Reduction of Ca^{2+} in the external solution to 0.2 mM or removal of K^+ from the pipette solution abolished activation of Ca_{in} or K_{out}, respectively (Fig. 2B), consistent with a K_{out} due to K^+ efflux and a Ca_{in} due to Ca^{2+} influx.

In intact biological systems, excess Cu^{2+} or ascorbate alone are also capable of generating some $OH^{\bullet}$, but are much less potent than Cu/a (Halliwell and Gutteridge, 1999). Nevertheless, prolonged (15 minutes) exposure to external 1 mM Cu^{2+} or ascorbate alone did not activate currents in *Arabidopsis* root protoplasts; moreover, Cu^{2+} alone almost completely blocked control Ca_{in} and halved K_{out} (corresponding to initial current decrease during the first 2-4 minutes after Cu/a addition; Fig. 2A). Only long-term exposure to 1-5 mM Cu^{2+} (30 to 90 minutes) activated currents resembling Cu/a-induced currents (in 19% of protoplasts tested; n=62). Activation of Ca_{in} and K_{out} was observed even at 3 μM Cu/a (15 minutes exposure; Ca_{in} measured at –160 mV increased from –41±4 pA to –63±9 pA; K_{out} measured at +100 mV increased from +107±18 to +177±34; n=11); maximal activation was achieved at 0.3 to 1 mM Cu/a. The potent response of Ca_{in} and K_{out} to $OH^{\bullet}$ contrasts markedly with that to the non-radical H_2O_2. In mature epidermal protoplasts, prolonged (15 minutes) exposure to 1 to 20 mM H_2O_2 had no significant effect on Ca_{in}, while K_{out} even decreased (at +100 mV) by 34±3% (n=17).

Permeability of FOR-activated conductances

Selectivity estimates were made for $OH^{\bullet}$-activated conductances observed in protoplasts derived from mature epidermis of Columbia. Since, in multi-cationic conditions, using ramps or reversal potentials for measuring plant cation channel selectivity can be unreliable (Demidchik and Tester, 2002), selectivity estimates were obtained from values of conductance calculated from currents at voltages between –80 mV and –160 mV (for Ca_{in}) or between 0 mV and +80 mV (for K_{out}). For the inward-rectifying conductance, this yielded a permeability sequence of K^+ (1.00) ≈ NH_4^+ (0.91±0.13; n=4) ≈ Na^+ (0.71±0.09; n=4) ≈ Cs^+ (0.67±0.09; n=4) > Ba^{2+} (0.32±0.01; n=10) ≈ Ca^{2+} (0.24±0.01; n=10) > TEA^+ (0.09±0.01; n=7). These data show that $OH^{\bullet}$-induced Ca_{in} was mediated by nonselective cation channels [NSCC (Demidchik and Tester, 2002; Demidchik et al., 2002)] rather than the root hyperpolarisation-activated Ca^{2+} channel (Véry and Davies, 2000; Miedema et al., 2001). The TEA^+ permeability of the $OH^{\bullet}$-activated Ca_{in} was lower than that of the *Arabidopsis* root plasma membrane NSCC described previously (Demidchik and Tester, 2002), suggesting that FORs may selectively activate a particular type of NSCC. To confirm that $OH^{\bullet}$-activated Ca_{in} was mediated by NSCC rather than an inwardly rectifying K^+ channel, protoplasts derived from mature epidermis of the Columbia *akt1* mutant [that lacks the root plasma membrane K^+ inward-rectifying channel (Hirsch et al., 1998)] were tested. No significant differences in the response to 1 mM Cu/a were found between wild-type and *akt1* (in *akt1* after 15 minute exposure to 1 mM Cu/a, Ca_{in} measured at –160 mV was –121±9 pA and K_{out} measured at +100 mV was +278±28 pA; n=14). The permeability sequence of the outward-rectifying conductance agreed with that previously determined for the *Arabidopsis* root K^+ outward rectifier (Maathuis and Sanders, 1995): K^+ (1.00) > Na^+ (0.31±0.03; n=10) > Ba^{2+} (0.06±0.01; n=4) ≈ TEA^+ (0.05±0.01; n=4).

Pharmacological analysis of FOR-activated conductances

Pharmacological analysis of $OH^{\bullet}$-activated conductances (Columbia mature epidermis; Fig. 2C) further confirmed that Ca_{in} and K_{out} were mediated by different populations of cation channels. The $OH^{\bullet}$-activated Ca_{in} was insensitive to the K^+ channel blocker TEA^+ (20 mM) but was blocked significantly by the cation channel blockers La^{3+}, Gd^{3+} (both 50 μM) and verapamil (20 μM). This response to TEA^+ and lanthanides resembles that of the plasma membrane NSCC conductance shown previously to mediate Na^+ influx to *Arabidopsis* and wheat roots (Demidchik et al., 2002; Demidchik and Tester, 2002). However, the verapamil sensitivity of the $OH^{\bullet}$-activated Ca_{in} is in marked contrast to the verapamil-insensitive Na^+ influx pathway and supports the premise that FORs activate a distinct population of root NSCC. In contrast to Ca_{in}, $OH^{\bullet}$-activated K_{out} was sensitive to TEA^+, less sensitive to lanthanides and insensitive to verapamil. The block of K_{out} by TEA^+, Ca^{2+} and Ba^{2+} agrees with previous observations on the constitutive K_{out} observed in *Arabidopsis* root epidermis/cortex protoplasts (Maathuis and Sanders, 1995) and tends to confirm that the latter is activated by FORs.

Cell-specific FOR responses

The response of root epidermal cells to FORs has great physiological significance since these cells are situated at the 'root/environment boundary'. These cells are exposed to the environment and are the first challenged by pathogens and abiotic stresses. Cells situated beneath the epidermis may have a less important role in FOR production and signalling as they do not interact directly with the rhizosphere. Accordingly, $OH^{\bullet}$-induced effects in an inner tissue (the pericycle) were examined. In pericycle protoplasts identified by cell-specific GFP expression (Kiegle et al., 2000a), only slight $OH^{\bullet}$-induced increases in currents were found, occurring after a prolonged lag-phase (23±6 minutes; n=10) between application of 1 mM Cu/a and activation (Fig. 3). These data show that the response to $OH^{\bullet}$ is tissue-specific and stronger in cells that directly interact with the environment.

A novel procedure of protoplast isolation specifically from the root epidermal elongation zone permitted comparison of epidermal responses from young and mature regions (Columbia). In elongation zone protoplasts under control conditions, the background currents were small until approximately 1 hour after establishing the whole cell configuration. At this time, constitutive hyperpolarisation-activated Ca^{2+} channel (HACC) activity appeared that resembled HACC activity previously described from the root elongation zone cortex, apical epidermis and root hair (Kiegle et al., 2000a; Véry and Davies, 2000; Demidchik et al., 2003). Properties of elongation zone epidermal HACC were dissimilar to the $OH^{\bullet}$-induced conductance (data not shown).

$OH^{\bullet}$-induced currents were two- to threefold larger than in mature epidermis (n=10; Fig. 4). These results were consistent with $OH^{\bullet}$-induced K^+ efflux from intact roots (peak magnitude 262±54 and 95±25 nmol m^{-2} $second^{-1}$ for elongation and mature zone respectively; n=5) measured non-invasively using the MIFE™ technique. Thus, FOR channel activation varies with the developmental state of a given cell type.

Biotic and abiotic stress stimulate FOR production by roots

The apical position of epidermal elongation zone cells and lack of protection from the root cap mean that such cells are amongst the first to respond to changing soil environment as the root descends. Their increased sensitivity to FORs as a regulatory/stress signal could make them 'antennae' for receiving and amplifying external stimuli. To establish that *Arabidopsis* root cells generate FORs in response to stress, FOR production in intact Columbia roots was measured using lucigenin-based chemiluminometry (Papadakis and Roubelakis-Angelakis, 1999). Exposure (30 minutes) to high salt (150 mM NaCl), cold shock (2°C) and elicitors (1% cellulase) increased lucigenin chemiluminescence (which directly reflects FOR formation) by 224±14%, 253±17% and 632±55%, respectively, in comparison with the resting level (84±9.3 counts $second^{-1}$ for 100 mg fresh weight; n=7). In in vitro tests, addition of 30-100 μM Cu^{2+}/asc to the lucigenin

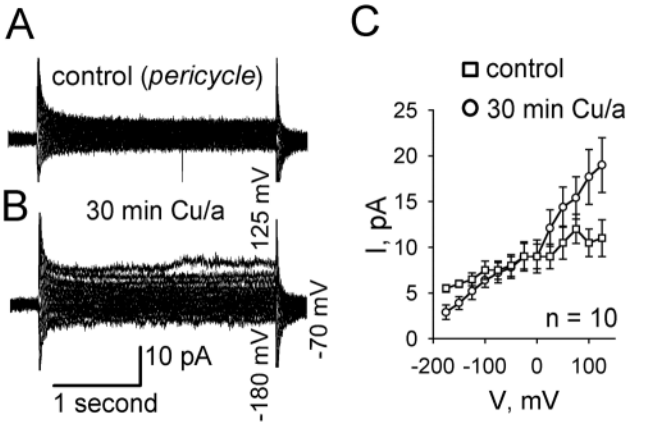

Fig. 3. Hydroxyl radical effects on *Arabidopsis* root pericycle protoplasts. (A) Whole cell currents before exposure (control). (B) Whole cell currents after 30 minutes exposure to 1 mM Cu^{2+}/ascorbate (Cu/a). (C) Mean (±s.e.m.) current-voltage curves.

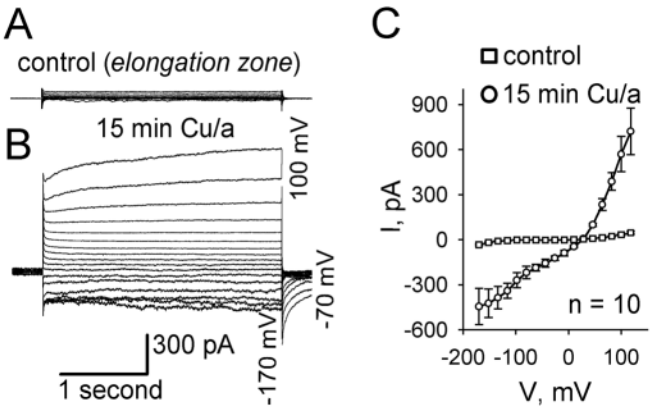

Fig. 4. Hydroxyl radical effects on *Arabidopsis* root elongation zone epidermal protoplasts. (A) Whole cell currents before exposure (control). (B) Whole cell currents after 15 minutes exposure to 1 mM Cu^{2+}/ascorbate (Cu/a). (C) Mean (±s.e.m.) current-voltage curves.

assay caused an increase in luminescence comparable with that caused by cellulase. These results (in conjunction with the patch clamp data) clearly demonstrate that *Arabidopsis* root cells generate an FOR signal in response to (a)biotic stress and FORs activate plasma membrane cation channels.

FORs and root growth

The enhanced sensitivity of elongation zone epidermis to FORs raises the possibility that the latter are involved in growth regulation. According to Cramer and Jones (Cramer and Jones, 1996), root growth (rate of cell elongation) approximately linearly depends on free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), significantly accelerating with even slight elevation in $[Ca^{2+}]_{cyt}$. Therefore the FOR activation of Ca^{2+} influx channels in the elongation zone should stimulate root growth. Comparative 12 day assays of Columbia root growth in medium containing additional Cu^{2+} [ascorbate is present in the cell wall in at least the sub-millimolar range (Smirnoff and Wheeler, 2000)] showed (mean±s.e.m.) 21.5±1.5, 39.4±1.9 and 29.2±2.1% higher growth rates in 0.3, 1 and 3 μM Cu^{2+}, respectively, than in control medium containing a non-growth-limiting Cu^{2+} concentration of 0.1 μM (6.2±0.2 mm day^{-1}; *n*=25). The cation channel blocker Gd^{3+} (5 μM added to growth medium) suppressed this Cu^{2+}-induced stimulation of root growth. As Gd^{3+} blocked the $OH^{\bullet}$-activated Ca_{in}, these results suggest that Cu^{2+}-induced increased growth rates may have been due to FOR-activated cation channels. Moreover, root growth was affected by the $OH^{\bullet}$ scavenging agent, dimethyl sulfoxide (DMSO; 0.1% induced a 26±2.2% decrease of root elongation rate; *n*=15). Although it was not possible to corroborate this result in patch clamp experiments (DMSO promoted loss of the whole cell configuration), the effect of the $OH^{\bullet}$ scavenging agent coupled with the particular $OH^{\bullet}$-sensitivity of root elongation zone protoplasts strongly suggest a role for FORs in root growth.

Verification of FOR-activated K^+ efflux and Ca^{2+} influx at the whole root level

In whole root studies to verify cellular responses at the whole organ level, Cu/a was still used to generate FORs as washing procedures and prolonged exposure to solutions was likely to deplete the cell wall of ascorbate (Smirnoff and Wheeler, 2000). Thus adding Cu^{2+} alone to generate FORs by reaction with wall ascorbate would not have been a fair test. Whole root K^+ efflux from Columbia induced by 1 mM Cu/a was measured photometrically over a 30 minute period. Mean (±s.e.m.) efflux was significantly enhanced by the presence of Cu/a at all time points, thus confirming the patch clamp data (*n*=5; Fig. 5A).

The $OH^{\bullet}$ effect on Ca^{2+} influx in whole roots was examined by applying chemiluminometry (Price et al., 1994; Kiegle et al., 2000b) to Columbia plants constitutively expressing aequorin targeted to the root cell cytosol. In agreement with the $OH^{\bullet}$-activated Ca_{in}, $OH^{\bullet}$ raised $[Ca^{2+}]_{cyt}$ (Fig. 5B). With 10 mM external Ca^{2+}, 1 mM Cu/a increased $[Ca^{2+}]_{cyt}$ from 0.114±0.01 μM to a peak of 19.15±0.89 μM (*n*=12). With 1 mM Ca^{2+} (Fig. 5B), the maximal $OH^{\bullet}$-induced $[Ca^{2+}]_{cyt}$ increase was four to five times smaller, demonstrating that the $[Ca^{2+}]_{cyt}$ increase was mainly due to Ca^{2+} influx from the external medium. The whole root $[Ca^{2+}]_{cyt}$ increase in response to Cu/a was more rapid than the current response of protoplasts. The slower activation time in protoplasts may result from the absence of cell wall that in vivo would provide an additional pool of reactants that would effectively increase the local concentration of hydroxyl radicals above that generated by Cu/asc. It is also feasible that protoplast isolation and recording conditions render channels less sensitive or responsive.

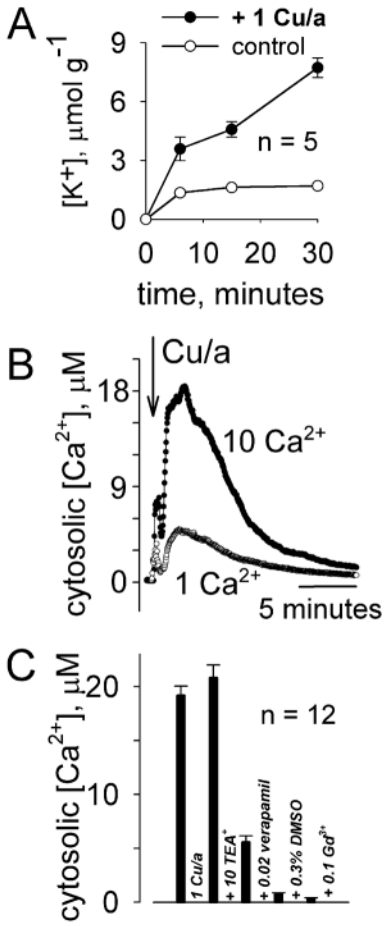

Fig. 5. Hydroxyl radical effects on *Arabidopsis* root fluxes. (A) Mean (±s.e.m.) time course of K^+ release from Columbia roots. (B) Root cell $OH^{\bullet}$-induced $[Ca^{2+}]_{cyt}$ increase measured using cytosol-targeted aequorin. Data were obtained with 1 and 10 mM ambient Ca^{2+}; 1 mM Cu/a was added as indicated by the arrow. (C) Effect of 30 minutes exposure to test compounds on $OH^{\bullet}$-induced $[Ca^{2+}]_{cyt}$ increase. Concentrations in mM.

Significantly, the $OH^{\bullet}$-scavenger DMSO reduced peak $[Ca^{2+}]_{cyt}$ increase in response to Cu/a (Fig. 5C), thus confirming $OH^{\bullet}$ as the activator FOR species (*n*=12). To confirm that the increase in $[Ca^{2+}]_{cyt}$ was the result of Ca^{2+} influx, roots were pretreated with cation channel blockers (Fig. 5C). TEA^+ (10 mM) did not inhibit the peak $OH^{\bullet}$-induced $[Ca^{2+}]_{cyt}$ increase but verapamil (20 μM) and Gd^{3+} (100 μM) were strongly inhibitory (*n*=12). Qualitatively these results match the effects of these agents on the $OH^{\bullet}$-induced Ca_{in} of mature epidermis and strongly suggest that the conductance observed in protoplasts was involved in the $OH^{\bullet}$ response of the whole root.

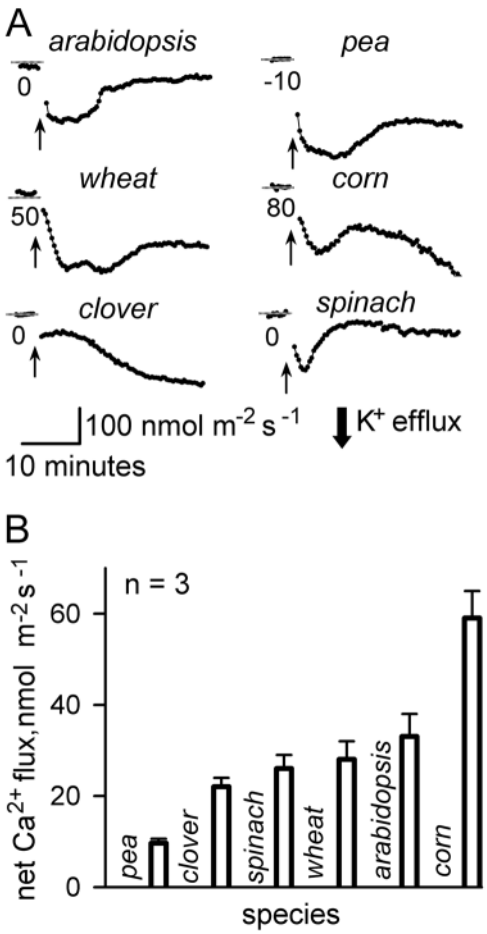

Fig. 6. Hydroxyl radical effects on epidermal root fluxes of different species. (A) Representative traces of $OH^{\bullet}$-induced net K^+ efflux measured by MIFE™ from mature epidermal cells of different species. Initial flux values are shown as dotted lines. The 1 mM Cu/a was added as indicated by arrows. (B) Hydroxyl radical-induced net Ca^{2+} influx measured by MIFE™ simultaneously with K^+ efflux. Control Ca^{2+} fluxes before Cu/a addition were negligible (up to 100 times smaller) in comparison with $OH^{\bullet}$-induced influxes. Mean values (±s.e.m.) are maximal Ca^{2+} influx between 2 to 40 minutes exposure to 1 mM Cu/a.

FOR epidermal flux activation across species

Having confirmed that channel activation can manifest at a multicellular level, the in planta time-courses of the $OH^{\bullet}$-activated net K^+ and Ca^{2+} fluxes of intact *Arabidopsis* (Columbia) root mature epidermal cells were measured simultaneously using MIFE™ (Fig. 6). Again, addition of 1 mM Cu/a caused significant K^+ efflux (Fig. 6A). Net Ca^{2+} flux shifted negative in the first 2 minutes after Cu/a addition suggesting a transient influx decrease corresponding to the Cu^{2+} suppression of Ca_{in} measured by patch clamp. This was followed by long-term Ca^{2+} influx increase consistent with the previously observed $OH^{\bullet}$-activation of Ca_{in} (Fig. 6B). The possible ubiquity of FOR responses was then confirmed as similar effects were measured in mature epidermal cells from a wide range of crop plants (including dicotyledons, monocotyledons, C3 and C4 plants). In all species tested, $OH^{\bullet}$ activated K^+ efflux and Ca^{2+} influx (Fig. 6). Moreover, flux amplitude and time-course varied between species, suggesting species-specific 'flux signatures' in response to $OH^{\bullet}$.

Discussion

It is recognised that in animal systems free oxygen radicals are not merely destructive; they can be signalling and regulatory agents (Sohal and Weindruch, 1996; Kourie, 1998; Halliwell and Gutteridge, 1999; Thannickal and Fanburg, 2000). It has become increasingly clear in plant biology that FORs can play 'positive' roles and are not simply harmful products of redox imbalance. The site of FOR production [which can be organellar, cytoplasmic or apoplastic (Bowler and Fluhr, 2000)], the local concentration and the duration of exposure will all determine whether FORs signal, regulate or damage. FORs (and the FOR precursor H_2O_2) are implicated in cellular responses to: abiotic stress and pathogen attack (Moran et al., 1994; Shen et al., 1997; Alvarez et al., 1998; Karpinski et al., 1999; Van Doorslaeder et al., 1999); cross tolerance (Bowler and Fluhr, 2000); gravitropism (Joo et al., 2001); development (Becana and Klucas, 1992; Shorning et al., 2000); and hormone action (Pei et al., 2000; Schopfer et al., 2002). Experimentally, two of the main challenges are to identify the radical effecting a response and identify its target(s). In this study it has been shown for the first time that free oxygen radicals ($OH^{\bullet}$) regulate plant ion channels; the effects of $OH^{\bullet}$ manifest at membrane, cell, tissue and whole root level.

The application of Cu/a (as a $OH^{\bullet}$-generating mixture) to the extracellular face of root epidermal protoplasts activated two distinct plasma membrane cation conductances – Ca_{in} and K_{out}. Two key lines of evidence demonstrate that channel activation was regulatory in nature and not the result of loss of membrane integrity: reversibility of activation and specificity of activation. $OH^{\bullet}$ are not membrane-permeable and most probably exerted their effect directly at the external membrane face, given that the pipette solution could not readily have supported membrane-delimited phosphorylation-based channel activation. Significantly, the FOR precursor H_2O_2 failed to activate conductances of the mature epidermis. This is in contrast to the sensitivity of guard cell Ca^{2+}-permeable NSCC to H_2O_2 (Pei et al., 2000) and confirms the premise of Mittler and Berkowitz (Mittler and Berkowitz, 2001) that H_2O_2 is not the sole oxygen-derived species capable of signalling and regulation in plants. Moreover, the results demonstrate that a given cell type can be responsive to specific oxygen-derived species.

It should be noted that, apoplastically applied, FOR species and H_2O_2 are capable of differential gene activation (Wisniewski et al., 1999; Bowler and Fluhr, 2000). It is therefore feasible that $OH^{\bullet}$-activated Ca_{in} comprises part of a signalling mechanism for Ca^{2+}-dependent gene regulation.

Analysis of the $OH^{\bullet}$-activated conductances revealed for the first time that a plant outwardly rectifying K^+ conductance (K_{out}) can be activated by FORs. Free-oxygen-radical-activated K^+ channels have been previously described in many animal preparations and play crucial roles in animal cell physiology (Kourie, 1998).

Permeation and pharmacological profiles demonstrated that the $OH^{\bullet}$-activated Ca_{in} is mediated by a specific group of NSCC that do not share identity with the *Arabidopsis* NSCC involved in toxic Na^+ influx (Demidchik and Tester, 2002;

Demidchik et al., 2002). Whether this group of NSCC comprises more than one channel type is a question that must now be addressed by extensive biophysical studies. The presence of rapidly and slowly activated components of $OH^{\bullet}$-activated currents strongly suggests that several cation channel types are activated. The finding of a novel group of channels points not only to differential regulation and physiological role of root plasma membrane NSCC but also to specific genetic identities.

The cell and tissue-specific responses to $OH^{\bullet}$ indicate precise functions for FORs and the target channels. The epidermis marks the boundary between the plant and its environment; the greater sensitivity of the epidermis (relative to the pericycle) suggests that its $OH^{\bullet}$-activated Ca_{in} plays a signalling role in the perception of and response to rhizosphere challenges. As whole root studies showed that $OH^{\bullet}$ generates $[Ca^{2+}]_{cyt}$ transients, it is reasonable to suppose that epidermal $OH^{\bullet}$-activated NSCC function in this context (a view supported by the similar blocker profiles of transients and Ca_{in}). Abiotic and biotic stresses (such as salinity and pathogen attack) are known to induce oxidative bursts which vary in time of onset, cellular origin and duration [from minutes to several hours (Bowler and Fluhr, 2000)]. That *Arabidopsis* roots respond to salinity, cold shock and elicitors by releasing FORs has been confirmed in the present study. Given the short half life of FORs and their detection in the recording medium, it is likely that the epidermis was involved in their production. The mechanism of stress-induced apoplastic FOR production can only be speculated on but could include the NADPH oxidase [involved in pathogen responses (Torres et al., 1998; Bowler and Fluhr, 2000)], cell wall amine peroxidases and the conversion of H_2O_2 to $OH^{\bullet}$ by cell wall Fe^{2+} or Cu^{2+}. The root is therefore capable of generating FORs in response to stress and the epidermis could respond with a $OH^{\bullet}$-activated Ca_{in}-mediated $[Ca^{2+}]_{cyt}$ elevation. Additionally, K^+ efflux is one of the earliest cellular stress responses (Babourina et al., 2000; Bowler and Fluhr, 2000). A mechanistic basis of that response has been demonstrated here: FOR-activated K^+ efflux through K^+ outward rectifying channels.

Calcium may lie upstream or downstream of stress-induced FOR production in plant tissues (Bowler and Fluhr, 2000), illustrating the complexity of the signalling networks. It is clear now that even if $[Ca^{2+}]_{cyt}$ changes were upstream, the $OH^{\bullet}$-activated Ca_{in} is competent to mediate any subsequent Ca^{2+} influx to amplify and propagate the signal. In this respect, it is significant that the NADPH oxidase thought to be responsible for pathogen-elicited oxidative bursts contains an EF-hand indicative of Ca^{2+} stimulation (Torres et al., 1998). Increased apoplastic superoxide anion production by a Ca^{2+}-stimulated NADPH oxidase would result in $OH^{\bullet}$ at the external face of the plasma membrane through superoxide/H_2O_2 interaction and H_2O_2 breakdown catalysed by cell wall transition metals (Fry, 1998; Halliwell and Gutteridge, 1999). This would activate Ca_{in}. Moreover, sustained $[Ca^{2+}]_{cyt}$ elevation caused by lanthanide-sensitive Ca^{2+} influx is required for defence-gene transcription associated with radical production (Blume et al., 2000), and Ca_{in} appears competent to mediate such a $[Ca^{2+}]_{cyt}$ elevation.

Plant roots grow in length by Ca^{2+}-dependent cell elongation at the root apex (Cramer and Jones, 1996). In the elongation zone, the $OH^{\bullet}$-induced Ca^{2+} currents were significantly larger than in mature cells, therefore differential channel sensitivity to FORs can define the zone of root Ca^{2+} uptake. That the $OH^{\bullet}$-scavenger DMSO inhibited root elongation supports the possibility of FOR involvement in regulating cell extension. Moreover, as $OH^{\bullet}$ are implicated in xyloglucan remodeling, which would increase wall extensibility in the elongation zone (Fry, 1998; Vissenberg et al., 2000), FORs may be a co-ordinating factor for the Ca^{2+} influx and wall extension necessary for growth. In this context, it is significant that hydroxyl radical involvement in auxin-induced extension growth of coleoptiles has been recently reported (Schopfer et al., 2002). Another possible FOR-regulated process (also centered at the elongation zone) is the root gravitropic response. Gravistimulated FOR production of the elongation zone greatly exceeds that of the mature region (Joo et al., 2001). Since elevation of $[Ca^{2+}]_{cyt}$ is implicated in the gravitropic response (Joo et al., 2001), the finding here of potent $OH^{\bullet}$ activation of Ca_{in} in the elongation zone is significant. Placing the $OH^{\bullet}$-activated K_{out} in these contexts is problematic as its activity would depend on the equilibrium potential for K^+ (E_K; which in turn depends on external K^+) relative to the resting membrane voltage. It has been proposed that K^+ efflux helps counteract the depolarizing effect of Ca^{2+} influx on membrane voltage (Miedema et al., 2001), thus allowing net Ca^{2+} influx to proceed. Harnessing both Ca^{2+} and K^+ conductances to FOR regulation in the elongation zone may ensure secure membrane voltage regulation in this critical region.

Overall, it is now clear from this study that root FORs have precise cellular targets and obvious functions. The effect of FORs is not restricted to the root cell of a model plant but is observed in those of monocots and dicots, C3 and C4 plants. The response of each species varies in time course and magnitude but the fundamental response of K^+ efflux and Ca^{2+} influx is conserved. The observed effects of $OH^{\bullet}$ at the cell, tissue and species level have begun to reveal an intricate regulatory network involving apoplastic FORs, in which channel-mediated events will depend on the FOR species, its local concentration and duration of exposure.

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