

***Arabidopsis* Annexin1 Mediates the Radical-Activated Plasma Membrane Ca²⁺- and K⁺-Permeable Conductance in Root Cells**^W

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Plant cell growth and stress signaling require Ca²⁺ influx through plasma membrane transport proteins that are regulated by reactive oxygen species. In root cell growth, adaptation to salinity stress, and stomatal closure, such proteins operate downstream of the plasma membrane NADPH oxidases that produce extracellular superoxide anion, a reactive oxygen species that is readily converted to extracellular hydrogen peroxide and hydroxyl radicals, OH[•]. In root cells, extracellular OH[•] activates a plasma membrane Ca²⁺-permeable conductance that permits Ca²⁺ influx. In *Arabidopsis thaliana*, distribution of this conductance resembles that of annexin1 (ANN1). Annexins are membrane binding proteins that can form Ca²⁺-permeable conductances in vitro. Here, the *Arabidopsis* loss-of-function mutant for annexin1 (*Atann1*) was found to lack the root hair and epidermal OH[•]-activated Ca²⁺- and K⁺-permeable conductance. This manifests in both impaired root cell growth and ability to elevate root cell cytosolic free Ca²⁺ in response to OH[•]. An OH[•]-activated Ca²⁺ conductance is reconstituted by recombinant ANN1 in planar lipid bilayers. ANN1 therefore presents as a novel Ca²⁺-permeable transporter providing a molecular link between reactive oxygen species and cytosolic Ca²⁺ in plants.

INTRODUCTION

Plant cells use cytosolic free Ca²⁺ ([Ca²⁺]_{cyt}) in signal transduction and growth, yet the molecular identities of the

Ca²⁺-permeable transporters that permit Ca²⁺ influx remain largely undiscovered (reviewed in Dodd et al., 2010). Two Pore Channel1 releases vacuolar Ca²⁺ in *Arabidopsis thaliana* (Dodd et al., 2010). The Cyclic Nucleotide-Gated Channel (CNGC) family is implicated, with CNGC2 forming a plasma membrane (PM) Ca²⁺-permeable channel in *Arabidopsis* for defense and senescence (Dodd et al., 2010; Ma et al., 2010). *Arabidopsis* loss-of-function mutants implicate glutamate receptor-like channels in stress signaling and pollen tube growth (Qi et al., 2006; Dodd et al., 2010; Michard et al., 2011). As yet, the genetic identities of the PM Ca²⁺ influx transporters that are regulated by reactive oxygen species (ROS) to elevate [Ca²⁺]_{cyt} are unknown. In root cell growth, adaptation to salinity stress, and stomatal closure, these operate downstream of the PM NADPH oxidases that produce extracellular superoxide anion, a ROS readily converted to extracellular hydrogen peroxide and hydroxyl radicals, OH[•] (Demidchik et al., 2003, 2010; Foreman et al., 2003; Kwak et al., 2003; Chung et al., 2008; Dodd et al., 2010; Laohavisit et al., 2010). In plants, extracellular OH[•] are thought to coordinate growth and stress responses (Foreman et al., 2003; Renew et al., 2005; Müller et al., 2009; Demidchik et al., 2010). The *Arabidopsis* root epidermal PM contains a Ca²⁺-permeable conductance (activated by

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extracellular OH[•]), operating in cell elongation and implicated in sodicity stress signaling (Demidchik et al., 2003; Foreman et al., 2003; Chung et al., 2008). In elongation, the conductance lies downstream of the Respiratory Burst Oxidase Homolog C (RBOHC) NADPH oxidase (Foreman et al., 2003) that is the initial source of ROS. The conductance resembles that formed by purified maize (*Zea mays*) annexins incorporated into artificial bilayers containing a lipid peroxidation product (Laohavisit et al., 2010). Hence, an *Arabidopsis* annexin could be responsible for forming the PM OH[•]-activated Ca²⁺ conductance.

Annexins are membrane binding proteins found in pro- and eukaryotes (Morgan et al., 2006). Several animal annexins have been reported to function in vitro as Ca²⁺ channels, including vertebrate annexins A1, 2, 5 to 7, and 12 (Burger et al., 1994; Liemann et al., 1996; Kourie and Wood, 2000). Loss-of-function mutants may have impaired ability to regulate [Ca²⁺]_{cyt}, for example, A5 (–/–) chicken DT40 cells (Kubista et al., 1999), A7 (+/–) murine brain cells (Watson et al., 2004), and A7 (–/–) murine cardiomyocytes (Schrickel et al., 2007). Annexin A5 binds to peroxidized membranes (Balasubramanian et al., 2001) and contributes to peroxide-induced Ca²⁺ influx in chicken DT40 cells (Kubista et al., 1999). However, no studies have causally linked in vitro annexin transport activity to that in native membrane (Konopka-Postupolska et al., 2011). Annexins are abundant throughout the plant kingdom (reviewed in Laohavisit and Davies, 2011a), and some contain the charged residues found in the hydrophobic pore of channel-forming animal annexins (Kourie and Wood, 2000; Laohavisit et al., 2009; Laohavisit and Davies, 2011a). Of these, *Capsicum annuum* annexin32/24 (ANN32/24) mediates Ca²⁺ influx in liposomes (Hofmann et al., 2000), while maize annexins ANN33 and ANN35 support a Ca²⁺-permeable conductance in planar lipid bilayers (PLBs) (Laohavisit et al., 2009, 2010), and *Arabidopsis* ANN1 forms a K⁺-permeable channel in PLB (Gorecka et al., 2007). More compelling evidence for annexins as ion transport pathways requires electrophysiological analysis of annexin mutants (Laohavisit et al., 2009; Konopka-Postupolska et al., 2011).

Expression and abundance of *Arabidopsis* ANN1 matches the occurrence of the OH[•]-activated PM Ca²⁺ conductance in the root epidermis and at the apex of root hairs (Clark et al., 2001, 2005; Dinneny et al., 2008). ANN1 transcript abundance decreases by 24% as root epidermal cells mature (Dinneny et al., 2008), and the magnitude of the root epidermal PM OH[•]-activated conductance decreases by ~75% (Demidchik et al., 2003). ANN1 (one of eight annexins; Clark et al., 2001) can exist as an integral PM protein (Alexandersson et al., 2004; Lee et al., 1998; Santoni et al., 2004; Benschop et al., 2007; Marmagne et al., 2007). This makes ANN1 a prime candidate for the root epidermal PM OH[•]-activated conductance. We tested ANN1 function by applying electrophysiological analyses to the homozygous *ann1* knockout mutant and the complemented mutant *ann1/ANN1* (Lee et al., 2004). Recombinant ANN1 was used to reconstitute an OH[•]-activated Ca²⁺-permeable conductance in PLBs. Cytosolic aequorin was deployed to assess the impact of *ann1* on OH[•]-activated [Ca²⁺]_{cyt} elevation. The results show that ANN1 supports the OH[•]-activated

conductance of the root epidermal PM, contributes to regulation of [Ca²⁺]_{cyt}, and plays a role in growth.

RESULTS

ann1 Lacks the PM OH[•]-Activated Conductance

Spheroplasts were released from the apices of young wild-type root hairs and the *ann1* homozygous single insert T-DNA knockout mutant (Lee et al., 2004). PM currents were obtained using the patch clamp whole-cell recording mode. In this, currents are generated as populations of channels open; negative current is influx of cations into the cytosol or efflux of anions. Ionic conditions were used previously to delineate OH[•]-activated currents in the wild type (Demidchik et al., 2003; Foreman et al., 2003). In controls, small inward and outward cation currents were observed at hyperpolarized and depolarized voltages, respectively, in both genotypes (Figures 1A and 1B). Mean values were not statistically significantly different between the wild type and *ann1* (see Supplemental Table 1 online; *n* = 6). Wild-type apical PM supported increased inward and outward cation currents in response to the generation of extracellular OH[•] by 1 mM CuCl₂ and 1 mM ascorbic acid (Cu-Asc; Demidchik et al., 2003) (Figure 1A; see Supplemental Table 1 online; *n* = 6). The conductance would be competent in Ca²⁺ influx across the PM at root hair apical voltages (Felle et al., 1992). OH[•]-activated currents did not occur in *ann1* (Figure 1B; see Supplemental Table 1 online; *n* = 6). Root hair spheroplasts from the complemented *ann1/ANN1* line were too fragile for use in patch clamp recordings.

Protoplasts were obtained from mature cells of wild-type and *ann1* root epidermis. Control PM currents were not statistically significantly different between the wild type, *ann1*, and *ann1/ANN1* (Figures 1C to 1E; see Supplemental Table 1 online; *n* = 6). In controls, Cu or Asc alone had no significant effect on currents (see Supplemental Figures 1A to 1D online; *n* = 3), in agreement with previous studies (Demidchik et al., 2003; Foreman et al., 2003). Rectifying inward and outward currents were evoked when OH[•] was generated by 1 mM Cu-Asc at the extracellular membrane face of the wild type (Figure 1C; see Supplemental Table 1 online; *n* = 6). Previously, removal of Ca²⁺ from the bath or K⁺ from the pipette abolished the inward current and outward current, respectively (Demidchik et al., 2003). Here, when Ba²⁺ replaced Ca²⁺ in the bathing solution, an inward current was still observed in response to OH[•] (see Supplemental Figure 1E online; *n* = 3), a response seen previously in root hair and epidermal PM (Demidchik et al., 2003; Foreman et al., 2003). As Ba²⁺ permeates Ca²⁺-permeable channels but not K⁺-inward rectifiers (Véry and Davies, 2000), these data confirm that an inward Ca²⁺ current was activated. At voltages more negative than the reversal potential for K⁺ (*E*_K), it is feasible that K⁺ made a small contribution to the inward current (see Supplemental Figure 1E online). Time dependency was more pronounced than reported previously for this cell type (Demidchik et al., 2003). The conductance would be competent in PM Ca²⁺ influx at normal root epidermal voltages (Demidchik et al., 2002). Currents were effectively abolished in *ann1* (even with increased observation

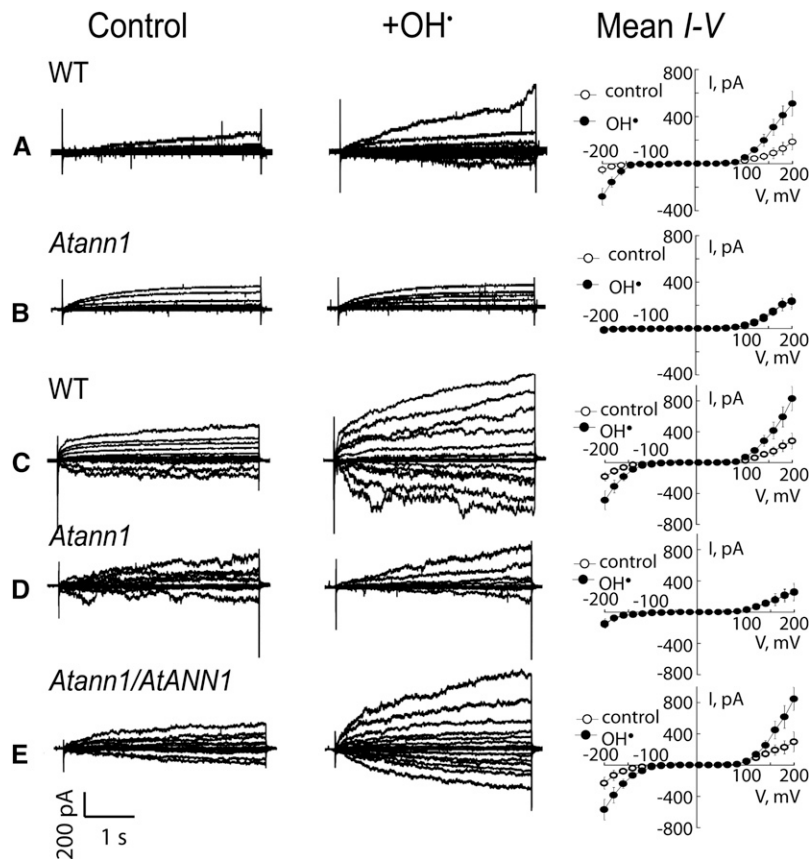


Figure 1. *ann1* Root Plasma Membrane Lacks the OH[•]-Activated Conductance.

(A) Whole-cell patch clamp recordings from wild-type (WT; Col-0) root hair apical spheroplast PM. Left: Control currents (*I*) elicited by step voltage (*V*) changes, from a representative spheroplast. Centre: *I* after exposure to extracellular OH[•] generated by 1 mM copper and 1 mM ascorbic acid (Cu-Asc). Holding potential was 0 mV. Right: mean ± SE, *I*-*V* relationships for control (open circles) and OH[•] exposure (*n* = 6). *I* below the *V* axis is cation entry into the spheroplast.

(B) As in **(A)** but for *ann1* (*n* = 6). Spheroplasts from the complemented mutant proved too fragile to patch clamp.

(C) As in **(A)** but for wild-type root epidermal protoplasts (*n* = 6).

(D) *ann1* root epidermal protoplasts (*n* = 6).

(E) Complemented mutant *ann1/ANN1* (*n* = 6). Bathing medium (mM): 20 CaCl₂, 0.1 KCl, 0.02 NaCl, and 5 MES-Tris, pH 5.6, adjusted to 270 mOsm with D-sorbitol. Pipette solution: 40 K-gluconate, 10 KCl, 0.4 CaCl₂, 1 mM BAPTA, and 2 MES-Tris, pH 7.2, adjusted to 270 mOsm with D-sorbitol.

time) but were restored in *ann1/ANN1* (Figures 1D and 1E; see Supplemental Table 1 online; *n* = 6). That *ann1/ANN1* currents were not greater than the wild type may indicate that ANN1 abundance or activity at the PM is tightly regulated in this cell type. Wild-type root hair apical PM and the root epidermal PM also contain a constitutive hyperpolarization-activated Ca²⁺ channel (HACC) that is involved in elongation (Véry and Davies, 2000; Demidchik et al., 2002). *ann1* spheroplasts still retained HACC activity, demonstrating that the HACC has a distinct molecular identity (Figure 2; *n* = 3). HACC-mediated current at -200 mV was approximately double that of the OH[•]-activated Ca²⁺ conductance. Accordingly, *ann1* root hairs still grew but were significantly shorter than the wild type on either low or replete nutrient medium. Growth was restored in *ann1/ANN1* (mean mature length, μm ± SD; low nutrient: wild type 383 ± 114, *ann1* 249 ± 117 [*P* = 0.001, Student's *t* test], *ann1/ANN1* 401 ± 129; replete medium: wild type 457 ± 128, *ann1* 404 ±

124 [*P* = 0.005], *ann1/ANN1* 435 ± 99; 100 root hairs per determination). These data are consistent with ANN1's operation in root hair elongation and the previous observation of short *ann1* primary roots (Clark et al., 2005).

***ann1* Is Impaired in [Ca²⁺]_{cyt} Elevation**

Extracellular OH[•] activate Ca²⁺ influx across the PM and cause elevation of [Ca²⁺]_{cyt} in root epidermis and root hairs (Demidchik et al., 2003; Foreman et al., 2003). As the PM OH[•]-activated Ca²⁺-permeable conductance, ANN1 should be capable of elevating [Ca²⁺]_{cyt} and its loss of function would be expected to manifest as an impaired OH[•]-induced [Ca²⁺]_{cyt} increase. To test this, the *ann1* mutant was transformed to express cytosolic (apo)aequorin constitutively. When single excised roots were challenged with extracellular OH[•] (generated by Cu-Asc), a transient increase in [Ca²⁺]_{cyt} was observed in both genotypes,

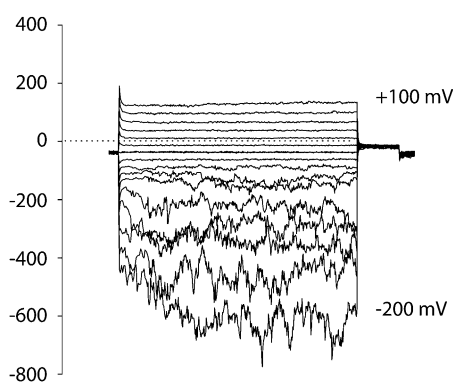


Figure 2. *ann1* Root Hair Apical PM Retains the Constitutive HACC Conductance.

Representative currents recorded in the whole-cell patch clamp configuration from an *ann1* root hair apical spheroplast PM showing the HACC conductance that is a characteristic of the root hair apex. No OH^{\bullet} were used in this experiment, and Ba^{2+} replaced Ca^{2+} in the bathing medium. Current flowing below the time axis is cation entry into the protoplast. Bathing solution comprised 10 mM BaCl_2 and 2 mM MES-Tris, pH 6, adjusted to 275 mOsm with D-sorbitol. Pipette solution comprised 0.5 mM CaCl_2 , 8.5 mM $\text{Ca}(\text{OH})_2$, 2 mM MgATP, 0.5 mM Tris-ATP, 10 mM BAPTA (final free Ca^{2+} , 1 μM), and 15 mM HEPES-Tris, pH 7.3, adjusted to 275 mOsm with D-sorbitol.

but there was no significant difference between maximum $[\text{Ca}^{2+}]_{\text{cyt}}$ of the wild type ($1.16 \pm \text{SE } 0.03 \mu\text{M}$, $n = 3$) and mutant ($1.16 \pm 0.06 \mu\text{M}$, $n = 3$; $P = 0.999$, Student's *t* test). $[\text{Ca}^{2+}]_{\text{cyt}}$ returned to its basal level ($\sim 0.2 \mu\text{M}$) after ~ 70 s in both genotypes. However, mathematical simulations suggest that cell-specific changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ might not manifest at the whole-organ level (Dodd et al., 2006). Therefore, protoplasts were isolated from *ann1* root epidermis to test for a lesion in $[\text{Ca}^{2+}]_{\text{cyt}}$ regulation.

Buffer alone applied to root epidermal protoplasts elicited a transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increase (touch response) of $0.82 \pm \text{SE } 0.15 \mu\text{M}$ in the wild type ($n = 4$) and $0.58 \pm 0.11 \mu\text{M}$ in *ann1* (Figure 3A; $n = 4$). Differences were not statistically different. Basal $[\text{Ca}^{2+}]_{\text{cyt}}$ was restored 10 s after the touch stimulus. The $[\text{Ca}^{2+}]_{\text{cyt}}$ increase evoked by extracellular OH^{\bullet} was greater and more prolonged than the touch response in both genotypes. The maximum OH^{\bullet} -induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ occurred after the maximum touch response (Figure 3A): $1.45 \pm 0.058 \mu\text{M}$ in the wild type (at 4 s after stimulus, $n = 4$) and $1.12 \pm 0.084 \mu\text{M}$ in *ann1* (at 5 s after stimulus, $n = 4$), a statistically significant 23% loss of signal in the mutant ($P = 0.018$). Basal $[\text{Ca}^{2+}]_{\text{cyt}}$ was regained more quickly in the mutant than the wild type, and when the area under the curve was calculated (36 to 75 s), $[\text{Ca}^{2+}]_{\text{cyt}}$ was statistically significantly lower in the mutant, consistent with a lesion in a Ca^{2+} influx pathway (wild type, $23.85 \pm \text{SE } 0.9 \mu\text{M}$; *ann1*, $17.08 \pm 1.51 \mu\text{M}$, $P = 0.0084$). That genotypic differences could be resolved at the population level of a single cell type rather than organ upholds the modeling predictions of Dodd et al. (2006). The lesion in the $[\text{Ca}^{2+}]_{\text{cyt}}$ response may be limited to the epidermis but does not appear to prevent propagation of the $[\text{Ca}^{2+}]_{\text{cyt}}$ response at the whole-root level.

To test if $[\text{Ca}^{2+}]_{\text{cyt}}$ increases came from PM Ca^{2+} influx, Gd^{3+} (300 μM) was applied prior to the stimulus to block influx

channels. In Gd^{3+} controls, the touch response was $0.24 \pm 0.083 \mu\text{M}$ in the wild type and $0.3 \pm 0.91 \mu\text{M}$ in *ann1* (Figure 3B; $n = 3$), suggesting ANN1 does not participate in the touch response. Aligning time courses (Figure 3B) indicated that the touch response would be completed when the maximum response to OH^{\bullet} occurred. Gd^{3+} delayed and weakened wild-type maximum response to OH^{\bullet} ($0.86 \pm 0.085 \mu\text{M}$, 11 s after application; $n = 3$; $P = 0.0019$), indicating block of PM Ca^{2+} influx. Wild-type maximum $[\text{Ca}^{2+}]_{\text{cyt}}$ response to OH^{\bullet} under Gd^{3+} block was not significantly different to that of *ann1* without block ($1.12 \pm 0.084 \mu\text{M}$), suggesting that Gd^{3+} -sensitive influx in the wild type was accounted for by ANN1 (Figure 3C). However, the presence of non-ANN1 transport routes was apparent in the response to OH^{\bullet} of *ann1* under Gd^{3+} block (Figures 3B and 3C). There, the maximum $[\text{Ca}^{2+}]_{\text{cyt}}$ response was $0.71 \pm 0.10 \mu\text{M}$ (12 s after application; $n = 3$), which was significantly lower than in *ann1* without Gd^{3+} ($1.12 \pm 0.084 \mu\text{M}$; $P = 0.025$). While consequences of non-ANN1 transporters are evident in both genotypes, overall, the data confirm ANN1's acting in PM Ca^{2+} influx to mediate the $[\text{Ca}^{2+}]_{\text{cyt}}$ response to OH^{\bullet} in a cell type known to support high levels of ANN1 expression and harbor the OH^{\bullet} -activated PM cation conductance.

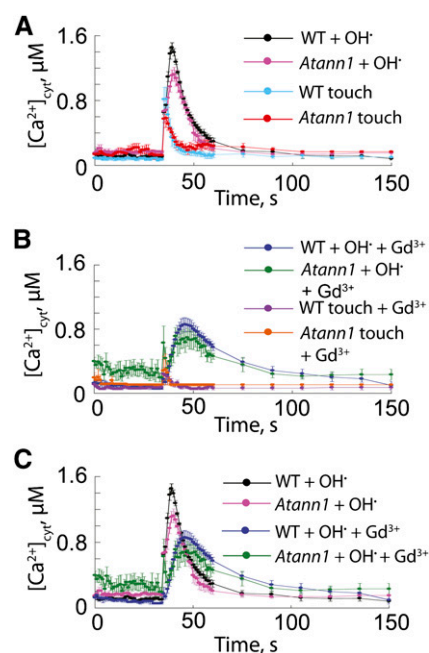


Figure 3. OH^{\bullet} -Activated $[\text{Ca}^{2+}]_{\text{cyt}}$ Increase in Root Epidermal Protoplasts Is Impaired in *ann1*.

(A) Root epidermal protoplast $[\text{Ca}^{2+}]_{\text{cyt}}$ was measured with cytosolic aequorin. Control touch response (buffer only at 35 s) was similar in the wild type (WT) and *ann1*. OH^{\bullet} was generated by 0.5 mM Cu-Asc. Data are mean $\pm \text{SE}$ ($n = 4$). Buffer comprised 10 mM CaCl_2 , 0.1 mM KCl, and 2 mM Tris/MES, pH 5.8, adjusted to 270 mOsm with D-sorbitol.

(B) As in (A) but protoplasts were incubated with 300 μM GdCl_3 for 30 min prior to $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement ($n = 3$).

(C) Summary plot of experiments from (A) and (B) showing the effect of Gd^{3+} on OH^{\bullet} -induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses of wild-type and *ann1* protoplasts.

ANN1-Mediated K⁺ Efflux Conductance Is Distinct to That of a Shaker-Like K⁺ Channel

Root epidermal PM contains an OH⁻-activated Shaker-like K⁺ efflux channel, GORK (for guard cell outward rectifying K⁺ channel; Hosy et al., 2003), implicated in stress-induced cell death (Demidchik et al., 2010). Normal OH⁻-activated currents were observed in *gork* root epidermal PM, using the same conditions as for *ann1* (Figure 4; see Supplemental Table 1 online; *n* = 6). The OH⁻-activated K⁺ efflux conductance seen in the wild type is most probably generated directly by ANN1; therefore, two distinct OH⁻-activated K⁺ efflux pathways operate in root epidermis. As *gork* root growth is normal (Hosy et al., 2003), these data support a specific role for ANN1 in root elongation.

K⁺ Efflux Is Impaired in Intact *ann1* Cells

The PM voltage of single, intact root epidermal cells in the elongation zone (measured with an impalement microelectrode) did not differ significantly between genotypes: the wild type ($-142 \pm \text{SE } 4 \text{ mV}$; *n* = 7), *ann1* ($-141 \pm 4 \text{ mV}$; *n* = 7), and *ann1/ANN1* ($-134 \pm 7 \text{ mV}$; *n* = 5). Respiratory inhibition with 0.1 mM NaCN and salicyl hydroxamic acid, to inhibit PM H⁺-ATPase-mediated H⁺ extrusion and reveal passive transport events (Leidi et al., 2010), evoked rapid membrane depolarization until the diffusion potential (E_D) was attained. E_D values were comparable between the wild type ($-90 \pm 4 \text{ mV}$; *n* = 7) and *ann1/ANN1* ($-87 \pm 4 \text{ mV}$; *n* = 5) but were more negative in the *ann1* mutant ($-103 \pm 3 \text{ mV}$; *n* = 7; *P* = 0.05). Extracellular OH⁻ evoked a small and transient hyperpolarization followed by a depolarization in the wild type (Figure 5A; *n* = 3) and *ann1/ANN1*. Hyperpolarization was not observed in *ann1*

(Figure 5A; *n* = 3), and no differences were found in E_D values 5 min after OH⁻ treatment. Lack of arrest and reversal of the hyperpolarization in *ann1* is consistent with ANN1-mediated K⁺ efflux (E_K was estimated to be approximately -156 mV) in response to extracellular OH⁻.

Loss of AtANN1 effectively abolished the net K⁺ efflux from the root elongation zone in response to extracellular OH⁻ (Figures 5B and 5C; *n* = 5; no respiratory inhibitors). Maximum net K⁺ efflux from *Atann1* was $-26 \pm \text{SE } 7 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (*n* = 5) compared with the wild type's $-765 \pm 149 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Figure 5B; *n* = 5). Wild-type K⁺ efflux was inhibited by 20 mM tetraethylammonium⁺ (TEA), leaving a residual net flux with a maximum of $-198 \pm 12 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (*n* = 5) at 9.4 min after OH⁻ generation. Given that *Atann1* did not sustain a K⁺ efflux response, this incomplete inhibition by TEA⁺ in the wild type may represent incomplete penetration of this blocker. Complementation restored OH⁻-induced net K⁺ efflux to 72% of the wild-type maximum (*n* = 5) but altered the time course of the response so that the peak flux occurred 6.3 min after the OH⁻ stimulus compared with the wild type at 16.8 min. Reasons for this are unclear at present. At the mature epidermis, kinetics and magnitude of net K⁺ efflux elicited by OH⁻ from the wild type differed from that at the elongation zone (Figure 5D; *n* = 5). Maximum efflux was lower in the mature epidermis, a pattern observed previously (Demidchik et al., 2003). TEA⁺ abolished the maximum component of the wild-type efflux response (wild type, $-310 \pm 46 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 21.6 min; TEA⁺, $-55 \pm 15 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 21.6 min, *n* = 5), lowering it to the response shown by *ann1* ($-87 \pm 8 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 21.6 min; Figure 5D; *n* = 5). Thus, the wild type's maximum component can be attributed to ANN1. Appreciable OH⁻-induced net K⁺ efflux remained in *ann1*, but this was effectively abolished by TEA⁺ ($-6 \pm 3 \text{ nmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 21.6 min; Figure 5D; *n* = 5).

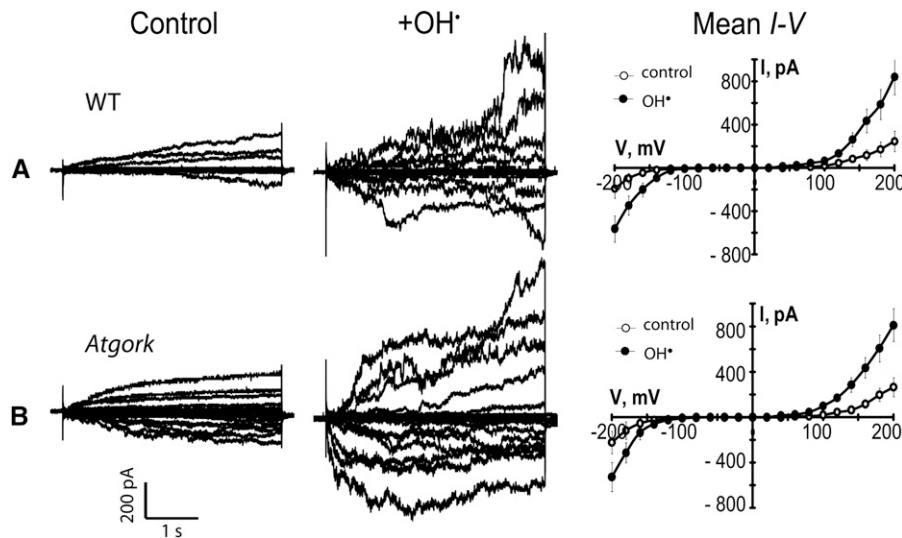


Figure 4. *Atgork* Root Epidermal PM Retains the OH⁻-Activated Conductance.

(A) Whole-cell recordings from wild-type (WT; Wassilewskija) root epidermal protoplasts. Left: Current traces under control conditions, elicited by step changes in voltage from a representative protoplast. Center: the same protoplast after exposure to extracellular OH⁻ generated by 1 mM Cu/Asc. Right: Mean \pm SE current-voltage (*I-V*) relationships for control (open circles) conditions and OH⁻ exposure (*n* = 6). Recording conditions are as in Figure 1. **(B)** As in **(A)** but with data gathered from *gork* (*n* = 6).

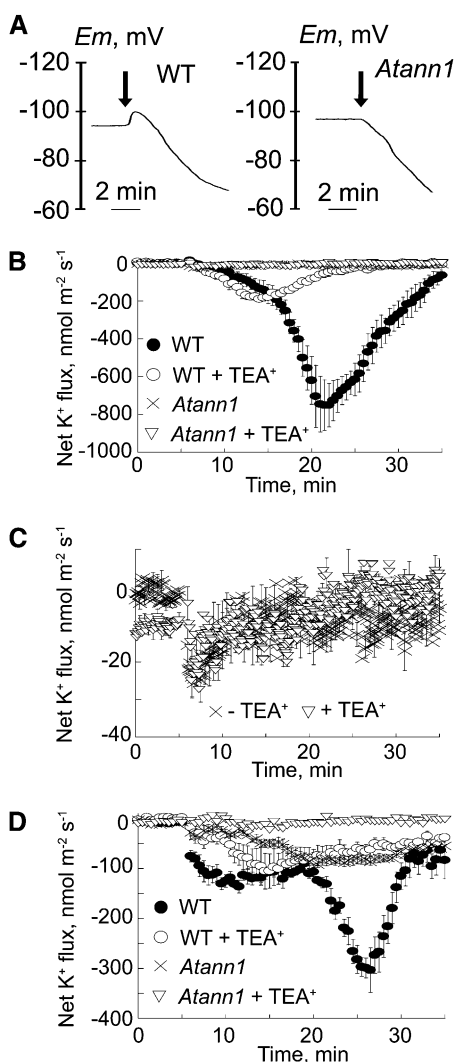


Figure 5. *ann1* Perturbs Diffusion Potentials and OH⁻-Activated Net K⁺ Efflux of the Root Epidermis.

(A) Representative recordings of PM potential from wild-type (WT; Col-0) and *ann1* root elongation zone epidermis. Respiratory blockade was imposed by 0.1 mM NaCN-salicyl hydroxamic acid to reveal the diffusion potential (E_D). Extracellular OH⁻ were generated by 0.5 mM Cu-Asc addition, as indicated by the arrow, resulting in a hyperpolarization in the wild type (left) but not *ann1* (right) followed by a similar depolarization in both lines ($n = 3$). Lack of arrest and reversal of the hyperpolarization in *ann1* is consistent with ANN1-mediated K⁺ efflux (E_K was estimated to be approximately -156 mV) in response to extracellular OH⁻.

(B) Net K⁺ efflux recorded from the wild-type (closed circles) and *ann1* (crosses) elongation zone epidermis using an extracellular vibrating K⁺ electrode. Extracellular OH⁻ were generated by 1 mM Cu-Asc addition at time = 0 min. TEA⁺ was 20 mM (wild type, open circles; *Atann1*, open triangles). Negative values signify net efflux. Data are mean \pm SE of five trials.

(C) *ann1* elongation zone response from **(B)** at greater resolution.

(D) As in **(B)** but values were recorded at the mature epidermis ($n = 5$). Recordings in the first 60 s after this addition were discarded to allow for establishment of diffusion gradients.

Purification of Recombinant ANN1

As a final test of the identity of the protein mediating the OH⁻-activated conductance, recombinant ANN1 was produced for incorporation in PLB. ANN1 was cloned from the wild type (Columbia-0 [Col-0]), and the predicted protein sequence matched that reported in the National Center for Biotechnology Information database for ANN1 (NP 174810.1). ANN1 was expressed in *Saccharomyces cerevisiae* (a null background for annexins; Creutz et al., 1992), and the protein was obtained by three cycles of Ca²⁺-dependent binding to asolectin liposomes (Laohavisit et al., 2009). ANN1 (37 kD, matching the predicted mass) was detected in the supernatant by Coomassie blue staining and confirmed by immunoblotting using an anti-ANN1 peptide antibody (Figure 6). Silver staining revealed several contaminant protein bands, including a predominant band at ~ 60 kD, which was also found previously (Creutz et al., 1992) using *S. cerevisiae* to produce recombinant mammalian annexins. Analysis of the 60-kD yeast protein using matrix-assisted laser desorption/ionization (MALDI) peptide mass fingerprinting (searched against the yeast protein database) suggested three possible identities. These were Nuclear Signal Recognition1 (gi: 1323271), FK506-Sensitive Proline Rotamase3 (gi: 530998), and Survival Factor1 (gi: 6320553). ANN1 was not detected in preparations from an empty vector control.

Size exclusion chromatography was used to remove contaminants, an approach used previously for maize annexin purification (Laohavisit et al., 2009). The resultant fraction contained only ANN1, as shown by Coomassie blue staining, silver staining for 30 min, and immunoblotting (Figure 6). MALDI fingerprinting and electrospray ionization mass spectroscopy confirmed that the protein was ANN1; peptide fragments yielded 72% coverage. Overdeveloping the silver-stained gel (40 min) revealed very-low-level contaminants that were mostly also present in the buffer (Figure 6). To exclude the possibility that these contaminants were contributing to transport activity in PLB, the highly purified ANN1 preparation was immunoprecipitated for use in control experiments (Laohavisit et al., 2009, 2010).

ANN1 Reconstitutes an OH⁻-Activated Conductance

Recombinant ANN1 reconstituted a macroscopic OH⁻-activated Ca²⁺-permeable influx conductance in PLB designed as a PM mimetic. These experiments were designed to resemble the patch clamp trials, so negative current is equivalent to cation influx to the cytosol. PLBs comprised 1-palmitoyl 2-oleoyl phosphatidylethanolamine, cholesterol, and 1-palmitoyl 2-oleoyl phosphatidylserine in a 5:3:2 ratio, respectively (Laohavisit et al., 2009). Phosphatidylserine binding is a conserved characteristic of annexin, while cholesterol stabilized the PLBs. With Ca²⁺ as the major cation, OH⁻ in the *trans*-chamber (equivalent to patch clamping with OH⁻ at the extracellular PM face) resulted in a cation-permeable conductance (after 35 ± 5 min) in six out of 10 attempts with ANN1 (3 μ g) in the *cis*-chamber (Figure 7A; difference currents, produced by subtracting the control from test, are reported). OH⁻ are membrane-impermeable hence lipid peroxidation most likely promoted conductance formation.

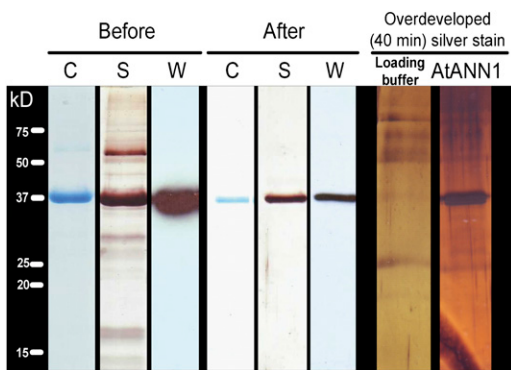


Figure 6. Purification of Recombinant ANN1.

ANN1 was expressed in *S. cerevisiae*, and the protein was purified by three cycles of Ca^{2+} -dependent binding to asolectin liposomes (Before) followed by size exclusion chromatography (After). The gel was either colloidal Coomassie blue stained (C), silver stained (S), or used to perform immunoblot analysis (W) to probe for ANN1 using anti-ANN1 peptide antibody. ANN1 is 37 kD. Overdeveloping the silver-stained gel revealed low-level contaminants that were also present in the buffer, including a predominant band at ~ 60 kD, which was also found when using *S. cerevisiae* to produce recombinant mammalian annexins. ANN1 was not detected in preparations from an empty vector control.

No conductance formed without OH^* (2-h observation; $n = 3$). Current was inwardly rectifying at hyperpolarized voltage (-14.5 ± 1 pA at -200 mV, $n = 6$; Figure 7A), consistent with Ca^{2+} influx from the *trans*- to *cis*-chamber and the inward Ca^{2+} current across the PM. Mean \pm SE reversal voltage (E_{rev}) was 33 ± 4 mV ($n = 6$). The permeability ratio (Véry and Davies, 2000) $P_{\text{Ca}^{2+}}:P_{\text{Cl}^-}$ was 34. The conductance was completely blocked by $50 \mu\text{M}$ Gd^{3+} (*trans*), confirming formation of a transport pathway by ANN1 (Figure 7A; $n = 6$). Thus, ANN1 reconstitutes an OH^* -activated Ca^{2+} -permeable conductance that is sensitive to a blocker that inhibits OH^* -induced PM Ca^{2+} conductance and $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in roots and protoplasts (Demidchik et al., 2003; Foreman et al., 2003).

With K^+ as the major cation, ANN1 supported an OH^* -activated K^+ conductance in PLB. A K^+ -permeable conductance was formed 53 ± 3 min after ANN1 addition ($3 \mu\text{g}$) under control conditions (four attempts out of six). OH^* in the *trans*-chamber caused transport activity in 26 ± 2 min (six out of eight attempts). Current was completely blocked by application of *trans* 50 mM TEA^+ (Figure 7B; difference currents reported, $n = 6$), consistent with block of At ANN1-mediated epidermal K^+ efflux. A $P_{\text{K}^+}:P_{\text{Cl}^-}$ of 53 was estimated for the OH^* -activated K^+ conductance ($E_{\text{rev}} = -30 \pm 5$ mV, $n = 6$).

With both Ca^{2+} and K^+ gradients present to reflect patch clamp recording conditions and pH 6.0 in both chambers, a rectifying OH^* -activated conductance was observed 39 ± 6 min after OH^* generation in nine out of 12 attempts (Figures 7C and 7D; difference currents reported). Even at lower voltages (± 20 mV), there was measurable current activation by OH^* (see Supplemental Figure 2 online). The E_{rev} was 4 ± 6 mV, close to the equilibrium potential (E) for H^+ (E_{H^+} 0 mV), suggesting that H^+ could be permeating rather than Ca^{2+} , K^+ , or Cl^- ($E_{\text{Ca}^{2+}}$ +62 mV; E_{K^+} -137 mV; E_{Cl^-} -15 mV). However, setting E_{H^+} at -62 mV (with

trans pH 7) had no significant effect on E_{rev} (5 ± 5 mV, $n = 5$; $P = 0.92$; Figure 7E). Using the $P_{\text{Ca}^{2+}}:P_{\text{Cl}^-}$ and $P_{\text{K}^+}:P_{\text{Cl}^-}$ values obtained for the individual OH^* -activated conductance, a $P_{\text{Ca}^{2+}}:P_{\text{K}^+}$ of 0.6 was deduced (Véry and Davies, 2000) from the experiments with both K^+ and Ca^{2+} present and symmetrical pH. This is lower than obtained for animal annexins in PLB, but the effects of ROS on animal annexin selectivity are unknown. Gd^{3+} ($50 \mu\text{M}$) in the *trans*-compartment blocked all current generated by ANN1, regardless of the *trans* pH (Figures 7C to 7E; $n = 6$). In all control experiments, heat-inactivated preparation was ineffective ($n = 3$), as was immunoprecipitated ANN1 (see Supplemental Figure 3 online; $n = 4$). This demonstrates that even in PLB (imperfect mimics of PM), ANN1 forms an OH^* -activated Ca^{2+} - and K^+ -permeable pathway.

DISCUSSION

ANN1 function has been examined here from protein to cellular level, demonstrating its transport activity at the root epidermal PM, ability to perturb both $[\text{Ca}^{2+}]_{\text{cyt}}$ and K^+ efflux, plus its involvement in growth. Even in the highly simplified PLB environment (which did not perfectly mimic the physico-chemical complexity of the PM), ANN1 was capable of reconstituting a rectifying OH^* -activated Ca^{2+} - and K^+ -permeable conductance. This strongly resembled the rectifying OH^* -activated Ca^{2+} - and K^+ -permeable conductance of the wild-type root epidermal and root hair apical PM that was absent in the *ann1* mutant but restored by complementation. The longer lag time required for conductance formation in PLB compared with native membrane and cells might reflect the need for ANN1 to be lying in close proximity to the bilayer for OH^* -activated transport events to occur or indicate that an interacting protein is required. Differences in ANN1 distribution between the PLB chamber and epidermal protoplast might also underpin the differences in the time dependency of the OH^* -activated currents in PLB and patch clamp studies, respectively.

Recombinant ANN1 K^+ current was blocked by Gd^{3+} and TEA^+ , which also block root epidermal OH^* -activated K^+ efflux (Cuin and Shabala, 2007). TEA^+ brought OH^* -activated net K^+ efflux from wild-type mature zone root epidermis to values supported by the *ann1* mutant, delineating the operation of ANN1 in intact cells. Moreover, OH^* -activated net K^+ efflux was lost completely from the elongation zone epidermis of *ann1*. The function of this OH^* -activated net K^+ efflux is at present unknown, but it could perhaps be involved in regulating membrane voltage. Results now point to two OH^* -activated net K^+ efflux pathways being present in the root epidermis: ANN1 and the previously characterized *Arabidopsis* GORK (Demidchik et al., 2010). The ANN1-mediated OH^* -activated K^+ efflux conductance was recorded in conditions that would suppress the Shaker-like GORK (low external K^+), suggesting that external K^+ may influence the operation of the two efflux pathways in vivo. For both, it can readily be envisaged that sustained production of OH^* could lead to their participation in K^+ loss leading to cell death (Demidchik et al., 2010).

Annexins have long been held to be a part of the plant cell's growth machinery, being abundant at polar growth points and being able to stimulate exocytosis (Carroll et al., 1998; reviewed

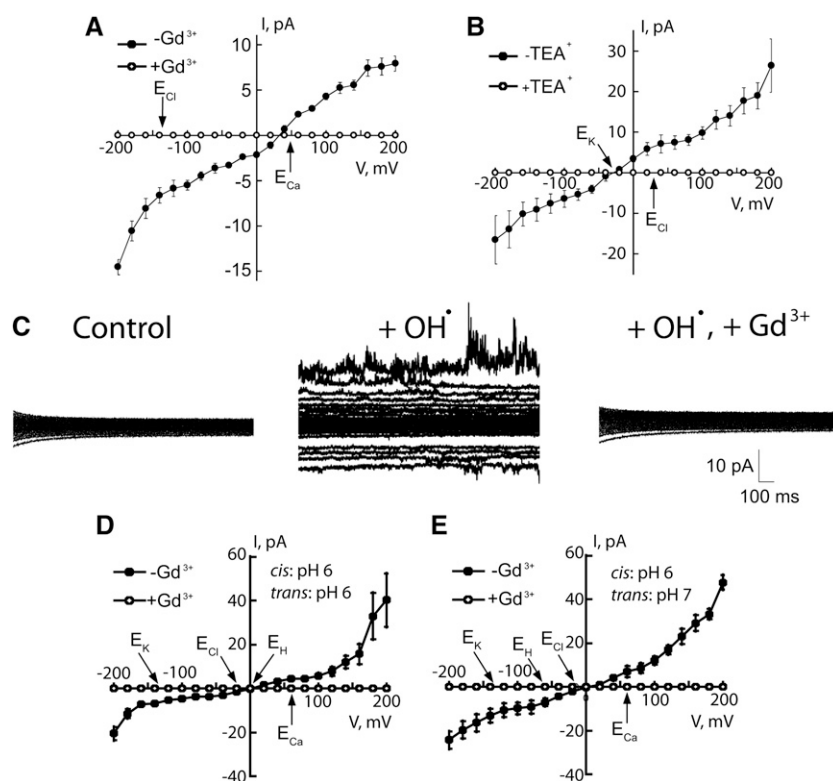


Figure 7. OH⁻-Activated Conductance Is Reconstituted by ANN1 in PLB.

(A) OH⁻-activated conductance mean \pm SE. *I-V* with Ca²⁺ as the major cation: *cis*-chamber (mM) 1 Ca²⁺, pH 6, ANN1 (3 μ g); *trans* 200 Ca²⁺, 1 Cu-Asc, pH 6. Positive *I* is positive charge movement from *cis* to *trans*. Current was blocked by 50 μ M Gd³⁺ *trans* (open symbols; *n* = 3).

(B) OH⁻-activated conductance *I-V* with K⁺ as the charge carrier: *cis*-chamber (mM) 200 KCl, pH 6, ANN1 (3 μ g); *trans* 50 KCl, 1 Cu-Asc, pH 6.0 (*n* = 4). Current was blocked by *trans* 50 mM TEA⁺ (open symbols; *n* = 3).

(C) Representative traces from one of six experiments with *cis* (mM) 200 K⁺, 1 CaCl₂, and *trans* 1 K⁺, 200 CaCl₂ both pH 6. Left: ANN1 (3 μ g) in the *cis*-chamber; *V* was changed in 20-mV steps. Middle: OH⁻ (1 Cu-Asc) in *trans* evoked inward and outward currents. Right: Block by 50 μ M Gd³⁺ *trans*.

(D) *I-V* relationships for the OH⁻-activated conductance of **(C)**, *n* = 5 and Gd³⁺ block (open symbols; *n* = 6). Equilibria are indicated. Expanded current traces are shown in Supplemental Figure 2 online.

(E) OH⁻-activated conductance *I-V* with *cis*, pH 6, *trans*, pH 7.0, to change *E_H*. All other conditions are as in **(C)**; *n* = 4 without *trans* Gd³⁺; *n* = 6 with 50 μ M Gd³⁺.

in Laohavisit and Davies, 2011a; Konopka-Postupolska et al., 2011). The ligan mutant of cotton (*Gossypium hirsutum*) is impaired in fiber elongation, and proteomic analysis revealed significant downregulation of five annexin isoforms when compared with the wild type, consistent with roles for annexins in growth (Zhao et al., 2010). ANN1's OH⁻-activated Ca²⁺ transport activity in PLB was consistent with a capacity for PM Ca²⁺ influx that could stimulate exocytosis. Accordingly, the Ca²⁺ transport lesion in *ann1* manifested in a significant impairment in epidermal cell PM-mediated OH⁻-activated [Ca²⁺]_{cyt} elevation. ANN1 supported 23% of the peak [Ca²⁺]_{cyt} response even though it is only one of over 20 possible channels in this cell type (Dinneny et al., 2008). Block of ANN1 by Gd³⁺ in PLB is consistent with Gd³⁺ inhibition of the wild type's OH⁻-stimulated [Ca²⁺]_{cyt} elevation in this and our previous study on root hairs (Foreman et al., 2003). Ca²⁺ influx at the root hair apex and elongation zone of the main root (where ANN1 is localized; Clark et al., 2001, 2005; Dinneny et al., 2008) is involved in growth and can be regulated by

extracellular OH⁻ (Foreman et al., 2003). ANN1 activation by OH⁻ at acidic pH is consistent with its contributing to Ca²⁺ influx in root hair elongation, in which extracellular ROS are sourced by the RBOHC NADPH oxidase (Foreman et al., 2003) and apical cytosolic acidification oscillates (Monshausen et al., 2007). The short root and root hair phenotype of the *ann1* mutant can thus be attributed to the absence of a PM OH⁻-activated Ca²⁺ influx conductance mediated by ANN1. That root and root hair growth could still advance highlights the importance of other root PM Ca²⁺ channels, such as the HACC, the molecular identity of which now need to be resolved. Additionally, the question now arises of whether annexins function as Ca²⁺ influx transporters in other tip-growing cells in which growth is linked to NADPH oxidase activity, such as pollen tubes and fungal hyphae (Cano-Domínguez et al., 2008; Liu et al., 2009).

Ca²⁺ influx mediated by ANN1 may also help explain the drought and salt sensitivity of the *ann1* mutant (Konopka-Postupolska et al., 2009; Huh et al., 2010), particularly as plant

annexins have been proposed to act as sensors of stress and stress-evoked ROS (Gorecka et al., 2007; Laohavisit et al., 2010; Laohavisit and Davies, 2011a). In roots of *Arabidopsis* under salt stress, preventing the formation of or scavenging OH[•] compromises the stability of the newly made mRNAs encoding for the PM Na⁺/H⁺ exporter Salt Overly Sensitive1 (SOS1; Chung et al., 2008). The ultimate source of the salt-induced ROS necessary for SOS1 mRNA stability is the NADPH oxidase RBOHC (Chung et al., 2008). This is also the NADPH oxidase that sources the ROS for the OH[•]-activated calcium conductance involved in root cell elongation (Foreman et al., 2003), shown here to be mediated by ANN1. Further studies are now required to ascertain whether ANN1 is involved in events leading to stabilizing SOS1 mRNA.

Animal annexins produce complex effects on ion transport across bilayers in vitro (reviewed in Konopka-Postupolska et al., 2011). In this study, ANN1 formed a macroscopic conductance, and single channel events were only rarely observed. Reasons for this are unclear. ANN1 was found previously to form single channel events in nonoxidized asolectin PLBs (Gorecka et al., 2007), and maize annexins ANN33/35 formed a 17 pS Ca²⁺-permeable channel in PLBs containing malondialdehyde (Laohavisit and Davies, 2011b). Plant Ca²⁺-permeable cation channel P_{Ca:P_K} values range from 0.14 for nonselective cation channels (Demidchik and Maathuis, 2007) to orders of magnitude greater for HACCs (e.g., 15 for *Arabidopsis* root hair HACC (Véry and Davies, 2000). ANN1's estimated P_{Ca:P_K} (0.6) is of similar magnitude to the conductance formed by maize ANN33/ANN35 in PLB (0.36; Laohavisit et al., 2009). Such values are lower than determined for animal annexins in PLB, albeit in nonoxidized conditions (e.g., 4.34 for annexin A5; Burger et al., 1994; Liemann et al., 1996) and indicate that residues other than those of the salt bridges may affect transport activity. PLBs and patch clamping are highly reductionist systems, and the permeability of ANN1 in vivo may well differ from values found here. The molecular identities of ROS-regulated Ca²⁺-permeable channels in plants have remained obscure, despite their manifest significance in growth, adaptation, and defense (Dodd et al., 2010). The Ca²⁺-permeable Stelar K⁺ Outward K⁺ Rectifier PM channel (Gaymard et al., 1998) has been shown to be activated by hydrogen peroxide in a heterologous expression system (Garcia-Mata et al., 2010), but the effects of ROS on its Ca²⁺ permeation, ability to affect [Ca²⁺]_{cyt}, and where it lies in relation to NADPH oxidase activity are unknown. Maize ANN33/ANN35 have previously been shown to form a hyperpolarization-activated macroscopic Ca²⁺ conductance in PLBs containing malondialdehyde (to mimic peroxidized PM; Laohavisit et al., 2010). A greater mechanistic understanding of how annexins form conductance pathways in membranes, particularly membranes subject to modification by ROS, is now required so that their contribution to Ca²⁺ signaling governed by ROS in development and adaptation (Mittler et al., 2011) can be better understood. With the demonstration that a Ca²⁺ influx pathway requires ANN1, annexins of crop plants (Laohavisit and Davies, 2011a) can now be addressed confidently as components of growth and signaling, alongside the more conventional CNGCs, glutamate receptor-like channels, and Ca²⁺-permeable high-affinity K⁺ transporters as Ca²⁺ influx routes (Qi et al., 2006; Dodd et al., 2010; Lan et al., 2010; Ma et al., 2010; Michard et al., 2011).

METHODS

Plant Culture

Arabidopsis thaliana Columbia (Col-0) and Wassilewskija were the wild-type parental lines for the *ann1* and *gork* homozygous insertional T-DNA lines, respectively (Hosy et al., 2003; Lee et al., 2004). The 35S-complemented ANN1 mutant was generated and described previously (Lee et al., 2004). For patch clamp, luminometry, and vibrating microelectrode experiments, plants were grown on Murashige and Skoog medium (Duchefa Biochemie) with 1% (w/v) Suc and 0.3% (w/v) Phytigel (Sigma-Aldrich). For impalement recordings and growth determinations, growth was on replete medium comprising (in mM) 5 KNO₃, 5 MgSO₄·7H₂O, 2 NaCl, 1 CaCl₂·2H₂O, 1 (NH₄)₂HPO₄, plus 0.1 g·L⁻¹ myo-inositol, 1 mg·L⁻¹ thiamine, 0.5 mg·L⁻¹ nicotinic acid, 0.5 mg·L⁻¹ pyridoxine, 25 μM KCl, 25 μM Fe-Na EDTA, 17 μM H₃BO₃, 10 μM MnSO₄·H₂O, 10 μM ZnSO₄·7H₂O, 2.5 μM CuSO₄·5H₂O, 1% (w/v) Suc, 5 mM MES/Tris, pH 5.7, and 2% (w/v) agar. Nutrient-poor medium was as described previously (Véry and Davies, 2000): 0.1 mM CaCl₂, 0.1 mM KCl, pH 5.7, and 2% (w/v) agar. Growth was for 4 to 10 d at 22°C in a 16-h day at 100 μmol·m⁻²·s⁻¹ irradiance. Mature root hair lengths were determined at day 4.

Patch Clamp Electrophysiology

The protoplasting protocol was adapted from Demidchik et al. (2003). Spheroplasts were released from young root hair apices using laser ablation (Véry and Davies, 2000). Standard patch clamp procedures were applied (Véry and Davies, 2000). For studies on epidermal protoplasts and the effects of hydroxyl radicals on root hair spheroplasts, bathing solution comprised 20 mM CaCl₂, 0.1 mM KCl, 20 μM NaCl, and 5 mM MES-Tris, pH 5.6, adjusted to 270 mOsm with D-sorbitol. The pipette solution comprised 40 mM K-gluconate, 10 mM KCl, 0.4 mM CaCl₂, 1 mM bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), and 2 mM MES-Tris, pH 7.2, adjusted to 270 mOsm with D-sorbitol. For trials on root hair spheroplast constitutive HACC, bathing solution comprised 10 mM BaCl₂ and 2 mM MES-Tris, pH 6, adjusted to 275 mOsm with D-sorbitol. Pipette solution comprised 0.5 mM CaCl₂, 8.5 mM Ca(OH)₂, 2 mM MgATP, 0.5 mM Tris-ATP, 10 mM BAPTA (final free Ca²⁺, 1 μM), and 15 mM HEPES-Tris, pH 7.3, adjusted to 275 mOsm with D-sorbitol.

Impalement and Vibrating Microelectrode Recordings

Seedlings (6 to 10 d old) were bathed in (mM) 0.2 KCl, 0.1 CaCl₂, 14 MES, and 5 Tris, pH 5.7. Membrane potential was measured in elongation zone epidermis using a 1-mm outer diameter microelectrode. Diffusion potential was achieved by superfusing with 0.1 mM NaCN and 0.1 mM salicyl hydroxamic acid (Leidi et al., 2010). Net fluxes of K⁺ were measured noninvasively using the MIFE technique (Cuin and Shabala, 2007) in (mM) 0.2 KCl, 0.1 CaCl₂, pH 5.6, and ±20 TEA-Cl. Apical root segments (8 to 10 mm) were equilibrated in bathing solution for 50 min. Recordings were made 2 to 3 mm (mature epidermis) or 120 μm (elongation zone) from the apex. To test for OH[•]-induced changes in net K⁺ flux, CuCl₂ and ascorbic acid were added simultaneously in bathing solution (1 mM final concentration). Recordings up to 60 s after this addition were discarded to allow for establishment of diffusion gradients. Significant differences in these and other trials were assessed with Student's *t* tests.

Protoplast [Ca²⁺]_{cyt} Determination

Col-0 and *ann1* were transformed using floral dip with *Agrobacterium tumefaciens* to express (apo)aequorin under a 35S promoter (Dodd et al., 2006). T3 generations were verified as *ann1*. Two independently generated lines per genotype yielding total photon counts in excess of 10⁵ in rosette leaf Ca²⁺ discharge assays were used. Protoplasts were

incubated in aequorin buffer (mM: 10 CaCl₂, 0.1 KCl, and 2 Tris/MES, pH 5.8, adjusted to 270 mOsm with D-sorbitol) containing 10 μM coelenterazine (Lux Biotechnology) for 4 h in the dark. Protoplasts were washed with osmotically adjusted aequorin buffer without coelenterazine and resuspended in this (70 per μL). Suspension (100 μL) was placed into the well of a white 96-well plate (Greiner Bio-One), transferred to a plate-reading luminometer (FLUOstar OPTIMA; BMG Labtech), and left in the dark for 30 min. After recording baseline luminescence for 30 s, 50 μL of buffer (with or without CuCl₂) and 50 μL of buffer (with or without ascorbic acid) were injected (0.5 mM final concentration of CuCl₂/ascorbic acid). Luminescence was recorded every second for 30 s and then every 15 s for a further 2085 s. Discharge solution was then injected (final concentration: 10% [v/v] ethanol, 1 M CaCl₂), and luminescence was recorded for a further 120 s. Calibration to convert luminescent values to [Ca²⁺]_{cyt} was performed as described previously (Dodd et al., 2006).

Cloning of ANN1

RNA was isolated from whole seedlings using the RNeasy mini kit (Qiagen) and used to synthesize cDNA using the Superscript III first-strand synthesis system (Invitrogen). cDNA was purified and cleaned using QIAquick (Qiagen). The PCR reaction consisted of 100 ng cDNA, 250 nM primers, 250 μM deoxynucleotide triphosphate, 2 mM MgCl₂, 0.625 units of Biotaq DNA polymerase (Bioline), and 17 mM NH₄SO₄, pH 8.8. The primers were as follows: Ann 1-5-1, 5'-ATGGCGACTCTTAAGGTTTCTGATTC-3'; Ann 1-3-5, 5'-ACTATCATTAAAGCATCATCTTCACCGAGAAGTGC-3'. The reaction was heated to 94°C for 2 min, followed by 30 cycles of denaturation (94°C, 20 s), annealing (50°C, 30 s), and extension (72°C, 2 min), followed by a final extension step (72°C, 10 min; iCycler, Bio-Rad). PCR products were analyzed by gel electrophoresis.

Heterologous Expression of ANN1

The pYES 2.1/V5-His Topo yeast expression vector (Invitrogen) was used for the expression of the *Arabidopsis* ANN1 construct under the control of a Gal-inducible promoter. Fast-growing diploid *Saccharomyces cerevisiae* strain INVSc1 (genotype *Mat a, his 3D1, leu 2, trp1-289, ura3-52*; Invitrogen) was transformed with yeast expression vector (pYES 2.1/V5-His Topo; Invitrogen) containing ANN1. Empty vector was used as a negative control. A single yeast colony was grown in liquid SC-U medium for 20 h at 30°C. Washed cells were resuspended in yeast extract/peptone YP medium to an OD₆₀₀ of 0.18 and grown at 30°C, until an OD₆₀₀ of 2 to 3 had been reached (18 h).

Purification of Recombinant ANN1

Cells were resuspended in extraction buffer (10 mM HEPES, 5 mM EGTA, 150 mM NaCl, and 0.01% [v/v] protease inhibitor cocktail [Sigma-Aldrich], pH 7.5) and disrupted by two cycles in an EmulsiFlex C5 homogenizer at 15,000 p.s.i. (Avestin). Debris were removed by centrifugation, the supernatant was incubated with DNase (5 μg mL⁻¹; Promega), RNase A (10 μg mL⁻¹; Roche Applied Science), and MgCl₂ (1 mM; 99.5% pure; Fisher Scientific) for 30 min at 180 rpm. Lysate was mixed with asolectin liposomes (200 mg soybean asolectin [Sigma-Aldrich] dissolved in (2:1) chloroform:methanol to give a final concentration of 13.3 mg/mL). CaCl₂ was added to a final concentration of 10 mM, and the mixture was incubated for 1 h. After centrifugation (30,000g, 30 min), the lipid pellet was resuspended in filter-sterilized wash buffer (50 mM HEPES, 150 mM NaCl, 10 mM CaCl₂, and 1 mM phenylmethanesulphonyl fluoride, pH 7.5). After a repeat centrifugation, the lipid pellet was resuspended in filter-sterilized wash buffer (50 mM HEPES and 10 mM CaCl₂, pH 7.5) and centrifuged (30,000 g, 30 min). The lipid pellet was resuspended in filter-sterilized elution buffer (50 mM HEPES and 10 mM EGTA, pH 7.5). After centrifugation (125,000g, 1 h), the final supernatant was mixed with asolectin

liposomes, and the purification process was repeated twice. The buffer was exchanged to 100 mM NaCl and 50 mM K-phosphate buffer, pH 7.5, and samples were concentrated using a Vivaspinn20 concentrator (molecular mass cutoff 10 kD; VivaScience). All steps were performed at 4°C. Samples were loaded onto a gel filtration column (Superose 12; GE Healthcare; equilibrated with 100 mM NaCl and 50 mM phosphate buffer, pH 7.5) attached to a fast performance liquid chromatography system. ANN1 was eluted at a flow rate of 0.4 mL/min. Fractions (1 mL) were tested for ANN1 by SDS-PAGE and immunoblotting. All proteins were exchanged into buffer (10 mM K-phosphate buffer, pH 7.4) prior to use. In immunoprecipitation studies, the ANN1 preparation was incubated for 30 min at room temperature with anti-At ANN1 peptide antibody (against NRYQDDHGEEIL, residues 204 to 215; Lee et al., 2004) in a 2:1 ratio (Laohavisit et al., 2009). Antibody alone was used as a control.

Gel Electrophoresis, Immunoblotting, and Protein Verification

Samples were subjected to SDS-PAGE as described previously (Laohavisit et al., 2009). In silver staining, gels were fixed overnight to enhance detection, washed twice for 20 min, and then stained for up to 40 min. For immunoblotting, membranes were incubated with primary antibody (1:5000 dilution) for 1 h, at room temperature, with shaking and then after washing incubated for 1 h with secondary antibody (1:5000 peroxidase-linked anti-rabbit IgG; Invitrogen). Proteins were visualized using enhanced chemiluminescence (ECL-plus; GE Healthcare). Mass spectrometry was used to determine protein identity. Samples were prepared for in-gel digestion as described previously (Laohavisit et al., 2009) and analyzed by MALDI peptide mass fingerprinting and electrospray ionization mass spectrometry. Identifications were based on multiple peptide matches.

PLBs

Lipids (25 mg mL⁻¹; Avanti Polar Lipids) were used to form a bilayer (1-palmitoyl 2-oleoyl phosphatidylethanolamine:cholesterol:1-palmitoyl 2-oleoyl phosphatidylserine) in a 5:3:2 ratio, respectively, across a 200-μm diameter aperture at room temperature (20 to 24°C) (Laohavisit et al., 2009, 2010). In Ca²⁺-permeability experiments, *cis*- and *trans*-chambers contained 0.5 mL 10 mM MES/*bis* Tris propane (BTP) and 1 mM CaCl₂, pH 6.0, and 2 mL 10 mM MES/BTP and 200 mM CaCl₂, pH 6.0, respectively. In K⁺-permeability experiments, the *cis*-chamber comprised 200 mM KCl and 10 mM MES/BTP, pH 6.0, and the *trans* comprised 50 mM KCl and 10 mM MES/BTP, pH 6. In combined experiments, *cis* was 1 mM CaCl₂, 200 mM KCl, pH 6; *trans* was 200 mM CaCl₂, 1 mM KCl, pH 6.0 or 7. Annexin (3 μg) protein was added to the *cis*-chamber. The bilayer was held at -150 mV (*cis* negative) to aid insertion. Hydroxyl radicals were generated by adding 1 mM Cu-Asc to the *trans*-chamber. Conductance was monitored for annexin activity for up to 2 h. GdCl₃ or TEA-Cl were also added to *trans*. Results were from at least three separate protein preparations.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank/National Center for Biotechnology Information RefSeq data library under accession number NP174810.1 for ANN1 (At1g35720; gb:AF083913).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Copper or Ascorbate Alone Do Not Elicit Plasma Membrane Currents; Copper and Ascorbate Together Elicit a Barium-Permeable Inward Conductance.

Supplemental Figure 2. Expanded Planar Lipid Bilayer Traces.

Supplemental Figure 3. Immunoprecipitated At ANN1 Does Not Support a Conductance in Planar Lipid Bilayers.

Supplemental Table 1. Root Plasma Membrane Currents.

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AUTHOR CONTRIBUTIONS

All authors contributed to research design and commented on the article. A.L., Z.S., L.R., A.-A.V., T.A.C., J.C.M., N.M., and K.M.C. performed the research and analyzed the data. J.M.D. wrote the article.

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