Tissue-specific root ion profiling reveals essential roles of the CAX and ACA calcium transport systems in response to hypoxia in Arabidopsis

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Received 18 November 2015; Accepted 18 January 2016

Abstract

Waterlogging is a major abiotic stress that limits the growth of plants. The crucial role of Ca²⁺ as a second messenger in response to abiotic and biotic stimuli has been widely recognized in plants. However, the physiological and molecular mechanisms of Ca²⁺ distribution within specific cell types in different root zones under hypoxia is poorly understood. In this work, whole-plant physiological and tissue-specific Ca²⁺ changes were studied using several ACA (Ca²⁺-ATPase) and CAX (Ca²⁺/proton exchanger) knock-out Arabidopsis mutants subjected to waterlogging treatment. In the wild-type (WT) plants, several days of hypoxia decreased the expression of ACA8, CAX4, and CAX11 by 33% and 50% compared with the control. The hypoxic treatment also resulted in an up to 11-fold tissue-dependent increase in Ca²⁺ accumulation in root tissues as revealed by confocal microscopy. The increase was much higher in stelar cells in the mature zone of Arabidopsis mutants with loss of function for ACA8, ACA11, CAX4, and CAX11. In addition, a significantly increased Ca²⁺ concentration was found in the cytosol of stelar cells in the mature zone after hypoxic treatment. Three weeks of waterlogging resulted in dramatic loss of shoot biomass in cax11 plants (67% loss in shoot dry weight), while in the WT and other transport mutants this decline was only 14–22%. These results were also consistent with a decline in leaf chlorophyll fluorescence (Fv/Fm). It is suggested that CAX11 plays a key role in maintaining cytosolic Ca²⁺ homeostasis and/or signalling in root cells under hypoxic conditions.

Key words: Ca²⁺-ATPase, calcium, Ca²⁺/proton exchanger, confocal microscopy, epidermis, hypoxia, potassium, sodium, stele, waterlogging.

Introduction

Waterlogging is a widespread abiotic stress to land plants, influencing nearly 10% of the global land area (Setter and Waters, 2003) and reducing up to 80% of crop yields (Shabala, 2011). There is a series of changes in physical, chemical, and biological properties in soil which ultimately inhibit growth of intolerant plants under waterlogging stress. The diffusivity of atmospheric oxygen (O₂) into soil pores is reduced by ~10 000-fold under waterlogging, which exposes the plant roots to
a hypoxic situation (Malik et al., 2001; Armstrong and Drew, 2002). The O₂ deficiency in the root zone depresses plant shoot and root growth, dry matter accumulation, and yield (Malik et al., 2001; Colmer and Greenway, 2011; Shabala et al., 2014). In addition, hypoxia limits the supply of ATP to plant H⁺-ATPase pumps and impairs ion transport processes, cell metabolism, and nutrient acquisition (Bailey-Serres and Voesenek, 2008; Elzenga and van Veen, 2010; Shabala et al., 2014).

Cytosolic free Ca²⁺ ([Ca²⁺]ₜ) has been widely recognized as the central regulatory signal for many processes in plant cells and as a key second messenger mediating plant adaptive responses to abiotic and biotic stimuli (Reddy et al., 2011; Steinhorst and Kudla, 2014; Zhu et al., 2015). These usually include multiple signal transduction pathways when plants are exposed to O₂ deficiency, salinity, drought, osmotic, oxidative, heat, and cold stresses, pathogens, and bacterial and fungal signals (Sanders et al., 1999; McAinsh and Pittman, 2009; Bose et al., 2011). Hypoxic stress causes a rapid elevation of [Ca²⁺]ₜ in the cells of Arabidopsis, maize, rice, and wheat (Subbaiah et al., 1994; Yemelyanov et al., 2011; Lindberg et al., 2012), and this elevation is fundamental for gene activation and acclimation responses at the cellular, tissue, as well as organismal levels (Subbaiah and Sachs, 2003; Shabala et al., 2014).

Potassium is not only essential for many plant metabolic processes, such as osmoregulation, enzyme function, stomatal movements, control of membrane polarization, and ionic balancing (Dreyer and Uozumi, 2011; Wang et al., 2013; Shabala and Pottosin, 2014), but also plays an important role in determining the fate of cells under stress conditions (Shabala, 2009; Demidchik et al., 2010; Anschutz et al., 2014; Demidchik et al., 2014). K⁺ homeostasis is essential to mediate plant adaptive responses to a broad range of abiotic and biotic stresses including drought, salinity, and oxidative stress (Chen et al., 2007; Cuin et al., 2008; Shabala et al., 2010; Bonales-Alatorre et al., 2013a; Anschutz et al., 2014; Demidchik, 2014). In rice seedlings, it was found that K⁺ effluxes in the coleoptile were down-regulated during prolonged anoxia (Colmer et al., 2001). The waterlogging-sensitive barley cultivar Naso Nijo showed a larger decline of K⁺ uptake in the mature zone after hypoxic treatment than waterlogging-tolerant TX9425 and CM72 cultivars. Also, the overall magnitude of the reduction of K⁺ efflux in the elongation zone was significantly higher in Naso Nijo than in TX9425 and CM72 (Pang et al., 2006; Zeng et al., 2014). Therefore, a root’s ability to retain K⁺ under hypoxic conditions is strongly correlated with waterlogging tolerance in barley, and highlights the root tissue specificity of plant-adaptive responses to hypoxia (Zeng et al., 2014). Salinity and waterlogging stress are abiotic stresses that often occur together in estuaries, river flood plains, agricultural land affected by secondary salinity (dryland and irrigated), or in areas irrigated with saline water (Smedema and Shiati, 2002; Qadir and Oster, 2004). When salinity occurs together with hypoxia, the transfer of Na⁺ and Cl⁻ from roots to shoots increases, whereas K⁺ transport decreases even further compared with salinity under normoxic conditions (Barrett-Lennard and Shabala, 2013). Very few studies have elaborated Na⁺ and K⁺ distribution in specific cell types during combined salinity and hypoxia stress.

As a ubiquitous denominator of cellular signalling networks, [Ca²⁺]ₜ signal is shaped by the balance of Ca²⁺ influx and efflux that is regulated by the membrane transport systems. The main Ca²⁺ sources entering into the cytoplasm are from the apoplast, vacuole, endoplasmic reticulum (ER), mitochondria, and chloroplast, through a large number of voltage-gated and ligand-gated Ca²⁺ channels at the plasma membrane, tonoplast, and endomembranes (Medvedev, 2005; Pottosin and Schiknnecht, 2007; McAinsh and Pittman, 2009). Reducing [Ca²⁺]ₜ to the resting level requires activation of Ca²⁺ transporters such as high-affinity Ca²⁺-ATPases [autoinhibited Ca²⁺-ATPases (ACAs) and ER-type Ca²⁺-ATPases] and the low-affinity Ca²⁺/H⁺ antiporter (CAXs) which are localized in both the plasma membrane and endomembranes (Sanders et al., 2002; Hetherington and Brownlee, 2004; Manohar et al., 2011; Huda et al., 2013). The different subcellular locations of these Ca²⁺ circuits may contribute to the unique code of Ca²⁺ signals and provide a mechanism for creating a stimulus-specific Ca²⁺ signature (Boursiac et al., 2010).

As [Ca²⁺]ₜ increases in response to environmental stress, the Ca²⁺ efflux system plays critical roles in restoring basal [Ca²⁺]ₜ levels and terminating stress-induced [Ca²⁺]ₜ signatures (Bose et al., 2011; Shabala, 2011). Analysis of Arabidopsis mutants, in particular acax4 and acax8, identified a variety of phenotypes including sensitivity to salt and cold stress, indicating these transporters play roles in plant stress responses (Geisler et al., 2000; Schiott and Palmgren, 2005). In yeast and tobacco BY-2 cells, a Ca²⁺/H⁺ antiporter, CAX4, is found to be localized on the tonoplast, and the CAX4 mRNA level increased after Mn²⁺, Na⁺, and Ni²⁺ treatment (Cheng et al., 2002). The loss-of-function mutant (cax4-1) and CAX4 RNA interference (CAX4 RNAi) lines indicated altered root growth and development in response to Cd²⁺, Mn²⁺, and auxin (Mei et al., 2009). These results demonstrated that CAX4 functions in root growth under heavy metal stress conditions. The function of CAX11 was also characterized in yeast, in which the fusion of CAX11 and green fluorescent protein (GFP) was found in the plasma membrane and nuclear periphery. Expressing CAX11 in a yeast mutant showed its role of mediating high-affinity K⁺ uptake and Na⁺ transport (Zhang et al., 2011). However, the exact role of these transporters is still elusive.

An increase in Ca²⁺ concentration in the cytosol and nucleus is detected by many Ca²⁺-sensing proteins [e.g. calmodulin (CaM), calmodulin-like proteins (CMLs), Ca²⁺-dependent protein kinases (CDPKs), and calcineurin B-like (CBL)/Ca²⁺-independent protein kinases (CIPKs)] (Dodd et al., 2010; Reddy et al., 2011). These proteins are able to decode the information present within the different Ca²⁺ spikes or oscillations and process this information into alteration of the cell function. For example, ZmCAP1, encoding Ca²⁺-ATPase, from maize has been shown to bind to and be stimulated by CaM following heterologous expression in yeast (Subbaiah and Sachs, 2000). Following an elevation in
[Ca\(^{2+}\)]\_cyr, CaM will interact with an N-terminal autoinhibitory domain of the ACAs, leading to activation of Ca\(^{2+}\) transport activity \((\text{Baekgaard et al.}, 2005\). These CaM-binding domains appear to be ubiquitous on all ACAs, but are highly divergent with little consensus sequence \((\text{Chung et al.}, 2000; \text{Baekgaard et al.}, 2006\). Different signalling pathways are regulated by the transcription machinery and expression of downstream target genes. The downstream reactions can cause an acclimation, helping the plant to survive the specific stress \((\text{Lindberg et al.}, 2012\). Thus, it is of importance to carry out investigations and screening of the interaction of the Ca\(^{2+}\)-sensing proteins with Ca\(^{2+}\)-ATPase or Ca\(^{2+}\)/H\(^{+}\) exchanger proteins under hypoxic stress.

Knowledge of hypoxia-induced transport processes is largely restricted to ion exchange at the root surface, which is easily accessible. However, little is known about the character of hypoxic signal transduction in specific cell types and tissues of roots \((\text{Pang et al.}, 2006; \text{Zeng et al.}, 2014; \text{Kotula et al.}, 2015\). We hypothesize that radial and longitudinal O\(_2\) profiles reported in plant roots under conditions of hypoxia have a major impact on the energy availability in various cell types, thus affecting their ionic homeostasis. We have also postulated that a lack of functional Ca\(^{2+}\) efflux systems may be detrimental to plant acclimation to hypoxic conditions. Both these hypotheses were confirmed in the current study. The objective of our research was to investigate anoxia-induced changes of Ca\(^{2+}\), K\(^{+}\), and Na\(^{+}\) in meristem, epidermal, and stelar cells of Arabidopsis in physiologically different root zones in wild-type (WT), and aca and cax mutants to reveal essential roles of the CAX and ACA calcium transport systems. Overall, our results suggest that Ca\(^{2+}\) efflux systems and especially CAX11 are essential to mediate plant Ca\(^{2+}\) homeostasis under hypoxic conditions.

**Materials and methods**

**Plant materials and treatments**

Seeds of *Arabidopsis thaliana* WT Columbia-0 (Col-0) and loss-of-function mutants (all in the Col-0 background) aca8 (SALK_0578770), aca11 (SALK_002747), cax4 (SALK_119863), and cax11 (SALK_013040) were obtained from the Arabidopsis Biological Resource Centre \((\text{http://www.arabidopsis.org/abrc})\). Seeds were surface sterilized with 1 ml of commercial bleach \((1\% \text{ (v/v) NaClO})\) for 10 min, and then washed five times with sterilized distilled water. Seeds were kept at 4 °C for 2 d and sown in Petri dishes containing 1% (w/v) phytogel, half-strength Murashige and Skoog (MS) medium, and 0.5% (w/v) sucrose at pH 5.7. Petri dishes containing seeds were sealed with 3M micro-pore tape and then transferred into a growth chamber with a 16h/8h day/night, 100 µmol m\(^{-2}\) s\(^{-1}\) photon flux density, and 22 °C. The Petri dishes were oriented upright, allowing the roots to grow down along the surface without penetrating into the medium. All chemicals were from Sigma-Aldrich (Castle Hill, NSW, Australia) in analytical grade unless individually specified.

Hypoxic treatment was imposed on the 10-day-old Arabidopsis seedlings submerged with 0.2% (w/v) agar solution pre-bubbled with high purity N\(_2\) (Coregas, Sydney, NSW Australia). For measurement of gene expression levels, WT Arabidopsis seedlings were treated with hypoxia for 1, 24, and 72 h; for measurement of K\(^{+}\) and Ca\(^{2+}\) distribution in roots, the WT and the four mutants were treated with hypoxia for 24 h. Combined hypoxia and salinity treatment was also imposed on the 10-day-old Arabidopsis seedlings in deoxygenated agar solution with addition of 50mM NaCl for 24 h, with measurement of Na\(^{+}\) distribution in roots. The whole seedlings were submerged in the bubbled solution and kept in the growth chamber with a 16h/8h day/night, 100 µmol m\(^{-2}\) s\(^{-1}\) photon flux density, and at 22 °C. Compared with hypoxia treatment or combined hypoxia and salinity treatment, the control condition was normoxic, non-saline, and without agar solution.

In the phenotyping experiments, aca8, aca11, cax4, cax11, and WT plants were grown in 0.2 litre pots filled with peat moss, perlite, vermiculite, and coarse sand, with a ratio of 2:1:1 (v/v), and watered with half-strength Hoagland’s nutrient solution. Plants were grown in a growth chamber at a 12h/12h light/dark regime and at 21 °C for 3 weeks. Plants were then subjected to waterlogging treatment by immersing pots in water and maintaining the water level 0.5 cm above the soil surface for another 3 weeks. The above-ground biomass of individual plants was determined as fresh weight, and dry weight was measured after drying in a Unitherm Drier (Birmingham, UK) for 2 d at 65 °C.

**Chlorophyll fluorescence**

Chlorophyll fluorescence was measured on the 6-week-old fully expanded leaves which had been subjected to the 3 week treatment with an OS-30p chlorophyll fluorometer (OPTI-Sciences, Hudson, USA). Plants were adapted in a dark chamber for 30 min before measuring. The maximum quantum efficiency of photosystem II (PSII; \(F_{v}/F_{m}\)) was recorded at a saturating actinic light \((660 nm)\) intensity of 1100 µmol m\(^{-2}\) s\(^{-1}\) \((\text{Zeng et al.}, 2013\). Measurements were taken in at least five replicates for each treatment.

**Quantitative real-time PCR (RT-PCR)**

Total RNA was extracted from 10-day-old Arabidopsis roots with TRIZOL reagent \((\text{Life Technologies, Mulgrave, VIC, Australia})\); \((\text{Lin et al.}, 2014; \text{Chen et al.}, 2016); First-strand cDNA synthesis was performed using the sensiFAST Kit \((\text{Bioline, Alexandria, NSW, Australia})\) with 1 µg of total RNA. RT-PCR was performed with SensiMix SYBR No-ROX Kit \((\text{Bioline, Alexandria, NSW, Australia})\) using a Rotor-Gene Q6000 \((\text{QIAGEN, Hilden, Germany})\). The specific primers are listed in Supplementary Table S1 at *JXB* online. The PCR program was two steps: one cycle of 95 °C, 10 min; and 40 cycles of 95 °C, 15 s; 60 °C, 15 s; and 72 °C, 15 s. The amplification of the target genes was monitored every cycle by SYBR-green fluorescence. Three technical and biological replicates were performed for each experiment and treatment. The transcripts of target genes were normalized to the control gene RNA polymerase II subunit \((\text{RPB2})\).

**Confocal laser scanning microscopy measurements**

CoroNa Green acetoxyethyl (AM) ester \((\text{Invitrogen, Eugene, OR, USA})\), Calcium Green-5N, AM \((\text{Invitrogen}, \text{Asante Potassium Green-2 (APG-2, TEFLabs, Austin, TX, USA})\) were employed to measure the Na\(^{+}\), Ca\(^{2+}\), and K\(^{+}\) concentration, respectively, in Arabidopsis root cells. The optimal CoroNa Green concentration was determined in our previous work \((\text{Bonales-Alatorre et al.}, 2013b; \text{Wu et al.}, 2015); All five types of root cells showed optimal fluorescence intensity when exposed to 15 µM Calcium Green-5N \((\text{Supplementary Fig. S1})\) and 20 µM APG-2. The indicators were dissolved in DMSO \((\text{Sigma})\) to a stock concentration of 1 mM. Then, 20 µM CoroNa Green, 20 µM Asante Potassium Green-2, and 15 µM Calcium Green-5N were added to measuring buffers \((\text{Na}\(^{+}\), 10mM KCl, 5mM Ca\(^{2+}\)-MES, pH 6.1; K\(^{+}\), 5mM NaCl, 5mM Ca\(^{2+}\)-MES, pH 6.1; Ca\(^{2+}\), 10mM KCl, 5mM Na\(^{-}\)-MES, pH 6.1), respectively. Arabidopsis seedlings were incubated in the dye-containing measuring buffers for 3 h in the dark. The stained seedlings were washed in distilled water for 3 min to remove residual dyes before measuring fluorescence intensity in meristem, elongation epidermal, elongation stelar, mature epidermal, and mature stelar root cells after 24h of hypoxic treatment. All dying steps in this work was done at room temperature.
A confocal laser scanning microscope fitted with a TCS SP1 II confocal head (SP.5, Leica Microsystems, Heidelberg, Germany) was used to measure fluorescent signals from roots. We used the 488 nm excitation line of an argon multiline laser and the tripledichroic TD 488/543/633 nm beam splitter. CoroNa Green fluorescence emission was detected in the photomultiplier at 505–525 nm. Calcium Green-5N fluorescence emission was detected in the photomultiplier at 520–550 nm. APG-2 fluorescence emission was detected in the photomultiplier at 530–550 nm. Images were analysed with Image J software (NIH, USA) to calculate cell fluorescence based on integrated density. The background signal was measured from an empty region with a similar size and subtracted from the whole-cell signal to obtain relative total cell fluorescence values (Bonales-Alatorre et al., 2013b). Relative total cell K⁺, Ca²⁺, and Na⁺ concentration data shown in Figs 3, 5, and 7 were divided by 1000.

The cytosolic Ca²⁺ concentration in Arabidopsis root cells was also determined using the fluorescent dye Calcium Green. For image analysis, several lines were drawn across the region of interest as shown in Supplementary Fig. S2A in an appropriate zone with Leica Application Suite X software (Leica Microsystems). Continuous fluorescence was quantified in arbitrary units by LAS AF software based on intensity and plotted in an Excel file (Supplementary Fig. S2B) (Wu et al., 2015).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21 (IBM, New York, USA). All data in the figures are given as means ±SE. The significant differences in relative expression level, relative total cell K⁺, Ca²⁺, and Na⁺ concentration, and phenotype were compared using Duncan’s multiple range test. Different lower case letters represent a significant difference between genotypes and treatments at P<0.05. The comparison of cytosolic Ca²⁺ concentration in different genotypes was done by paired samples t-test. The significance levels are *P<0.05, **P<0.01, and ***P<0.001.

Results

Hypoxia-induced changes in Ca²⁺ transporter transcript levels in Arabidopsis roots

We investigated the effects of hypoxia on transcript levels of ACA8, ACA11, CAX4, and CAX11 in the roots of Arabidopsis WT after 1, 24, and 72 h of hypoxia treatment (Fig. 1). With the exception of ACA11, all genes were down-regulated after 1 h of hypoxia treatment (Fig. 1). Hypoxia had the highest impact on transcript levels of ACA8, CAX4, and CAX11 in Arabidopsis roots at 24 h, with some transcripts reduced to only 33% of the control. Extending the hypoxia period to 72 h produced no significant differences in expression levels of these genes in roots as compared with the 24 h treatment.

Hypoxia-induced K⁺ distributions show tissue- and zone-specific responses in roots of Arabidopsis mutants

We studied changes of cellular K⁺ concentration in functionally different root zones (meristem, elongation, and mature) and tissues (epidermis and stele) in roots of Arabidopsis WT and four Ca²⁺ transporter mutants after 24 h of hypoxia treatment (Figs 2, 3). In root epidermis, hypoxia led to an increase in the cellular K⁺ concentration in the root meristem but a decrease in the mature zone cells in WT Col-0 plants (Fig. 3A). In the root stele, the cellular K⁺ concentration was increased by 1.5- to 2-fold in both apex (elongation) and mature root tissues (Fig. 3B). Mutant plants showed similar trends to the WT, but with several exceptions. Hypoxia caused a dramatic increase in K⁺ concentration in meristem and stelar tissue in the elongation zone of roots of aca8, aca11, and cax11 (Fig. 3), but led to a significant decrease in K⁺ concentration in these cell types in cax4 (Fig. 3B). Interestingly, elongation zone cells in the epidermis in cax4 had a significant decrease in K⁺ concentration after hypoxia, while there was no significant difference among the other four genotypes. Compared with the control, there was a 20% decrease in K⁺ in the mature zone in root epidermis in the WT and a 50% decrease in K⁺ in aca8, and no significant difference among the other lines (Fig. 3A; Table 1). Also in the stelar cells of

![Fig. 1](image-url) Relative expression of ACA8, ACA11, CAX4, and CAX11 in roots of the Arabidopsis wild type (WT) under normoxic or hypoxic treatments for 1, 24, and 72 h. RPB2 was used as a reference gene. Data are the mean ±SE (n=3 separate experiments each involving 3–5 biological replicates). Different lower case letters indicate significant differences between treatments at P<0.05.
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mature zones, after 24 h of hypoxia treatment, the K⁺ concentration increased dramatically in the WT, aca11, cax4, and cax11, but was not changed in aca8 (Fig. 3B).

Hypoxia induces an elevation of Ca²⁺ in the mature root zone

A clear difference in Ca²⁺ concentration was found in cells of the meristem, elongation zone, and mature zone after 24 h of hypoxia treatment, showing genotypic difference after O₂ deprivation (Figs 4, 5). In the root meristem (Fig. 5A), epidermal cells of WT plants showed a 40% loss of Ca²⁺ concentration under 24 h of hypoxic conditions. However, no significant changes in Ca²⁺ concentration were detected in aca8, aca11, cax4, and cax11 mutants compared with normoxic conditions (Fig. 5A; Table 1). In epidermal cells of the elongation zone (Fig. 5A), the Ca²⁺ concentration in the WT was much higher under hypoxic conditions, while a 30–40% decrease was measured in aca11, cax4, and cax11 mutants, and no changes in aca8. Epidermal cells in the mature zone showed a dramatic increase in Ca²⁺ level in the WT (3.3-fold) and cax mutants (a 1.3- to 1.7-fold increase) and no changes in the aca mutants (Fig. 5A; Table 1). Comparing the stelar cells in the elongation and mature zones, hypoxia had no significant effect on Ca²⁺ in the elongation zone of any genotype, but resulted in marked increases in Ca²⁺ for stelar cells.
in the mature zone of \textit{aca11}, with a 10.8-fold rise of Ca$^{2+}$ compared with the control and a 5.6-fold increase in \textit{cax11}, while the increase was only 2.7-fold in the WT (Fig. 5B).

**Combined hypoxia and salinity stress cause higher Na$^+$ accumulation in stelar cells of the elongation zone and mature zone**

NaCl treatment (50 mM) with hypoxia caused dramatic increases in the Na$^+$ concentration, especially in the root stele in both the elongation zone and mature zone (Fig. 7B). \textit{aca11} showed a huge 16.1-fold increase in Na$^+$ concentration in the mature zone in the root stele, while the changes for the WT and other mutants were from 3.3- to 4.5-fold higher than in the control. Also in stele, the Na$^+$ concentration increased moderately from 1.5- to 2.9-fold in the elongation zone of all these genotypes (Fig. 7B). Hypoxia and salinity stress caused complex changes in the root epidermis (Figs 6, 7A). In root meristem cells, no significant differences of Na$^+$ accumulation were found among WT, \textit{aca8}, and \textit{cax4}, while there were 2.6- and 2.4-fold higher levels of Na$^+$ in \textit{aca11} and \textit{cax11}, respectively (Fig. 7A; Table 1). In epidermal cells of the
Table 1. Effects of 24 h of hypoxia on quantitative characteristics of K⁺, Ca²⁺, and Na⁺ concentration measured from the meristem, elongation epidermal cell, elongation stelar cell, mature epidermal cell, and mature stelar cell in Arabidopsis wild type (WT), and mutants acaca8, acaca11, cax4, and cax11 (ratio of hypoxia ion concentration to control ion concentration)

|          | Meristem | Elongation epidermis | Mature epidermis | Elongation stele | Mature stele |
|----------|----------|----------------------|------------------|------------------|-------------|
| WT       | K⁺       | 1.5                  | 1.0              | 0.8              | 1.7         |
|          | Ca²⁺     | 0.6                  | 1.4              | 3.3              | 1.0         |
|          | Na⁺      | 1.1                  | 0.9              | 1.1              | 1.5         |
| acaca8   | K⁺       | 2.1                  | 1.2              | 0.5              | 2.0         |
|          | Ca²⁺     | 0.8                  | 1.0              | 1.0              | 1.1         |
|          | Na⁺      | 1.3                  | 1.0              | 1.1              | 1.8         |
| acaca11  | K⁺       | 2.8                  | 1.2              | 1.2              | 2.3         |
|          | Ca²⁺     | 0.8                  | 0.7              | 1.3              | 1.0         |
|          | Na⁺      | 2.6                  | 2.2              | 2.2              | 2.9         |
| cax4     | K⁺       | 0.8                  | 0.6              | 0.9              | 0.7         |
|          | Ca²⁺     | 1.0                  | 0.6              | 1.7              | 0.8         |
|          | Na⁺      | 1.4                  | 2.0              | 1.1              | 2.7         |
| cax11    | K⁺       | 2.0                  | 0.9              | 1.1              | 1.5         |
|          | Ca²⁺     | 1.0                  | 0.6              | 1.3              | 1.2         |
|          | Na⁺      | 2.4                  | 1.5              | 1.0              | 1.6         |

Data are the ratio of hypoxia over control. Measurements were collected from the meristem, elongation zone epidermal cell, elongation zone stelar cell, mature epidermal cell, and mature stelar cell of five Arabidopsis lines.

elongation zone, there were no changes of Na⁺ distribution in the WT and acaca8, but 2.2-, 2-, and 1.5-fold increases were found in acaca11, cax4, and cax11. Interestingly, in the mature zone in the root epidermis, all the five genotypes showed no significant Na⁺ concentration changes compared with the control (Fig. 7A).

**More Ca²⁺ is accumulated in the cytosol of stelar cells in the mature zone**

Hypoxic stress-induced changes in the [Ca²⁺]_{cyt} have been rarely considered in the context of the tissue-specific expression and regulation of appropriate Ca²⁺ transporters. Significantly (P<0.01) higher quantities of Ca²⁺ were accumulated in the cytosol of meristematic cells in acaca8 and acaca11 after 24 h of hypoxia, while no changes were observed in the WT, acaca8, and cax4 (Fig. 8A). In the elongation zone in root epidermis, [Ca²⁺]_{cyt} increased significantly in the WT (P<0.05) and acaca8 (P<0.05) and highly significantly in acaca11 (P<0.001), but no changes were found in cax4 and acaca11 (Fig. 8B). Epidermal cells in the mature zone showed significant elevation in [Ca²⁺]_{cyt} in all genotypes except acaca11 (Fig. 8C). Stelar root cells in the elongation zone had a higher Ca²⁺ concentration in the cytosol under hypoxic conditions in three of the mutants but not in the WT and acaca8 (Fig. 8D). In stelar cells of the mature zone, a significant (P<0.001) increase in the [Ca²⁺]_{cyt} was found in all the five genotypes (Fig. 8E).

A loss of CAX11 results in a sensitive phenotype to waterlogging stress

Growth of the Arabidopsis WT and all the mutants was significantly (P<0.05) reduced after 3 weeks of waterlogging stress (Fig. 9). The observed decline in the shoot fresh weight, dry weight, and shoot Fv/Fm was highly genotype specific, with cax11 more sensitive to waterlogging stress than the other genotypes. The knock-out mutant cax11 showed the strongest growth phenotype and exhibited a 60% reduction in shoot fresh weight and 67% loss in shoot dry weight after 3 weeks of waterlogging. Meanwhile, the average waterlogging-induced reductions in the shoot fresh and dry weight in the WT and other mutants were only 42% and 18%, respectively (Fig. 9A, B, D, E). These results were consistent with the significant decrease in the maximal photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values) in leaves of cax11 (Fig. 9C, F).

**Discussion**

**Ca²⁺ efflux systems play important roles in hypoxic stress**

A higher concentration of cytosolic Ca²⁺ can cause cytotoxicity owing to the precipitation of Ca²⁺ with phosphate and other anions, or binding to negatively charged macromolecules in the cytosol (Williams, 2006); it may also interfere with the cell’s ability to respond to various stimuli (Bose et al., 2011). To restore the low resting concentration in the cytosol, Ca²⁺ has to be transported out against an electrochemical gradient into the apoplast, or into intracellular organelles by Ca²⁺-ATPases and/or Ca²⁺/H⁺ antiporters. It is suggested that Ca²⁺-ATPases with high-affinity (K_m=0.1–2 μM) and low-capacity transporters are responsible for termination of [Ca²⁺]_{cyt} signalling; while Ca²⁺ exchangers with low-affinity (K_m=10–15 μM) and high-capacity transporters may be involved in the removal of [Ca²⁺]_{cyt} when its elevations are high (Sze et al., 2000; Bose et al., 2011; Shabala et al., 2015). Recently, CAX11 was reclassified as CCX5 (Cation Calcium
eXchanger) due to higher homology to mammalian K$^+$-dependent Na$^+$/Ca$^{2+}$ antiporters which catalyse the electrogenic countertransport of four Na$^+$ for one Ca$^{2+}$ and one K$^+$ (Dong et al., 2001; Cai and Lytton, 2004; Shigaki and Hirschi, 2006). Expression of AtCCX5 in a yeast mutant restored its growth in low K$^+$ (0.5 mM) medium and also suppressed its Na$^+$ sensitivity (Zhang et al., 2011). These findings suggest that CAX11 not only has the ability to remove Ca$^{2+}$ into the vacuole but also plays a role in Na$^+$ transportation. In the present study, cax11 was more sensitive to 3 weeks of waterlogging stress than the WT and other mutants tested (Fig. 9). We speculate that mutation of ACA8, ACA11, CAX4, and CAX11 in Arabidopsis potentially reduces the ability to terminate [Ca$^{2+}$]$_{cyt}$ signalling or to remove [Ca$^{2+}$]$_{cyt}$ disturbance of Ca$^{2+}$ homeostasis. Knock out of the multiple functional CAX11 might lead to sensitivity to waterlogging stress.

Ca$^{2+}$ efflux systems play important roles in stress tolerance (Qudeimat et al., 2008; Bose et al., 2011). The role of ACAs in shaping [Ca$^{2+}$]$_{cyt}$ signatures under salinity stress was first demonstrated in yeast (Anil et al., 2008). Knocking out AtACA4 and AtACA2 resulted in an increased NaCl sensitivity, while their overexpression in Arabidopsis seedlings led to increased stress tolerance in comparison with WT plants (Geisler et al., 2000). After 2-4 h of low O$_2$ treatment, ZmCAP1 encoding Ca$^{2+}$-ATPase in maize roots showed a 2- to 3-fold increase in

Fig. 4. Distribution of Ca$^{2+}$ in the root meristem, elongation, and mature zone in Arabidopsis wild-type (Col-0), aca8, aca11, cax4, and cax11 under normoxic or hypoxic treatments for 24 h. (A) Representative images of the root apex in control and hypoxic treatment. (B) Representative images of the root mature zone in control and hypoxic treatment. The relative Ca$^{2+}$ concentration in roots of 10-day-old seedlings was visualized using a confocal imaging system with Calcium Green fluorescent dye. One out of nine typical images is shown for each line. Scale bar=50 µm.
expression levels (Subbaiah and Sachs, 2000). In contrast, 1h and 24h hypoxic treatments reduced the expression of ACA8, CAX4, and CAX11 in WT roots to 33–50% of the control (Fig. 1), but not for ACA11. Reports on the functional role of ACA11 in plants are rather scarce, to say nothing of its modulation by hypoxic stress. ACA11 was identified as localized on the tonoplast and preferentially expressed in Ca-rich mesophyll; the simultaneous knock out of ACA4 and ACA11 does not appear to reveal a phenotype associated with perturbed apoplasmic Ca\(^{2+}\) homeostasis compared with cax1/cax3 (Conn et al., 2011). In the current study, after 3 weeks of waterlogging stress, the aca11 mutant did not show significant changes in shoot dry weight and maximum photochemical efficiency of PSII (chlorophyll fluorescence) compared with the control, consistent with the insignificant changes of ACA11 expression in the WT, which suggests that ACA11 is

![Fig. 5](image-url). Relative Ca\(^{2+}\) concentration in the meristem, elongation, and mature zone in root epidermis (A) and stele (B) of Col-0, aca8, aca11, cax4, and cax11 under normoxic or hypoxic treatments for 24h. The relative Ca\(^{2+}\) concentration was calculated by the fluorescence integrated density using Image J software. Data are the mean ±SE (n=30–40 cells from one individual plant with at least nine replicate plants). Different lower case letters indicate significant differences at P<0.05.
unlikely to play an important role in hypoxic stress responses. In rice, Ca^{2+} was reported to act as a physiological transducer for the expression of the alternative oxidase 1a (AOX1a) gene under O_2 deficiency (Tsuji et al., 2000). Here we suggest that hypoxia induces a rapid elevation of Ca^{2+} concentration as a signal, which probably inhibits the expression of Ca^{2+} efflux transporters. The Atcax1 mutant showed enhanced tolerance to freezing following cold acclimation (Catala et al., 2003). In contrast, Atcax3 has increased sensitivity to salt stress (Zhao et al., 2008). In our present work, cax11 was more sensitive to waterlogging than the WT (Fig. 9). Ca^{2+}/H^+ exchangers may be involved in resetting the [Ca^{2+}]_cyt elevation following different kinds of stress induction with specific characteristics (Dodd et al., 2010). The differential stress sensitivities of the cax mutants may be a consequence of specific responses by AtCAX1, AtCAX3, and AtCAX11 to the individual stresses (McAinsh and Pittman, 2009).

**Fig. 6.** Distribution of Na^+ in the root meristem, elongation, and mature zone in Arabidopsis wild type (Col-0), aca8, aca11, cax4, and cax11 under normoxic or hypoxic treatments for 24 h. (A) Representative images of the root apex in control and hypoxic treatment. (B) Representative images of the root mature zone in control and hypoxic treatment. The relative Na^+ concentration in roots of 10-day-old seedlings was visualized using a confocal imaging system with CoroNa Green fluorescent dye. One out of nine typical images is shown for each line. Scale bar=50 µm.

_Hypoxia stress leads to cell- and tissue-specific changes in K^+, Ca^{2+}, and Na^+ homeostasis_

Over the last few decades, increasing numbers of reports showed that plant stress tolerance is conferred by cell type-dependent processes (Roberts and Tester, 1995; Nylander et al., 2001; Ahmad and Maathuis, 2014). In this study, hypoxia had significant impacts on tissue distributions and cellular concentrations.
Calcium transporters in hypoxia responses

of K⁺ and Ca²⁺ in roots of Arabidopsis WT and Ca²⁺ transporter mutants. The combined hypoxia and salinity stress also had significant effects on the distribution of Na⁺ in different tissues and cell types in Arabidopsis WT and Ca²⁺ transporter mutants. Correspondingly, O₂ gradients have been found along both longitudinal (Pang et al., 2006) and radial (Armstrong, 1979; Armstrong et al., 1994; Gibbs et al., 1998) root profiles under hypoxic stress. It was demonstrated that with the shoot in the air, different barley root zones had different O₂ requirements for uptake from the medium, with higher O₂ influx in the elongation zone than in the mature zone (Pang et al., 2006). An excised maize root showed a steep O₂ concentration decline from the outer layers of cells to the stelar cells when in hypoxic solution (Gibbs et al., 1998), showing more severe hypoxic stress within the stele than in the cortex. Intact barley roots in deoxygenated stagnant solution and relying on internal O₂ movement into and along the root aerenchyma had slightly higher O₂ in various tissues in the subapical region than just behind the root tip. At the subapical region clear radial gradients in O₂ were seen, with the stele having the lowest O₂ concentration (Kotula et al., 2015). Therefore, it is suggested that different cell types in roots with diverse access to, and potential sensitivity to, O₂ operate differently under hypoxic stress. Our present data further support this suggestion that various cell types in different zones regulate concentrations of K⁺, Ca²⁺, and Na⁺ in distinct ways when faced with hypoxia.

Calcium is a universal secondary messenger response to abiotic and biotic stress (Chen et al., 2010; Shabala et al., 2011;...
but the changes in Ca\textsuperscript{2+} in different tissue-specific cells in roots under hypoxia required elucidation. The interesting finding in our data is that in the WT and all of the four Ca\textsuperscript{2+} transporter mutants (*aca8*, *aca11*, *cax4*, and *cax11*), the stelar cells in the elongation zone showed no changes in Ca\textsuperscript{2+} concentration, while in the mature zone they had higher Ca\textsuperscript{2+} than in normoxic controls (Fig. 5). This situation is similar to the stelar cells in the mature zone which also had significant increases of [Ca\textsuperscript{2+}]\textsubscript{cyt} in these five genotypes when treated with hypoxia (Fig. 8E). There have been very few studies on the Ca\textsuperscript{2+} concentration changes in different tissue-specific cells in roots under hypoxia. Studies on Arabidopsis leaf showed that there is more Ca\textsuperscript{2+} accumulated in the vacuoles of mesophyll cells than in those of the epidermis (Conn et al., 2011), which has been consistently observed across all eudicots so far examined. Conversely, the cereal monocots barley and wheat preferentially accumulate Ca\textsuperscript{2+} in epidermal cells and not within mesophyll or vascular bundle cells (Conn and Gilliham, 2010). In general, the simple presence or absence of ion transporters could not explain cell type-specific differences in ion accumulation. However, we might suggest that the higher concentration of Ca\textsuperscript{2+} observed in stelar cells in the mature zone than in stelar cells in the elongation zone in hypoxic roots provides evidence for the necessity for energetically expensive Ca\textsuperscript{2+} accumulation in the root stele tissue (Subbaiah and Sachs, 2003).

Fig. 8. Relative Ca\textsuperscript{2+} concentration in the cytosol in the meristem, elongation, and mature zone in root epidermis and stele of Col-0, *aca8*, *aca11*, *cax4*, and *cax11* under normoxic or hypoxic treatments for 24 h. Data are the mean ±SE (n=60 cells from one individual plant with at least nine replicate plants). Asterisks indicate significant differences at *P*<0.05, **P*<0.01, and ***P*<0.001.
Calcium transporters in hypoxia responses

There are numerous studies reporting perturbation in intracellular K+ homeostasis in response to hypoxia in plants (Wiengweera and Greenway, 2004; Mugnai et al., 2011; Barrett-Lennard and Shabala, 2013). Hypoxia increased K+ efflux in 6-day-old wheat roots due to membrane depolarization and the increase of membrane permeability of K+ (Pang and Shabala, 2010). In Vitis riparia roots, an anoxia-tolerant variety, severe K+ imbalance is avoided during anoxia stress by decreasing membrane permeability of K+; whereas Vitis rupestris, an anoxia-sensitive variety, had a strong decrease of K+ (Mancuso and Marras, 2006). Accordingly, in aerobic roots, xylem loading of ions from the external medium is achieved with energy provided by the plasma membrane H+-ATPase (De Boer and Volkov, 2003) through the depolarization-activated outwardly rectifying K+ (SKOR) channels. Since the stele is the first root tissue suffering O2 deficiency, the H+-ATPase activities in the xylem would be inhibited, leading to the closure of SKOR channels for a substantial decline of xylem K+ concentration (Colmer and Greenway, 2011). However, the present data in our experiment showed that there was more K+ accumulation in the stele than in the epidermis in both the elongation zone and mature zone in the WT, aca11, and cax11, which is contrary to the former theory. We speculate that the K+ accumulation in stele could probably flow via NORCs (non-selective outward-rectifying channels) (Chen et al., 2007; Zepeda-Jazo et al., 2008). Unfortunately, the molecular identity of NORCs (as other non-selective cation channels) remains elusive (Demidchik and Maathuis, 2007), and their expression levels cannot thus be thus directly measured. In addition, cells in hypoxic tissues
would still have produced some ATP by oxidative phosphor-
ylation when O₂ was above zero, or from glycolysis linked to
ethanolic fermentation (Kotula et al., 2015).

Although waterlogging is a widespread stress in its own
right, its impact on a plant performance is often confounded
by soil salinity (Barrett-Lennard and Shabala, 2013). Even
a brief period of root O₂ deprivation might result in a pro-
longed Na⁺ accumulation even after return to normoxic con-
ditions (Marschner, 2011). Thus, it was of interest to measure
the Na⁺ concentration under both hypoxia and salinity con-
ditions. After 24h of combined salt and hypoxic treatment,
there was a significant increase in Na⁺ in the elongation zone
stele and a dramatic increase in mature stelar cells in the WT
and all of the four Ca²⁺ transporter mutants (Fig. 7B). H⁺-
ATPase pumps are responsible for the maintenance of the
highly negative membrane potential at the root plasma mem-
brane, and the low energy in O₂-deficient tissues leads to a
substantial depolarization of the plasma membrane potential
and the closure of SKOR, which is highly selective for K⁺
over Na⁺ (Dietz et al., 2001; Koizumi et al., 2011; Zeng et al.,
2014). In contrast, the NORC, transporting K⁺, Na⁺, and
anions could open during hypoxia, and NORC is also consid-
ered as the main transporter for loading Na⁺ into the xylem
(Wegner and Raschke, 1994; Wegner and DeBoer, 1997; Pang
and Shabala, 2010), which probably explains the increase of
Na⁺ in cells of both the elongation zone and mature stele.
Interestingly, we also found that there was a 2.6-fold increase
in Na⁺ concentration in mature stelar cells relative to elon-
gation zone stelar cells under hypoxia, while there was no
difference between the two kinds of cells in the WT under
normoxic conditions (Supplementary Table S2). This may be
suggestive that even under hypoxia there is still Na⁺ flow
in the xylem from the root to the shoot via other pathways.
Some studies found that protein kinase SOS2 (Salt Overly
Sensitive 2)/CIPK24, involved in salt tolerance (Cheng et al.,
2004), could bind to the autoinhibitory domain of CAX1 to
activate the Ca²⁺ transport into the yeast vacuole, making
the cells tolerant to the high Ca²⁺ in the media. This result
implies the co-regulation of CAX1 and the SOS1 antiporter
by SOS2/CIPK24 in the signalling pathway for salinity toler-
ance (Shigaki and Hirschi, 2006). Therefore, a possible con-
vergence of salinity and hypoxia tolerance via the link to Ca²⁺
signalling and Ca²⁺ efflux systems is highly likely. This ques-
tion requires a detailed study in the future.

Conclusions

The results presented here allow a new hypothesis concern-
ing the hypoxic signal transduction in Arabidopsis roots
linked with CAX and ACA Ca²⁺ transporter systems. The
tendency in past research on hypoxic stress has been to focus
on whole roots, but the key challenge now is to try to explain
root function in terms of the different root zones (meristem,
elongation, and mature), tissues (epidermis and stele), and
specific cell types. In this study, unique cell types in differ-
ent zones regulate concentrations of K⁺, Ca²⁺, and Na⁺ in
distinct ways when faced with hypoxia. In the WT and all
of the four Ca²⁺ transporter mutants, the stelar cells in the
elongation zone showed no changes in Ca²⁺ concentration,
while in the mature zone they had higher Ca²⁺ than normoxic
controls. We also speculate that more K⁺ and Na⁺ accumu-
lation in the stele than in the epidermis in both the elongation
zone and mature zone could probably flow via NORC which
could open during hypoxia. In addition, Ca²⁺ efflux systems
and in particular CAX11 were shown to mediate plant Ca²⁺
homeostasis under hypoxic conditions, with an influence on
waterlogging tolerance.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Dose dependence of Calcium Green loading
into five types of Arabidopsis root cells.

Figure S2. Illustration of the quantification procedure
for Ca²⁺ distribution in the cytosol.

Table S1. The primers for quantitative RT-PCR experiments.

Table S2. Effect of 24h of hypoxia on Na⁺ relative concen-
trations in stelar cells of the elongation zone and mature zone
in Arabidopsis WT, aca8, aca11, cax4, and cax11 (ratio of
Na⁺ concentration in mature stelar cells relative to elongation
stem cells under control or hypoxic conditions).

Acknowledgments

This project is supported by an Australian Research Council (ARC)
Discovery Project to TDC and SS. Z-HC is supported by an ARC Discovery
Early Career Researcher Award (DE140101143) and a 1000-Plan project. XL
is the recipient of a China Scholarship Council award. We thank Dr Rong
Liu, Linda Westmoreland, Liz Kabonoff, Dr Sumedha Dharmaratne, and
Rosemary Freeman for their technical assistance. We thank especially Dr
Anya Salih for her support on imaging at the Confocal Bio-Imaging Facility
at Western Sydney University.

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