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# Leaf mesophyll $K^+$, $H^+$ and $Ca^{2+}$ fluxes are involved in drought-induced decrease in photosynthesis and stomatal closure in soybean

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ABSTRACT

Understanding the roles of chemical signals for drought tolerance is important for improving plant water use efficiency. Microelectrode ion flux measurement (MIFE), leaf gas exchange, and stomatal imaging were employed to assess the impact of short-term, PEG-induced and prolonged drought stress on soybean plants. We developed a new method to record steady-state $K^+$, $H^+$ and $Ca^{2+}$ fluxes from leaf mesophyll of soybean plants grown in a glasshouse over a long time period. Long-term $K^+$, $H^+$ and $Ca^{2+}$ fluxes under drought condition differed significantly from short-term PEG-induced drought stress. Moreover, the magnitude of changes differed between the ion fluxes and the physiological and growth traits. For instance, in the severe drought treatment, differences in the magnitude of $Ca^{2+}$ efflux between the drought-stressed plants and the control were greater than the changes in aperture width, guard cell width and leaf temperature. In addition, $H^+$ influx and $K^+$ and $Ca^{2+}$ efflux of leaf mesophyll were highly significantly ($P < 0.01$) correlated with many physiological traits. In summary, our results suggest that a large $K^+$ efflux, alkalisation of apoplastic pH ($H^+$ influx), and an early response of $Ca^{2+}$ efflux from leaf mesophyll are likely to serve as chemical signals and significant indicators for levels of drought stress in soybean.



## 1. Introduction

Drought is a meteorological term for water scarcity and imposes a range of stresses on plants. Drought stress is often associated with high temperature and irradiance, high soil salinity, low soil nutrient availability, and mechanical damage to roots in hot and hard soil (Ingram and Bartel, 1996; Zhu, 2002; Wilkinson and Davies, 2010). Global climate change is projected to increase the frequency and intensity of drought occurring due to increasing temperatures (Easterling et al., 1997) resulting in reduced global food and feed supplies (Schmidhuber and Tubiello, 2007). The recent year-long drought in 2012 in the Midwest/Plains in the United States is estimated to have cost US$35 billion including a large loss of soybean production. Therefore, the characterisation of underlying mechanisms of drought tolerance is a major goal of soybean production and breeding programmes (Pathan et al., 2007; Manavalan et al., 2009).

Chemical signals, essential for plant adaptation to water deficits, dominate during drought stress before hydraulic signals (Davies et al., 2002; Christmann et al., 2007). Although abscisic acid (ABA), pH, $Ca^{2+}$, malate and other factors have all been implicated in root to shoot signalling during drought stress, the role of these signals is still controversial (Schachtman and Goodger, 2008). Potassium is a limiting macronutrient for crop yield and quality. Maintaining $K^+$ homeostasis is essential for enzyme activation, stabilisation of protein synthesis, neutralisation of negatively charged proteins, formation of membrane potentials, and cytosolic pH homeostasis (Shabala and Cuin, 2008; Dreyer and Uozumi, 2011). Also, for a range of abiotic stresses in plants, a direct causal link has been found between the control of $K^+$ flux and programmed cell death (PCD) due to increased activities of proteases and endonucleases at low cytosolic $K^+$ (Davies et al., 1992; Shabala et al., 2007; Demidchik et al., 2010). Changes in apoplastic pH have a fundamental role in drought-induced chemical signalling and regulation. Apoplastic pH modulates ABA metabolism resulting in elevated leaf ABA concentrations and directly affects leaf water status that can alter guard cell turgor or sensitivity to leaf ABA concentration. Also, apoplastic pH regulates ion fluxes through the plasma membrane and alters distribution of ABA in the leaf cell compartments (Wilkinson and Davies, 1997; Wilkinson, 1999; Schachtman and Goodger, 2008). $Ca^{2+}$ is a key second messenger for drought stress responses, and drought stress-induced stomatal closure is achieved by dynamic waves of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_{cyt}$) regulated via

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ABA (Blatt, 2000; McAinsh and Pittman, 2008; Dodd et al., 2010). ABA-regulated, repetitive $[Ca^{2+}]_{cyt}$ elevations are suggested to be responsible for the inhibition of $H^+$-ATPase, inwardly rectifying $K^+$ and anion channels (Grabov and Blatt, 1999; Chen et al., 2010, 2012; Hills et al., 2012). Ultimately, plants need to regulate $K^+$, $Ca^{2+}$ and pH levels in leaf tissue to maintain photosynthetic competence, growth and yield. Thus, the ability of leaf mesophyll cells to control $K^+$, $H^+$ and $Ca^{2+}$ fluxes across the plasma membrane may be essential components of tissue tolerance, contributing to overall plant performance under extended drought.

Experimentally, drought is not easy to mimic in the laboratory, and many studies have used chemicals such as polyethylene glycol (PEG) and ABA for this purpose. Studies have measured ion fluxes (Pandolfi et al., 2012) and the expression of genes and proteins (Mohammadi et al., 2012; Fan et al., 2013) in leaves by applying polyethylene glycol (PEG). However, few relevant genes and proteins involved in $K^+$, $H^+$ and $Ca^{2+}$ transport and homeostasis were detected in the above studies on soybean. Also, few reports of ion fluxes in plants grown under drought treatments in the glasshouse or field have been published. In addition, changes in $K^+$, $H^+$ and $Ca^{2+}$ fluxes resulting from simulated, transient drought treatment have only been measured over relatively short periods, typically around 1 h in plants, but never determined over sustained periods (days or even weeks) of drought treatment. Therefore, there are still significant gaps in our understanding of the roles of chemical signals in drought, particularly in changes in long-term ion fluxes and their possible regulation of drought-induced physiological and growth responses.

In order to fill these gaps, we employed a variety of physiological techniques, including microelectrode ion flux measurement (MIFE), to assess the impact of prolonged drought on the performance of soybean plants. MIFE is an ideal technique for studying the tolerance of plants to some abiotic stresses (Shabala and Cuin, 2008), but its use in plant drought research is limited. Our overarching hypothesis was that changes in ion fluxes due to long-term drought affect stomatal behaviour, photosynthesis and growth of soybean plants and are different from those due to short-term treatments. Therefore, this study aimed to determine whether MIFE was applicable for following changes in ion fluxes in long-term drought experiments; to reveal potential regulation of leaf mesophyll ion fluxes on other physiological traits; and to explore supporting evidence for $K^+$, $H^+$, and $Ca^{2+}$ fluxes as chemical signals in drought stress tolerance of soybean.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Soybean (*Glycine max* L. cv. Galaxy [Global Food Group, Villawood, Australia]) was used for all experiments. The seeds used to produce seedlings for transient ion flux measurements in root epidermis were sterilised with commercial bleach for 10 min and germinated on Petri dishes. The germinated seeds were then transferred to a hydroponic growth system as described in Chen et al. (2005). Roots of 10-day-old seedlings were used for ion flux measurements at the mature zone ~10 mm from the apex.

Two experiments were conducted from 2012 to 2013 in a glasshouse with natural light, a day/night air temperature of $26/24 \pm 1\,^{\circ}C$ and ~60% relative humidity. Five seeds were sown in 4 L pots containing potting mixture (Debco Pty Ltd, Victoria, Australia) with a slow-release Osmocote® fertiliser (Scotts Australia, Sydney, Australia) and thinned to two healthy and uniform seedlings two weeks after germination. The plants were irrigated with half-strength Hoagland's solution and water to maintain a full water holding capacity (~50%, v/v) before the mild and severe drought treatments were applied at five weeks after sowing. The mild drought treatment was achieved by supplying 50 mL of water in each pot every other day, and watering was withheld from the onset of the treatment for severe drought. There were 10 pots (20 plants) each for the control, mild drought and severe drought treatments, and plant height, leaf number, and biomass were measured in both experiments. In Experiment 1, leaf samples were collected for short-term PEG-induced and long-term steady-state ion flux measurements. In Experiment 2, leaf samples were used for stomatal assay, gas exchange, pigment, and osmolality measurements and yield was determined.

### 2.2. Ion flux measurement

Net fluxes of $K^+$, $H^+$, and $Ca^{2+}$ were measured noninvasively using ion-selective vibrating microelectrodes (the MIFE technique) essentially as described in Shabala et al. (1997). Roots and the third fully expanded leaves were collected and placed in a 5 mL Perspex-glass measuring chamber with 2.5 mL control solution (0.5 mM KCl and 0.1 mM $CaCl_2$). The root and leaf segments were centred within the chamber and fixed horizontally by immobilising the segments using transparent rubber cross-bars within the chamber. Three electrodes were filled with ion-selective cocktails (Sigma, Buchs, Switzerland), and their tips aligned and positioned ~40 μm above the surface of the tissue. During measurements, electrodes were moved towards and away from the sample in a slow (10 s cycle, 40 μm amplitude) square-wave by a computer-driven micromanipulator. Net ion fluxes were calculated from the measured differences in electrochemical potential for these ions between two positions (Newman, 2001).

$K^+$, $H^+$ and $Ca^{2+}$ fluxes of root and leaf segments were measured for 10 min in the control to ensure steady initial values before adding 50 g $L^{-1}$ PEG 8000 for another 30 min. For drought-induced steady-state leaf mesophyll ion fluxes, a 40 mm × 2 mm segment was cut from the leaf and immediately immersed in the control solution. A standardising procedure was conducted to validate that the ion fluxes were steady and no large net influx or efflux occurred over a relatively long time. We found that 1 h pre-incubation in the control solution was sufficient for leaf segments to recover from cutting-induced, mechanical damage. Based on this procedure, we only collected 5 min ion flux data for each sample after 1 h of pre-incubation. Therefore, on each day of measurement, all samples were measured in a relatively short timeframe (~4 h) in order to minimise the effects of drought over a long time.

### 2.3. Gas exchange measurement

Net $CO_2$ assimilation ($A$, μmol $m^{-2}$ $s^{-1}$), stomatal conductance ($g_s$, mol $m^{-2}$ $s^{-1}$), intracellular $CO_2$ concentration ($C_i$, μmol $mol^{-1}$), transpiration ($T_r$, mmol $m^{-2}$ $s^{-1}$), and leaf temperature ($T_{leaf}$, °C) of the third fully expanded leaves and vapour pressure deficit (*VPD*, kPa) were determined over 24 days with an LI-6400XT infrared gas analyser (Li-Cor Inc., Lincoln, NE, USA). The conditions in the measuring chamber were controlled at an air flow rate of 500 mol $s^{-1}$, a saturating photosynthetically active radiation (PAR) of 1500 μmol $m^{-2}$ $s^{-1}$, 400 mmol $mol^{-1}$ $CO_2$, and a relative humidity of 60–70%.

### 2.4. Pigments and leaf sap osmolality measurement

Chlorophyll a, b and carotenoid content were measured according to Arnon (1949). Briefly, 0.1 g disks of the third fully expanded leaves were placed in 5 mL of extraction solution containing 80% acetone and 20% ethanol (v/v) and were then kept in the dark for 48 h. The absorbance of the extraction solutions at 645, 663 and 470 nm were measured with a UV–visible spectrophotometer (Cary, Mulgrave, VIC, Australia). The pigment contents