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Early response to salt ions in maize (*Zea mays* L.)☆

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ABSTRACT

Abscisic acid (ABA) regulates leaf growth and transpiration rate of plants exposed to salt stress. Despite the known fact that cell dehydration is instrumental for the modulation of ABA concentrations when NaCl is high in the external environment, it was never tested as to whether sodium (Na) or chlorine (Cl) also modulate ABA concentrations. To answer this question, a hydroponic study on maize (*Zea mays*) was established, by exposing plants to 50 mM of sodium glucosamide or glucosamine chloride. The effect of both ions on ABA was investigated in an early stage before (*i*) the salt ions accumulated to toxic tissue concentrations and before (*ii*) cells dehydrated. This allowed studying early responses to Na and Cl separately, well before plants were stressed by these ions. Gas chromatography–mass spectrometry analysis was used to quantify ABA concentrations in roots and in leaves after a period of 2 h after ion application. The transcript abundance of the key regulatory enzyme of the biosynthesis of ABA in maize, the 9-*cis*-epoxycarotenoid dioxygenase gene *viviparous 14*, was quantified via real-time quantitative-reverse-transcriptase-polymerase-chain-reaction. The results reveal that Cl and Na induce the increase of leaf tissue ABA concentrations at two hours after plants were exposed to 50 mM of the ions. Surprisingly, this effect was more pronounced in response to the Cl component. The increase in the guard-cell regulating ABA concentration correlated with a reduced transpiration. Mainly because of this result we suggest that the early accumulation of ABA is useful in maintaining cell turgor.

1. Introduction

Abscisic acid (ABA) is a plant hormone that is synthesized from carotenoids (Seo and Koshiba, 2002). Together with other regulating factors, it controls leaf growth and transpiration. In *Arabidopsis thaliana*, approximately 1–10% of the genome is regulated by ABA, being either induced or repressed by the 15-C weak organic acid (Finkelstein, 2013). Many of those ABA-responsive genes are also related to the response to abiotic or biotic stress (Choudhury and Lahiri, 2011; Finkelstein, 2013). It was shown for different plant species, that a considerable amount of the stress-related genes that are under control of ABA contribute to adaptive aspects of induced tolerance towards dehydration, *i.e.* by encoding enzymes of the compatible solute synthesis, of the detoxification of reactive oxygen species, or water channels (Ingram and Bartels, 1996; Yamaguchi-Shinozaki and Shinozaki, 2006; Finkelstein, 2013). Moreover, the tissue concentration of ABA is relevant for the stress response to salinity as well as for a possible tolerance mechanism. As a result of a salt-stress induced reduction in the osmotic potential of the root solution, shoot ABA concentrations increase in different plant species (Munns and Termaat, 1986; Munns, 2002; Zörb et al., 2013; Geilfus et al., 2015). An increase in ABA inhibits leaf elongation rates in maize caryopses (Cramer et al., 1998). Kutschera and Schopfer (1986) argued for maize coleoptiles that this retardation of growth is based on an ABA-mediated inhibition of the capacity of the cell walls to loosen, rather than by a reduction of turgor. Under salinity-induced water stress, this ABA-mediated growth reduction is thought to counteract wilting which would otherwise be the consequence of ongoing expansion growth under conditions of limited water availability (Kutschera and Schopfer, 1986).

Another important mechanism to avoid wilting under conditions of salinity-induced water stress is the regulation of the transpiration rate via ABA-based effects on stomatal aperture. A change of the ABA

Abbreviations: (ABA), abscisic acid; (DW), dry weight; (PEG), polyethylene glycol; (RT), relative turgidity; (qRT-PCR), real-time quantitative reverse transcriptase-polymerase chain reaction; (NCED), 9-*cis*-epoxycarotenoid dioxygenase

☆ The influence of chloride on the accumulation of ABA highlight the importance of integrating chloride into models that elucidate early response during the establishment of salt stress.

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concentration is well known to promote stomatal closure or inhibit opening (McAinsh et al., 1991; Leckie et al., 1998; Hetherington, 2001; Kwa et al., 2008), a process that is relevant for the water balances under salinity or drought stress (Iuchi et al., 2001; Davies et al., 2002; Zhang et al., 2006; Bauer et al., 2013; Geilfus et al., 2015).

But what are the environmental stress conditions that activate ABA biosynthesis when plants were exposed to salinity? Drought and salt (*i.e.* NaCl) are the two environmental stress events that result in the most pronounced accumulation of ABA, being a result of an activation of ABA-biosynthesis genes (Xiong and Zhu, 2003). The common feature between both stress events is the osmotic stress component. Water availability is reduced, which can result in a reduced leaf turgor and reduced water content. Water stress is known to activate the expression of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) which is a key regulatory enzyme for the production of ABA (Seo and Koshiba, 2002; Xiong and Zhu, 2003). Thus, a reduction in the plant water content is a critical factor that activates ABA *de novo* synthesis (Thompson et al., 1997; Wilkinson and Davies, 2002) that might also be instrumental for an accumulation of ABA during NaCl-based salinity. However, irrespective of a reduction in shoot water content, *viz.* fresh weight, it was never tested as to whether the salt ions Na^+ or Cl^- represent environmental stress factor that are also instrumental in the modulation of ABA tissue concentration. We studied maize that was subjected to a two-hour treatment with both ions to clarify this, by analysing (*i*) ABA concentration and (*ii*) the transcript abundance of the maize *NCED* ortholog *viviparous 14* (*vp14*) in correlation to root and leaf sodium and chloride concentration, transpiration and relative leaf turgidity. Our experimental design that allowed to discriminate between early responses of the Na- and the Cl-component revealed that Cl is besides Na an additional environmental factor that correlates with an increase of ABA in leaves of corn that were exposed for two hours to salts.

2. Materials and methods

2.1. Plant cultivation

Zea mays (cultivar STABIL, KWS Saat SE, Einbeck, Germany) was grown in hydroponic culture in a controlled environment chamber. Seeds were imbibed in 2 mM $CaSO_4$ for 1 d with additional aeration followed by a germination period of 6 d in moistened quartz sand. Afterwards, seedlings were transferred in 5-L plastic pots containing one-quarter-strength nutrient solution. After 2 d of cultivation, the nutrient concentration was increased to half-strength and, after 4 d of cultivation to full-strength. This was done to adapt young plants stepwise to nutrient concentration in the root medium. The solution was changed every 84 h (3.5 days) to avoid nutrient depletion. The nutrient solution had the following composition: 2.5 mM $Ca(NO_3)_2$, 1.0 mM K_2SO_4, 0.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.5 mM NaCl, 1.0 µM H_3BO_4, 2.0 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.005 µM $(NH_4)6Mo_7O_{24}$, 200 µM Fe-EDTA; pH, 6.8. Plants grew under a 14 h (22 °C): 10 h (18 °C) dark: light cycle (photoperiod 07:00-21:00 h) with an atmospheric water vapour pressure deficit of 0.58 kPa (75% RH) during photoperiod. Light intensity was 320–350 µmol s^{-1} m^{-2} above leaf canopy of the growing leaf number 4. Plants grew 10 days in full-strength nutrient solution before being subjected to different short-term salt treatments over a period of 2 h.

2.2. Experimental design

The separate effect of both ionic components of NaCl, *viz.* sodium and chloride, on abscisic acid (ABA) abundance and *viviparous 14* (*vp14*) mRNA abundance was investigated at 2 h after salt ions were added into the nutrient solution of the plants. At this early phase, plants were not yet stressed by the salt ions, but investigations during this early time point may allow to elucidate early mechanisms of adaptations to increasing concentrations of salt ions. The combined effect of Na^+ and Cl^- was tested by adding 50 mM of NaCl into the nutrient solution (experimental group 1). In order to test for sodium-associated effects that are conferred when chloride is absent, 50 mM Na^+ were given together with the membrane impermeable glucosamide$^-$ as counter anion (experimental group 2). Chloride-associated effects were investigated by the substitution of Na^+ by using the membrane impermeable glucosamine$^+$ as accompanying counter cation (experimental group 3). Osmotic effects that are not related to ionic effects were investigated by treating the roots with 93 g PEG 6000 l^{-1} nutrient solution, a dose that has the same lowering effect of the osmotic potential as 50 mM NaCl (Sümer et al., 2004) (experimental group 4). Control plants were not treated with salts or PEG (experimental group 5). At two hours after salt or PEG was added, respectively, the growing leaf number four that emerged from the sheath two days ago was harvested, as were the roots. Material was frozen in liquid nitrogen being stored at −80 °C, either for ion analysis, ABA quantification or quantification of the mRNA abundance of *vp14*. Five biological replicates were taken for each experimental group.

2.3. Ion analysis

The analysis of Na^+ and Cl^- was performed with 15 mg of dried leaves that were boiled for 5 min in 1.6 ml of deionized water. After cooling, samples were centrifuged and proteins were precipitated in the supernatant by washes in chloroform. Thereafter, samples were cleaned by passage through C-18 column. Na^+ and Cl^- concentrations were analysed using ion chromatography (Dionex ICS-5000+, Life Technologies GmbH, Darmstadt, Germany). Before roots were subjected to ion analysis, roots were thoroughly washed for 30 s with 1 mM Ca_2SO_4 to remove adhering salts from the surface.

2.4. Analysis of free ABA

At least 10 mg dry powder was used for one sample to determine the plant hormone ABA. For each biological replicate two technical replicates were performed. The samples were extracted with a mixture of isopropanol and acetic acid (95:5, v/v) for 2 h under continuous shaking at 4 °C. Before the start of the extraction procedure 100 ng of heavy H labelled ABA were added to each sample. [$^2H_6$]-ABA was from the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada. Further sample preparation was performed according to Meixner et al. (2005). Briefly, the samples were centrifuged for 10 min at 10,000*g*, the supernatant removed and evaporated to dryness under a stream of N_2. The residue was resuspended in methanol, centrifuged again for 10 min at 10,000*g*, the supernatant was removed and transferred in a glass vial. The compounds in the methanolic extract (20 µl) were methylated by addition of equal amounts of a 1:10 diluted solution (in diethylether) of trimethylsilyldiazomethane (Sigma-Aldrich, Germany) for 30 min at room temperature. The mixture was then evaporated and resuspended in 50 µl ethyl acetate for GC–MS analysis. Gas chromatography–mass spectrometry analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer using electron impact ionization at 70 eV, connected to a Varian *CP*-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Walnut Creek, CA, USA). For the analysis 1 µl of the methylated sample was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex (Aschaffenburg, Germany) ZB-5 column (30 m x 0.25 mm x 0.25 µm) using Helium as carrier gas at 1 ml min^{-1}. Injector temperature was 250 °C and the temperature program was 60 °C for 1 min, followed by an increase of 25 °C min^{-1} to 180 °C, 5 °C min^{-1} to 250 °C, 25 °C min^{-1} to 280 °C, then 5 min isothermically at 280 °C. For higher sensitivity, the µSIS mode (Varian Manual) was used. The endogenous hormone concentrations were calculated by the principles of isotope dilution (Cohen et al., 1986), using the ions at *m/z* 190/194 (endogenous and labelled standard; while the molecular ion of ABA would have six deuterium incorporated, during