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Apoplastic pH and growth in expanding leaves of *Vicia faba* under salinity

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ABSTRACT

Salinity affects water availability in the soil and subsequently the plant uptake capacity. Upon exposure to salt stress, leaf growth in monocot plants has been shown to be reduced instantaneously, followed by a gradual acclimation. The growth reactions are caused by an initial water deficit and an accompanied osmotic effect, followed by an IAA-induced sequestration of protons into the apoplast that increases leaf growth again as explained by the acid growth theory. In this study, we investigated the dynamics of growth reactions and apoplastic pH in leaves of the dicot *Vicia faba* in the presence of NaCl during the initiation of salt stress. Concurrent changes in apoplastic pH were detected by ratiometric fluorescence microscopy using the fluorescent dye fluorescein tetramethylrhodamine dextran. To elucidate the possible relation between the dynamics of leaf growth and apoplastic pH, results of the ratio imaging technique were combined with an *in vivo* growth analysis imaging approach. Leaf growth rate of *V. faba* was highest in the dusk and the early night phase; at this time a concomitant decrease of the apoplastic pH was observed. Under salinity, the apoplastic pH in leaves of *V. faba* increased with a simultaneous decrease of leaf growth towards increasing developmental stages, but with complex aberrations in the 24-h-leaf-growth pattern compared to control leaves. In conclusion, these results show that salt stress leads to an increase in apoplastic pH and to a declined leaf growth activity with complex 24-h-interactions of growth and pH in *V. faba*.

1. Introduction

Soil salinity is one of the major environmental constraints limiting agricultural production worldwide (FAO, 2008). NaCl is the predominant salt species that causes growth reduction in non-resistant crop plants. The decline in shoot growth on salt-affected soils is due to an inhibition of cell division and cell elongation caused by osmotic effects, ion toxicity, and mineral disturbances in plants (Tester and Davenport, 2003). Glycophytes such as faba beans (*Vicia faba* minor) show a high sensitivity to salinity (Delgado et al., 1994). However, the various deleterious effects of salinity on growth are still not completely understood. According to the acid-growth theory, the acidification of the leaf apoplast is the major requirement to increase cell-wall extensibility which controls extension growth (Hager et al., 1971). This theory is supported by the finding that auxin mediates the acidification of the apoplast below a pH of 5.5 thus stimulating cell elongation in oat coleoptiles (Hager, 2003), maize and pea (Jacobs and Ray, 1976). A lowered apoplastic pH is presumed to activate wall-loosening enzymes such as expansins, thus enhancing cell growth (Cosgrove, 2000b; Geilfus et al., 2010). Several environmental conditions may affect plant growth by altering apoplastic acidification, for example, growth inhibition by water stress is accompanied by an increase in apoplastic pH and a decrease in acidification rate (Van Volkenburgh and Boyer, 1985). Apoplastic alkalization can be understood as a general response to various stresses such as drought or salinity (Felle and Hanstein, 2002). Leaf growth reduction of sensitive plants in the first phase of salt stress is partly caused by a reduced plasma membrane H^+-ATPase pumping activity (Hatzig et al., 2010; Pitann et al., 2009; Zörb et al., 2005) by which H^+ extrusion into the apoplast decreased. The dynamics, with which leaf growth is decreased upon exposure of plants to salt stress was mainly investigated with monocotyledonous plants (Munns et al., 2000a). As the environmental control of leaf growth in monocots and dicots strongly differs (Poiré et al., 2010; Walter et al., 2009) it is important to elucidate the dynamics of growth response and its physiological background also in relevant dicot species. We hypothesize that (i) a reduced apoplastic acidification is one key factor for salt sensitivity of faba beans which is already published for many monocots, (ii) but the general leaf growth pattern of faba beans is not affected by salt stress. In order to reflect the conditions of living *V. faba* leaves adequately, two *in vivo* techniques were used. The ratio imaging technique offers the opportunity to measure the apoplastic pH in leaves with a high temporal and spatial resolution (Hoffmann et al., 1992; Mühling et al., 1995). By using a second non-invasive approach, namely the digital image sequence processing (DISP), it

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is possible to monitor the growth process of plant leaves at realtime conditions (Walter et al., 2002; Walter and Schurr, 2005). The objective of this investigation was to characterize the salt stress induced inhibition of leaf growth in the context of changes in apoplastic pH. For the first time, these results provide insights into the dynamic relation of apoplastic acidification and plant growth under salinity in a dicotyledonous plant.

2. Materials and methods

2.1. Plant cultivation and NaCl treatment

Faba beans (*V. faba* minor cv. Scirocco) seeds were soaked in aerated 0.5 mM $CaSO_4$ solution for 12 h and germinated at 25 °C in the dark in coarse quartz sand moistened with 1 mM $CaSO_4$. After 7 d, seedlings were transferred to containers with 5 L of a one-fourth concentrated nutrient solution. After 2 and 4 d of cultivation the concentration of the nutrient solution was increased to a half and a full strength, respectively. The full strength nutrient solution had the following composition: 2.0 mM $Ca(NO_3)_2$, 0.1 mM KH_2PO_4, 1.0 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.2 mM KCl, 0.1 mM KH_2PO_4, 1 mM NaCl, 10 μM H_3BO_4, 2.0 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.05 μM $(NH_4)_6Mo_7O_{24}$, 0.01 μM $NiSO_4$, 60 μM Fe-EDTA. The NaCl treatment was started 6 d after the full nutrient concentration was applied. NaCl was added in 25 mM increments daily until a final concentration of 100 mM NaCl was reached. Plants were grown in a growth chamber under controlled conditions. The day/night temperature was 20/15 °C under a 14 h photoperiod with a light intensity of 500 μE m^{-2} s^{-1}. The relative humidity was 50–60%. To investigate the effect of salinity on shoot growth, plant cultivation was extended to 30 d. Shoot growth was recorded on a daily basis by measuring leaf length and shoot height. Additionally, the leaf area of excised leaves was measured using an area meter (ADC BioScientific Ltd., Herts, England).

2.2. Ratio imaging device

After cultivation for 30 d, the youngest fully developed leaves were harvested. Excised leaves were washed with water and thereafter, the leaf apoplast was infiltrated with the pH-sensitive fluorescent dye fluorescein tetramethylrhodamine (FTMR)-dextran (20 μM, MW, 10,000, Sigma–Aldrich, Germany) using a vacuum infiltration technique (Mühling and Läuchli, 2000). Subsequently, leaves were washed again with deionised water to remove adhering dye and cut into segments of approximately 4 cm^2. Leaf segments were then placed upside down between an object plate and a cover slide. Subsequently, fluorescence emission intensity was measured. The pH was measured using an inverse microscope (Leica DM IRB, Solms, Germany) coupled to a high sensitive CCD-camera (CoolSNAP, Photometrics, Tucson, Arizona, USA). Data acquisition and calculation of images was carried out with the Meta Fluor® imaging system (Visitron, Puchheim, Germany) by using the program Meta Series (version 6.2). Applying the dual excitation technique (Mühling and Läuchli, 2000; Mühling et al., 1995) the adaxial side of the leaf was used and excited at 490 nm/440 nm for the imaging of the pH (Leica pH 1; 20×/0.40 objective). The fluorescence signal resulting from the apoplast of the epidermal and stomatal cells of the intact leaf was detected and used for ratio imaging. Ratios were converted to pH values by an *in vivo* calibration for different $[H^+]$ concentrations (Mühling et al., 1995).

2.3. Leaf growth analysis

For leaf growth analysis, a digital image sequence processing method (Schmundt et al., 1998) was used to monitor growth of the 6th leaf as soon as its leaf blade was developed. During the image acquisition, leaves were fixed to the focal plane of a CCD-camera (640 × 480 pixels; Scorpion IEEE1394, Point Grey, Vancouver, Canada) by using 12 g weights and illuminated with infrared diodes (940 nm). Near-infrared images were captured every 3 min on the adaxial side of the leaves using an interference filter (940 nm, Schott, Mainz, Germany). Image sequences were evaluated with algorithms based on a structure-tensor approach (Bigün and Granlund, 1987; Schmundt et al., 1998) that calculates velocities from all structures that are consistently moving within an image sequence of a growing leaf. Relative growth rates (RGRs) were obtained by tracking time dependent deformation of a polygonal area of interest selected within the image. Subsequently, RGR was calculated as

$$\mathrm{RGR}(t) = \left(\frac{1}{A(t)}\right)\left(\frac{dA(t)}{dt}\right), \quad (1)$$

in which $A(t)$ is the area of interest at time t. The mean RGR maps were obtained by calculating a 3-min mean of RGR for small area elements within the image. The resolution of RGR on a given image depends on the selected filter set for interpolation of velocities from different positions within the scene (Scharr, 2005) and on the number of sequential images used for calculation of velocities. Mean RGR-maps calculated from a sequence of 30 min have a resolution of several hundred spots per image (see Fig. 1).

2.4. Statistical treatment

Values are given as means ± standard error of at least four replicates if not stated otherwise. Significant differences were calculated using the Student's *t*-test.

3. Results

3.1. Effect of salinity on shoot growth and ion concentration

A 9 d application of 100 mM NaCl reduced the biomass of shoots to 67% compared to control plants (Table 1) caused by a decrease of the RGR of faba beans under saline conditions. Visual damages of leaves due to ion toxicity or other deficiency could not be observed although the leaves of the salt treated plants appeared dark-green (not shown). During salt treatment, *V. faba* showed a significant decrease in K^+ and Ca^{2+} concentrations and an increase of Na^+ concentration in leaves (Table 1). Despite lower concentrations, no K^+ or Ca^{2+} deficiency occurred at the short time treatment (according to Bergmann, 1993). Since no toxicity symptoms appeared on the leaves, it can be assumed that Na^+ concentration was within the range of non-toxic concentration.

3.2. Characterization of spatio-temporal patterns in leaf growth influenced by apoplastic pH during 24 h (diel cycle)

Fig. 1A illustrates the typical diel variations in relative growth rate (RGR) of *V. faba* under control (1 mM NaCl) and salt stress (100 mM NaCl) conditions. The RGR of the youngest leaf grown under control conditions, monitored with the DISP technique (Fig. 1C) increased towards the first hours of dark period, and thereafter gradually decreased during the night. Minimum growth activity was observed at dawn and the following light period. The abrupt light–dark and dark–light transition due to cultivation in the climate chamber resulted in a transient increase and decrease in RGR, respectively. Similar results were obtained for leaf growth under saline conditions. There, the RGR peaked in the early hours of the dark period, and then abruptly decreased within the next 4 h. This was followed by a transient increase in RGR, which was in total contrast to growth under control conditions. Salt treated