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Microscopic and macroscopic monitoring of adaxial–abaxial pH gradients in the leaf apoplast of *Vicia faba* L. as primed by NaCl stress at the roots

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ABSTRACT

The pH is a basic chemical requirement in cellular and apoplastic compartments that influences various physiological processes in plants. Apoplastic pH shifts can modulate the apoplastic and symplastic distribution of plant hormones or influence proton motive force-driven uptake processes over the plasmamembrane. Changing environments are known to effect on the apoplastic H^+-concentration in leaves and roots. The onset of NaCl-stress at the roots for instance primes a chloride-specific systemic alkalinization of the leaf apoplast. By means of microscopy- and macroscopy-based *in planta* ratio-imaging we surprisingly found that large adaxial–abaxial pH gradients were established throughout the leaf apoplast during the formation of the NaCl-induced alkalinization. Moreover, the root system is necessary to ensure the transient nature of the leaf apoplastic alkalinization.

1. Introduction

The pH is a basic chemical requirement [1] and alterations of the apoplastic proton concentrations influence various physiological processes. The pH of the apoplast of growing cells is acidic (pH < 6.5; [2,3]) typically spanning from 4.0 to 6.5 [4–6], being favorable for improving expansin activity. Expansins are cell-wall-loosening agents [7] that are thought to mediate acid-induced growth [8]. According to the acid growth theory, an auxin-mediated acidification of the leaf apoplast is the major requirement for initiating growth [9]. Besides endogenous factors such as hormones, the apoplastic pH can also be influenced by exogenous factors such as the nitrogen form NH_4^+ or NO_3^- in the soil and its uptake [10,11].

Felle [1] reports that pH shifts can cause changes in protein conformation and hypothesizes, in his review, that these pH shifts have pronounced effects, for example, on membrane transporters [12]. Monshausen et al. [13] even associate the extracellular pH to be a 'signaling element' that can modulate auxin signaling by altering the chemiosmotic proton gradients that drive auxin transport. There are number of evidences in literature proving that shifts in the apoplastic proton concentration change the protonation state of plant hormones [14]. This in turn affects the concentration and apoplastic/symplastic-distribution of the hormone ABA [15–17]. Besides affecting the concentration of organic acids, changes in the apoplastic pH are known to influence various uptake processes over the plasmalemma membrane as e.g. the proton motive force-driven uptake of inorganic ions [18–20].

Changing environments were also shown to effect on the apoplastic H^+-concentration. During the onset of drought or salinity, apoplastic pH changes have been reported [21–23]. With regard to salinity, NaCl stress at the roots is known to induce transient pH changes in the apoplast of distant leaves [2,22]. In this context, Geilfus and Mühling [24] found that chloride anions and not sodium cations act as the factor that primes the alkalinization in the apoplast of distant leaves of salt-sensitive *Vicia faba*.

Our data revealed, that these transient pH changes are by no means equally distributed within the apoplast of leaves. The novelty of this study is, that we firstly report about large adaxial–abaxial pH gradients that were established throughout the leaf apoplast during the formation of the NaCl-induced alkalinization. In addition, data revealed that the root system is necessary to ensure the transient nature of the leaf apoplastic alkalinization in intact plants.

Abbreviations: ROI, region of interest; PM, plasma-membrane.

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2. Materials and methods

2.1. Plant material and growth conditions

V. faba L. minor cv. Espresso and cv. Scirocco (Saaten-Union GmbH, Isernhagen, Germany) were grown under hydroponic culture conditions in a climate chamber (Vötsch VB 1514, Vötsch Industrietechnik GmbH, Stuttgart, Germany) as described elsewhere [6,24]. After 30 days, in vivo pH recording was performed on growing leaves.

2.2. Analysing the specific effect of Cl^- ions on the formation of the alkalinization

The effect of Cl^- was examined by using L-cysteinium$^+$ as a chloride-accompanying counter cation. By this means, the separate effect of Cl^- can be differentiated from the combined effect of Cl^- given as NaCl [24]. Both salts were added as stress stimulus to the roots of hydroponically grown plants.

2.3. In planta imaging of leaf apoplastic [H^+]-dynamics

2.3.1. Loading of fluorescent pH indicator

For microscopic ratiometric *in planta* measurement of leaf apoplastic pH dynamics, 25 μM of the fluorescent pH indicator Oregon Green 488-dextran (Invitrogen GmbH, Darmstadt, Germany) were loaded into the apoplast of intact plants as described in detail by Geilfus and Mühling [23]. For macroscopic analysis, 50 μM Oregon Green 488-dextran were used. Confocal laser scanning microscopy proved that the apoplastic dye is not able to enter the cytosol [6].

2.3.2. Microscopic live imaging of leaf apoplastic [H^+]-dynamics

Microscopic fluorescence images were collected as a time series with a Leica inverted microscope (DMI6000B; Leica Microsystems, Wetzlar, Germany) as described elsewhere [23].

2.3.3. Macroscopic live imaging of leaf apoplastic [H^+]-dynamics

Macroscopic fluorescence images were collected as a time series with a Leica macroscope (Z6 APO A; Leica Microsystems, Wetzlar, Germany) connected to a DFC camera (DFC 345FX; Leica Microsystems) using a fully planapochromatic zoom system and objective (ocular, HC PLAN s 10×/22 Br. M; objective, 0.5× Planapo Z-series; zoom, 6.3:1; zoom range 0.57–3.6×; magnification, 7.1–45×) together with a built-in iris diaphragm for depth of field adjustment. The macroscope was operated using a ProScan III controler box (Prior Scientific, Jena, Germany). A Lumen LM200Bi mercury lamp (Prior Scientific) was used for illumination at excitation wavelengths of 440/20 and 495/10 nm. The exposure time was 250 ms for both channels. Hg-lamp and external wheel with excitation filters were controlled within a Lumen 200Pro Series unit (Prior Scientific). The dye fluorescence at both excitation channels was collected using a 535/25-nm emission band-pass filter (BP 535/25; ET535/25M; Leica Microsystems) and a dichromatic mirror (LP518; dichroit T518DCXR BS; Leica Microsystems).

2.3.4. Ratiometric analysis

The fluorescence ratio F_{495}/F_{440} was obtained as a measurement of apoplastic pH. Microscopic image analysis and data processing was carried out using the LAS AF software (version 2.3.5; Leica Microsystems), macroscopic image analysis and data processing was carried out using the MM fluor software (version 1.8.5; Leica Microsystems). For conversion of the fluorescence ratio data into apoplastic pH values, an in vivo calibration was conducted as described elsewhere [23]. The Boltzmann fit was chosen to fit sigmoidal curves to the calibration because, as explained in detail by Schulte et al. [3], the Boltzmann equation can be derived directly from the Grynkiewicz equation [25] describing the relationship of the analyte concentration to the fluorescence and fluorescence ratios.

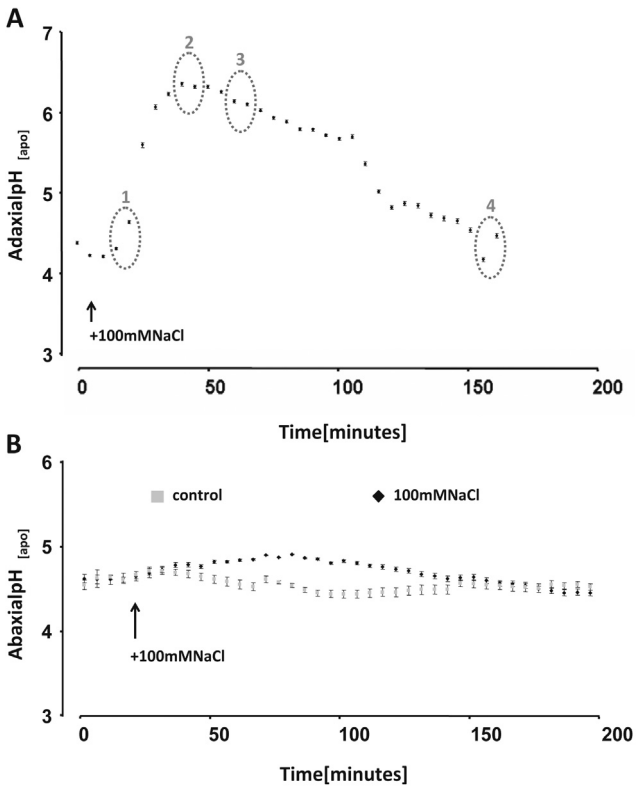

Fig. 1. Ratiometric microscopy-based leaf apoplastic pH recording in response to the addition of 100 mM NaCl to the roots of *Vicia faba* L. with special regard to the differences in the magnitude of the pH response between the (A) adaxial and the (B) abaxial leaf apoplast. pH as plotted over time (diamonds, black kinetics). Fluorescence images were collected as a time series every 5 min. Arrows indicate time point of addition of 100 mM NaCl to the nutrient solution. For the sake of comparison, the pH course of non treated control plants was displayed as measured in the abaxial leaf apoplast (quadrangles, gray curve). Leaf apoplastic pH quantitation as averaged (n = 6 ROIs per ratio image and time point with each ROI = 210 μm × 235 μm; mean ± SE of ROIs). Representative kinetic of 6 equivalent recordings of plants gained from independent experiments (n = 6 biological replicates). Light-gray dotted and numbered ellipses refer to time points mentioned in Section 3.1.

2.3.5. Statistics and presentation of ratiometric pH measurements

All pH curves are kinetics of the respective leaf apoplastic responses. According to Felle [26], curves representing biological replicates are not suitable for statistical treatment, however, all curves shown are representative recordings of six to eight independent experiments.

3. Results

3.1. Magnitude of the NaCl-induced pH response varies between the adaxial and abaxial leaf apoplast

In response to the initiation of 100 mM NaCl stress at the root site (starting point labeled by black arrow), the pH of the palisade parenchyma apoplast located below the adaxial leaf surface started to increase about 10–15 min after the stress had been initiated (Fig. 1A, see 1st dotted ellipse), reaching the most alkaline point after a period of 35–40 min (2nd dotted ellipse). At 55–60 min after the stress had been applied to the roots, the leaf apoplastic pH began to continuously re-acidify (3rd dotted ellipse), finally reaching the pH range at which the alkalinization had started (4th dotted