RATIO IMAGING OF TEMPORAL CHANGES IN LEAF APOPLASTIC PH OF VICIA FABA (L.) PLANTS UNDER DROUGHT STRESS BY FLUORESCENCE MICROSCOPY TECHNIQUE

MĚŘENÍ ZMĚN PH APOPLASTU LISTŮ ROSTLIN VICIA FABA VYSTAVENÝM SUCHU POMOCÍ POMĚROVÉ FLUORESCENČNÍ METODY

Thirupathi Karuppanapandian[1], Christoph-Martin Geilfus[2], Karl Hermann Mühling[2], Vít Gloser[1]

[1]Faculty of Science, Department of Experimental Biology, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic, tkpandian@mail.muni.cz, vitgloser@sci.muni.cz

[2]Institute of Plant Nutrition and Soil Science, Christian Albrechts University, Hermann-Rodewald-Str. 2, 24118 Kiel, Germany

Summary

We observed temporal dynamics of changes in apoplastic pH as a general stress response signal in fully developed leaves of *Vicia faba* (L.) plants both in diurnal cycle and during progressive soil drying. Use of H^+-sensitive fluorescence probe in combination with ratio imaging microscopy allowed in situ measurements of intact plants with fine time resolution. Gradual increase of pH in apoplast started after 2 d of drought stress simultaneously with significantly decreased soil water content whereas changes in leaf water potential occurred later – after 4d of drought stress. The maximum change of 1.32 pH units was observed in leaf apoplast after 11 d of soil drying. The changes of pH were observed both in younger (top) leaves and in older (bottom) leaves but the amplitude was bigger in older leaves. The leaf apoplastic pH proved as a useful marker of stress signaling in plants.

Key words: drought stress, leaf apoplastic pH, ratio imaging, *Vicia faba* L.,

Souhrn

Pozorovali jsme dynamiku změn pH v apoplastu jako obecný stresový signál u plně vyvinutých listů bobu německého (*Vicial faba*) v denní cyklu i při vysychání půdy. Pomocí pH citlivého fluorescenčního indikátoru a poměrové fluorescenční mikroskopie jsme měřili změny v intaktních rostlinách *in situ* v krátkých časových intervalech. Postupný vzestup pH začal po dvou dnech sucha současně s průkazným poklesem obsahu vody v půdě, zatímco ke změnám ve vodním potenciálu listů došlo až po 4 dnech sucha. Maximální změna pH o 1:32 nastala po 11 dnech sucha. Změny pH apoplastu byly pozorovány jak v mladších (horních), tak ve starších (dolních) listech, avšak ve starších listech byla amplituda změn větší. Detekce časné reakce rostlin na stresové faktory prostředí pomocí analýzy změn pH apoplastu *in situ* se ukázala jako užitečná metoda při objasňování procesů dálkové signalizace v rostlinách.

Klíčová slova:

Stres suchem, pH apoplastu, bob německý, fluorescenční indikátor, poměrové měření

INTRODUCTION

The apoplastic space plays a prominent role in the communication of plants with the outer environment and enables the plants to adapt for survival within the changing environmental conditions /10, 11/. The apoplastic pH of the leaf controls various metabolic processes and is related to signaling cascades. Diurnal variations as well as environmental conditions e.g. drought and salinity stresses /5, 8, 11/ can alter the leaf apoplastic pH, consequently affecting processes that depend upon the apoplastic H^+ concentration.

Plants experiencing soil water deficits frequently generate a high apoplastic pH, which drives abscisic acid (ABA) partitioning into the apoplast and away from the symplast. Leaf apoplastic pH can have a direct physiological impact on guard cells functioning and growing cells but it will also significantly interaction with ABA. Increase in apoplastic pH will result in greater accumulation of ABA in the apoplast, which can ultimately close stomata /11/ and limit plant growth rates /1/, since it is considered that an apoplastic acidification is needed for cell wall extensibility. Under drought, pH can increase prior to changes in plant water status or growth rate and stomatal conductance /4/.

Precise measurement of pH in leaf apoplast is, however, challenging and precise information about dynamics of changes are very limited /6/. Also pH measurements based on sampling of xylem sap from leaves may not truly reflect the status of apoplast in leaf mesophyll. Therefore, the objective of this study was to analyze *in situ* the temporal changes of leaf apoplastic pH of *Vicia faba* plants under drought stress by ratio imaging fluorescence microscopy technique that provides clear insight into processes in intact plant leaf /8/.

MATERIALS AND METHODS

Vicia faba L., minor cv. Fuego; Saaten-Union GmbH, Isernhagen, Germany) was grown in 2L of plastic pots (14x14x14 cm) filled with 1.82 kg of soil in a climate chamber (14/10 h L/D; 23/15°C; 60% humidity). After 30 d of growth, the young and/or fully developed leaves were used for *in situ* apoplastic pH analysis and various physiological parameters. Soil water content (SWC) was measured using ThetaProbe connected to HH2 meter (AT Soil Moisture Kit, Delta-T, Cambridge, UK). Leaf water potential (LWP) was estimated by pressure chamber technique (Skye Instruments Ltd, UK). Twenty five µM of H^+ sensitive fluorescent probe Oregon green 488 dextran (Invitrogen GmbH, Darmstadt, Germany) was used for apoplastic pH measurement. The Leica inverted microscope (DMI6000B; Leica Microsystems, Wetzler, Germany) connected to a DFC camera (DFC 360FX, Leica microsystems) and coupled to a computer was used for ratio imaging analysis. The fluorescence ratio F_{490}/F_{440} was obtained as a measurement of pH on a pixel-by-pixel basis. Image analysis was carried out using LAS AF software (Version 2.3.5. Leica microsystems). The Boltzmann fit was chosen for fitting sigmoidal curves to calibration data as described by /9/.

RESULTS AND DISCUSSION

Changes of apoplastic pH have been proposed as one of the key factors in transmitting information regarding stress to distant unaffected plant organs (shoots, leaves) as a part or complex signalling network /1, 11/. Ratio imaging by pH-sensitive fluorescent probe is an adequate technique for *in vivo* pH measurements /8/. We showed it can be useful for examination of diurnal changes of leaf apoplastic pH and time-course changes under progressive soil drying. Analysis of diurnal dynamics of young and old leaves disclosed the 0.4 pH unit increase in younger leaves compared to older leaves for 24 h after 6 d of drought (Fig. 1A). The decrease of 0.2 pH unit was observed during entire dark period compared to light period, both in younger and older leaves (Fig. 1A).

The maximum change of 1.3 pH unit was observed in leaf apoplast of *V. faba* 11 d under drought stress, and this increase was already significant after 2 d of drought (Fig. 1B) under controlled conditions. This is in line with findings of /7/ that confirmed an apoplastic alkalization in bean plants under various stress conditions by pH micro electrodes. Proton pumps (ATPases) are hypothesized to play a key role in control of apoplastic pH by driving H^+ ions into the sap in well-watered conditions. Transport and metabolism of nitrate anions has also an effect on apoplastic pH in drying soil /10/.

Under drought, water potential (WP) becomes more negative throughout the plant and is considered as a reliable marker for quantifying plant water stress response. The SWC in our experiment was significantly reduced after 2 d of soil drying (Fig. 2A) and was followed by significant decrease in LWP after 4 d (Fig. 2B). This is in agreement with previous results /2/. /3/ also found that similar decrease of LWP in pepper plants in response to drought stress. The changes in LWP might be attributed to a change in osmotic pressure–the osmotic component of water potential.

Acidification of the leaf apoplast by the plasma membrane H^+-ATPase is a premise to cell wall extensibility and cell growth. Thus alkalinization of leaf apoplast under stress by less than 0.5 unit is sufficient to inhibit plant growth, particularly leaves. Drought-induced growth reduction was consistent with apoplastic alkalinization in maize leaves /5/ or resulted in apoplastic alkalinization of up to half pH units in barley and bean leaves (Felle and Hanstein, 2002).

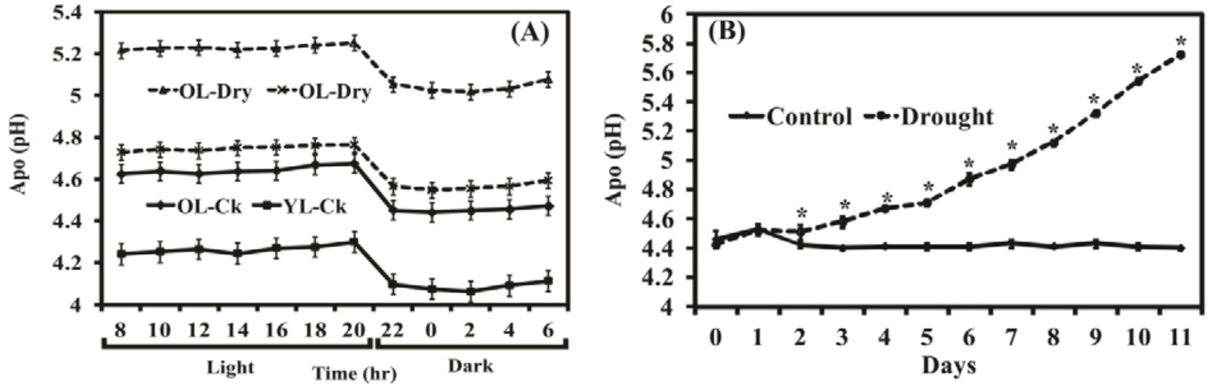

Fig. 1: Effect of drought stress on apoplastic pH in intact leaves of V. faba. Diurnal dynamics of young and old leaves after 6 d of drought (A), and temporal changes in leaf apoplastic pH 11 d of drying (B). Means ± SE (n=5). Significant differences (p ≤0.05) indicated by (*). OL-Ck; old leaf-control; YL-Ck; young leaf-control; OL-Dry; old leaf-drought, YL-Dry; young leaf-drought.

Our results show that ratio imaging fluorescence microscopy technique allows sensitive detection of changes of pH in leaf apoplast of *Vicia faba* plants. The time-course analysis of pH indicates pH increase starting with significant reduction in SWC on day 2. This suggests very early stress perception. Hence, early increase of pH in apoplast likely belongs to the early long -distance signaling sequence in plants under progressive soil drying.

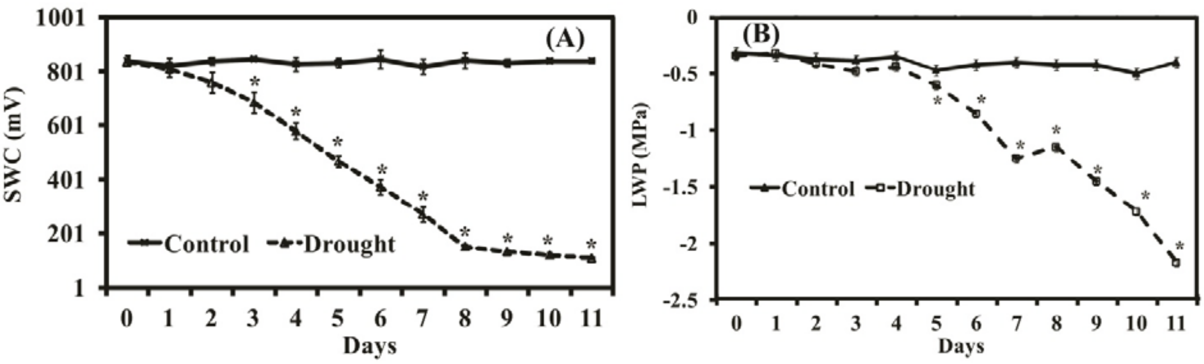

Fig. 2. Effect of drought on soil water content (SWC) (A) and leaf water potential (LWP) (B) in V. faba plants under controlled conditions. Means ± SE (n=5). Significant differences (p ≤0.05) are indicated by (*).

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