Determination of apoplastic K\(^{+}\) in intact leaves by ratio imaging of PBFI fluorescence

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Abstract

The tetraammonium salt of the K\(^{+}\) binding fluorescent dye benzofuran isophthalate (PBFI) was used to investigate the influence of potassium nutrition (0.1–2.1 mol m\(^{-3}\)) on apoplastic K\(^{+}\) in Vicia faba leaves by means of ratio imaging. As a reference the infiltration-centrifugation method was used. Both methods reflected the influence of K\(^{+}\) supply on apoplastic K\(^{+}\) concentration. The abaxial leaf side revealed significantly higher K\(^{+}\) concentrations (20–25 mol m\(^{-3}\)) than the adaxial side (5–8 mol m\(^{-3}\)). Application of CCCP led to an immediate increase in apoplastic K\(^{+}\) demonstrating the reliability of the PBFI method.

Key words: Vicia faba, leaf, apoplast, K\(^{+}\), PBFI, ratio imaging, ratiometric fluorescence microscopy.

Introduction

Knowledge of apoplastic ion concentrations in the leaves is important for the understanding of transport processes, enzymatic reactions and cell expansion (Grignon and Sentenac, 1991). The great variation of data published for K\(^{+}\) in this plant compartment (Grignon and Sentenac, 1991) may be explained by fluctuations in export and import rates to the leaf apoplast as well as by different methods and plant species used.

One of the major problems in any study dealing with apoplastic ion concentration is the method by which apoplastic solution is obtained. For leaves several indirect methods have been suggested including the elution procedures (Long and Widders, 1990), the vacuum perfusion of leaf discs (Bernstein, 1971), a pressure technique (Jachetta et al., 1986), and different centrifugation techniques (Rohringer et al., 1983; Pfanz and Oppmann, 1991; Dannel et al., 1995; Mühling and Sattelmacher, 1995). A more direct approach are in situ methods such as the X-ray microanalysis (Pihakaski-Maunsbach and Harvey, 1992) or the application of ion-selective micro-electrodes (Blatt, 1985). However, the former requires complex preparation of the specimen, which is likely to disturb native ion distribution (at least of mobile ions such as K\(^{+}\)) the latter gives access only to the apoplast in the immediate vicinity of injured cells. The ratio-imaging technique offers the unique possibility to determine the activity of ions at a high temporal and spatial resolution without interferences encountered with single-wavelength dyes such as photobleaching or the dependency on dye concentration (Bright et al., 1989).

So far the K\(^{+}\) binding fluorescent dye PBFI-AM (cell permeant) was used to measure intracellular K\(^{+}\) in animal cells (Jezek et al., 1989; Kasner and Ganz, 1992) and leaf and root protoplasts (Lindberg, 1995). To the authors' knowledge, the use of PBFI-salt (cell impermeant) has not yet been tested for its suitability to determine K\(^{+}\) concentration in the leaf apoplast. Ion concentrations and ion relations (for example K\(^{+}\)/Ca\(^{2+}\)) in this plant compartment may differ significantly from those in the cytosol. Thus, substantial methodological efforts were required to adapt the PBFI method to this plant compartment. In the present study the applicability of this dye to the ionic conditions of the apoplast was investigated. Additionally, the relationship between potassium supply to the roots and apoplastic K\(^{+}\) in leaves is described.

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Abbreviations and symbols: CCCP, carbonylcyanide-3-chlorophenylhydrazone; IWF, intercellular washing fluid; MES, 2-[W-morpholino]ethanesulphonic acid; PBFI-AM, tetraacetoxymethyl ester of the K\(^{+}\)-binding ratio fluorescent dye benzofuran isophthalate; PBFI-salt, tetraammonium salt of the K\(^{+}\)-binding ratio fluorescent dye benzofuran isophthalate; TRIS, tris (hydroxymethyl)aminomethane.

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Materials and methods

*Vicia faba* L cv. Troy seeds were imbibed in aerated 0.5 mol m$^{-2}$ CaSO$_4$ for 1 d and then germinated in perlite. After 1 week seedlings were transferred to nutrient solution (4/4 plants per pot). The nutrient solution was replaced every 3 d. The standard nutrient solution had the following composition: 2.0 mol m$^{-2}$ Ca(NO$_3$)$_2$, 2.0 mol m$^{-2}$ KCl, 0.1 mol m$^{-2}$ KH$_2$PO$_4$, 0.5 mol m$^{-3}$ MgSO$_4$, 10 mmol m$^{-3}$ H$_3$BO$_3$, 0.2 mmol m$^{-3}$ CuSO$_4$, 0.5 mmol m$^{-3}$ ZnSO$_4$, 0.5 mmol m$^{-3}$ MnSO$_4$, 0.01 mmol m$^{-3}$ (NH$_4$)$_6$ MO$_2$O$_7$, and 30 mmol m$^{-3}$ Fe-EDTA.

The plants were grown in a growth chamber at 20/15 °C day/night temperature and a 14 h photoperiod at 300 $\mu$mol m$^{-2}$ s$^{-1}$ PAR.

The ratio imaging process was preferred because it allowed much faster loading than the time-consuming transpiration-driven loading technique via the petiole. Additionally, the infiltration facilitate an equal dye concentration within the leaf apoplast which is recommended when using PBFI because the ratio is not completely independent of dye concentration (data not shown).

Fluorescence emission was determined on four positions of each leaf. Backgrounds were measured using leaves infiltrated only with the pH buffer and subtracted before the ratio procedure.

The spectra for dye characterization were acquired with a fluorescence spectrastarefluorophotometer RF-5001 PC (Shimadzu, Japan).

### Results and discussion

Probably due to the autofluorescence of the green leaf tissue and possible due to light scattering through the intact leaf, higher PBFI concentrations (50 mmol m$^{-2}$) than used in cytosolic studies (Jezek *et al.*, 1989; Kasner and Ganz, 1992; Venema *et al.*, 1993; Lindberg, 1995) were required to obtain an acceptable signal to noise ratio. As reported before (Jezek *et al.*, 1989) this resulted in a shift in the excitation spectra to longer wavelengths (Fig. 2) possible due to an internal filter effect.

For the determination of apoplastic K$^+$ the ratio 340/400 nm was used inspite of the fact that 340 nm does not reflect the excitation maxima. This was due to the

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**Table 1.** Effect of H$^+$, NH$_4^+$, Ca$^{2+}$, and Mg$^{2+}$ concentration (mol m$^{-3}$) on PBFI-K ratio 340/400 nm

<table>
<thead>
<tr>
<th>Concentration</th>
<th>H$^+$</th>
<th>NH$_4^+$</th>
<th>Ca$^{2+}$</th>
<th>Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 $\times$ 10$^{-7}$</td>
<td>—</td>
<td>1.3</td>
<td>14</td>
<td>1.3</td>
</tr>
<tr>
<td>1 $\times$ 10$^{-6}$</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1 $\times$ 10$^{-5}$</td>
<td>1.3</td>
<td>—</td>
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<tr>
<td>1 $\times$ 10$^{-4}$</td>
<td>1.3</td>
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<td>1.3</td>
<td>—</td>
</tr>
<tr>
<td>5 $\times$ 10$^{-4}$</td>
<td>1.3</td>
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<td>5 $\times$ 10$^{-3}$</td>
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<tr>
<td>1 $\times$ 10$^{-2}$</td>
<td>1.3</td>
<td>1.3</td>
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</tr>
</tbody>
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$\rho$ 0.96 0.29 0.10 0.07
lack of a more appropriate filter. Nevertheless, the $K^+$ concentration was quasi-linearly correlated to the 340/400 nm ratio up to 50 mol m$^{-3}$ (insert, Fig. 3). For further studies, however, the use of the 360/400 nm ratio is suggested if possible.

For in vivo calibration leaf discs ($\varnothing$ 0.5 cm) were treated for 60 min with the ionophore nigericin (5 mmol m$^{-3}$) in order to achieve equilibrium between cytoplasmic and apoplastic $K^+$. Thereafter, nigericin and $K^+$ in the external solution were removed with deionized water in

![Excitation spectra of 10, 50 and 100 mmol m$^{-3}$ PBFI in 25 mol m$^{-3}$ MES + 10 mol m$^{-3}$ TRIS + 10 mol m$^{-3}$ $K^+$ at pH 6.0 measured at the emission at 520 nm.](https://academic.oup.com/jxb/article-abstract/48/8/1609/501906)

![Excitation spectra of 50 mmol m$^{-3}$ PBFI in 80 mol m$^{-3}$ MES + 35 mol m$^{-3}$ TRIS (pH 6.0) at different $K^+$ concentrations (0–80 mol m$^{-3}$) measured at the emission at 520 nm. Relationship between $K^+$ concentration in the apoplast of Vicia faba leaves and PBFI fluorescent ratio (340/400 nm), (for methodological details see text).](https://academic.oup.com/jxb/article-abstract/48/8/1609/501906)
three washing steps. Leaf discs were infiltrated with solutions containing PBFI as well as K⁺ at defined concentrations and ratios were calculated after excitation at 340 and 400 nm (insert, Fig. 3).

Application of the protonophore carbonylcyanide (10 mmol m⁻³ CCCP, 50 mol m⁻³ MES-TRIS, pH 6.5) on to the leaf surface is expected to induce K⁺ loss from the symplast. This treatment resulted in a quasi-immediate increase of PBFI fluorescence (Fig. 5), suggesting that PBFI was not previously accessible to endocellular K⁺. Such an assumption is supported by the observation that PBFI fluorescence in all experiments was observed at the cell periphery of the epidermal cells (Fig. 4).

Previous experiments with PBFI have revealed that this dye is not exclusively selective for K⁺ but reveals affinity for Na⁺ (Minta and Tsien, 1989) as well as for H⁺ while

![Image of false color image of a Vicia faba leaf (adaxial side) loaded via the petiole (5 h) with 50 mmol m⁻³ PBFI.](image)

Fig. 4. False colour image of a Vicia faba leaf (adaxial side) loaded via the petiole (5 h) with 50 mmol m⁻³ PBFI. (A) Excited at 340 nm, (B) excited at 400 nm, (C) ratio reflecting apoplastic K⁺. The emission was measured through a wide bandpath filter (520-560 nm). False colour ratio image represents apoplastic K⁺ concentration (mol m⁻³).

![Graph of Apoplastic K⁺ concentration over time](image)

Fig. 5. Effect of 10 mmol m⁻³ CCCP on K⁺ concentration in the leaf apoplast. The leaves were infiltrated with 50 mmol m⁻³ PBFI and a pH buffer (50 mol m⁻³ MES-TRIS, pH 6.5). 50 s after onset of the experiment 100 μl CCCP (10 mmol m⁻³, 50 mol m⁻³ MES-TRIS, pH 6.5) was sprayed on to the leaf surface.
bivalent cations like Ca\(^{2+}\) or Mg\(^{2+}\) do not significantly influence the ratio (Table 1). The very weak effect of \(\text{NH}_4^+\) does not interfere significantly with apoplastic K\(^+\) measurements due to the very low concentration of this monovalent cation under our experimental conditions (Mühling and Sattelmacher, 1995). Thus, it is concluded that PBFI may be used to study apoplastic K\(^+\) if plants are cultivated in a Na\(^+\) poor medium and if the H\(^+\) effect is corrected. The latter may be achieved either by buffering the infiltration solution or by mathematical approaches.

The significance of controlling the apoplastic pH becomes even more important as one realizes that the H\(^+\) concentration may affect measurements of apoplastic K\(^+\) in several ways other than influencing fluorescence yield. (i) PBFI may change from a tetravalent anion to a neutral form which may be permeant. Since the cytoplasmic K\(^+\) concentration is high, even small amounts of sequestred PBFI could contaminate the fluorescence signal from apoplastic PBFI. (ii) Protonation may effect dye concentration in the Donnan phase were one may expect K\(^+\) to be more abundant. (iii) Since H\(^+\) buffer capacity of cell walls differs between plant species, it may be anticipated that the relevance of the above factors is dependent to the plant species used.

From Table 2 it is apparent that apoplastic K\(^+\) reflects K\(^+\) supply to the root system (concentration of the nutrient solution: 0.1 and 2.1 mol m\(^{-3}\)). This applies to both the PBFI and the centrifugation method. This is in good agreement with earlier findings by Long and Widders (1990) who demonstrated increasing apoplastic K\(^+\) concentration as a result of raising K\(^+\) supply (2 and 10 mol m\(^{-3}\)) with peeled Pisum leaves. The fact that two independent methods led to a comparable set of data makes us confident that the PBFI method does reflect ion concentrations in the apoplast of intact leaves.

Contrary to other methods, the ratio imaging approach does permits to study localized concentration differences of the apoplastic solution. The uneven distribution of K\(^+\) within the leaf apoplast may be taken as an example. Independent of K\(^+\) nutritional status the abaxial leaf side revealed significantly higher apoplastic K\(^+\) concentrations compared to the adaxial side (Table 2).

This is probably a reflection of stomatal closure induced by the infiltration process as Vicia faba leaves have an amphistomatous structure with a slight higher stomata dominance at the abaxial side (Yera et al., 1986). Similar results were obtained by Bowling (1987) who found a rise of apoplastic K\(^+\) when stomatal closure was induced by K\(^+\) selective microelectrodes. Apoplastic K\(^+\) gradients were measured in the vicinity of stomata (Fig. 4).

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**References**


