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Journal of Chromatography A, 809 (1998) 231–239

JOURNAL OF CHROMATOGRAPHY A

Determination of inorganic cations and anions in single plant cells by capillary zone electrophoresis

A. Bazzanella[a], H. Lochmann[a], A.D. Tomos[b], K. Bächmann[a,*]

[a]*Darmstadt University of Technology, Department of Chemistry, Petersenstr. 18, 64287 Darmstadt, Germany*
[b]*School of Biological Science, University of Wales, Bangor, Gwynedd LL57 2UW, Wales, UK*

Received 22 December 1997; received in revised form 16 February 1998; accepted 20 February 1998

Abstract

Investigation of the solute transport in plants on a molecular level requires the knowledge of the solute distribution between different cells within a tissue. Capillary electrophoresis is demonstrated as a useful technique for the determination of a number of solutes in single plant cell vacuoles. The procedures of sampling vacuolar sap, dilution of the sample, internal standardization and subsequent injection into the separation capillary are described. Extracted vacuolar sap volumes have been in the range of 20 to 60 pl. For obtained sap volumes >20 pl division of the sample droplet into subsamples is possible. This allows repeated injections into the CE and thus high reliability of the obtained data and, using different CE systems, the possibility of determination of many different species in one vacuolar sample. This is demonstrated with the determination of inorganic cation and anion concentrations in the same vacuole of individual wheat epidermal cells using indirect UV detection. The applicability of CE for vacuolar analysis is illustrated with the identification of intercellular solute patterns between different wheat epidermal cell types.

Keywords: Plant materials; Inorganic cations; Inorganic anions

1. Introduction

Compartmentation of nutrients and other solutes between vacuole and cytoplasm of higher-plant cells is important in the response of plants to changes in nutrient availability and to their ability to withstand stresses [1–3]. However, it is now becoming clear, that solutes are also differentially distributed between different cell types [4] and even between cells of the same tissue [5–7]. Therefore, the knowledge of local solute distribution between different cells is a prerequisite for the understanding of intercellular communication and regulation of solute transport. This requires the investigation of solute concentrations on a single-cell or even subcellular level.

Several approaches have been made for this purpose, such as the direct measurement of ions in single plant cells by ion-selective microelectrodes [8–10], energy dispersive X-ray (EDX) analysis of frozen tissue sections [11–13] or extracted vacuolar sap [7,14] and microfluorometric enzymatic assays of cell sap [7,14].

For measurement of time-dependent processes the use of ion-selective microelectrodes is advantageous but their application is limited to a small number of ions and the determination of more than one solute needs the elaborate construction of double, triple or five barrel electrodes. EDX analysis only allows

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00144-7

determination of elements, hence the corresponding chemical species or the soluble and insoluble parts cannot be distinguished, which reduces the observed information. For microspectrofluorometric analysis enzymatic assays for a wide variety of solutes are available. However, simultaneous determination of analytes is not possible and the important ratio of different solutes within one cell or compartment is not obtained by this method.

In the last few years, capillary electrophoresis (CE) has been introduced as a powerful technique for phytochemical analysis [15–22]. Using the high efficiency of CE, the simultaneous determination of many compounds can be achieved even in complex plant matrices. The low injection volumes required (in the nl range for 75- and 50-μm capillaries and even less for capillaries with smaller I.D.) opened the possibility of analyses on the single-cell level as shown by various approaches like the analysis of whole single snail neurons [23–26], human erythrocytes [27–32] or other mammalian cells [33–36] and analysis of cytoplasm extracted from individual cells [24,37–40]. Several excellent reviews on this topic have been published [41–45].

However, though a rather high number of publications on single-cell analysis by CE exist, no application for single plant cells has been reported yet.

In this work the applicability of capillary electrophoresis for the analysis of single plant cell vacuoles is demonstrated for the first time. Injections of whole plant cells are generally not possible because they are surrounded by a cell wall. In addition due to the presence of a cell turgor, cell-sap sampling differs considerably from animal cells. The procedures of sampling vacuolar sap, dilution of the sample, internal standardization and subsequent injection into the separation capillary are described. The determination of inorganic cation and anion concentrations in single plant cell vacuoles is shown.

2. Experimental

2.1. Apparatus

The experiments were carried out on a laboratory-built CE system, equipped with a Lambda 1000 UV detector (Bischoff, Leonberg, Germany) and a high-voltage power supply type HCN 6 M-30000 from FUG (Rosenheim, Germany).

Untreated fused-silica capillaries (Chromatographie Service, Langerwehe, Germany) of 75-μm I.D.×360-μm O.D. were used. The total lengths of the capillaries varied from 70 to 100 cm. On the injection side, 2 cm of the polyimide coating was removed, and the outer diameter of the capillary tip was conically reduced to about 150 μm by manual grinding on a fast rotating corundum plate.

Two types of microcapillaries were used for sampling and for measuring identical aliquots of sample and internal standard. Capillaries for cell sap sampling were pulled from borosilicate glass capillaries of 1.0 mm O.D.×860 μm I.D. (Clark Electromedical Instruments, Pangbourne, Reading, UK) to a tip diameter of about 1 μm using a vertical pipette puller Model 700C (David Kopf Instruments, Tujunga, CA, USA). Constriction capillaries were prepared from the normal sampling tips with a microforge.

Droplets of volumes in the nl-range were produced using an ultra micro pump (World Precision Instruments, Berlin, Germany) equipped with a modified microsyringe (ILS, Stützerbach, Germany) with luer connected glass microcapillary.

Sample injection was carried out by moving the end of the separation capillary with a micromanipulator (Leitz, Wetzlar, Germany) towards the sample droplet. All operations were observed with a stereoscopic microscope (Nikon SMZ-1B, Düsseldorf, Germany).

2.2. Chemicals

For sample storage and treatment AS 4 silicon oil (Wacker, Burghausen, Germany) and paraffin oil (Type 76235, Fluka, Buchs, Switzerland) were used. Both were ultrasonically extracted 20 times with Milli-Q water to remove ionic impurities and centrifuged.

For the determination of cations, an electrolyte consisting of 5 m*M* imidazole (Fluka) and 2 m*M* 18-crown-6 (Merck, Darmstadt, Germany) was used, the pH was adjusted with sulfuric acid to 4.5.

The electrolyte for the determination of anions

contained 2.5 m*M* pyromellitic acid in MicroSelect quality (Fluka), 15 m*M* Tris(hydroxymethyl)aminomethane (Fluka) and 1 m*M* DoTAOH. The latter was prepared from the bromide salt (Fluka) using an anion exchanger in the hydroxide form. Cation standards were prepared from their chloride salts, anion standards from their sodium salts. Both were purchased from Merck in the highest quality available and used as received. Cesium bromide (Merck) was used as internal standard for both cation (Cs) and anion (Br) analysis. All electrolytes and standard chemicals were dissolved in Milli-Q water.

2.3. Plant material

Wheat seedlings were grown under hydroponic conditions (modified Long Ashton medium with 7 m*M* sulphate and 0.9 m*M* nitrate) and 70 $\mu mol/m^2$ s light intensity. Vacuolar samples were taken from the upper epidermis of the third leaf of three-week-old plants.

3. Results and discussion

3.1. Procedure of sampling, creation of subsamples and internal standardization

The procedure of extracting cell sap from a single vacuole is shown in Fig. 1. A microcapillary with a tip diameter of 1 μm is filled with purified and water-saturated silicon oil. Using a micromanipulator, the microcapillary is inserted into the vacuole of a plant cell. Due to the cell turgor, vacuolar sap immediately enters the tip of the microcapillary. The microcapillary is then rapidly removed from the cell since the collapsing turgor will draw water from the surrounding tissue into the cell osmotically, which can lead to sample dilution. The capillary tip remains less than 1 s in the cell. After sampling the tip is

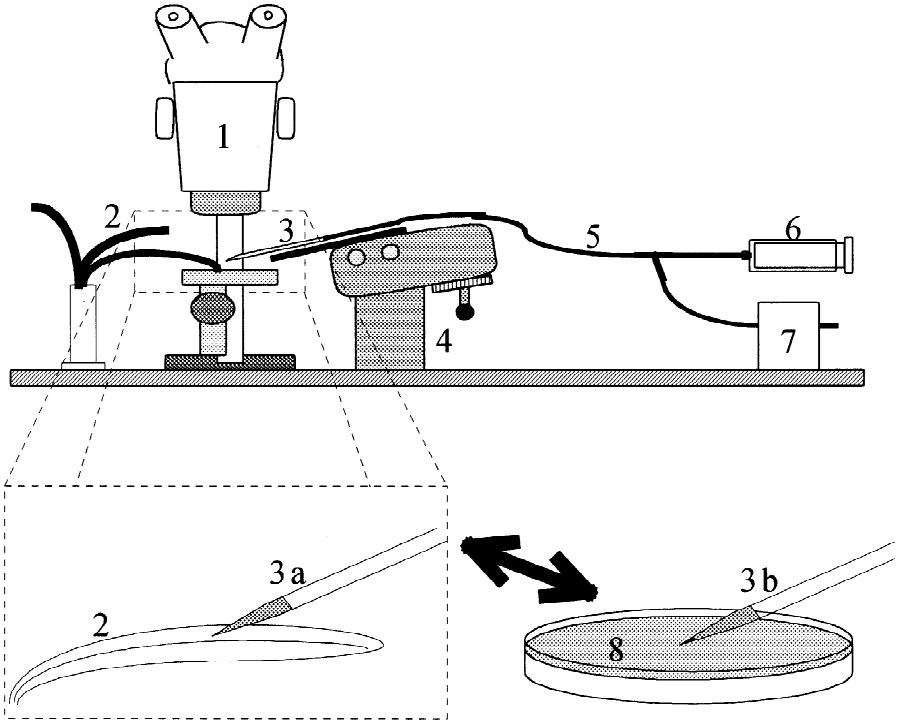

Fig. 1. Experimental setup for sampling single vacuoles; 1: microscope; 2: plant material; 3: sampling capillary; (3a: filled with silicone oil before sampling; 3b: with vacuolar sample in the tip); 4: micromanipulator; 5: tubing; 6: syringe; 7: valve; 8: petri dish filled with paraffin oil.

immediately immersed in a polyethylene petri dish filled with paraffin oil in order to protect the sample droplet from evaporation. The sample droplet, which has a volume of about 10 to 60 pl, is ejected onto the bottom of the petri dish, which contains several 5-nl water droplets (created using the ultra micro pump), and a droplet of 200 m*M* CsBr. The latter is used as internal standard for cation and anion analysis respectively, as neither cesium nor bromide are present in plant samples. The sample droplet remains, covered with paraffin oil, on the bottom of the petri dish, in its ideal spherical form, as the surface of the petri dish material is not wetted by the sample droplet.

The following sample handling procedure has three goals: (1) division of the vacuolar sample into several subsamples of identical (but unknown) volume; (2) dilution of the sample to a volume and ionic strength which is suitable for CE; and (3) addition of an internal standard which allows recalculation of the subsample volume and the solute concentrations.

Fig. 2 explains the entire procedure of dividing the vacuolar sample into subsamples, dilution with water and internal standardization. From the original vacuolar sample droplet several subsamples of about 10 pl can be taken using a constriction capillary. This is a glass microcapillary with a sharp constriction at a certain position in the inner diameter. These capillaries contain a volume of about 10 pl from the tip to the constriction and are used for measuring identical aliquots of sample and internal standard. The constriction capillary is filled with silicon oil and connected to a 50-ml syringe via PE tubing to apply a slight vacuum or pressure respectively. With

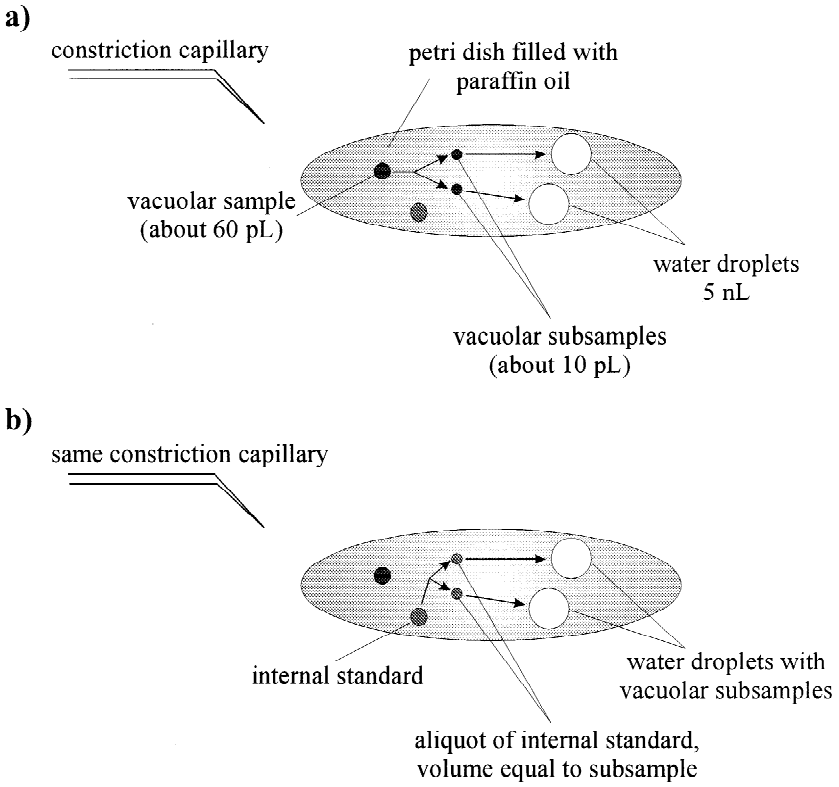

Fig. 2. Sample handling procedure including (a) the creation of subsamples and injection into water droplets and (b) internal standardization, all with the same constriction capillary.

an opening valve, the pressure difference can be reduced to zero immediately. The tip of the constriction capillary is inserted into the sample droplet and the sample is sucked in until the constriction is reached, then the valve is opened. The tip is removed out of the remaining sample droplet and a small zone of paraffin oil is sucked in. Consequently, the constriction capillary contains a subsample (about 10 pl) encapsulated with oil. A second subsample is injected the same way, until several subsamples of identical (but unknown) volume are positioned in the constriction capillary, each separated from the others by a zone of paraffin oil. Each subsample is expelled into one of the 5 nl water droplets (positioned in the same petri dish) in order to dilute the concentrated cell sample and thus provide concentrations and droplet volumes suitable for injection into the CE. The constriction capillary is washed with water and filled with internal standard aliquots of the exact subsample volume using the same procedure. The internal standard is also expelled into the water droplets containing the subsamples. Thus the need for knowledge of the absolute subsample volume is eliminated and analyte concentrations can directly be determined using the internal standard. Using this procedure, up to about 40 samples can be prepared in one petri dish, a ground plan on the bottom of the petri dish allows orientation and unambiguous identification of the sample droplets. The whole process of sampling and sample handling is observed under a stereomicroscope with 200-fold magnification.

3.2. Injection into the CE capillary

In Fig. 3, the injection of the 5-nl droplets containing the vacuolar subsamples and the identical volume of internal standard into the CE is shown. It is managed by positioning the end of the separation capillary into the petri dish with the sample droplets, moving the capillary tip towards the sample droplet with a micromanipulator and sucking it completely into the capillary by vacuum. In order to facilitate the injection into the CE capillary the outer diameter of the injection end of the CE capillary is conically grinded to about 150 μm. Using such a prepared capillary, the complete injection of the droplets into the CE is easily observed under the microscope. After exchanging the petri dish against an electrolyte vial with the electrode connected to the high voltage, the separation is started.

3.3. Sample storage

Storage of the prepared samples (subsamples and internal standard positioned in the 5-nl water droplets) is possible for several weeks by freezing the closed petridish at −20°C. The sample droplets do

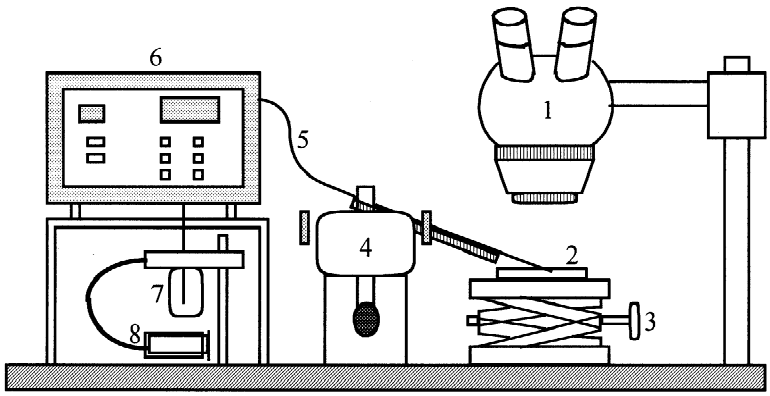

Fig. 3. Setup for injection of the prepared vacuolar subsamples into the CE; 1: microscope; 2: petri dish with samples/inlet buffer vial; 3: sample lift; 4: micromanipulator; 5: CE capillary; 6: UV detector; 7: outlet buffer vial; 8: syringe.

not change their original position during the freezing or melting processes.

3.4. Accuracy of the sample handling

The accurate measurement of the volume of subsample and internal standard using the constriction capillary is a prerequisite for determining the concentrations of vacuolar solute concentrations. Thus an experiment was performed to examine the error of repeated volume determinations with the same constriction capillary. Eleven water droplets of about 5 nl and one droplet of 200 m*M* CsBr were prepared in a petri dish filled with paraffin oil. Using the procedure described above each water droplet was loaded with the same volume of CsBr (about 10 pl) with a constriction capillary. Table 1 shows the obtained peak areas for Cs using indirect UV detection with imidazole electrolyte. Correction of peak areas for the migration time was not necessary as the latter was highly reproducible. The values indicate an error for the creation of subsamples with a constriction capillary of ±6% (3.4% R.S.D.), including any injection errors into the CE capillary. This is contrary to the normal sample handling procedure, where CsBr is employed as internal standard on the one hand to calculate analyte concentrations and on the other hand to exclude CE injection errors. The high reliability of the volume measurement with constriction capillaries was further confirmed with the analyses of the subsamples of real vacuolar samples, where almost identical migration times and peak areas for the subsamples were observed respectively.

Table 1
Accuracy of volume measurement with a constriction capillary; CE conditions as in Fig. 4B

Run No.	Peak area for Cs (μV s)	Run No.	Peak area for Cs (μV s)
1	1752	7	1805
2	1696	8	1626
3	1653	9	1663
4	1762	10	1681
5	1766	11	1722
6	1782		
Mean peak area		1719	
R.S.D.		3.4%	

3.5. Determination of cations and anions in vacuolar samples

The concentrations of inorganic solutes in the vacuoles of different epidermal cell types of the 3rd leaf of a 3-week-old wheat plant were determined using the described procedure for sampling, sample handling and transfer into the CE capillary. Fig. 4 shows typical electropherograms for the cation and anion determination in subsamples from the same vacuole. The corresponding vacuolar sample had a volume of about 60 pl, measured under the microscope against the scale of an eyepiece graticule. Subsamples of about 10 pl were created and internal standardization with 200 m*M* CsBr was performed using the same constriction capillary. Thus, repeated injections of samples from the same vacuole were possible for both the cation and anion system.

Subsequently the intercellular solute patterns between different epidermal cells were examined. In Fig. 5, the topography of the upper epidermis of a cereal leaf is shown. The different cell types analysed are indicated as ‘trough’ cells (in the troughs of the wave-like leaf surface), ‘ridge’ cells (on the top of the ridges) and ‘inter-stomatal’ cells (between adjacent stomata). The solute distribution in these cell types was found to follow distinct patterns such that it is possible to identify the epidermal cell type from the ratio of observed solute concentrations. Fig. 6 shows one example for the obtained cation data. The interstomatals accumulate calcium to significant higher levels than ridge and trough cells, indicating a strong gradient towards the stomata. This has previously been shown for barley by Fricke et al. [7] using EDX analysis, and is thought to relate to the pharmacological role of calcium in the stomatal function. This example illustrates the use of CE to identify such fine scale patterns between adjacent cells. Moreover, using single-vacuole analysis with CE, it is possible to study the accumulation of solutes in this compartment at different ambient conditions. For example under conditions of saturating sulphate but low nitrate nutrition, usually high amounts of sulphate are accumulated in the epider-

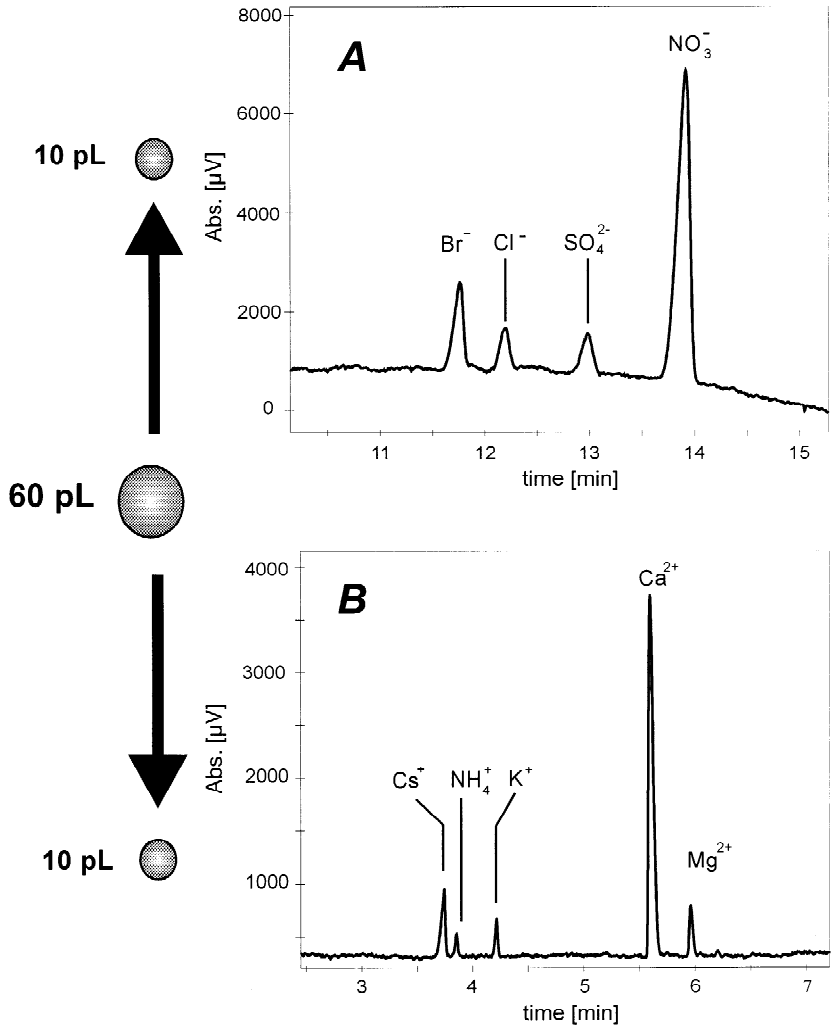

Fig. 4. Determination of (A) inorganic anions and (B) cations in subsamples from the same vacuolar sample. Electrolytes: (A) 2.5 m*M* pyromellitic acid, 15 m*M* Tris, 1 m*M* DoTAOH, pH 8.1; (B) 5 m*M* imidazolesulphate, 2 m*M* 18-crown-6, pH 4.5. Capillaries: (A) length 99.5 cm (80 cm to detector)×75-μm I.D.×360-μm O.D., 150 μm O.D. at the capillary inlet; (B) length 92 cm (70 cm to detector)×75-μm I.D.×360-μm O.D., 150 μm O.D. at the capillary inlet. Conditions: (A) voltage −25 kV, current 9 μA; (B) voltage 30 kV, current 8 μA. Detection: indirect UV, (A) 254 nm; (B) 214 nm.

mal vacuoles of wheat leaves, as nitrate drops to negligible levels [46]. However, we were able to show, that under very low light illumination, when osmotic requirements can be met by the low concentrations of nitrate, very little sulphate is accumulated in the vacuoles.

The entire results of our cation and anion measurements will be presented elsewhere, but they in principle indicate, that even a tissue like the leaf epidermis, which is generally considered to have a uniform solute distribution, is not homogeneous at all. Consequently one has to realize, that solute and metabolite concentrations obtained from larger bulk samples (even within the same tissue) only give mean values of all compartments and different cell types, and do not reflect processes that are regulated

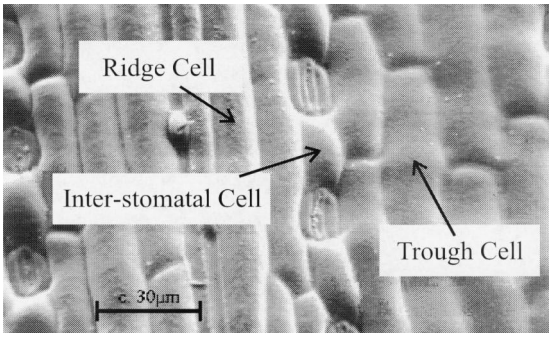

Fig. 5. Scanning electron micrograph of a (positive) replica of the upper epidermis of a cereal leaf with the different analysed cell types.

on the single-cell level. The investigation of such processes essentially requires single-cell analyses.

4. Conclusion

Capillary electrophoresis is a powerful method for the analysis of solutes in individual plant cell vacuoles. The described procedure for the creation of subsamples from vacuolar samples opens the possibility for determination of a large number of solutes and metabolites in the same vacuole. This is the first report on single-vacuole analysis by capillary electrophoresis and to date the only reliable method for the determination of sulphate in single vacuoles. In future work, the determination of other species in vacuolar samples will be presented.

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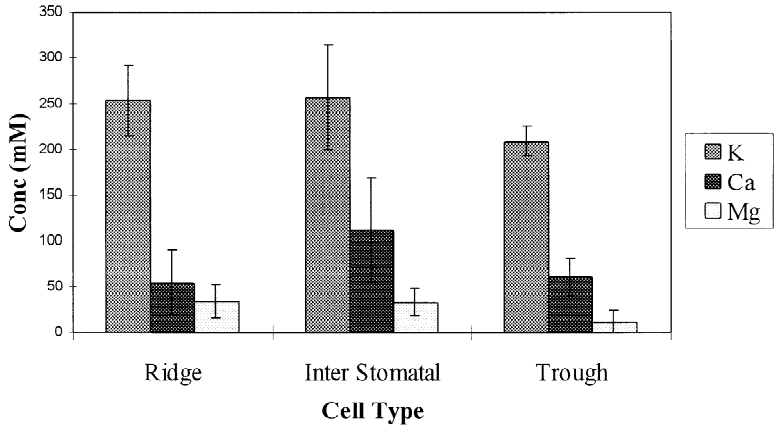

Fig. 6. Cation concentrations in vacuoles of different cell types of the upper epidermis of wheat; the error bars indicate the range of the observed concentrations corresponding to at least 10 different cells for each cell type.

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