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Cell sampling and analysis (SiCSA): metabolites measured at single cell resolution

A. Deri Tomos[1] and Ruth A. Sharrock[2]

Ysgol Gwyddorau Biolegol, Prifysgol Cymru Bangor, Bangor, Gwynedd LL57 2UW, Wales, UK

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Abstract

By using a fine oil-filled glass microcapillary mounted on a micromanipulator, the solutes of individual plant cells can be sampled. These samples can then be analysed using a range of physical and chemical methods. Hydrostatic pressure (cell pressure probe), osmotic pressure (picolitre osmometer), organic solutes (enzyme-linked fluorescence microscope spectrometry or capillary electrophoresis), inorganic solutes (X-ray microdroplet analysis or capillary electrophoresis), ^{14}C (mass spectrometry), proteins (microdroplet immunoblotting), and mRNA (rt PCR) have been measured. Collectively, the battery of techniques is called single cell sampling and analysis (SiCSA) and all of the techniques have relevance to the study of plant metabolism at the resolution of the individual cell. This review summarizes the techniques for SiCSA and presents examples of applications used in this laboratory, in particular those relating to cell metabolism.

Key words: SiCSA, Single Cell Analysis, sucrose, malate, barley leaves, carrot, maize, *Arabidopsis*.

Introduction

The behaviour of leaves, shoots, roots, and other plant organs represents the integrated sum of that of their individual cells. While it is probably self-evident that the contributions of different organs and tissues to the overall plant will vary, it is not so clear how this is manifested at the level of the individual cell. Gene expression is predominantly 'quantized' at the resolution of the individual protoplast. In principle, each cell in a tissue could be differently programmed. Moreover, in the case of the vectorial distribution of metabolites and other solutes concentration gradients must be set up within tissues. Analysis of metabolites in whole tissue homogenates is, therefore, of limited value as this provides only an average of the contribution made by many different types of cell. In recent years techniques designed to look at plant physiology with a resolution of the individual cells are becoming more widely used. These include the use of reporter genes (Berger *et al*., 1998), *in situ* hybridization (McFadden, 1994), ion-sensitive fluorescent probes (Oparka and Read, 1994; Patrick and Offler, 2001), ion-sensitive microelectrodes (Miller, 1994; Miller *et al*., 2001), X-ray microanalysis (van Steveninck and van Steveninck, 1991), immunocytochemistry (Harris, 1994; Tobin and Yamaga, 2001), single-cell dissection and analysis (Outlaw and Zhangi, 2001), and nuclear magnetic resonance (Verscht *et al*., 1998; Köckenberger, 2001).

The approach described in this paper is derived from the use of the cell pressure probe to study plant organ water relations at the single cell level (Tomos and Leigh, 1999; Tomos, 2000). This review describes the technique of single cell sampling and analysis (SiCSA) as developed and used in this laboratory with particular reference to metabolic compartmentation. Interested readers are referred to Tomos *et al*. (Tomos *et al*., 2001) who have compared SiCSA with other techniques in the context of the variation in vacuolar contents. Other quantitative approaches are described by Cannon *et al*. (Cannon *et al*., 2000).

The measurement and manipulation of the turgor pressure of individual cells within tissues has revolutionarized our understanding of water relations and led to considerable interest in the cellular details (Steudle and Peterson, 1998). While a lot can be learned from this high-resolution study of water behaviour, the application

[1] To whom correspondence should be addressed. Fax: +44 1248 382362. E-mail: a.d.tomos@bangor.ac.uk
[2] Present address: Ysgol Amaethyddiaeth a Choedwigaeth, Prifysgol Cymru Bangor, Bangor, Gwynedd LL57 2UW, Wales, UK.

of this approach to metabolites introduces additional challenges. The most significant being due to the metabolic interconversions within cells and the greater variety of transport at each membrane. The principle, though, is the same. What is needed is *quantitative* information of the activities and concentrations of the components at the resolution of the individual cell and a quantitative analysis of the metabolic interconversions that distinguish cell-specific activities from the universal housekeeping activities of all cells.

In this paper is described a battery of techniques collectively termed Single Cell Sampling and Analysis (SiCSA) that is designed (as far as possible) to provide quantitative information on the roles of individual cells in intact organs under physiological conditions. The techniques are used to study a range of biophysical as well as biochemical parameters. Those related to metabolized solutes will be discussed here. This includes organic analysis but also, briefly, biophysical and inorganic measurements since the behaviour of major organic solutes cannot be isolated from that of the inorganic as both contribute colligatively to osmotic pressure. This parameter, in turn, determines cell turgor. There is ample evidence that this is a controlled characteristic—either being regulated homeostatically or altered to perform a specific function—as in movement (Irving *et al*., 1997).

Materials and methods

Single cell sampling

Samples in the volume range of 10–100 pl are obtained by inserting a fine oil-filled glass microcapillary (tip diameter 1–3 μm) into the cell. In the case of mRNA measurements, the oil is replaced by an extraction buffer containing an RNAse inhibitor. The capillary is mounted on a micromanipulator and the operation is viewed under a stereomicroscope. Cell turgor pressure is generally considerable (0.1–1.0 MPa) and the cell sap is driven by it into the capillary tip. The capillary is removed from the cell and the sample is then expelled from it by increasing the pressure in the oil—either by using a full cell-pressure probe set-up (Malone *et al*., 1989) or by connecting the capillary to a hand-operated hypodermic syringe barrel (Tomos *et al*., 1994). In most procedures the samples are initially expelled under water-saturated paraffin oil in order to prevent evaporation. Constriction pipettes of suitable volumes are then used to manipulate these micro-droplets for the various analyses (Tomos *et al*., 1994).

The ideal cells for this technique are those of the epidermis growing in the air. These can be pierced directly and samples are obtained without contamination from other cells (Malone *et al*., 1991). Root cells of hydroponic plants require care to avoid contaminating or diluting the sample with the nutrient medium (Pritchard *et al*., 1996). When cells beneath the epidermis are sampled, such as mesophyll or root cortex, care is needed to avoid contamination from overlying cells. In the case of barley mesophyll this is achieved by inserting the capillary through a stomatal pore (Fricke *et al*., 1994; Koroleva *et al*., 1997, 1998). For other cells, the tip is pushed through the overlying cells until it is close to the target cell. Any sap in the microcapillary from punctured cells is then expelled by pressurizing the oil. The tip is then advanced into the target cell and the sample obtained as described above (Pritchard *et al*., 1996). Use of a confocal laser microscope may assist targetting deep cells in future, but currently a combination of position (an electronic position transducer can be employed; Pritchard *et al*., 1989) and cell sap characteristics are used. Examples of the latter are the large volume that is obtained from bundle-sheath cells (Fricke *et al*., 1994; Koroleva *et al*., 1997, 1998) and the characteristic very low Ca^{2+}/P ratio of mesophyll cells (Fricke *et al*., 1994). The S-cells of *Arabidopsis* (see below) have both a characteristic volume and a unique sulphur signal (Koroleva *et al*., 2000*b*). Ideally, cell markers, analogous to those used for subcellular fractionation, need to be developed. Single-cell gene expression measurements may provide the solution in some cases.

Difficulties arise with small cells of low turgor pressure. Small cells below 0.1 MPa turgor pressure and cells below some 50 pl present particular difficulties. The major drawback of the approach in the study of metabolism, however, is that it cannot currently distinguish intracellular compartments of the cell. The microsamples are a mixture of vacuole and cytoplasm This was demonstrated by the presence of a cytoplasmic enzyme in barley mesophyll samples (Fricke *et al*., 1994). Here again, it is hoped that ways may be devised to use compartmental markers for quantitative correction of local concentrations.

Organic components

Two techniques to quantify organic solutes in the microdroplets are currently used. These are fluorescent microscope photometry (generally linked to an enzymatic redox reaction involving NAD(P)H; Zhen *et al*., 1991; Tomos *et al*., 1994; Koroleva *et al*., 1998) and capillary electrophoresis (Bazzanella *et al*., 1998; Lochmann *et al*., 1998).

The former has been more widely used. In this laboratory the solutes analysed are nitrate (using nitrate reductase; Zhen *et al*., 1991), glucose and fructose (using glucose-phosphate dehydrogenase; Fricke *et al*., 1994; Koroleva *et al*., 1997), sucrose and fructans (using glucose-phosphate dehydrogenase, Koroleva *et al*., 1998), malate (using malate dehydrogenase; Fricke *et al*., 1994; Koroleva *et al*, 2000*a*), mannitol (using mannitol dehydrogenase; Pritchard *et al*., 1996), and glucosinolates (using thioglucosidase; Koroleva *et al*., 2000*b*). Amino acids have been analysed using a fluorescence assay linked to *o*-phthaldialdehyde (Fricke *et al*., 1994; Pritchard *et al*., 1996). Quantification is by comparison with standard samples prepared using the same constriction pipettes (Tomos *et al*., 1994).

In order to use capillary electrophoresis on such small samples, it is necessary to apply the microdroplets—diluted to a final volume of some 3 nl—directly to the capillary column (Bazzanella *et al*., 1998; Lochmann *et al*., 1998). Custom-made equipment has been built for this purpose (Lochmann *et al*., 1998). The electrophoretogram is then run typically at 500 V cm^{-1}. Various configurations are possible. The simplest being direct cation or anion zone electrophoresis. Sugars (rendered cationic by complexing with Cu^{2+}), carboxylic and amino acids from single cells have been separated (Lochmann *et al*., 1998). In many cases the peaks can be detected by a simple indirect method in which displacement of a UV-absorbing anionic buffer, such as pyromellitic acid, is measured using an absorption spectrometer (Bazzanella *et al*., 1998). Greater sensitivity can be achieved using laser activated fluorescence detectors (Lochmann *et al*., 1998). In preliminary experiments (AD Tomos, Larsen and Brooks, unpublished data) more complex molecules, such as catechins, have been detected from

individual cells using micellar electrokinetic chromatography (Barroso and van de Werken, 1999) in which mixtures are separated using micelles of sodium dodecyl sulphate moving in water subject to the electro-osmotic flow inherent in the capillary technique. Quantification of the results of capillary electrophoresis is greatly improved by the ability to introduce internal standards into the sap samples. Using the same constriction pipettes, identical volumes of known concentrations of anions or cations absent from the sap can be mixed with the analysate (Bazzanella *et al.*, 1998).

Pressure, osmotic pressure and inorganic solutes

Hydrostatic (turgor) pressure is measured using the cell pressure probe (Hüsken *et al.*, 1978; Tomos and Leigh, 1999; Tomos, 2000). This consists of an oil-filled microcapillary, attached to an electronic manometer, that can be inserted into individual cells. The equipment can detect changes in pressure to a resolution of 0.01 MPa or better. In the context of metabolism, this corresponds to an uncompensated change of solute concentration of 4 mOsmolal (i.e. 4 mM of a non-electrolyte). Individual cell osmotic pressure is measured using a picolitre osmometer (Malone *et al.*, 1989; Tomos *et al.*, 1994). This measures the melting point depression of the microdroplets obtained from cells. The resolution is again in the range of 4 mOsmolal. The smallest volumes measurable with current equipment can be illustrated by the successful measurement of the osmotic pressure of the Buller's drop of fungal ballistospores (Webster *et al.*, 1995). These possess a volume of less than 1 pl.

Currently two methods are applied to measure inorganic solute concentrations. These are X-ray microanalysis of the dried microdroplets (Malone *et al.*, 1991; Tomos *et al.*, 1994; Fricke *et al.*, 1994) and the capillary electrophoresis discussed above (Bazzanella *et al.*, 1998). The former cannot distinguish between the molecular species of the elemental signals (e.g. the molecular origin of S or P signals). Both techniques can exploit the use of internal standards that can be added to the sample before analysis. Rubidium nitrate and caesium bromide are suitable for the two techniques, respectively. Capillary electrophoresis has the advantage of being able to measure simultaneously both organic and inorganic electrolytes, while X-ray analysis has the advantage of being able to measure elemental anions and cations simultaneously.

Enzyme and protein analysis

Reversal of the enzymatic assays of single cell solutes allows analysis of the activity of the enzymes themselves. Fricke *et al.* measured malate dehydrogenase levels in order to demonstrate the presence of cytoplasm in cell extracts of mesophyll and its absence from those of epidermis (Fricke *et al.*, 1994). Koroleva *et al.* measured acid invertase activity in epidermal, mesophyll and bundle sheath cells and compared the values with those of a whole leaf extract (Koroleva *et al.*, 1997). The activity of the whole leaf could not be accounted for by the relatively low levels of the three cell types and it was concluded that acid invertase was located within the vascular bundle and/or in the apoplast.

In situ immunolocalization has proved a powerful technique for the cellular localization of a range of proteins (Harris, 1994). Such a technique is difficult to quantify, however. Koroleva *et al.* showed the feasibility of challenging microdroplets dried on nylon membranes with enzyme-linked antibodies (Koroleva *et al.*, 2000*a*). In this study Rubisco protein was detected in microdroplets extracted from mesophyll and bundle-sheath cells, but not in those from epidermal cells. Moreover, the size of the reaction spot (horseradish peroxidase) was proportional to the number of cell samples added to each spot, providing evidence that the approach has the potential of being quantifiable; although this has not been tested.

mRNA

The most recent technique employed is the use of rt PCR to detect the presence of specific mRNA species in the microdroplets. Procedures for single plant cells have been published by Karrer *et al.* (Karrer *et al.*, 1995) who demonstrated the feasibility of measuring Rubisco, cyclophilin and actin from individual tomato cells and Brandt *et al.* (Brandt *et al.*, 1999), who measured single cell peroxidase and Rubisco from cucumber, starch phosphorylase from potato and GFP from tobacco transformed with its gene under the control of a companion-cell specific promoter from *Arabidopsis*. This approach has been repeated in barley mesophyll and bundle sheath, using modified techniques for Rubisco small subunit (RSSU), actin and sucrose: fructan fructosyltransferase (SFT) (OA Koroleva, J Gallagher, C Lu, AD Tomos, unpublished data). Both RSSU and SFT gene expression appear to be induced by light at the level of the individual cell, as well as at the whole leaf level.

^{14}C-labelling at single cell level

The use of radiolabelled tracers continues to contribute enormously to metabolic studies. Koroleva *et al.* have applied these to the contents of individual cells (Koroleva *et al.*, 2000*a*). Microdroplets of epidermal, mesophyll and bundle sheath cells of barley leaves that had been exposed to $^{14}CO_2$ were analysed using BAMS (biological accelerated mass spectroscopy). By combining single-cell measurements of the major carbohydrates and malate with the $^{14}C/^{12}C$ ratios, Koroleva *et al.* were able to demonstrate that the specific activity of all three cell types was similar to that of the CO_2 supplied within 45 min of onset of application (Korolev *et al.*, 2000*a*). This would indicate a rapid turnover of assimilate in all three cell types. This is especially significant for the epidermis, the malate in which is currently assumed to be derived from the sucrose of the mesophyll cells.

Applications

Mapping the carbohydrate metabolism of the barley leaf

The behaviour of photoassimilates in the barley leaf has been extensively studied by SiCSA. That sucrose accumulates in the mesophyll and bundle sheath during the day and decreases during the night has been confirmed (Table 1) (Koroleva *et al.*, 1998). If the overall load of carbohydrates in the leaf is increased, by either increasing illumination or by decreasing the activity of sink tissues elsewhere in the plant, this accumulation is amplified (Koroleva *et al.*, 1998). Following the increase in sucrose is a synthesis of fructan (Tomos *et al.*, 1992). Both mesophyll and bundle sheath cells accumulate fructan (Koroleva *et al.*, 1998). Moreover, there is an indication that the threshold of sucrose concentration may be lower in the bundle sheath than in the mesophyll. If this is indeed the case it might be the basis of how a sugar gradient is maintained between the mesophyll and

Table 1. *Solute mapping of malate, sucrose and fructan in barley leaves*

Effect of location and treatment. Data from epidermal, mesophyll and bundle sheath cells are shown following a period in the dark (dawn), after 11/12 h at 500 μmol m^{-2} s^{-1} illumination (light), after 2 d photoperiod at 1000 μmol m^{-2} s^{-1} (high light) and after suppressing leaf export by cooling roots and shoot apex to 10 °C (mean ± sd, *n*=3 plants; n/a, not analysed). (Data adapted from Koroleva *et al*., 1997, 1998.)

	Dawn	11/12 h light	12 h high light	2 d high light	Cooling
Epidermis (trough)					
Malate (mM)	168 ± 26	178 ± 11	n/a	n/a	230 ± 17 (48 h)
Sucrose (mM)	6 ± 8	6 ± 3	n/a	n/a	0 (48 h)
Mesophyll					
Malate (mM)	29 ± 19	72 ± 17	n/a	n/a	47 ± 15 (48 h)
Sucrose (mM)	34 ± 19	88 ± 18	225 ± 31	131 ± 12	165 ± 45 (96 h)
Fructan (mM)	7 ± 20	0	0 ± 1	148 ± 25	189 ± 35 (96 h)
Bundle sheath					
Malate (mM)	31 ± 17	70 ± 19	n/a	n/a	19 ± 5 (48 h)
Sucrose (mM)	14 ± 4	(12)	89 ± 15	109 ± 25	95 ± 17 (96 h)
Fructan (mM)	25 ± 37	0	119 ± 12	413 ± 150	174 ± 74 (96 h)

bundle sheath cells even under conditions of reduced sugar export from the leaf. Polymerization of sucrose would also address the question of osmotic pressure regulation during carbohydrate accumulation (Tomos *et al*., 1992). However, the earlier suggestion that mesophyll turgor pressure is maintained constant despite increased sugar accumulation (Tomos *et al*., 1992) seems not to be borne out by more thorough investigation (OA Koroleva and AD Tomos, unpublished data).

The light-induced appearance of sucrose : fructan fructosyltransferase mRNA is assumed to be related to the increase in mesophyll and bundle sheath fructan concentrations following sucrose accumulation. Patterns of gene expression in the two cell types are being related to their carbohydrate content (OA Koroleva, unpublished data).

The most unexpected result has been the behaviour of the epidermis during the various changes in the mesophyll. Fricke *et al*. showed that at a lower light intensity (150 μmol m^{-2} s^{-1}) the vacuole of the epidermis accumulated negligible organic solutes (Fricke *et al*., 1995). The measured osmotic pressure could be fully accounted for by inorganic solutes. At higher light intensities (450 μmol m^{-2} s^{-1}) a considerable concentration of malate (> 150 mM) was found in the cells closest to the stomatal complex (Fig. 1). The larger cells away from the stomata, termed ridge and trough cells (Fricke *et al*., 1995), accumulate lower concentrations. This accumulation is associated with the accumulation of Ca^{2+} by the same cells and the authors suggest that it may be a mechanism by which excess Ca^{2+} and malate are kept away from the stomatal guard cells for which both compounds play a signalling role (Ruiz *et al*., 1993; Hedrich *et al*., 1994).

Under conditions of induced high leaf carbohydrate load, the malate content of the epidermal trough cells sincreases considerably (Table 1) (up to > 250 mM) (Koroleva *et al*, 2000*a*). Even under these conditions,

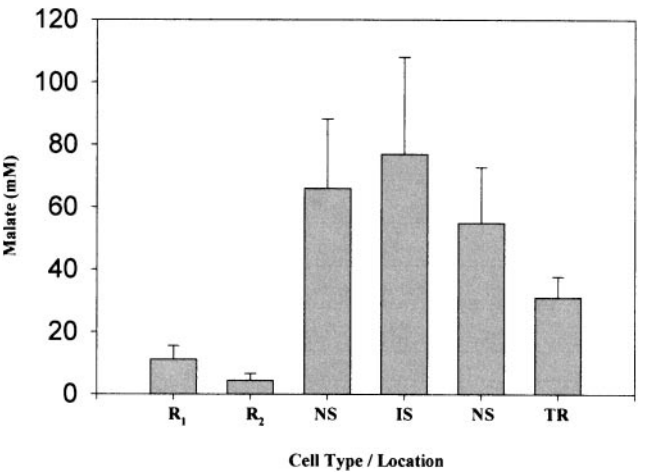

Fig. 1. SiCSA mapping of malate in barley epidermal cells. A transverse transect of adjacent cells across the region of a stomatal pore. Plants grown at an illumination of 400 μmol m^{-2} s^{-1}. Cells were located midway down (R_1) and at the base (R_2), the ridge overlying an intermediate vein, near (NS) and axially-between (IS) the stomata and in the trough region (TR) (mean ± se, *n*=5); (adapted from Fricke *et al*., 1995).

however, negligible quantities of sugars are found in the epidermis (< 4 mM). The rapid equilibration of the radioactive specific activity of this malate with both the sucrose of the mesophyll and the applied $^{14}CO_2$ in tracer experiments suggests that the two pools are in close contact. Considering this, it is therefore surprising that the induced accumulation of malate (to 135 mM) during high illumination is only partially reversible. On keeping these high malate-containing leaves in darkness for 6 d the only change in epidermal trough cell malate is a 20% drop over the first 24 h, followed by maintenance of an average of 95 mM for the rest of the period (Koroleva *et al*., 2000*a*). This might suggest two different epidermal malate pools, one rapidly, the other only slowly, equilibrating with the mesophyll. More extensive application of the single cell ^{14}C measurements are needed to determine this. The preliminary observation of Koroleva *et al*.

(Koroleva *et al*., 2000*a*) suggests a rapid equilibration of all the epidermal malate.

The metabolic relationship between mesophyll sugars and epidermal malate remains unclear. In particular, it is not known in which form organic carbon is imported by the epidermal cells. The malate content of the epidermis is also influenced by external factors. Richardson *et al*. (Richardson *et al*., 1997) showed that epidermal malate concentrations increase in response to infection of the cells by powdery mildew (*Blumaria (Erisiphe) graminis*).

It is clear, however, that barley epidermis cannot be used as a paradigm for all leaves. In wheat, for example, capillary electrophoresis of epidermal cells revealed a low concentration of malate, but a large anionic peak of lower mobility—probably citrate (AD Tomos, A Bazzanella, H Lochmann, unpublished data). Kehr *et al*. demonstrated significant concentrations of sucrose in potato epidermis that responded to a transgenic insertion of a sucrose transporter antisense gene (Kehr *et al*., 1999). Recently significant quantities of sucrose, glucose and fructose (20–60 mM total hexose equivalents) have been measured in the epidermis of the dicotyledonous species *Alonsoa meridionalis* and *Asarina barclaiana* (OV Voitsekhovskaja, OA Koroleva, AD Tomos, unpublished data).

Apart from malate and sugars, few leaf metabolites have so far been measured by SiCSA. Fricke *et al*. used a fluorescent *o*-phthaldialdehyde technique to measure 11 mM, 7 mM and 0.2 mM total amino acids in mesophyll, bundle sheath and epidermal cell extracts, respectively (Fricke *et al*., 1994).

Mapping of organic solutes in roots

Maize root: The distribution of the organic solutes that contribute significantly to the cell osmotic pressure of maize roots has been described previously (Pritchard *et al*., 1996). Radial measurements 12 mm from the tip (newly fully expanded cells) demonstrated that inorganic and organic solutes followed reciprocal gradients across the cortex, under a range of nutrient conditions. Inner cells were higher in hexoses and amino acids (Table 2), while the outer cells were higher in K^+ and Cl^-. The (artificial) exception was the distribution of mannitol following 24 h of incubation of the roots in 400 mM mannitol—when 65 mM and 35 mM were measured in the outer and inner cortex, respectively (Table 2).

The largest (proportional) step between any tissues analysed was that in amino acids across the endodermis. Cortical cells just outside the endodermis were compared with stellar parenchyma cells. Samples of the former contain 62 mM total amino acid, while the latter contain 13 mM. No attempt was made in this study to interpret the metabolic significance of these solute distributions.

Carrot tap root: The mature storage taproot of carrot has a different structure and function to that of the primary root of maize described above. However, it shares the reciprocal radial distributions of solutes. Korolev *et al*., using SiCSA, demonstrated that the tissues furthest from the cambium (and active phloem) have the highest inorganic and the lowest soluble sugar concentrations (Fig. 2) (Korolev *et al*., 2000*a*). Malate concentrations follow those of K^+ and are highest away from the cambium. Use of $^{14}CO_2$ as a tracer of photoassimilate distribution showed that the pattern of sugar distribution followed the same patterns as the movement of ^{14}C from the active phloem (Korolev *et al*., 2000*b*). This does not explain the reciprocal nature of the K-malate distribution. Unlike the maize root, where it was argued that the reciprocal gradients reflected osmotic regulation across the root radius (Pritchard *et al*., 1996), osmotic pressure is far from constant across the mature carrot radius (Tomos *et al*., 2000). It is more likely that the malate : sugar ratio reflects the developmental age of the cells. Korolev *et al*. (Korolev *et al*., 2000*a*) showed that during maturation, the storage organ progresses through several stages, each with its own set of major

Table 2. *Solute mapping of sugars, amino acids and mannitol in maize root tips*

Sugars were measured as hexose after enzymatic hydrolysis of sucrose. Individual cells were sampled at 5, 180 and 360 μm below the surface, 12 mm from the root tip. These depths correspond to the outer and inner cortex and stele parenchyma respectively. Three treatments are illustrated. Growth in 0.5 mM $CaCl_2$, full strength Hoagland solution and following 24 h in 400 mM mannitol solution (in 0.5 mM $CaCl_2$); (mean ± sd, n=10–25; n/a, not analysed) (adapted from Pritchard *et al*., 1996).

	Outer cortex	Inner cortex	Stele parenchyma
0.5 mM $CaCl_2$			
Amino acids (mM)	22 ± 20	53 ± 6	14 ± 14
Hexoses (mM)	76 ± 43	180 ± 29	200 ± 32
Hoagland's solution			
Amino acids (mM)	24 ± 12	62 ± 1	13 ± 9
Hexoses (mM)	93 ± 16	138 ± 24	150 ± 29
400 mM mannitol			
Amino acids (mM)	n/a	n/a	n/a
Hexoses (mM)	171 ± 40	223 ± 47	250 ± 28
Mannitol (mM)	66 ± 10	36 ± 22	50 ± 18

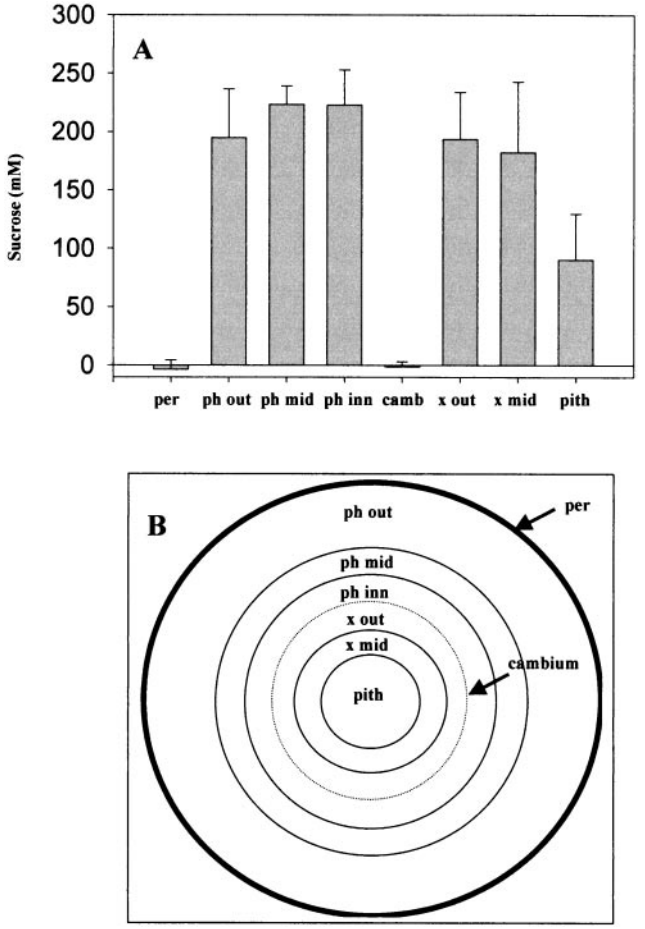

Fig. 2. SiCSA mapping of sucrose in a mature carrot root. (A) A transverse transect from the periderm (per) to the pith. For illustrative purposes, the periderm and cambium data are from 50-d-old roots, the remaining data from 90-d-roots. The samples were taken from cut sections. (B) Diagram illustrating location of sampled tissues. ph, phloem; camb, cambium; x, xylem. Out, outer; mid, middle; inn, inner (mean $\pm$ sd, $n = 4$–8); (adapted from Korolev *et al.*, 2000*a*).

osmotica. Immediately post germination these appear to be amino acids (116 mM) which account for up to 50% of the osmoticum in the extract of 10-d-old seedlings. These are replaced first by inorganic and organic salts then by hexoses before sucrose finally becomes the predominant organic osmoticum. The outer, malate-rich cells may, therefore, represent cells that were laid down in an earlier developmental stage than that of the cells nearer the cambium.

One cell type, however, stands out against the overall trend of sugar concentrations (Fig. 2). These are the cells closest to the cambium. Due to the size limitations on sampling described above, the data presented for the sap composition of cambial cells (Korolev *et al.*, 2000*a*; Tomos *et al.*, 2000) will not include unexpanded nascent cells and describe expanded vacuolated cells. However, very little of the osmotic pressure (0.8 MPa; 320 mOsmolal) can be accounted for by sugars. Potassium is also negligible—ruling out K-malate. Amino acids were not measured, but are the most likely candidate osmoticum. Korolev *et al.* (Korolev *et al.*, 2000*a*) suggest that this situation is due to rapid metabolism of the sugars that reach the cambium from the phloem outside it. These are either converted to amino acids, oxidized for energy or included in the maturing cell walls. Such a process would maintain a steep sugar gradient between the inner phloem cells and the cambium that would maintain a passive diffusion of sugars towards the rapidly developing tissue.

S-cells of Arabidopsis

Koroleva and colleagues (Koroleva *et al.*, 2000*b*) have recently applied the SiCSA technique to the distribution of solutes in the flower stalk of *Arabidopsis thaliana*. The analysis of one cell type illustrates the power of combining different analytical techniques within SiCSA. This cell type was first identified due to its very high sulphur signal on X-ray microanalysis of microdroplets extracted from cells in the vicinity of the vascular bundles. On analysis of freeze-dried sections of flower stem, these cells were identified as large, elongated cells situated between the phloem of every vascular bundle and the endodermis. A SiCSA solute analysis indicated glucose concentration of some 10 mM. The independently measured osmotic pressure, however, could be accounted for by the potassium and sulphur-containing species. The concentrations of both K^+ and S were dependent on the light intensity under which the plants were grown. Enzymatic hydrolysis of the droplets with thioglucosidase (an enzyme that hydrolyses the thiol-esters of glucosinolates) released a quantity of glucose equivalent to more than 100 mM glucosinolate. This was consistent with the more than 200 m equivalent of S measured by X-ray analysis of the microdroplets as each glucosinolate possesses two S atoms. In this analysis results of osmotic pressure, X-ray and enzymatic analysis of the samples were integrated to describe their composition. The location of the S-containing cells would suggest that they are component of a system to discourage herbivores.

Conclusions

SiCSA will contribute considerably to our quantitative understanding of the interactions between individual cells within tissues and organs. Improvement of separation techniques and detection limits will increase its value in metabolic research, although the obstacle of vacuolar/cytoplasmic mixing remains to be solved. Its major power is in enabling the integration of a range of different metabolites and linking these to the physical behaviour of tissues, notably turgor pressure. Inorganic solutes and metabolites can also interact in other ways, for example, in maintaining solubility. In studies of vacuolar berberine

in *Coptis japonica* it was found that it had a solubility of 3 mM in the presence of nitrate, but of 640 mM in the presence of 168 mM malate (Sato *et al*., 1992). Particularly valuable information will be derived from analysis of single-cell gene expression. This transcends the problem of vacuolar/cytoplasmic mixing, for example, and opens up the considerable opportunities of exploiting current advances in genomics. It represents functional genomics at the level of the individual cell.

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