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Ion distribution in cereal leaves: pathways and mechanisms

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SUMMARY

Measurement of ion concentrations in the vacuoles of different cell types in cereal leaves using a variety of techniques indicates that ions are differentially distributed between different cell types. Thus mesophyll cells are enriched in P but contain relatively little Ca^{2+} or Cl^-, whereas the reverse is true for epidermal cells. Solutes reach the leaf via the transpiration stream and we consider three possible pathways which they could follow from the xylem to leaf cells. The first is a fully apoplastic mesophyll pathway in which both water and solutes move together through the leaf apoplast passing bundle sheath, mesophyll and epidermis in turn. The second is a partly symplastic mesophyll pathway in which ions and water pass into the symplast at the mestome/bundle sheath cells. Water continues to sites of evaporation via either a transcellular or symplastic pathway, but ions may be secreted back to the mesophyll apoplast and move to the epidermis along an extracellular route. The third is a vein extension pathway which provides a diffusional pathway for ions to the epidermis. A testable hypothesis for the roles of the pathways in supplying solutes to the mesophyll and epidermis is proposed and the implications of each of these pathways for transport systems in individual cell types is discussed.

1. INTRODUCTION

Plant cells use mineral ions for a variety of purposes, including as substrates in biochemical reactions (e.g. PO_4^{3-}, NO_3^- and SO_4^{2-}), cofactors in enzymes (e.g. Mg^{2+}, Zn^{2+} and Mn^{2+}), osmotica (e.g. K^+, Na^+ and NO_3^-) and for intracellular signalling (Ca^{2+}) (Marschner 1986). For optimal growth, the concentrations of ions in plant cells need to be maintained within particular limits and failure to do so results in reduced productivity due to either nutrient deficiencies or ion toxicities (Leigh & Wyn Jones 1986). Although some recycling may occur via the phloem, ultimately leaves are dependent on the xylem for inorganic ions. Therefore, the composition of leaf cells will depend on the mixture of ions delivered to the xylem by the roots and the ability of different leaf cells to absorb particular ions from the transpiration stream as it moves through the leaf.

The processes that determine the selectivity of xylem loading in the root are discussed elsewhere in this volume (see Clarkson, this volume). Here we will describe recently gained insights into the composition of different cell types in cereal leaves and will discuss the implications these have for the pathways along which the transpiration stream moves through the leaf, and for the mechanisms of ion transport into different cells. The discussion is confined to ions present at relatively high concentrations (e.g. K^+, Ca^{2+}, NO_3^-, P_i and Cl^-) but the conclusions should also apply to micronutrient ions. We will restrict our discussion to results obtained for epidermal, mesophyll and bundle sheath cells in cereal leaves as the greatest body of information exists for these. We will not discuss stomatal guard cells in any detail as they have been reviewed by others (e.g. Mansfield *et al.* 1990).

2. THE COMPOSITION OF DIFFERENT CELL TYPES IN CEREAL LEAVES

(*a*) *Solutes in epidermal, mesophyll and bundle sheath cells*

Chemical analysis of bulk leaf extracts provides little information about the composition of individual cells because the results are the average for all cells in the tissue. To gain cell-specific information it is necessary to use techniques that can be applied at the level of individual cells or cell types. Here we discuss results obtained with X-ray microanalysis (for description of method see van Steveninck & van Steveninck (1991)), isolation and analysis of protoplasts from different cell types (Dietz *et al.* 1992*b*) and single-cell sap analysis (Tomos *et al.* 1993*a*). Ion-selective microelectrodes (Vaughan-Jones & Aickin 1987) also have the potential to provide information at the single cell level but have mainly been applied to studying cells in stomatal complexes (e.g. Penny *et al.* 1976; MacRobbie & Lettau 1980). Unlike the other methods they have not been used to compare mesophyll, epidermal and other

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Table 1. *Concentrations of ions in mesophyll and epidermal protoplasts from the primary leaves of barley*

(Protoplasts were isolated enzymically from 10-d-old barley leaves grown in a standard nutrient solution. Data are from Dietz *et al.* (1992*b*).)

	concentration/(mol m^{-3})	
ion	epidermal protoplasts	mesophyll protoplasts
K^+	169 ± 5	299 ± 5
P_i	2.8 ± 0.9	75.5 ± 8.2
Mg^{2+}	2.0 ± 0.2	17.1 ± 1.4
Na^+	1.0 ± 0.2	3.0 ± 1.9
Ca^{2+}	9.4 ± 2.2	1.6 ± 0.2
NO_3^-	182 ± 48	226 ± 66
Cl^-	61.8 ± 21.5	22.6 ± 6.6
SO_4^{2-}	0.8 ± 0.1	2.4 ± 0.6

cell types. In the main, all of these methods measure the composition of the vacuole but as this compartment dominates the intracellular volume (Leigh & Wyn Jones 1986) the results give a meaningful estimate of cell composition. X-ray microanalysis can also give information about ion concentrations in subcellular compartments other than the vacuole (e.g. Storey *et al.* 1983; Leigh *et al.* 1986) but results for these compartments are not discussed here and we confine ourselves to vacuolar composition. For simplicity we have assumed that all of the techniques measure ion concentrations even though some methods (e.g. X-ray microanalysis) measure only the total concentrations of elements. This assumption is reasonable for K^+, Na^+, and Cl^-, but for NO_3^-, P_i, SO_4^{2-} and Ca^{2+} other chemical forms may also be measured.

Leigh & Storey (1993) used X-ray microanalysis of bulk-frozen hydrated tissue to survey the vacuolar composition of mesophyll and epidermal cells from barley leaves grown with a range of nutrient treatments including K-deficiency and salinity. They found that while the majority of mesophyll cells contained detectable P, none was detectable in epidermal cells. In contrast the reverse was true for Cl^- and Ca^{2+} while Na^+ and K^+ were more evenly distributed between the two cell types. Similar results have been found in other X-ray microanalytical studies of cereal leaves (Leigh *et al.* 1986; Hodson & Sangster 1988; Huang & van Steveninck 1989; Boursier & Läuchli 1989; Williams *et al.* 1991). These differences in composition are not confined to epidermal and mesophyll cells. Williams *et al.* (1991) found that some bundle sheath cells in barley leaves were enriched in Mg and S, elements which were not detected in vacuoles of other cells.

Problems with X-ray microanalysis are that it is difficult to quantify and is a relatively insensitive technique having minimum detection limits of 20 mol m^{-3} or more (Lazof & Läuchli 1991; van Steveninck & van Steveninck 1991). None the less, the general veracity of cell compositions measured with this method has been confirmed by analysis of isolated protoplasts and by single-cell sap sampling. Martinoia *et al.* (1986) showed that mesophyll protoplasts of barley leaves contained 43% of the total leaf Cl^-, 65% of the NO_3^-, 88% of the P_i and 88% of the SO_4^{2-}. This approach has been extended by Dietz *et al.* (1992*b*) who measured concentrations of ions in both mesophyll and epidermal protoplasts. They found that epidermal protoplasts contained much lower P_i concentrations than mesophyll protoplasts which, by contrast, had lower concentrations of Ca^{2+} and Cl^- (table 1). Potassium and NO_3^- were present at high concentrations in both cell types.

Single-cell sap sampling is a modification of the pressure probe technique used to measure turgor pressure in higher plant cells (Hüsken *et al.* 1978). When the probe is inserted into a cell, a sample of vacuolar sap (volume 30–150 10^3 μm^3) is driven by turgor pressure into the tip of the probe's microcapillary and can be removed for analysis. Initially this technique was used to compare turgor with sap osmotic pressure and provide information on cell water potential (Shackel 1987; Nonami & Schulze 1989; Malone *et al.* 1989; Malone & Tomos 1992) but micro-scale analytical techniques have now been developed to allow the chemical composition of the sap to be measured. These techniques include quantitative X-ray microanalysis of dried sap droplets to measure inorganic elements (Malone *et al.* 1991; Tomos *et al.* 1993*a*,*b*) and microscope-based fluorescence assays to measure organic solutes and inorganic ions using NAD(P)H-dependent enzyme reactions (Zhen *et al.* 1991; Tomos *et al.* 1993*a*). With these techniques it is now possible to measure quantitatively the sap composition of different cell types, to follow changes in cell composition as nutrient supply or some other environmental parameter is altered and to compare the composition of neighbouring cells of the same type.

The ionic composition of sap taken from upper epidermal, mesophyll and bundle sheath cells of barley leaves is shown in figure 1. These data confirm that epidermal cells have higher concentrations of Ca^{2+} and Cl^- than the mesophyll or bundle sheath cells, that the latter two cell types both have greater concentrations of P_i than the epidermis, and that all three cell types contain similar concentrations of K^+, Na^+ and NO_3^-. An advantage of the single-cell sap analysis technique is that it is not restricted to inorganic solutes. Thus measurement of sugar concentrations in epidermal and mesophyll cells has shown that these are also differentially distributed between the two cell types. After 24 h illumination, detached wheat leaves accumulated more than 100 mol m^{-3} sugars in mesophyll cells, principally as sucrose, but only about 4 mol m^{-3} in epidermal cells, mainly as glucose (Tomos *et al.* 1992). Free amino acids are virtually undetectable in sap from barley epidermal cells but are present at about 10 mol m^{-3} in mesophyll cell sap and 7 mol m^{-3} in bundle sheath cells (Fricke *et al.* 1993). These results indicate that, as well as having a different ionic composition from the mesophyll, the epidermis uses only inorganic ions as its principal osmotica whereas the mesophyll and the

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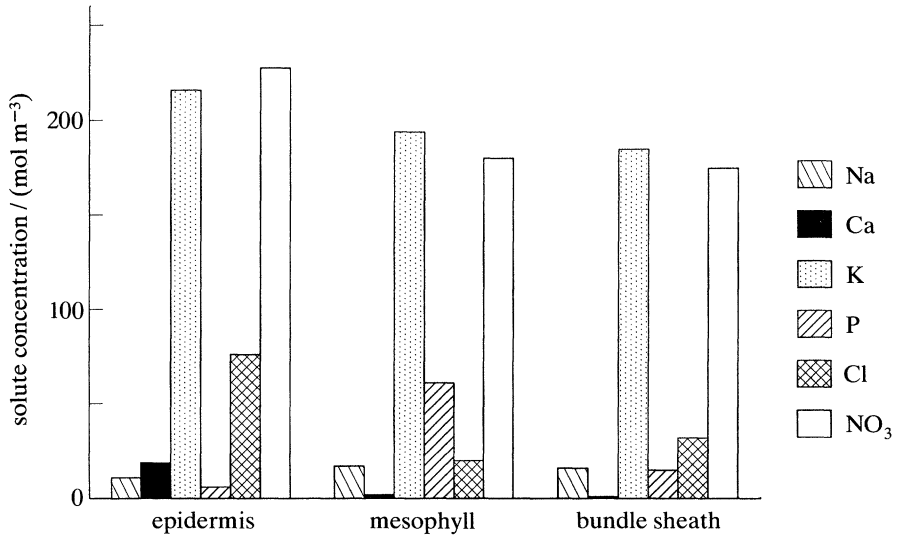

Figure 1. Ion concentrations in sap extracted from individual upper epidermal, mesophyll and bundle sheath cells of barley leaves. Results are means for 4–7 cells analysed per cell type. From Fricke *et al.* 1993.

bundle sheath use a mixture of organic solutes and inorganic salts.

The physiological reasons for these distributions of ions and solutes between cells in cereal leaves remain unclear but they may be related to the requirements of the mesophyll cells for a particular ionic composition in order to perform optimally. Thus the storage of P_i to high concentrations in mesophyll cells, but not in the epidermis, may be related to the requirement for P_i in the export of fixed carbon from the chloroplast (Flugge & Heldt 1991) and may be a mechanism for maximising its availability to the chloroplast during periods of P deficiency. Similarly, the exclusion of Ca^{2+} and some of the Cl^- from the mesophyll may indicate a particular sensitivity of mesophyll processes to these ions and, perhaps, an inability of the tonoplast of mesophyll cells to retain these ions effectively in the vacuole. An extension of these ideas is that mechanisms exist to prevent excessive accumulation of certain ions in mesophyll cells and to ensure these ions are partitioned to the epidermal layers. If this is so, the maintenance of these ion distributions between epidermis and mesophyll may be important in the ability of the plant to withstand nutrient stresses.

(*b*) *The effects of nutrient supply*

Leigh & Storey (1993) found that Ca^{2+} and Cl^- were detectable in epidermal cells at all shoot concentrations of these ions but Ca^{2+} was never detected in mesophyll cells, while Cl^- became detectable only at the highest shoot Cl^- concentration (figure 2). Potassium and Na^+ were found in both cell types at all shoot K^+ or Na^+ concentrations and they were both generally at higher concentrations in the mesophyll cells (figure 2). In contrast to Cl^-, Na^+ showed no propensity to accumulate specifically in the epidermis even under salt stress (figure 2). The trends for Cl^- have been confirmed by Dietz *et al.* (1992*b*). Using the more sensitive technique of protoplast analysis, the showed that some Cl^- is always present in mesophyll cells even at low tissue Cl^- leaves (see also figure 1).

Dietz *et al.* (1992*b*) also studied the effects of variation of P_i, NO_3^- and SO_4^{2-} supply on the composition of epidermal and mesophyll protoplasts. Increasing the P_i supply increased P_i concentrations in the mesophyll, but not in the epidermis. Even at the highest P_i concentration supplied (50 mol m^{-3}) the epidermal protoplasts contained only 5 mol m^{-3} P_i. In contrast, changes in NO_3^- or SO_4^{2-} supply had effects on anion concentrations in both cell types and there was evidence for substitution of NO_3^- for Cl^-. Further evidence for this substitution has been obtained using samples of sap from single cells. When NO_3^- and Cl^- concentrations in the growth medium were altered, these anions showed reciprocal variations in concentration in barley leaf epidermal cells but the concentrations of P_i and SO_4^{2-} were unaffected (table 2). In contrast, when the major available anion was SO_4^{2-}, both P_i and SO_4^{2-} concentrations increased in some of the epidermal cells. The changes in cell composition occurred without any major effects on turgor or sap osmotic pressure indicating that these parameters are more conserved that the concentrations of individual solutes. The increase in P_i concentrations in the epidermis in the SO_4^{2-}-grown plants indicates that the restriction of P_i to the mesophyll is not universal and that when turgor generation in the epidermis demands it, this nutrient can be stored in the epidermal cells. No organic solutes were detected in epidermal cells even when ion supplies were altered widely. This further emphasizes the dependence of the epidermis on inorganic osmotica.

(*c*) *Variations between and within epidermal layers*

Measurement of the composition of cells in the adaxial and abaxial epidermal layers of barley leaves

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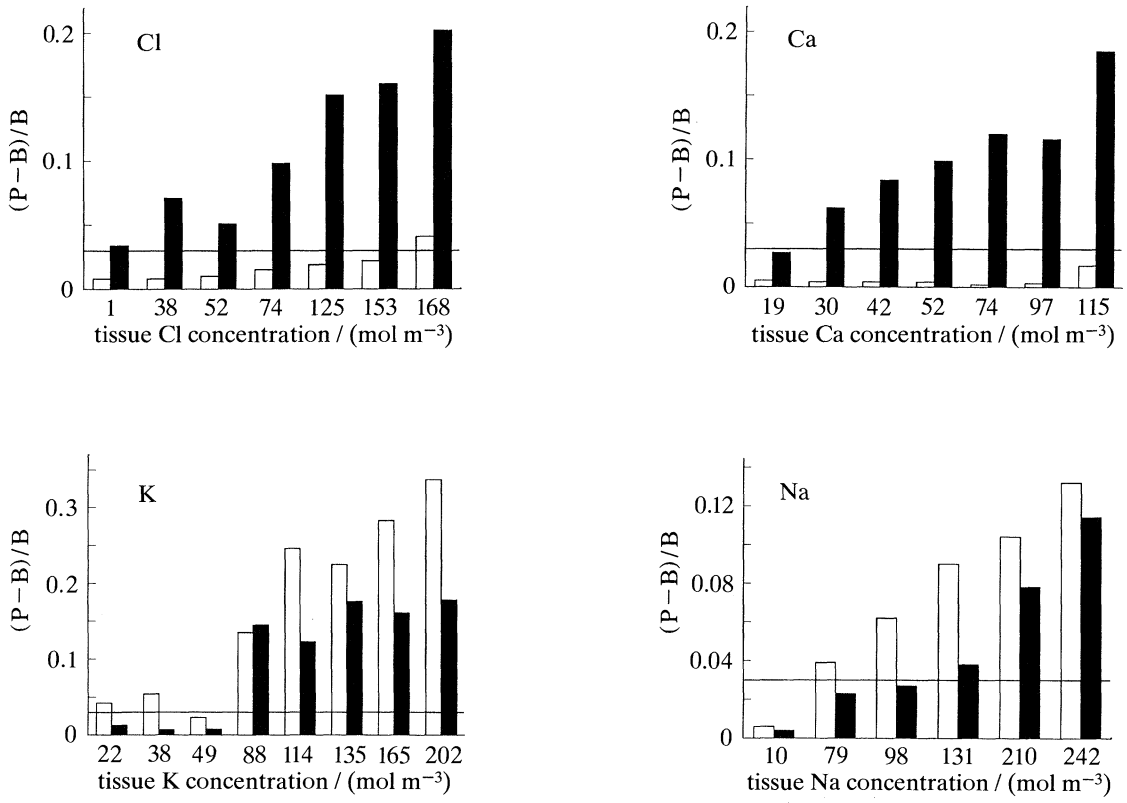

Figure 2. Relative levels of Cl^-, Ca^{2+}, K^+ and Na^+ in vacuoles of mesophyll (open bars) and epidermal (filled bars) cells of barley leaves as a function of the shoot concentrations of these ions. The elements were measured by X-ray microanalysis of bulk-frozen hydrated tissue and their relative levels expressed as a peak (P) to background (B) ratio (P-B)/B. The horizontal line indicates the minimum detectable level for each element (about 20–25 mol m^{-3}; see Lazof & Läuchli 1991). Adapted from Leigh & Storey (1993) with the permission of Oxford University Press.

using X-ray microanalysis suggested that there was no differential distribution of Ca^{2+}, Cl^- or Na^+ between the two layers whereas K^+ showed a slight preferential accumulation in the adaxial epidermis (Leigh & Storey 1993). This is similar to the situation in *Commelina communis* where Ca^{2+} is found at equal concentrations in the abaxial and adaxial epidermal layers despite a sixfold greater rate of transpiration from the abaxial surface (Atkinson 1991; Ruiz *et al.* this volume). In *Sorghum bicolor* leaves, however, more asymmetric distributions of elements have been measured with X-ray microanalysis. Leaves from salt-grown plants had more Ca^{2+} and Cl^- in adaxial than abaxial epidermal cells while P_i accumulated to relatively high concentrations in the abaxial cells but not in the adaxial ones (Boursier & Läuchli 1989).

Measurements of ion concentrations in files of epidermal cells indicates that concentrations can very widely. Leigh & Storey (1993) found that in both the adaxial and abaxial epidermal layers there were groups of K^+-rich cells separated by K^+-depleted cells. In K^+-deficient leaves, Ca^{2+} or Na^+ replaced K^+ and these replacement cations showed similar patterns of variation to those measured for K^+. The concentration of Cl^- generally varied in parallel with that of the dominant cation. Leigh & Storey (1993) were unable to determine whether the spatial variations in cation concentrations were related to any particular anatomical feature of the leaves because such information is unobtainable after the tissue has been prepared for X-ray microanalysis. However, using single-cell sap sampling, it has been possible to demonstrate that in primarly leaves of barley the K^+-depleted cells are furthest from the vascular strands and that there is a reciprocal variation in the concentrations of K^+ and Ca^{2+} in the epidermis as leaves approach senescence, even when K^+ is in plentiful supply (Tomos *et al.* 1993*b*). In general, Ca^{2+} concentrations in the epidermis increase with age and are highest near the leaf tip which is also where K^+ depletion is first measurable (W. Fricke, R. A. Leigh & A. D. Tomos, unpublished results).

Thus the general picture that emerges from these various approaches is one of a controlled distribution of ions and other solutes between the different cell types in cereal leaves. The consistency of the distributions measured by different techniques and their relative constancy under different conditions suggest that mechanisms exist for establishing and maintaining these patterns. In the next sections we will explore the implications of these observations both for the mechanisms that distribute solutes from the xylem to the cells and for the membrane transport mechanisms in individual cell types.

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Table 2. *The effect of variations in anion supply on the concentrations of ions, turgor and osmotic pressure (π) in epidermal cells of wheat leaves*

(Wheat plants were grown in nutrient solutions with anions present at 7 mol m^{-3} (NO_3^- and Cl^-) or 3.5 mol m^{-3} (SO_4^{2-}) when they were the major anions and 0.1 mol m^{-3} or less at other times. Results are given as mean ± s.d. for four measurements in each treatment. From Richardson *et al.* (1993).)

	major anion in nutrient solution		
parameter measured	NO_3^-	Cl^-	SO_4^{2-}
NO_3^-/(mol m^{-3})	222 ± 61	< 10	low[a]
Cl^-/(mol m^{-3})	18 ± 15	113 ± 51	80 ± 43
SO_4^{2-}/(mol m^{-3})	18 ± 12	26 ± 11	49 ± 32
P_i/(mol/m^{-3})	10 ± 18	10 ± 12	47 ± 32
K^+/(mol m^{-3})	227 ± 60	224 ± 34	234 ± 42
turgor/MPa	0.82 ± 0.31	0.94 ± 0.20	0.87 ± 0.29
π/MPa	1.03 ± 0.07	1.01 ± 0.12	0.97 ± 0.09
calculated π/MPa	1.20	0.93	0.99

[a] Not measured but assumed to be low as all measured osmotic pressure accounted for by the concentrations of other ions.

3. IMPLICATIONS FOR DISTRIBUTION PATHWAYS FROM THE XYLEM

Any descriptions of the pathways that ions follow from the xylem to the various cell types in a cereal leaf must be able to explain the patterns of cell composition described above and be consistent with the architecture of the leaf. In principle these pathways can be apoplastic, symplastic or transcellular (i.e. crossing all membranes in series; Tomos & Wyn Jones 1988). In cereal leaves, the xylem and phloem and associated elements are surrounded by the mestome sheath and bundle sheath cells. Beyond these are the mesophyll cells and the vein extensions (Canny 1990*a*). In the lateral and larger intermediate veins, the vein extensions make contact with the epidermis. Thus ions moving from the xylem to the epidermis could, in principle, move via the mesophyll cells or via the vein extensions.

(*a*) *Fully apoplastic mesophyll pathway*

The simplest pathway to envisage is one where the transpiration stream moves through the apoplast from the xylem sequentially passing the bundle sheath and mesophyll to the point of evaporation, with ions and water being absorbed by cells along the route. We term this the fully apoplastic mesophyll pathway and it is shown as pathway 1 in figure 3. Ion movement along it will be by mass flow of solution driven by the water potential gradient between the xylem and the sites of evaporation, and by diffusion down ion concentration gradients. Cell composition along the pathway will be determined by the nature of the transport characteristics of each cell type and the availability of any solute in the local apoplast. For example, the high levels of Ca^{2+} in the epidermis could reflect its low rate of uptake by the upstream bundle sheath and mesophyll cells, while the absence of P in epidermal vacuoles could indicate either the lack of a P uptake mechanism in these cells or a low supply of P to the epidermal apoplast.

The fully apoplastic pathway is consistent with the known general properties of walls and membranes, i.e. that walls have a high passive permeability and no solute selectivity while membranes have low passive solute permeability, high selectivity and the option for energy-linked active transport. It could account for any distribution of solutes that is observed and thus can be consistent with any pattern of cell composition. Experimental evidence for this pathway is provided by work with tracers such as that of Tanton & Crowdy (1972) who found that lead chelate applied to the base of transpiring, detached shoots accumulated in and around guard cells, which they took to be the

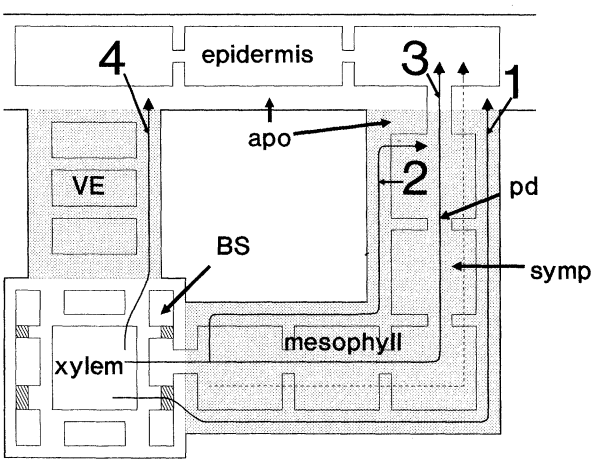

Figure 3. Diagram of the possible pathways for water and solute distribution from the xylem to different cell types in cereal leaves. The numbered pathways are: 1, fully apoplastic mesophyll pathway; 2, partly symplastic mesophyll pathway; 3, fully symplastic mesophyll pathway; and 4, vein extension (VE) pathway. The dotted line is in the proposed transcellular pathway for water flow. The hatched boxes between the bundle sheath cells indicate the suberized layers which it is proposed can prevent direct access of the transpiration stream to the mesophyll apoplast. apo = apoplast; symp = symplast; pd = plasmodesmata; BS = bundle sheath.

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Table 3. *Turgor pressure, sap osmotic pressure (π), cell solute contents and apoplastic water potential in mesophyll and epidermal cells of dark-adapted wheat leaves immediately after excision (0 h) and after 24 h illumination of the detached leaves*

(All parameters were measured on sap removed from single cells with a modified pressure probe. From Tomos *et al.* (1992).)

	hours	
parameter	0	24
mesophyll cells		
turgor/MPa	0.35	0.38
π/MPa	0.91	$\gg 1.0$
water potential/MPa	-0.56	< -0.62
sugars/(mol m^{-3})	10	> 100
epidermal cells		
turgor/MPa	0.75	0.76
π/MPa	0.86	1.26
water potential/MPa	-0.1	-0.5
sugars/(mol m^{-3})	< 2	4
K^+/(mol m^{-3})	140	220
Cl^-/(mol m^{-3})	75	200

sites of evaporation. This result implies there is no barrier to the movement of the chelate from the xylem to the point of evaporation. It would, however, require the accumulation of any residual solutes near the site of water evaporation where they would either remain or be absorbed by the local cells.

Attractively simple though this pathway is, there are several lines of evidence that question its veracity:

1. In a series of papers, Canny (summarized in Canny 1990*a*, this volume) has argued that on leaving the vascular bundle the transpiration stream immediately crosses a membrane into the symplast. His argument is based on the lack of penetration of the dye, sulphorhodamine G, into the mesophyll apoplast as would be expected if a fully apoplastic pathway were followed. The anatomical basis for this observation is probably wall suberization in the mestome/bundle sheath and this has been observed in wheat (Kuo *et al.* 1974) and *Aegilops comosa* (Eleftheriou & Tsekos 1979). Kuo *et al.* (1974) drew attention to the parallel between this organization and that of the endodermis of the root. Such an arrangement clearly prevents the free movement of water and solutes from the xylem to the leaf apoplast, and to gain access to the mesophyll from the xylem they would have to pass into cells at the mestome/bundle sheath.

2. Single-cell sap sampling indicates that the mesophyll apoplast has an osmotic pressure (i.e. solute content) considerably higher than that of the xylem sap. In leaves of both wheat (table 3) and *Tradescantia virginiana* (Nonami *et al.* 1990), the mesophyll apoplast has a water potential of -0.5 MPa or more negative. This is considerably lower than the xylem water potential which is -0.2 MPa or less negative depending on the rate of transpiration (Smith 1991; Zimmermann *et al.* this volume). There is no hydrostatic component to these apoplastic water potentials as the measurements were made at low transpiration rates and thus they represent the apoplast and xylem osmotic pressures. These results indicate that there is a large increase in solute concentration between the xylem and the mesophyll apoplast which can be interpreted as indicating that they are separated by a barrier that prevents the free movement of solutes and water between them. However, it has not yet been possible to measure water potentials of files of mesophyll cells. Therefore, the possibility that there are solute concentration gradients within the mesophyll apoplast, and that the step in water potential between the xylem and mesophyll apoplasts is spread over the entire pathway rather than at the xylem–bundle sheath interface, cannot be ruled out.

3. By using a pressure bomb, Tyree & Cheung (1977) measured the resistances to water flow to the xylem in intact leaves of beech, *Fagus grandifolia*, as well as in leaves infiltrated with water or with their edges cut to allow the xylem direct access to the bomb. They found that the resistances to the back flow of water from the leaf to the xylem were similar in the first two cases, and both were much higher than that in the third. They concluded that the resistances to water flow were too high to be explained by a purely apoplastic route and, from measurements of the activation energy for water flow, they concluded that water had to cross membranes to reach the xylem. It seems reasonable to assume that this would also be true for water flow from the xylem to the leaf.

4. Atkinson (1991) has shown that movement of Ca^{2+} to the epidermal layers of *Commelina communis* is independent of the rate of transpiration from each of the leaf surfaces. This cannot be reconciled with a model in which water and solutes move together through the apoplast. Such an arrangement should result in greater Ca^{2+} accumulation in the adaxial epidermis. The selective accumulation of some nutrient ions in the adaxial epidermis of *Sorghum* and others in the abaxial is also inconsistent with a fully apoplastic pathway for both water and solutes (Boursier & Läuchli 1989).

5. If this pathway extends to the epidermis, it would require continuity of the apoplast between the mesophyll and epidermis. However, there is a step in osmotic pressure between the apoplast of the mesophyll and epidermis similar to that between the mesophyll and xylem (table 3; Nonami *et al.* 1990). As argued above, this could indicate the presence of a physical barrier to water and solute movement.

These various results are inconsistent with a fully apoplastic pathway and suggest that the transpiration stream must cross cell membranes at some point on its journey from the xylem to the epidermis.

(*b*) *Partly symplastic mesophyll pathway*

The simplest alternative hypothesis that is compatible with the above objections is one in which the transpiration stream enters the symplast at the mestome/bundle sheath (Canny 1990*a*). All transport could then be symplastic to the epidermis. However,

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the high osmotic pressure of the mesophyll apoplast (see above) and the presence of apoplastic P_i (Mimura *et al.* 1992) and cations (Flowers *et al.* 1991) suggest that some solutes must enter the apoplast after the xylem–mesophyll barrier has been crossed. The apparent control of apoplastic P_i levels (Mimura *et al.* 1992) suggests that this release is a regulated process. This is also supported by the observation that cell and apoplastic osmotic pressures change in parallel to maintain a constant turgor in both mesophyll and epidermal cells (table 3). This could not be achieved without controlled release and uptake of solutes. Therefore it is necessary to postulate that some solutes are secreted back into the apoplast once they have crossed the barrier at the mestome/bundle sheath, and consequently follow a route that is only partly symplastic (pathway 2 in figure 3). This pathway differs from the fully apoplastic mesophyll pathway in that solutes are selectively ejected by cells rather than being selectively *absorbed*. Moreover, the presence of P_i in the apoplast of barley leaves (Mimura *et al.* 1992) suggests these secreted solutes are not just those destined for storage in the epidermis.

The data of Atkinson (1991) on Ca^{2+} distribution between epidermal layers, and the results in table 3 demonstrating a high osmotic pressure in the mesophyll apoplast, may indicate that water and solutes follow different pathways after they have entered the symplast at the mestome/bundle sheath. If all the transpirational water were to re-enter the apoplast it would not be possible to explain either the independence of Ca^{2+} distribution from adaxial or abaxial transpiration rates or the increase in apoplastic osmotic pressure between the xylem and the mesophyll apoplast. Therefore, once water has entered cells at the mestome/bundle sheath, it may follow a transcellular (vacuole-to-vacuole) or a symplastic pathway to the sites of evaporation (shown as dashed line in figure 3). The high hydraulic conductivity of leaf cell membranes and their large surface area normal to the flow (Tomos *et al.* 1981) would permit rapid water flow via a transcellular pathway, and so such a pathway would not prevent a high rate of transpirational water loss from leaves. Nor is this directional, transcellular movement of water necessarily opposed to the counter movement of solutes to the phloem, because the velocity of water flow through the leaf is slow. Canny (1990*b*) has estimated that the residence time of water in wheat leaves is 20 minutes. If the water is following through a transcellular pathway in the mesophyll and this tissue comprises some 60% of the leaf volume (Dietz *et al.* 1992*a*) then to traverse a mean water path of 100 μm from the xylem to the epidermis (see Jellings & Leech 1982) it would have to travel at a mean velocity of only 8 μm min^{-1}. In contrast, sucrose diffusing freely through the symplastic pathway would take 2.5 s to cover the same distance if the diffusion coefficient is the same as that in water (Nobel 1991).

Thus water and solutes could follow different pathways after their initial uptake by the mestome/bundle sheath with water flowing transcellularly while solutes return to the mesophyll apoplast and move to the epidermis by diffusion. The lower apoplastic osmotic pressure in the epidermis may indicate that the required concentration gradients exist to drive this apoplastic solute diffusion. However, the difference in osmotic pressure between the epidermal and mesophyll apoplasts (table 3) may indicate a barrier between these two compartments that forces solutes to re-enter the symplast to reach the epidermis, but this remains to be established. The low concentrations of organic solutes in the epidermis (see above) may indicate that there is not a major symplastic route between the epidermis and mesophyll in cereal leaves although functional mesophyll–epidermis plasmodesmatal connections are present in *Commelina cyanea* (Erwee *et al.* 1985). The role of apoplastic and symplastic pathways at this interface in cereals needs to be clarified.

If solutes do follow this partly symplastic pathway to the epidermis with symplastic steps at the bundle sheath–mesophyll and mesophyll–epidermal interfaces and an apoplastic pathway in between, this raises the question of why such a complex route is followed and why a fully symplastic pathway (number 3 in figure 3) is not utilized. One reason might be that high concentrations of many of the solutes destined for the epidermis are incompatible with optimal mesophyll functioning (see above) and temporary transfer to the apoplast minimizes their effects on mesophyll processes. Thus the symplastic movement of Ca^{2+}, the ion most strictly confined to the epidermis (figures 1 and 2), could be incompatible with the need to maintain cytosolic concentrations of this ion in the pmol m^{-3} range (Miller & Sanders 1987) and transfer to the apoplast could be a way of avoiding uncontrolled effects of elevated Ca^{2+} concentrations on mesophyll processes including photosynthetic sucrose synthesis (Brauer *et al.* 1990). Alternatively, if a fully symplastic route is followed, then an explanation is needed for why there is no significant diversion of Ca^{2+} into the vacuoles of the mesophyll cells. The reasons for high concentrations of solutes in the apoplast of mesophyll cells would also have to be sought, although one possibility is that they function in mesophyll turgor regulation, for example during cycles of accumulation and export of sugars produced by photosynthesis (table 3; Tomos *et al.* 1992).

(*c*) *The vein extension pathway*

In many leaves, the mesophyll is not the only anatomical link between the xylem and the epidermis. Canny (1990*a*) has demonstrated that sulphorhodamine G can diffuse from the xylem to the epidermis in the apoplast of cells extending from the bundle sheath, and has referred to these as vein extensions. The dye did not diffuse laterally from these extensions implying that their apoplast is not continuous with that of neighbouring mesophyll cells. This apoplastic vein extension pathway (number 4 in figure 3) could provide a route for solutes to move to the epidermis without entering the mesophyll symplast or apoplast. The movement of sulphorhodamine G along the vein extensions was towards both epidermal layers and its

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kinetics were slower than free diffusion in solution indicating that it was not driven by bulk transpirational flow (Canny 1990*b*). Thus, as with the partly symplastic mesophyll route, the vein extension pathway is one that appears to allow solutes to move separately from water. Interestingly, sulphorhodamine G did not diffuse into the cell walls between the individual vein extension cells and this may indicate that there is no transcellular water flow between them to create a hydrostatic pressure that would draw the dye into the intercellular walls. Thus water may not be moving to the epidermis at any significant rate along these vein extensions. It remains unclear whether there is a symplastic route for ions to move from these extensions to the mesophyll.

The vein extensions provide a potential pathway for the delivery of epidermis-specific solutes (e.g. Ca^{2+}) to the epidermis without the need to enter the mesophyll apoplast or symplast. The separation of these solutes from water and mesophyll-specific solutes (e.g. P_i) could occur at the mestome/bundle sheath after which they pursue completely separate paths. Thus partitioning of solutes between these two pathways will be determined by the rate at which the mestome/bundle sheath can absorb those solutes destined for the partly symplastic mesophyll pathway. The rate of solute movement to the epidermis along the vein extension pathway will be determined by the concentration gradient between the xylem and the epidermal apoplast. This will depend on the relative rates of uptake into the partly symplastic mesophyll pathway, recycling directly from the xylem to the phloem, and accumulation within cells of the epidermis. Differential changes in the rates of these processes could provide a mechanism for reversing flow along the vein extensions by lowering concentrations of solutes at the xylem end of the pathway. This could be important in the recycling of ions such as K^+ which decline in concentration in the epidermal cells during leaf aging (P. Hinde, R. A. Leigh & A. D. Tomos, unpublished data) and osmotic adjustment in the epidermis during sugar accumulation (Tomos *et al.* 1992).

(*d*) *An hypothesis*

Although our current understanding does not allow us to determine whether any of the above pathways dominates ion delivery to different cell types in the cereal leaf, we propose the following model as a speculative, but testable, hypothesis that is in accord with the available evidence. The main features of the model are, firstly, that all ions reaching the mesophyll of cereal leaves must be selectively absorbed from the xylem by the mestome/bundle sheath cells and then transferred to the mesophyll either symplastically or apoplastically. Secondly, that the vein extensions are the major routes for delivery of ions to the epidermis although water does not flow along this pathway to any significant extent but follows a transcellular pathway through the mesophyll. Thirdly, that the mesophyll pathway does not represent a significant route for the delivery of ions to the epidermis of cereal leaves and that suberization at the mesophyll–epidermis interface prevents significant apoplastic movement of solutes between these two cell types while plasmodesmatal connections may be lacking or largely inoperative. Thus in this model selectivity of transport systems at the mestome/bundle sheath cells is the main determinant of the mix of ions that is delivered to either the mesophyll or the epidermis.

A number of approaches could be used to test this model. Firstly, investigations of transport systems in the mestome/bundle sheath cells (possibly by patch clamping, see below) could indicate whether the necessary array of transporters is present to explain the required selectivity of transport at this interface. Secondly, extensions of Canny's studies with fluorescent dyes (e.g. Canny 1990*a*,*b*, this volume), particularly utilizing ones that can cross into the mesophyll from the transpiration stream, might indicate whether these are able to reach the epidermis via the mesophyll. Thirdly, micro-injection of dyes into epidermal cells could indicate whether there are functional plasmodesmata between epidermal and mesophyll cells and therefore whether there is the potential for symplastic transfer between these cell types. Fourthly, further studies with single cell sampling should indicate whether there are sudden and unique steps in water potential between different cell types or whether the differences shown in table 3 are due to a more gradual gradient that is spread evenly between the xylem and the epidermis. If the changes are abrupt this would argue for physical barriers separating the apoplast of different cell types. Finally, the use of non-invasive techniques such as nuclear magnetic resonance imaging might be able to demonstrate the pathways that water and solutes follow.

4. IMPLICATIONS FOR TRANSPORT MECHANISMS IN CELLS

The different compositions of different cell types indicate either that they are selective in the ions they absorb from (or return to) the transpiration stream as it moves through the leaf, or that cells earlier in the pathway have removed certain solutes and therefore they are not available to cells further downstream. However, each of the pathways discussed above demands different arrangements of transport systems in the various cell types. Studying the transporters present in leaf cells might thus provide evidence for or against these pathways.

(*a*) *Fully apoplastic mesophyll pathway*

For this pathway, it is proposed that the transpiration steam would sequentially pass the bundle sheath, mesophyll and epidermal cells as it moves through the leaf apoplast. Thus to account for the observed distributions of ions, the bundle sheath and mesophyll cells must be able to exclude Ca^{2+} very effectively and Cl^- partly, while selectively absorbing P_i and, in the case of some bundle sheath cells, also SO_4^{2-} and Mg^{2+}. The affinity and storage capacity of the mesophyll for P_i must be such that the amount reaching the epidermis is sufficient for the metabolic

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needs of these cells but not enough to initiate storage in epidermal vacuoles, except when it is demanded for turgor generation in the absence of other anions (e.g. table 2). However, NO_3^-, K^+, and Na^+ are still available to the epidermis in relatively large quantities and so all leaf cells must have good supplies of these ions and possess the ability to transport them.

The negative electrical potential across the plasma membrane of leaf cells (Jeschke 1976) provides a large inwardly-directed driving force for the uptake of cations into mesophyll cells. The low concentrations of Ca^{2+} in these cells indicate that either they lack ion channels that permit passive inward Ca^{2+} transport or they possess a very efficient active Ca^{2+} extrusion system. This extrusion could be mediated either by a Ca^{2+}-transporting ATPase or by a Ca^{2+}/H^+ antiporter (Evans *et al.* 1991). The presence of both has been demonstrated in plasma membrane vesicles prepared from *Zea mays* leaves (Kasai & Muto 1990) but their relative contributions to Ca^{2+} exclusion from the mesophyll remain to be established. Uptake of K^+ and Na^+ is probably mediated by K^+ channels and non-specific cation channels (e.g. Moran *et al.* 1984; Kourie & Goldsmith 1992; Spalding *et al.* 1992).

Uptake of Cl^- and NO_3^- is against the membrane potential and accumulation of high internal concentrations probably requires active transport. The distributions of Cl^- suggest that mesophyll cells may lack an active Cl^- transporter or they may have Cl^- channels that permit efflux of any Cl^- that is absorbed. Active transporters for Cl^- would have to be present in epidermal cells to account for their high internal Cl^- concentrations and both epidermal and mesophyll cells would have to possess active NO_3^- transporters. Similarly, mesophyll cells would need a very efficient active transport system for the uptake of P_i. Such a system could also be present in the epidermis as these cells can accumulate P_i under certain conditions (table 2; Boursier & Läuchli 1989). Therefore there is no need to invoke the lack of a P_i transport system in the epidermal cells and, indeed, a system will be needed for accumulating the low amounts of P_i needed for metabolism. Thus the low concentrations of P_i in epidermal cells are probably due to low availability because of the prior accumulation of P_i in bundle sheath and mesophyll cells, although in certain conditions the mechanisms responsible for this must be overcome (see table 2).

(*b*) *Partly symplastic mesophyll pathway*

This is a more complicated pathway than the fully apoplastic mesophyll pathway and it is less easy to predict the precise transport properties required in different cell types as they will depend on the contributions that symplastic transport makes to movement to the epidermis, and whether some ions move along the vein extensions. If all solutes reaching the epidermis have followed the partly symplastic mesophyll pathway then all must pass through the cells at the mestome/bundle sheath. Therefore, for this pathway, these cells would have to possess passive transport systems (channels) for cations and active uptake systems for most anions. Some of these ions may then be effluxed back to the mesophyll apoplast and the cells that are responsible for this export would have to possess the appropriate active (cations) and passive (anions) outwardly directed transport systems. If the bundle sheath cells are responsible for both uptake from the xylem and efflux to the mesophyll apoplast then they would have to possess spatially separated transporters for achieving this. Alternatively, the transport to the apoplast may be achieved after symplastic transfer to the mesophyll and the transporters required for export would then be present in the mesophyll cells. The presence of P_i in the leaf apoplast (Mimura *et al.* 1992) indicates that the export of ions to the apoplast is not restricted just to ions that accumulate in the epidermis at high concentrations.

If transport to the epidermis is fully apoplastic once ions have been exported from the bundle sheath or mesophyll then the transport systems required in the epidermis will be the same as those for the fully apoplastic mesophyll pathway. However, as indicated above, there is possibly a barrier between the mesophyll and epidermal apoplasts which would require a symplastic transport step and, therefore, the cells involved would have to possess the appropriate transport systems to achieve this. This raises the intriguing possibility that mesophyll cells possess distinct suites of transporters depending where they are located in the leaf. Those close to the bundle sheath could be involved in the excretion of certain ions to the apoplast while those nearer the epidermis would be responsible for absorbing ions for symplastic transfer to the epidermis. Measurement of the composition of mesophyll cells in barley leaves did not reveal any obvious differences related to position (R. A. Leigh & R. Storey, unpublished results) which suggests that the symplastic transfer of ions to the epidermis would have to be efficient with little diversion of ions to the vacuoles of the mesophyll cells involved in this process.

(*c*) *The vein extension pathway*

As with the partly symplastic mesophyll pathway, the transport features of the bundle sheath cells are an important aspect and will determine the nature and proportions of different ions that move along the vein extension pathway. If the vein extension pathway is the only route to the epidermis, then cells at the mestome/bundle sheath would have to possess very effective mechanisms for selecting ions destined for the mesophyll, and for rejecting those to be transported to the epidermis. Thus the effectiveness with which Ca^{2+} seems to be excluded from the mesophyll would require very efficient Ca^{2+} exclusion or extrusion mechanisms at the bundle sheath to ensure this ion was restricted to the vein extension pathway. In contrast P_i would be efficiently taken up into the bundle sheath. Other ions which accumulate in both the mesophyll and epidermis would be less efficiently absorbed at the bundle sheath. Selectivity need not be restricted to the cells immediately adjacent to the xylem as it is possible that cells further along the

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extensions could also be effective in absorbing ions from the apoplast for transfer to the mesophyll. The transport properties required in mesophyll and epidermal cells would be similar to those required for the partly symplastic pathway. Extrusion of certain ions to the mesophyll apoplast would still have to occur to account for the high osmotic pressure in this compartment but ions destined for the epidermis might not be involved and hence a simpler array of transporters could be present. If the vein extensions are the sole route to the epidermis then the transfer to the symplast at the mesophyll–epidermis interface would not be required as there would be no need for ions to cross the putative apoplastic barrier at this point.

5. THE TRANSPORT PROPERTIES OF DIFFERENT LEAF CELLS

The transport properties of mesophyll and epidermal cells have not been compared frequently so there is little evidence for or against the presence of differing arrays of ion transport systems in each of these cell types. Dietz *et al.* (1992*b*) measured Cl^- uptake by mesophyll and epidermal protoplasts of barley and found that influx was higher into epidermal than into mesophyll protoplasts when compared on a 'per protoplast' basis but not when related to membrane area. This suggests that there may be little difference in Cl^- transport capabilities of epidermal and mesophyll cells and favours a scenario in which the lower concentrations of Cl^- in mesophyll cells compared with epidermal cells arise because mesophyll cells are not exposed to high concentrations of Cl^-. This would result if Cl^- moves to the epidermis mainly along the vein extensions.

The technique of patch clamping (Hedrich & Schroeder 1989) provides the most obvious approach to compare epidermal and mesophyll ion channels and other charge-carrying transporters because this technique can be applied to individual cells. Although one of the first applications of this technique in plants was on mesophyll protoplasts (Moran *et al.* 1984) it has not been used extensively on leaf cells other than stomatal guard cells (e.g. Schroeder 1992). Comparisons of different cell types from the same leaf have not yet been made. The few studies that have applied patch clamping to leaf cells have largely concentrated on mesophyll protoplasts and have characterized cation channels.

Spalding *et al.* (1992) found three cation channels in the plasma membrane of *Arabidopsis thaliana* mesophyll protoplasts. Two were selective for K^+ over Na^+ and Cl^- whereas the third did not discriminate between K^+ or Na^+. One of the K^+-selective channels showed inward rectification, was light-activated and could be involved in the passive uptake of K^+. The other two channels tended to be closed at negative membrane potentials and probably do not play any role in K^+ uptake at physiological membrane potentials. From the effect of light on the membrane potentials, Spalding *et al.* (1992) concluded that there must also be other channels in the plasma membrane of *A. thaliana* that can depolarize the membrane potential beyond the K^+ equilibrium potential. They suggested these could be either by Cl^- or Ca^{2+} channels but did not explore this further. Oat mesophyll protoplasts possess a voltage-gated K^+ channel that is probably involved in inward K^+ transport at physiological membrane potentials (Kourie & Goldsmith 1992). Recently, Ding & Pickard (1993) described a mechanosensitive Ca^{2+} channel in epidermal cells of the leaf sheath of *Allium cepa*. This may be involved in Ca^{2+} influx in response to physical disturbance of the cells (e.g. in wind) but it is unclear whether it also operates in general Ca^{2+} influx under other conditions.

6. CONCLUDING REMARKS

The asymmetric distribution of ions and other solutes between different cell types in cereal leaves indicates that any description of solute distribution which assumes that all cells have the same average composition is unacceptable. Further, the pathways and mechanisms of solute and water distribution from the xylem to the various cells in a leaf must be able to account for the patterns of ion composition seen and their maintenance under a variety of stress conditions. Although we have concentrated on the implications for cereal leaves, it is likely that the general principles will also apply to leaves of other plants as differential distributions of ions are also seen in leaves of *Vicia faba* (Outlaw *et al.* 1984), *Lupinus luteus* (Treeby *et al.* 1987; Treeby & van Steveninck 1988) and *Atriplex spongiosa* (Storey *et al.* 1983) although the patterns are not necessarily the same as those seen in cereal leaves. Thus in *Vicia faba* leaves (Outlaw *et al.* 1984) the P concentration is higher in epidermal cells (60 mol m^{-3}) than in spongy parenchyma (5 mol m^{-3}) or palisade parenchyma (4 mol m^{-3}) while in lupin leaflets Cl^- is found at high concentrations in both the mesophyll and epidermis of salt-grown plants (Treeby & van Steveninck 1988). It will now be interesting to see whether these different distributions are a reflection of different pathways of water and solute flow through the leaf or of differences in transport properties of the leaf cells. Elucidating these pathways will be important not just to gain a better understanding of the ionic relations of leaves but also for determining how signal molecules derived from the xylem (e.g. abscisic acid; Gowing *et al.* this volume) reach their intended target cells.

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