Mechanical stabilization of desiccated vegetative tissues of the resurrection grass *Eragrostis nindensis*: does a TIP 3;1 and/or compartmentalization of subcellular components and metabolites play a role?

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Abstract

During dehydration, numerous metabolites accumulate in vegetative desiccation-tolerant tissues. This is thought to be important in mechanically stabilizing the cells and membranes in the desiccated state. Non-aqueous fractionation of desiccated leaf tissues of the resurrection grass *Eragrostis nindensis* (Ficalho and Hiern) provided an insight into the subcellular localization of the metabolites (because of the assumptions necessary in the calculations the data must be treated with some caution). During dehydration of the desiccant-tolerant leaves, abundant small vacuoles are formed in the bundle sheath cells, while cell wall folding occurs in the thin-walled mesophyll and epidermal cells, leading to a considerable reduction in the cross-sectional area of these cells. During dehydration, proline, protein, and sucrose accumulate in similar proportions in the small vacuoles in the bundle sheath cells. In the mesophyll cells high amounts of sucrose accumulate in the cytoplasm, with proline and proteins being present in both the cytoplasm and the large central vacuole. In addition to the replacement of water by compatible solutes, high permeability of membranes to water may be critical to reduce the mechanical strain associated with the influx of water on rehydration. This is the first report of a possible TIP 3;1 in vegetative tissues (previously only reported in orthodox seeds).

Key words: Aquaporin, desiccation-tolerance, non-aqueous fractionation, water stress.

Introduction

Many plants are subject to water-deficit stress at some stage during their life and, consequently, a diverse array of strategies to survive this stress has developed. A few unusual so-called resurrection plants are able to survive extreme loss of water (desiccation) from their vegetative tissues (Gaff, 1971). Mechanical damage is proposed to be one of the major causes of irreversible desiccation-induced damage in plants (Iljin, 1957). Extreme loss of water results in a loss of cell volume as tissue water content declines. Water plays an important structural role in plant cells and it has been proposed that cells cannot contract beyond a certain minimum ‘critical volume’ without irreparable damage to membranes (Meryman, 1974). Vegetative tissues which are able to survive desiccation to an air-dry state have mechanisms that cope with this potentially lethal mechanical stress. Angiosperm desiccation-tolerant plants, relying predominantly on protective mechanisms during dehydration, use two different approaches to minimize this stress: water replacement or a reduction in cellular volume (Gaff, 1971).

The desiccated cells of some resurrection plants become filled with vacuoles containing non-aqueous contents,
replacing the volume occupied by water in the hydrated state. This water replacement strategy has been proposed to reduce the mechanical stress on the cellular substructure (Farrant, 2000). The presence of numerous small vacuoles, similar in appearance to the protein storage vacuoles found in orthodox seeds, has been reported in a number of resurrection species (Dalla Vecchia et al., 1998; Farrant, 2000; Vander Willigen et al., 2001a). Neither the formation of these small vacuoles nor their contents have been investigated and hence their importance in reducing mechanical stress is unclear.

An alternative strategy to prevent lethal mechanical stress on cellular desiccation is to reduce cellular volume. Extensive cell wall folding in some desiccation-tolerant species has been recorded to cause as much as a 78% reduction in cellular volume on dehydration (Farrant, 2000; Vander Willigen et al., 2001a). Unlike cytorrhysis (irreversible cell wall collapse) observed in many desiccation-sensitive tissues, cell wall folding is associated with changes in the biomechanical properties of the cell walls brought about by changes in the biochemical properties of the wall structure on dehydration (Vicre et al., 1999). Vander Willigen et al. (2001a) proposed that this controlled reduction in cellular volume on dehydration was associated with unusual water relations in these tissues. This possibly prevented the development of negative turgor and, thereby, reduced the potential for irreparable mechanical damage in these resurrection plants.

Resurrection plants which are able to minimize mechanical stress during dehydration must also have mechanisms to minimize similar stresses associated with the influx of water into the cells on rehydration. Since aquaporins play a crucial role in water movement, their behaviour under water stress conditions is starting to receive attention (Yamada et al., 1995, 1997; Kirsh et al., 2000; Mitra et al., 2001). There are now some reports of changes in the expression of aquaporins under stress conditions which influence not only the hydraulic conductivity of membranes but also water potential via aquaporin phosphorylation (Liu et al., 1994; Johansson et al., 1996, 1998; Sarda et al., 1997; Barrieu et al., 1999).

Not long after the discovery of water channel proteins in plants, their role in desiccation tolerance was suggested (Hartung et al., 1998). Five aquaporins have been isolated from resurrection angiosperms: three PIPs (plasma membrane intrinsic proteins) and a TIP (tonoplast intrinsic protein) from Craterostigma plantagineum (Mariaux et al., 1998) and one TIP 1;3 from Sporobolus stapfianus (Neale et al., 2000). Changes in the expression of these five genes during desiccation were reported; however no protein studies on these TIPs or their role in desiccation tolerance have been undertaken. Most of the studies on the role of aquaporins in water stress have focused on the roles of PIPs; very few studies have considered the function of TIPs in water-limited environments.

The focus of this paper was to investigate the processes that may be involved in dealing with mechanical stresses associated with desiccation in the resurrection grass, Eragrostis nindensis. The accumulation of small vacuoles and metabolites on dehydration was investigated, and the compartmentalization of the metabolites was characterized using non-aqueous fractionation. In addition, the protein expression and immunolocalization of a proposed TIP 3;1 (formerly α-TIP) were undertaken. The possible roles of these phenomena in reducing mechanical stress are discussed.

Materials and methods

Plant material
Mature Eragrostis nindensis (Ficalho and Hiern) plants from an inselberg (granitic outcrop) located in a north-western region of South Africa were potted in the sandy soil from the site and translocated to a greenhouse at the University of Cape Town. All plants remained hydrated for at least one month prior to any experimentation.

The second leaf on a tiller from at least five different E. nindensis plants was harvested at intervals during desiccation (withholding of watering) from plants which were initially hydrated to full turgor. Prior investigation (Vander Willigen et al., 2001b) showed that the outermost leaf on a tiller is sensitive to desiccation and so these leaves have not been included in this study. After 2 weeks in the dry state, plants were rewatered and the soil was held at field capacity during regular sampling over a rehydration period. Relative water contents (RWC) were determined gravimetrically at each point, as described previously (Vander Willigen et al., 2001b). The mean RWC at each sampling point was calculated from leaf segments either side of that used in each assay. Because diurnal changes in the expression of TIPs and metabolites have been reported (Henzler et al., 1999; Norwood et al., 2000), tissue was always harvested at 09.00 h, immediately plunged into liquid nitrogen, and stored at −80 °C. Since the sampling intervals during rehydration were less than 24 h, sampling times are indicated in the relevant legends.

TIP antibodies
The TIP antibodies used in this study were kindly provided by Dr JC Rogers, (Institute of Biological Chemistry, Washington State University, USA). The TIP 3;1 was isolated from Phaseolus vulgaris seed. Antibodies were raised in rabbits against polypeptides synthesized according to the following sequence: HQLAPEDY, by Jauh et al. (1998). This polypeptide forms part of a conserved domain from the carboxyl-terminal, cytoplasmic tails of this TIP isoform and has been found to be conserved across many species (Jauh et al., 1998). In addition, antibodies raised against the entire TIP 3;1 isolated from P. vulgaris (Johnson et al., 1989) were also used. Preliminary experiments confirmed that these antibodies only cross-reacted with proteins of an expected size (c. 26 kDa) in E. nindensis.

Transmission electron microscopy
Hydrated and desiccated leaves were prepared using conventional and freeze-substitution techniques, respectively. Conventional preparation methods are described in Sherwin and Farrant (1996).

For preparation of material by freeze-substitution, leaf segments (c. 0.5 mm²) were first cryofixed by immersion in dry, precooled
Mechanical stabilization in desiccated tissues

Leaf segments (c. 5 mm² pieces) from 2 cm distal to the leaf sheath of E. nindensis and whole seeds of P. vulgaris were ground in liquid nitrogen and homogenized in Trizol (Life Technologies, GIBCO BRL) for 5 min at room temperature with vortexing (c. 0.1 g tissue in 1 ml Trizol per microcentrifuge tube) according to the methods of Chomzynski and Sacchi (1987). The protein extraction method was modified slightly by washing the pellets three times with cold 0.1 M ammonium acetate and once with cold acetone (centrifugation at 12 000 g for 10 min at 4 °C and 5 min incubation periods between washes). Air-dried protein samples were stored at −20 °C.

**Protein quantification**

Resuspended proteins were quantified according to the method of Bradford (1976) using a protein assay kit (Pierce, Rockford USA). The absorbance of 40 μl protein samples that had been incubated with 2 ml of the diluted Bradford reagent for 2 min, was read at 595 nm (Beckman DU 530 spectrophotometer, Fullerton, CA, USA). Protein concentrations were calculated from a standard curve, constructed using bovine serum albumin (BSA).

**Western blot analysis**

Proteins (15 μg per lane) were incubated at 37 °C for 10 min and electrophoresed on 20% SDS-PAGE gels containing 0.1% bis acrylamide. The gel, PVDF membrane, Whatman 3MM paper, and foam pads (all cut to the same size as the gel) were soaked in transfer buffer (192 mM glycine, 25 mM TRIS, 3.5 mM SDS, 20% (v/v) methanol). The proteins were transferred to the PVDF western blotting membrane (Boehringer Mannheim) for 1 h at 30 V in a precooled western blotting tank (Hoefer, San Francisco, USA). The membrane was rinsed with TBS (50 mM TRIS, 150 mM NaCl, pH 7.5) and non-specific binding sites were blocked with 0.5% (w/v) skimmed milk powder in TBS for 1 h at room temperature with shaking. The membrane was then incubated overnight with the primary antibody (1:2000 dilution (polyepitope); 1:3000 dilution (entire protein)) diluted with 0.5% (w/v) skimmed milk powder at 4 °C with shaking. After two washes with TBS containing 0.1% Tween 20 for 10 min, the membrane was incubated with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) at 1:1000 dilution for 1 h at room temperature with shaking. The membrane was thoroughly washed before detection using chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia, Buckinghamshire, UK). Blots were developed on high performance chemiluminescence film (Amersham Pharmacia, Buckinghamshire, UK), scanned and analysed using Biorad Quantity One 4.4 software (Biorad, California, USA). Duplicate gels were routinely stained with Coomassie Brilliant Blue R-250 (0.25% (w/v) in 50% (v/v) methanol, 7% acetic acid (v/v)) to confirm equal protein loading. All blots were repeated at least tree times on separately extracted protein samples from different plants.

**Immunofluorescence studies**

Leaf segments (c. 5 mm² pieces) from 2 cm distal to the leaf sheath, collected during numerous stages of dehydration and rehydration, were fixed in 4% (w/v) paraformaldehyde in 0.1 M Sørensen’s phosphate buffer (pH 7.2) overnight at 4 °C. Specimens were then dehydrated through an alcohol series (50%, 70%, 95%, and 100% ethanol, 100% isopropanol, 100% butanol) and embedded in paraffin wax at 60 °C overnight. Thick wax-embedded transverse sections (10 μm) were cut on a Leitz rotary microtome (Vienna, Austria) and attached to APTES- (3-aminopropyltriethoxysilane) coated glass microscope slides by heating to 60 °C for 1 h. After dewaxing with xylol and rehydrating through an ethanol gradient, the sections were washed with PBS (50 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) and blocked with 1% BSA in PBS for 1 h in a moist chamber at room temperature. Sections were incubated with the TIP 3;1 polypeptide antibody (1:100 dilution) or 1% BSA (control) in PBS overnight at 4 °C in a moist chamber. Thereafter, the sections were jet-washed with 0.1% Triton X-100 in PBS and then rinsed for a further 15 min in the buffer before incubating in the fluorochrome tagged secondary goat anti-rabbit IgG antibody (Alexa fluor 568, Molecular Probes, Oregon USA: dilution 1:1000) for 2 h in a moist chamber at room temperature in the dark. The slides were then thoroughly washed with PBS containing Triton X-100. Sections were mounted with ProLong Antifade (Molecular Probes, Oregon, USA) and viewed with an inverted fluorescent microscope (Nikon, Tokyo, Japan) under oil emersion with a B2 DM510 epi-fluorescence filter (Zeiss, Tokyo, Japan). The approximate absorption and fluorescence emission maxima for Alexa fluor 568 are 578 nm and 603 nm, respectively (Molecular Probes Inc., 2000). A confocal laser scanning microscopy (550 Confocal Microscope, Leica, Cambridge) was used to confirm the results, using a narrower range of wave lengths (600–610 nm). Images were captured with an AxioCam digital camera (Zeiss, Hallbergmoos, Germany) using AxioVision 2.05 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Care was taken to use the identical exposure times and images were not adjusted thereafter for comparative purposes.

**Non-aqueous fractionation of dry leaf tissues**

The subcellular compartments of dry leaf tissues (4.2±0.6% RWC) were separated using slight modifications of the non-aqueous fractionation method described by Stitt et al. (1989). Dry leaves (c. 2 g) were ground in liquid nitrogen and dried in a lyophilizer for 24 h. Care was taken at all stages after lyophilization to avoid any condensation forming in the samples. Each sample was resuspended in a precooled tetrachloroethylene:heptane solution (66:34 (v/v), density: 1.3 g cm⁻³) which had been dried over molecular sieve beads. The samples were ultrasonicated on ice (Sonorex TK 52H, Bandelin, Berlin) for a total of 60 s in 10 s pulses to prevent overheating. The homogenate, diluted 3-fold with dry, precooled heptane, was then passed through a glass microfibre filter and centrifuged for 10 min at 2200 g (Sigma 3K10 centrifuge, Germany). The supernatant was discarded while the sediment was resuspended in 3 ml of the 66:34 tetrachloroethylene:heptane solution. Aliquots
(of the suspension) (200 µl) were kept aside for measurement of enzyme activity and metabolite assays in the unfraccionated material and the remainder was layered onto a prepared linear density gradient (12 ml between 1.3 and 1.62 g cm⁻³). The gradient was prepared in polyallomer ultracentrifuge tubes immediately prior to use from dried solutions of heptane and tetrachloroethylene using a gradient former connected to a peristaltic pump (Miniplus 3, Gilson, France). The sample with the preformed gradient was centrifuged for 2 h at 15 000 g at 5 °C (swing-out rotor SW28, ultracentrifuge (Beckman L5-65, Fullerton, USA)). The content of the centrifuge tube was then collected in eight fractions and each fraction separated into two portions: 1/3 for marker enzyme assays and 2/3 for metabolite assays. All aliquots were diluted 3-fold with heptane and centrifuged for 10 min at 2200 g. The supernatant was discarded and the pellets were dried in a desiccator over silica gel for 24 h and then extracted for the various assays.

**Marker enzyme assays**

Extracts were prepared by adding 500 µl of a solution containing 0.25 mM potassium phosphate (pH 7.5), 0.5 mM dithiothreitol (DTT), and 0.5 mM EDTA to the dried pellets. After vigorous shaking, another 500 µl of the same solution was added. The enzyme activities were measured in the supernatant of the solution after centrifugation for 2 min in a bench-top centrifuge. NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) was measured by the oxidation of NADPH at 340 nm using the method described by Stitt et al. (1989). This assay was used as a cytosolic marker. Soluble acid invertase was used as a lytic (central) vacuole marker, assayed as the hydrolysis of sucrose to glucose according to the methods of Kingston-Smith et al. (1998). The TIP 3;1 was found to be associated with the small vacuoles (see below) and so this subcellular compartment was identified by plotting the fraction extracts onto nitrocellulose membranes which were then incubated with TIP 3;1 antibodies as described previously. Care was taken to blot equal amounts of extract and spot intensity was recorded using Biorad Quantity One 4.4 software (Biorad, California, USA). All samples were loaded onto the same five replicate membranes.

**Metabolite assays**

The following assays were performed on bulk leaf tissue samples at various stages of dehydration and rehydration as well as on aliquots of the dry leaf samples after non-aqueous fractionation. In both cases (bulk leaf samples and fractionated samples) at least three replicate assays from different plants were performed, with two internal replicates for each assay.

**Sugars:** Ground frozen leaf samples (and the fractionated samples) were extracted in cold 100 mM NaOH (50% (v/v) ethanol/water). Chloroform (15%, v/v) was added and the samples incubated on ice for 10 min. Thereafter the pH was adjusted to 7.5 with 100 mM HEPES in 100 mM glacial acetic acid. After centrifugation at 28 000 g for 20 min at 4 °C, the supernatant was removed. A repeat extraction was performed on the pellets. The supernatants were pooled and then centrifuged. Sucrose, glucose, and fructose in the supernatant were calculated from the spectrophotometric measurement of NADPH production using a D-glucose/D-fructose sugar assay kit (Boehringer Mannheim, Germany).

**Free proline:** Free proline was measured spectrophotometrically according to the methods of Magne and Larher (1992) for samples with high sugar contents. Frozen ground leaf samples (and the fractionated samples) were extracted in 100% toluene. A 1% ninhydrin solution in 60:40 (v/v) glacial acetic acid/water was then added and the samples heated in a boiling water bath for 1 h. Twice the sample volume of toluene was added to the cooled samples. After vigorous shaking, the upper phase was read at 520 nm. Concentrations were calculated from a standard curve derived from known concentrations of proline.

**Protein:** Bradford (1976) assays, as described previously, were performed on the fractions.

**Determination of cellular and subcellular volumes**

Desiccated leaves were hand-sectioned dry and viewed immediately in an iso-osmotic solution of PEG 6000 using a light microscope (Ascopscope; Zeiss, Hallbergmoos, Germany). Preliminary experiments were undertaken to ensure that no swelling of the tissues occurred prior to photographing the sections. Hydrated leaves were sectioned and viewed in deionized water. Cellular cross-sectional areas of more than 40 digital images from at least eight individual plants from both the hydrated and dehydrated leaves were measured using an image analysis program (AnalySIS, Soft Imaging Software), and it was assumed that cross-sectional area is an unbiased estimate of volume (Winter et al., 1993). Volumes of subcellular compartments in dry leaves were estimated from cross-sectional areas measured from digital images of transmission electron micrographs of the dry leaves. At least 15 grid blocks from each of four different sections from at least two different specimens per sample were analysed.

Preparation of these images was as previously described (see "Transmission electron microscopy").

**Results**

**Formation of vacuoles during desiccation**

During dehydration of the desiccation-tolerant inner leaves of *E. nindensis*, numerous small vacuoles formed in the bundle sheath cells (BSC) of these leaves (Fig. 1A). Unlike the mesophyll cells in which extensive cell wall folding was observed (Fig. 1B), the thicker-walled BSC maintained most of their volume and shape in the desiccated state, comprising 37% and 33% of the cross-sectional area of the leaves in the hydrated and dehydrated states, respectively. The folding of the thin walls of the mesophyll cells led to a considerable reduction in volume on dehydration, such that, in the dry state, they comprised only 22% of the leaf cross-sectional area compared with 49% in the hydrated state.

The characteristics of the small vacuoles in the BSC during dehydration are summarized in Table 1. As dehydration occurred, the large central vacuole became reduced in size and small vacuoles started to form from the periphery of the cell. In the air-dry state no central vacuole was apparent; the cells were packed with numerous small vacuoles. It is unclear whether this change in size and number of vacuoles was due to fragmentation of the central vacuole or *de novo* synthesis of the smaller ones. However, although total vacuolar volume of the BSC did not change during desiccation, the length of tonoplast visible on the micrographs increased (Table 1). After a 6 h rehydration period, to a RWC of 15%, the number of vacuoles had been reduced by 66% compared with the number in the dry state (Table 1).
Tonoplast intrinsic protein

Western blots using polypeptide antibodies to TIP 3;1 suggested that the protein was present only (or recognized the antigen only) in the desiccated leaves of *E. nindensis* (Fig. 2). It thus seemed that a TIP 3;1 appeared very late during dehydration, once the leaves had less than 0.43 ± 0.22 g H₂O g⁻¹ dry mass (11% RWC) and that it was degraded rapidly on rehydration. This result was confirmed using the TIP 3;1 antibodies raised against the entire protein (Johnson *et al.*, 1989; data not shown). Like all other vegetative tissues studied to date (Jauh *et al.*, 1998), TIP 3;1 was not found in the desiccation-sensitive outer leaves of *E. nindensis* (data not shown).

Immunofluorescence studies suggested that TIP 3;1 was present only in dry inner leaves of *E. nindensis* (Fig. 3). The localization of the fluorescent probe to the abundant small vacuoles present in the BSC (Fig. 1) of this tissue in the dry state suggests the presence of a TIP 3;1 in these small vacuole tonoplasts in *E. nindensis*. Unfortunately the wavelength and intensity of the background fluorescence changed with tissue hydration state and thus could not be eliminated completely. This is likely to be a consequence of the changes in pigment composition and concentration with desiccation (Vander Willigen *et al.*, 2001b).

Subcellular distribution of metabolites

During dehydration of the inner leaves, an accumulation of proline, sucrose, and protein was recorded (Fig. 4A1, B1, C1). Proline and sucrose contents decreased early on rehydration (Fig. 4A2, B2), whereas a higher concentration of proteins was maintained during the rehydration process (Fig. 4C2). Non-aqueous fractionation of the dry leaves enabled an analysis of the subcellular compartmentation of the accumulated metabolites without any rehydration of the tissue, although the analysis is complicated by the presence of two tissue types, BSCs and mesophyll/epidermal cells. Only vacuolar and cytoplasmic (which included mitochondria, chloroplasts, and nuclear compartments) fractions were analysed separately.

Figure 5 shows the distribution of enzyme markers (reaction to TIP 3;1 antibody for small vacuoles) and hence the distribution of subcellular compartments (cytoplasm, large central vacuole, small vacuoles) in the dry leaf tissue fraction from the non-aqueous density gradient. From
these data the proportional distribution of metabolites among the subcellular compartments was calculated using a method similar to that described by Riens et al. (1991) (Table 2). This table also shows the initial concentration of the metabolites in the dry leaf tissue. From the distribution of metabolites among compartments and the initial concentration of metabolites in whole leaf tissue, the mass percentage of each metabolite in each compartment was calculated (Table 2). To allocate the metabolites to subcellular compartments in the two tissue types (BSCs and mesophyll/epidermal cells) requires that the contribution of each compartment in each tissue type to total leaf volume be known. This was achieved by multiplying the volume contribution of each compartment to each tissue type (Table 3) by the proportion that each tissue type contributed to the whole dried leaf (33.6% for the BSCs and 22.6% for mesophyll/epidermal cells). The results of these calculations are also shown in Table 3 (the volumes of vascular tissues or other cellular constituents not analysed by non-aqueous fractionation were not measured directly). The mass percentage of a metabolite in a compartment (Table 2) was then allocated to a tissue type according to the ratio of that compartment in each tissue type (for example, 6.4 g protein in the cytoplasm per 100 g whole leaf tissue is distributed in the ratio 1:9 between BSC cytoplasm and mesophyll/epidermal cytoplasm). The resultant distribution of metabolites among compartments in the tissue types is given in Table 4. Allocating metabolites amongst compartments in this manner requires two assumptions: (i) the concentration of a metabolite in a compartment is the same in both tissue types, and (ii) all the metabolites measured in bulk leaf tissue are accounted for in the analysis of the subcellular compartments (i.e. concentrations of protein, proline, and sugars in structural material and vascular tissue are low). In addition, combining proportions based on mass (Table 2) and volume (Table 3) to yield concentrations (Table 4) invokes the assumption that the density of the dry leaf material is homogeneous. This assumption is not true (otherwise separation on a density gradient would not occur), but the range of densities on the gradient (1.3–1.6 g cm⁻³) was not large and would not appreciably affect the patterns of metabolite distributions calculated.

A large proportion of the small vacuoles were packed in the BSCs (Table 3), and these structures contained approximately equal amounts of protein and free proline, and somewhat more sucrose. These constituents total nearly 13% of the dry mass of the dehydrated leaves; however, since these small vacuoles occupy over 30% of the leaf tissue (Table 3) there must be many more, as yet unidentified, metabolites in these small vacuoles. From the insert to Fig. 1, it was clear that many of the small vacuoles are of different electron density and hence it is suggested that the distribution of the metabolites is not uniform in each vacuole. It is interesting to note that most of the proline was found in the small vacuoles and not the cytoplasm (Table 4) and hence may not act as an osmolyte in this environment. Sucrose concentration in the dry leaf was high (Table 2) and most of this was located in the cytoplasm of the mesophyll/epidermal cells (Table 4). Unfortunately, differences in the composition of the cytoplasm of the different cell types or the composition of individual organelles could not be determined using these techniques.
Discussion

Resurrection plants are thought to survive the mechanical stresses associated with desiccation using one of two strategies. Extensive cell wall folding, which should reduce tensions on structures enclosed by the wall, has been reported in some resurrection plants (Hallam and Luff, 1980; Vicre et al., 1999). Alternatively, cells maintain their original volume and become packed with vacuoles filled with non-aqueous matter (Dalla Vecchia et al., 1998; Farrant, 2000). In E. nindensis, both strategies are employed; the thicker-walled BSCs maintain volume and abundant small vacuoles replace the water of the hydrated cells (Fig. 1A), and the thinner-walled mesophyll and epidermal cells become reduced in volume and extensive cell wall folding is observed (Fig. 1B).

The importance of cell wall folding in the mechanical stabilization of desiccated tissues, as observed in the mesophyll and epidermal cells (Fig. 1B) have been investigated in both this and other resurrection plants.
(Vicre et al., 1999; Vander Willigen et al., 2001a). However, the significance of abundant small non-aqueous vacuoles in desiccated tissues such as those observed in the BSCs of *E. nindensis* (Fig. 1A; Table 1) has not been studied. Due to difficulties in investigating the ultrastructure of partially hydrated or dry tissues (Platt et al., 1997), it was not possible to determine how and from where these vacuoles originated. However, it was evident that at least some de novo membrane synthesis must have occurred since the total tonoplast length per cell increased on dehydration (Table 1). A relatively large surface area-to-volume ratio has been suggested to be important to the mechanical stabilization of dehydrated cells (Iljin, 1957). Since this author reported that small cells were reported to be more tolerant to drying than larger cells, it is probable that in desiccated vegetative tissues numerous small vacuoles are mechanically more stable than one large one in desiccated tissues.

There are numerous reports of an increase in metabolites in resurrection plants on drying (Fig. 4; Tymms and Gaff, 1979; Bianchi et al., 1991; Ghasempour et al., 1998; Whittaker et al., 2001), but the subcellular localization of these has not been established previously. Non-aqueous fractionation has become a useful technique for analysing subcellular biochemistry (Heineke et al., 1997; Farre et al., 2001) and it is, in fact, particularly well suited to desiccated material in which the addition of aqueous solvents is undesirable (to avoid rehydration). This technique has not yet been validated in heterogeneous tissues and a number of assumptions were necessary before any final metabolite distributions could be calculated. Thus there is a greater degree of uncertainty in the results obtained and caution in their interpretation is necessary.

Using non-aqueous fractionation, it was found that there were almost equal amounts of proline and protein and slightly more sucrose in the small vacuoles in *E. nindensis* (Table 4), but there is a significant proportion of other, as yet unidentified, constituents as well. Proline accumulation

![Fig. 5. Distribution among fractions 1–8 of the marker enzymes (solid bars, cytosolic marker; grey bars, vacuolar marker) and TIP 3.1 antibodies (open bars) after non-aqueous fractionation of dry desiccation-tolerant inner *E. nindensis* leaves (5% RWC).](https://academic.oup.com/jxb/article-abstract/55/397/651/594248)

Table 2. Subcellular distribution of metabolites in dry (5% RWC) desiccation-tolerant inner leaves of *E. nindensis* separated using non-aqueous fractionation

Metabolites in each fraction were measured spectrophotometrically and the subcellular distribution calculated by comparing the metabolite and marker enzyme distribution. The results represent the means ± standard deviation of the measurements on three different fractionations with different tissue samples, each pooled from at least four individual plants.

<table>
<thead>
<tr>
<th></th>
<th>Proportional distribution in each compartment</th>
<th>Concentration in leaf (g/100 g)</th>
<th>Mass percentage in each compartment</th>
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<tr>
<td></td>
<td>Cytoplasm</td>
<td>Small vacuole</td>
<td>Central vacuole</td>
</tr>
<tr>
<td>Protein</td>
<td>51 ± 4</td>
<td>30 ± 3</td>
<td>19 ± 3</td>
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<tr>
<td>Free proline</td>
<td>18 ± 2</td>
<td>53 ± 3</td>
<td>29 ± 2</td>
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<tr>
<td>Glucose</td>
<td>59 ± 2</td>
<td>17 ± 2</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Fructose</td>
<td>60 ± 2</td>
<td>16 ± 1</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>72 ± 4</td>
<td>15 ± 3</td>
<td>14 ± 4</td>
</tr>
</tbody>
</table>

Table 3. The relative volumes of the various tissue types in the dry desiccation-tolerant *E. nindensis* leaves calculated from transmission electron micrographs

The ratio of BSC to mesophyll and epidermal relative volume was 32.7 ± 3.8:21.6 ± 2.6 calculated from light micrographs. The results represent the means ± standard deviation of measurements from more than 30 micrographs from four different sections from at least two different specimens per sample. It was assumed that none of the vacuoles in the BSC were central vacuoles.

<table>
<thead>
<tr>
<th></th>
<th>Contribution of each compartment to tissue type</th>
<th>Contribution of each compartment in each tissue to total leaf volume</th>
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<tbody>
<tr>
<td></td>
<td>Bundle sheath cells</td>
<td>Mesophyll and epidermis</td>
</tr>
<tr>
<td>Central vacuole</td>
<td>0</td>
<td>39 ± 5</td>
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<tr>
<td>Small vacuole</td>
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<td>Cytoplasm</td>
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is thought to act as an osmolyte during water deficits and has also recently been shown to be associated with the formation of small vacuoles in *Saccharomyces cerevisiae* (Maggio et al., 2002). Since the accumulation of proline (Fig. 4A) was at a similar stage in drying to the appearance of the small vacuoles (Table 1), it is possible that this solute was incorporated into the vacuoles as they were forming, and hence may not be acting as an osmolyte in this circumstance. Although proline is generally associated with the cytoplasm, it has been observed in the lytic vacuoles of potato tubers (Farre et al., 2001). Of the metabolites measured, sucrose was found to contribute the highest mass in the small vacuoles of the BSC. However, it was in the cytoplasm of the mesophyll and epidermal cells where most of the sucrose was located, making up more than all the total mass of all the other measured metabolites in these tissues. Sugars are known to be important in the formation of glasses and the stabilization of membranes. While the importance of vitrification in conferring desiccation tolerance has been questioned (Buitink et al., 2002), it is still believed to contribute towards the stabilization of tissue once they have become dry, and hence to longevity in the desiccated state (Leopold et al., 1994).

The protein storage vacuoles found in orthodox, desiccation-tolerant seeds (Pernollet, 1978), are very similar in appearance to the small vacuoles found in desiccation-tolerant vegetative tissues (Fig. 1). Unlike the seed protein vacuoles, it seems that the non-aqueous vacuoles of *E. nindensis* are filled with a number of different constituents. Although their composition does differ from that of the cytoplasm and lytic vacuoles (Table 4), no particular metabolites investigated in this study were exclusively associated with them.

Although the formation of small vacuoles and the maintenance of cell volume on dehydration is not exclusive to poikilochlorophyllous resurrection plants such as *E. nindensis*, it is more commonly recorded in this group of desiccation-tolerant plants (Farrant, 2000; Vander Willigen et al., 2001a). These plants lose chlorophyll on dehydration and hence this must be resynthesized on rehydration before photosynthesis and autotrophism can resume (Hambler, 1961). Although the accumulation of metabolites has been reported for both poikilochlorophyllous and homoiochlorophyllous (retain chlorophyll and chloroplast structure on drying) resurrection plants, the formation of non-aqueous vacuoles in the poikilochlorophyllous species may act not only in membrane stabilization and water replacement, but as a store of energy for the initial stages of rehydration. In *E. nindensis*, it is in the BSC in which abundant small vacuoles form (Fig. 1; Table 1). Since it is in the vascular bundles where the majority of photosynthetic carbon reduction will occur, it is possible that the accumulation of the metabolites illustrated in Fig. 4, are necessary to reconstitute the subcellular structure of these cells. It is likely that this may be necessary for metabolism in the BSCs before those of the mesophyll and epidermal tissues.

Not only are the BSCs the site of photosynthetic carbon reduction, but they are also in closest proximity to the vascular tissues. As a consequence, these cells will be the first to experience the inrush of water on rehydration. Membrane tearing on rehydration is thought to be a cause of irreparable damage in many desiccation-sensitive tissues (Pammenter and Berjak, 1999). The presence of a TIP 3;1 on the membranes of the small vacuoles in the dehydrated BSC cell of *E. nindensis* (Fig. 3) may be important to ensure high membrane water permeability for rehydration. The TIP 3;1 from *P. vulgaris* (the antibody which cross-reacts with the protein from desiccated inner leaves of *E. nindensis*) is an aquaporin since it is permeable to water, the activity of which is regulated at the protein level by turgor-dependent phosphorylation (Maurel et al., 1995). Other TIP 3;1s expressed during the dehydration stages of seed maturation have been proposed to be involved in maintaining membrane permeability during this phase (Maurel et al., 1995). Since the expression of TIP 3;1 in seeds is developmentally regulated, appearing coincident with the onset of desiccation tolerance, it has been presumed to play a protective/stabilizing role in those tissues (Johnson et al., 1990). However, since the *E. nindensis* TIP 3;1 is found only in very dry tissues, its

<table>
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<th>Table 4. The relative distributions of the measured metabolites among the subcellular compartments of the bundle sheath cells and mesophyll and epidermal tissues of <em>E. nindensis</em></th>
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<tbody>
<tr>
<td><strong>Bundle sheath cells</strong></td>
</tr>
<tr>
<td><strong>Cytoplasm</strong></td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Free proline</td>
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<tr>
<td>Glucose</td>
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<td>Fructose</td>
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<td>Sucrose</td>
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role in desiccation tolerance cannot yet be conclusively deduced.

There have also been suggestions that this TIP isoform may be permeable to glycerol and small proteins (Maurel and Chrispeels, 2001). During germination, TIP 3;1s from seeds are thought to be associated with the mobilization of the contents of the protein storage vacuoles with which they are associated (Johnson et al., 1990; Maurel et al., 1997). The TIP 3;1 found in E. nindensis is associated with the abundant small vacuoles (Fig. 3) and may thus be important in mobilizing the proteins, sugars, proline, and other solutes which accumulate therein (Table 4). Jauh et al. (1999) proposed that some TIPs play a role in determining the function of a vacuole; the TIP 3;1 seen on protein storage vacuoles are gradually replaced by TIP 1;3s during seed germination in P. vulgaris, redefining the function and structure of those vacuoles. The proposed TIP in E. nindensis (Figs 2, 3) might have a similar function.

The appearance of this possible TIP 3;1 in desiccated inner leaves of E. nindensis is the only account of this TIP isoform in vegetative tissues. To date, no expression of all other TIP 3;1s known in the seeds of numerous plants have been reported to be expressed subsequent to seed maturation (Maurel et al., 1997), although desiccated plant tissues were not examined in those studies. However, this antibody has been shown to cross-react with high specificity to TIP 3;1s in seeds, but not vegetative tissue, from a number of species. This TIP isoform may well be absent from hydrated vegetative tissues, but it may be important in desiccation-tolerant vegetative tissue.

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