Conservation of Cell Order in Desiccated Mesophyll of *Selaginella lepidophylla* ([Hook and Grev.] Spring)

WILLIAM W. THOMSON* and KATHRYN A. PLATT

Department of Botany and Plant Sciences, University of California, Riverside, CA 92521–0124, USA

Received: 19 August 1997  Accepted: 19 November 1997

Understanding of the basis of desiccation tolerance in mature plant tissues that survive extreme dehydration requires knowledge of the degree of cellular order in the dry state. Generally, aqueous fixatives have been used in ultrastructural studies of such material, and these are known to be inadequate in the preservation of dry material. Cryopreservation provides a more assured level of fixation fidelity than aqueous fixatives, particularly with dry material. Using freeze substitution and electron microscopy, we examined the ultrastructure of dry mesophyll cells of *Selaginella lepidophylla* ([Hook and Grev.] Spring). In this material the cells were condensed and had highly folded walls. The plasmalemma was bounded on both sides by layers of granular material, and the membrane was in close and continuous apposition to the walls. The conformation and position of organelles and their structure appeared to be influenced by being compacted within the shrunken cells, but the ultrastructural integrity of all organelles and cellular membranes, including mitochondria, chloroplasts and vacuoles, was maintained in the dry state. These cells had numerous small vacuoles clustered in aggregates, and the tonoplast membranes appeared to be coated on the internal side by a fine granular layer. The vacuoles contained osmiophilic material of varying degrees of condensation and had embedment holes suggesting the presence of salt crystals within the vacuoles. The general conclusions from these studies are that a critical level of cell order is maintained in the dry state in these desiccation-tolerant plants, and a high degree of effective packing and shape fitting of cellular constituents with the compaction forces of dehydration underlies this conservation of cell order.

Key words: Freeze substitution, *Selaginella lepidophylla* ([Hook and Grev.] Spring), ultrastructure, membrane structure, desiccation tolerance, resurrection plants.

INTRODUCTION

It is well known that many seeds, as well as pollen, can survive nearly complete dehydration. Acquisition of desiccation tolerance by seeds and pollen occurs during seed ripening and pollen maturation and is integral to the developmental processes involved (Bewley, 1979, 1995; Hoekstra, 1986). However, many of the bryophytes, club mosses and some angiosperms are poikilohydric, in that mature tissue with highly vacuolated cells undergo severe desiccation and remain viable for long periods of time in the dry state (Bewley, 1979). A general theme in the literature concerns the concept that desiccation tolerance involves some essential level of preservation of cell integrity with dehydration and/or, if damage is limited during dehydration, repair mechanisms can re-establish cellular integrity with rehydration (Bewley, 1979). Cellular integrity, cellular order and compartmentation, as well as membrane integrity are not just functional considerations, but have a structural basis. To establish the nature of the structural integrity of desiccation-tolerant plant materials and estimate the contribution of cellular structures to desiccation tolerance, information on the ultrastructure of dry, desiccation-tolerant cells is fundamental. There are many published studies on the ultrastructure of dry seeds and pollen, and some on poikilohydric plants (see Bewley, 1979, 1995, for reviews). In most of these, aqueous chemical fixation procedures have been used. Chemical fixatives penetrate tissues rather slowly (Mersey and McCully, 1978), and their stabilization of cellular constituents is also slow (Mersey and McCully, 1978; McCully and Canny, 1985; Dong, McCully and Canny, 1994). This can unfortunately result in hydration swelling of dry cells and osmotic swelling of organelles prior to chemical stabilization (Swift and Butrose, 1973; Butrose, 1973; Fellows and Boyer, 1978; Bartley and Hallam, 1979; Oliver and Bewley, 1984) and often cause differential swelling, for example, of the wall (Butrose, 1973; Gaff, Zee and O’Brien, 1976; Hallam, 1976; Vigil et al., 1984; Opik, 1985). These limitations tend to obscure assessments of ultrastructural organization. Most investigators acknowledge that anhydrous osmium-vapour fixation provides a reliable, ‘life-like’ preservation of dry cells (Hallam, 1976; Opik, 1980, 1985; Smith, 1991). Vapour fixation requires a long time, weeks to months, and even then, osmium does not always fully penetrate the tissue (Opik 1980, 1985; Singh et al., 1984; Smith, 1991). Also, infiltration of resins into anhydrously fixed dry plant material is noticeably poor, sectioning of the embedded material is difficult, and often only small fragments of sectioned material are recoverable for examination with the electron microscope (Hallam, 1976; Opik, 1980).

Freeze substitution has been established as an alternative to chemical fixation and is acknowledged as superior in preserving the life-like organization of plant cells (Browning and Gunning, 1977; Tiwari, Polito and Webster, 1990; Ding, Turgeon and Parthasarathy, 1991; Lancelle and
We report here on the ultrastructural features and organization of dry, desiccation-tolerant cells of the resurrection plant *Selaginella* that had been preserved using this approach. The physical process of cryopreservation (Backmann and Mayer, 1987; Knoll, Verkleji and Plattner, 1987; Sitte, Edelmann and Neuman, 1987) avoids the difficulties in delineating the ultrastructural features of dry cells after chemical fixation.

**MATERIALS AND METHODS**

**Plant Material**

*Selaginella lepidophylla* plants purchased from Carolina Biological Supply were received in plastic bags in a dry condition. The water content (8–10%) of these plants was determined gravimetrically with use of a microwave oven.

**Cryofixation and Freeze Substitution**

Small pieces (1–3 mm²) of microphylls were plunged rapidly into liquid propane, cooled to near its freezing point with liquid nitrogen. The frozen samples were then transferred to fixative and substitution solvent (see following) in vials. The vials were maintained in a −80 °C freezer for 1 to several weeks and then removed, placed on dry ice and left in a −20 °C freezer overnight, during which the dry ice sublimed. Samples were then placed in an ice bath for 8 h and slowly brought to room temperature. Several changes of 100% solvent were then made, followed by infiltration and embedding in Spurr’s resin.

The fixatives used were 2% osmium tetroxide, 1% osmium tetroxide and 2% anhydrous glutaraldehyde in combination, or 2% anhydrous glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) prepared in the appropriate solvent. Acetone and ethanol were tried as substitution fluids, but proved unsuitable in that infiltration of both fixative and resin into the samples was incomplete, and thin sections were virtually unobtainable. Also, since the material had a very low water content, fixation and substitution at room temperature was tried but resulted in serious extraction of cellular content by the organic solvents. However, fixation in methanol at −80 °C (Ristic and Ashworth, 1993) resulted in fairly good infiltration of fixative and resin into the material, and thin sections were readily obtained. Sections were stained with lead citrate and uranyl acetate before examination in a Philips EM400 electron microscope.

**RESULTS**

In the freeze-substituted dry material, the mesophyll cells of the microphylls were collapsed and had highly folded walls (Fig. 1). The cell contents were highly condensed but not contracted from the walls (Figs 1 and 2), and the plasmalemma had a generally smooth, even profile in continuous apposition to the cell wall. A fine, granular layer, approx. 20–30 nm thick, coated the inner surface of the plasmalemma. A thinner, granular layer also bounded the outer surface of the plasmalemma (Figs 2 and 3). Internal cellular constituents and compartments were densely packed together but definable as separate entities, particularly by membrane boundaries (Figs 4 and 5), although the nuclear, mitochondrial and chloroplast envelope membranes lacked a high degree of definition. In most instances, the plasmalemma and the tonoplast membranes were highlighted as electron-translucent bands against the high background mass density of the compact cell contents. However, high magnification examination of these membranes showed the electron-translucent band to be the central, translucent component of a tripartite membrane (Figs 3 and 6). Within the chloroplasts, the entire photosynthetic membrane continuity (i.e. the granal-fretwork system) was organized in the form of folds or pleats (Figs 1, 4 and 5). The granal compartments were compressed, and the thylakoid membranes had an even parallelism within the granum, with a uniform spacing of compartments across the grana (Fig. 7). The stroma of the chloroplast had a uniform density and texture, and the chloroplast ribosomes were predominantly clustered in small to large groups within the stroma (Figs 4 and 5). Similarly, the plastoglobuli were clustered in groups, often in numbers of 50 to 100 or more (Fig. 4). In the osmium-treated material, the plastoglobuli often had a doughnut-like appearance with an electron-dense periphery and a central translucent dot (Figs 4 and 7). In the glutaraldehyde-only treated material, the plastoglobuli were round to ovoid in shape, but had a low degree of contrast (Fig. 5).

Mitochondria were recognizable in all preparations, but were more clearly delineated in the osmium-fixed material. Their general form appeared to be configured by the collapsed, compacted nature of the protoplasmic material. The mitochondrial matrix had an even texture and density, although a central, more electron-dense component was common (Fig. 8). The cristae appeared to be uniformly compressed, relatively numerous per mitochondrion, and often quite lengthy forms occurred (Figs 7 and 8). The amount of clearly identifiable cytoplasmic matrix material lacking ribosomal entities was quite small and interspersed between the aggregated organelles. The most noticeable components of the cytoplasmic matrix were highly clustered zones of ribosomes that virtually filled the regions between the organelles (Figs 2, 4–6). In total, the cytoplasmic matrix, including that containing ribosomes and cytoplasmic membranes, was sparse. Cytoplasmic lipid bodies were observed, often in regions where the small vacuoles were clustered, and the vacuolar contour was indented, apparently by being pressed against the lipid bodies with compaction during dehydration (Fig. 1). The nuclei of these cells had irregular outlines (Fig. 9).

Although the protoplasmic material was densely compacted in these cells, it did not appear to be a completely random aggregate of cellular constituents, particularly in regard to vacuolar structures. A relatively large number of moderately sized vacuoles occurred in these dry cells, and these were frequently bunched in groups in different regions of the cells. Most vacuoles were generally oblong in form, although round, and other variably shaped forms were not uncommon. In osmium-treated material, the content of the vacuole varied. In some vacuoles, the content was highly condensed (Fig. 4) and had a high density, whereas in others, the contents varied considerably in condensation of
Figs. 1, 2, and 3. Electron micrographs of mesophyll cells of dry Selaginella prepared by freeze substitution in methanol with 2% OsO₄. The entire cell has collapsed during dehydration. The cell wall (CW) is highly folded, and the cell contents are condensed. Chloroplasts (C) contain organized compact thylakoids (T). V, vacuole; L, lipid droplet. x 22,000.

Fig. 2. High magnification of the cell wall-plasmalemma interface of a cell from dry Selaginella that was freeze substituted in methanol with OsO₄ and anhydrous glutaraldehyde. The wall (CW) is highly folded, and the plasmalemma maintains close apposition to it (arrows). x 75,000.

Fig. 3. High magnification of a portion of a cell of dry Selaginella prepared as in Fig. 2 illustrating the close apposition of the plasmalemma to the cell wall (CW) and the tripartite image of this membrane. Also shown are the electron-dense layers apposed to both the inner and outer surfaces of the plasmalemma. PG, plastoglobuli in a chloroplast. x 136,000.

dense material (Figs 1, 4 and 7). All types could be found in the same cell. In the glutaraldehyde-prepared material, this condensation of vacuolar content was not observed, and the vacuolar contents consisted primarily of a dispersed granular material in a lightly stained background (Fig. 5). In both preparations, small, often polygonal-shaped holes with dense boundaries occurred in the vacuoles (Fig. 5). These holes in the embedment plastic have a similar appearance to those observed in vacuoles of other plant cell types for which there is evidence that they are actual outlines or ‘ghosts’ of salt precipitations that had not been retained through sectioning, staining, and examination under the
beam of the electron microscope (Arnott and Pautard, 1970; Franceschi and Horner, 1980).

**DISCUSSION**

Ultrastructural studies on dry plant material, such as poikilohydric plants, have been vexed by difficulties in adequately preserving the material. Aqueous chemical fixatives have been shown to have serious limitations in that changes in cellular and membrane organization often occur before stabilization by the fixatives. Cryopreservation is a physical process that confers rapid stabilization of cellular constituents and structures, and with desiccated material of low water content ice-crystal formation is minimal, if ice forms at all. Artifacts induced by ice are not an apparent problem (Bliss, Platt-Aloia and Thomson, 1984; Platt-Aloia et al., 1986; Platt, Oliver and Thomson, 1994). We have found that the ultrastructure of cells of the dry microphylls...
of *Selaginella* that were cryofixed and freeze substituted in either osmium tetroxide or anhydrous glutaraldehyde were well preserved, and sections of entire cells were obtained. The following general observations were made: (a) as is consistent with observations on dry seeds and other poikilohydric plants (Hallam and Luff, 1980; Bergstrom, Schaller and Eichmeier, 1982; Webb and Arnott, 1982), the cell content was highly condensed and the cell walls were highly convoluted; (b) the plasmalemma had a smooth, even, continuous appearance and was firmly associated with, and followed the contours of, the pleated walls; and (c) all cellular compartments were delineated by membranes as previously noted using freeze-fracture electron microscopy (Platt et al., 1994). No apparent disorganization of these membranes was observed, nor was there any apparent swelling or distortion of the various compartments, other than that attributable to compaction forces that probably occur with dehydration. These observations are counter to studies in which severe structural disorganization of dry cells of desiccation-tolerant material has been reported (Gaff et al., 1976; Hallam and Gaff, 1978a, b; see Bewley, 1979, 1995; Oliver and Bewley, 1984). However, these latter observations were derived from observations of material prepared with aqueous fixatives.
Thomson and Platt—Conservation of Cell Order in Dry Selaginella

Webb and Arnott (1982) have suggested that with the concomitant shrinkage of the wall and protoplast during dehydration, the retention of the cell wall-plasmalemma association is probably essential for the maintenance of the structural integrity and, thus, viability of cells (i.e. loss of this contact engenders loss of cell viability). This would also appear to apply to our present observation with Selaginella. We further suggest that with the infolding of the walls during dehydration and the maintenance of the plasmalemma in parallel with the walls, the surface area of the plasmalemma probably does not decrease significantly with the volumetric decrease in the cells with dehydration.
Similarly, with rehydration and expansion of the cells, no significant increase in new membranes would be required as the volume of the cells returned to the normal hydrated state. We note, however, that the situation might be different with some mosses such as Tortula, since Tucker, Costerton and Bewley (1975), using Nomarski optics, found cells in the dehydrated state to be highly plasmolysed.

Gaff et al. (1976) observed that the cell vacuole became fragmented during dehydration of the leaves of Borya nitida, but the resulting small vacuoles retained an intact tonoplast. Our observations are similar in that vacuolar compartmentation was retained in the dry cells of Selaginella in the form of many small vacuoles delineated by an intact membrane. This is of interest because there is substantive evidence that the vacuole in many mature plant cells contains a complement of hydrolytic enzymes (Wink, 1993). The retention of vacuolar compartmentation and tonoplast integrity with dehydration would seem to be critical to dehydration tolerance, if only to retain such enzymes, if present, within the vacuoles. A corollary postulate would be that maintenance of vacular integrity is critical to normal recovery with rehydration.

Two aspects of the vacuolar ultrastructure in the dry cells must be mentioned. After substitution with osmium, the vacuolar contents showed a varying degree of osmiophilia, with some vacuoles having a uniform, condensed content of high contrast, while others in the same cell had regions of variable levels of densely aggregated materials. We can only speculate as to the possible basis of this osmiophilia—proteins, amino acids, phenols etc.; however, the high density of vacuolar contents probably reflects a concentration and condensation effect resulting from the fragmentation of a large central vacuole into smaller units, coupled with the increase in solute concentrations of vacuolar contents due to the loss of water during dehydration. Similarly, removal of water from the vacuole with dehydration would effectively raise the internal ionic concentration to saturation level, and salt precipitation would not be unexpected. We suggest that the sectional ‘ghosts’ of salt crystals in the vacuoles in the non-osmium-fixed material confirms this process.

The typical tripartite pattern of densities common to bilayered, lipid membranes was observed for the plasmalemma and tonoplast. However, the mitochondrial, chloroplast, nuclear and cytoplasmic membranes had a ‘negative image’. This is a common feature of dry material, particularly after anhydrous fixative procedures (Opik, 1980). This ‘negative image’ term is inadequate and inaccurate because it identifies only a high general contrast differential between the bulk, compact cytoplasm and a region within membranes that is normally electron translucent (see Platt-Aloia and Thomson, 1983, for discussion). The absence of a tripartite image pattern does not necessarily indicate that such membranes have other than a bilayer organization (Opik, 1980; Platt-Aloia and Thomson, 1983). That membranes in desiccated, but viable, plant cells have the basic bilayer organization common to all biological membranes has been shown in a number of freeze-fracture electron microscopy studies (Vigil et al., 1985; Platt-Aloia et al., 1986; Thomson and Platt-Aloia, 1987; Platt et al., 1994). Results from experimental studies have led to suggestions that a variety of factors could maintain or protect membranes, other cellular constituents, and general cell order during dehydration. These include sugars as substitutes for water (Crowe et al., 1988), osmoregulation compounds (Hansen and Hitz, 1982; Ingram and Bartels, 1996), the glassy state of water (Koster, 1991), and protective proteins (Close, 1993). All these factors may be involved. Extrapolating from our observations that the desiccation-tolerant cells, although highly condensed, retained fundamental levels of cell order, we suggest that the process by which the various organelles and cell structures are compacted during dehydration must be orderly. We suggest that the process involves a high degree of packing or fitting, in a space-filling sense, of diversely shaped geometric structures with the decrease in volume of the cells. Obviously, the cell wall folds inward with dehydration, and there is a reduction in cell volume. Similarly, organelles such as mitochondria, chloroplasts and the nucleus are condensed with dehydration and assume space-filling shapes, apparently moulded by the compression of cellular constituents together with dehydration. For less deformable elements, maximal effective packing could require a degree of subcellular sorting and aggregation to maximize geometric fitting in a limited space. The closely packed aggregates of cytoplasmic ribosomes, the clustered pattern of chloroplast ribosomes and plastoglobuli, the clustered nature of the small vacuoles, and the folded, pleated form of the chloroplast photosynthetic membranes all appear to be features of effective, space-filling packing.

Several reports suggest that the rate of dehydration is critical to survival with rehydration. That is, desiccation-tolerant tissues that are dried slowly tend to survive rehydration, whereas rapidly dried material does not (Bewley, 1979, 1995). Even though the conformation of cell structures might change with dehydration, the process of the physical sorting and adequate shape-fitting association of these and larger elements would probably be relatively slow. Configuring agents, conferred from gene expression, may also be required to maximize effective and efficient packing of various cell constituents. Thus, if dehydration occurred too rapidly, maintenance of the necessary degree of order would not occur. Close, progressive packing during dehydration could, in itself, preserve order in neighbouring structures. For example, if membranes are confined at both surfaces by densely packed, but closely associated, molecular structures such as proteins, major internal rearrangements and disorganization of membrane structure would not be expected. This could explain the presence of a fine lamina observed on both surfaces of the plasmalemma in the dehydrated material. Also, a highly ordered packing of cell constituents possibly establishes a structural resistance to physical disruption of membranes and compartments with the massive insurge of water during rehydration.

Bewley (1979) suggested that in order to survive desiccation, poikilohydric plants must, at the cellular level (a) limit damage during desiccation to repairable levels; (b) maintain functional integrity in the dry state; and (c) have a system that becomes active during rehydration that repairs damage, particularly to membranes. Earlier, Keilin...
(1959), in a scholarly summary, identified the retention of structural integrity as fundamental to the capacity to recover to an active state. Maintenance of physiological integrity in the desiccated state as pointed out by Bewley (1979) and structural order as emphasized by Keilin (1959), are probably intrinsically linked properties. The hypothesis for our research is that an elastic, integral hierarchy of structural and functional relationships differentiate the living cell from the dysfunctional and dying. In regard to dry, desiccation-tolerant material, our observations support Keilin’s view that a critical level of cellular order is maintained in the dry state, and this structural framework predicates normal recovery of metabolic activity with rehydration. In a broad sense, we find that this assumption appears to be implicit in most published studies directed at eliciting protection mechanisms, whether they be substances that confer stability to cytoplasmic or nuclear constituents, or agencies that maintain membrane organization at very low hydration levels.

Finally, some comments on methods are needed. Freeze-substitution procedures with acetone or ethyl alcohol resulted in very poor image qualities, and infiltration of resin into the sections was inconsistent, or at least not uniform or complete. Substitution and fixation of the dry material at room temperature not only produced poor preservation but also resulted in extraction of cell constituents by the organic solvents. Even with methanol freeze-substituted material, sectioning of the blocks was a demanding task, and tears and splits commonly occurred in the sections. Others have noted these problems with dry substitution and reviving leaves of Borya nitida Labill.—A desiccation tolerant plant. Australian Journal of Botany 24: 225–236.


