Isolation and Characterisation of Chloroplasts from *Myrothamnus flabellifolius* Welw.

Priyum K. Koonjul\(^1\), Wolf. F. Brandt\(^1\), George G. Lindsey\(^1\) and Jill M. Farrant\(^2\) *

\(^1\) Department of Biochemistry,
\(^2\) Department of Botany, University of Cape Town, Private Bag, Rondebosch 7701, South Africa

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**Summary**

Chloroplasts isolated from the resurrection plant *Myrothamnus flabellifolius* using trehalose gradients had a higher buoyant density than chloroplasts isolated from another resurrection plant *Craterostigma wilmsii*. The latter had the same buoyant density as those isolated from the desiccation-sensitive (DS) plant *Pisum sativum*. The increased buoyant density in *M. flabellifolius* was ascribed to the unusual ultrastructure of the thylakoid membranes. Standard chloroplast isolation protocols resulted in membrane damage in both resurrection plants. Trehalose rather than sucrose gradients were required for isolation of intact chloroplasts. Immunological studies showed that epitopes related to the desiccation stress protein dsp21 from *Craterostigma plantagineum* were present in *M. flabellifolius*. Several small (10–15 kDa) unique stromal proteins were also present. Polyphenolics, including anthocyanins, accumulated in leaves and chloroplasts of *M. flabellifolius* during drying. Envisaged functions for these are maintenance of membrane integrity, chlorophyll masking and antioxidant protection. Metal (Ca\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\)) concentrations declined and thylakoid membranes separated upon drying. We propose that this might be a mechanism to put a stasis on photosynthesis and minimise photo-oxidation damage under water stress conditions.


**Abbreviations:** DEPC = diethylpyrocarbonate; DS = desiccation sensitive; dsp = desiccation stress protein; DT = desiccation tolerant; dw = dry weight; EDTA = Ethylenediaminetetra-acetic acid; HEPES = 4-(2-Hydroxyethyl)-1-piperazineneethane sulphonic acid; HSP = heat shock protein; LEA = late embryogenesis abundant; PMSF = phenylmethyl-sulfonylfluoride; PS I/II = photosystem I/II; PVP = poly[1-vinylpyrrolidone-2]; RWC = relative water content; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; TEM = Transmission electron microscope.

**Introduction**

Resurrection plants have the ability to survive near complete dehydration and revive from an air-dried state (Gaff, 1971, 1989). They therefore provide excellent physiological systems for investigating the mechanisms of desiccation tolerance. Resurrection plants have been divided into 2 distinct classes, homoiochlorophyllous and poikilochlorophyllous plants, which retain and lose their chlorophyll upon drying, respectively (Hambler, 1961, Tuba et al., 1993, 1994). *Myrothamnus flabellifolius* Welw. (*Myrothamnaceae*) is a woody homoiochlorophyllous shrub that grows throughout the dry summer rainfall regions of Southern Africa. In conditions of limited water, the plant can lose up to 95 % of its relative water content (RWC). This is accompanied by the loss of approximately 30–50 % (depending on light levels during
drying) of the chlorophyll content (Farrant et al., 1999; Sherwin and Farrant, 1996). The retention of chlorophyll in the dry state may have severe consequences for plants growing under high light conditions since free radical subcellular damage (Navari-Izzo et al., 1995; Seel et al., 1991; Sgherri et al., 1993, 1994; Smirnoff, 1993; Van Camp et al., 1996) might be enhanced. The homiochlorophyllous resurrection plant *Craterostigma wilmsii* is able to minimise such damage using a number of mechanisms (Sherwin and Farrant, 1998). In the dry state leaves are folded such that only the abaxial surfaces of the outermost whorl of rosette leaves are exposed to light. Anthocyanins accumulate in those leaves serving to mask chlorophyll and as antioxidants (Quartacci and Navari-Izzo, 1997). Activities of key antioxidant enzymes also increase during drying (Sherwin and Farrant, 1998). Some of these mechanisms also appear to occur in *M. flabellicolus* (Farrant and Sherwin, 1998). During drying the leaves fold vertically along the stem, shading adaxial surfaces from light. Exposed abaxial surfaces become brown, possibly due to anthocyanin accumulation (Goldsworthy, 1992). In addition, *M. flabellicolus* has a unique chloroplastic structure in which the chloroplast thylakoid membranes are organised in a staggered conformation, termed the staircase arrangement (Wellburn and Wellburn, 1976). Although not found in other resurrection plants, this unusual thylakoid arrangement may be implicated in protection of the chloroplasts under conditions of water deficit. We have thus undertaken to characterise the chloroplasts of *M. flabellicolus*.

To date, apart from ultrastructural observations of chloroplast organisation in intact leaves, there have been no studies reported on the biochemical characteristics of chloroplasts from resurrection plants. In this report we have isolated chloroplasts from *M. flabellicolus* and compared their properties with chloroplasts isolated from the resurrection plant *Craterostigma wilmsii* Engl. (Scrophulariaceae) and the desication sensitive (DS) plant *Psam nitens* L. (Fabaceae). In addition, ultrastructural and some biochemical changes that occur in chloroplasts of *M. flabellicolus* during drying are given.

**Materials and Methods**

**Plant material**

The resurrection plants *M. flabellicolus* and *Xerophyta humilis* were collected and maintained as described previously (Sherwin and Farrant, 1996; Dace et al., 1998). *P. nitens* plants were sown from seed and were maintained in a greenhouse under a 30% shade cloth with no supplementary lighting. Fully expanded young leaves from hydrated plants were used for experimental purposes. Certain experiments were also performed on dry (5% RWC) leaves of *M. flabellicolus*. Whole plants of this species were dried to an air-dry state by withholding water from the soil. Rose (*Rosa* spp.) and violet (*Sant species*) plants were purchased from a local nursery and leaves were used immediately for polyphenolic extraction.

**Isolation of chloroplasts and thylakoid membranes**

The modification of the protocol described by Kut and Flick (1986) was used. Leaves were ground on ice using a mortar and pestle in cold isolation medium (0.35 mol/L mannitol, 5 mmol/L EDTA, 0.1% 2-mercaptoethanol, 50 mmol/L Tris-HCl, pH 8.0) supplemented with 1% (w/v) caffeine, 1% (w/v) PVP poly[1-vinylpyrrolidone]-2, 1 mmol/L MgCl₂, 1 mmol/L MnCl₂, 5 mmol/L ascorbic acid, and 0.1% (w/v) PMSE. The ground leaf preparation was filtered through four layers of sterile cheesecloth and the crude filtrate was centrifuged at 1,000 g to remove cellular debris and nuclei. The supernatant was loaded onto a sugar gradient that was centrifuged for 1 h at 81,000 g. A variety of sugar gradients in 25 mmol/L EDTA and 50 mmol/L Tris-HCl (pH 8.0) buffer were utilized. Amongst these were discontinuous sucrose gradients consisting of 17 mL 30% (w/v) and 8 mL 52% (w/v) sucrose, discontinuous sucrose gradients consisting of 17 mL 52% (w/v) and 8 mL 80% (w/v) sucrose and linear 30% (w/v) to 100% (w/v) trehalose gradients. Chloroplast bands were carefully removed from the gradient by aspiration and the sugar was removed by washing 5 times with 25 mmol/L EDTA, and 50 mmol/L Tris-HCl, pH 8.0.

Thylakoid membranes were prepared from chloroplast samples that were lysed for 30 min at 4°C in 10 mmol/L Tris-HCl, pH 7.4, and 150 mmol/L KCl (Sgherri et al., 1993). The sample was centrifuged at 12,000 g, for 20 min and the pellet resuspended in 10 mmol/L Tris-HCl, pH 7.4, 150 mm KCl and 1 mmol/L EDTA. The suspension was divided into two aliquots, one of which was applied to a 52% (w/v) to 100% (w/v) linear sucrose gradient in the same buffer.

**Chlorophyll fluorescence studies**

The activity of photosystem II (PSII) in isolated chloroplasts was measured using chlorophyll fluorescence techniques using OS-55 modulated fluorometer. Chloroplasts in isolation buffer were dark adapted by maintaining in a foil wrapped container for 5 min. They were then exposed to a saturating light intensity of 4 mmol photons m⁻² s⁻¹ for 1 s, and the initial (F₀) and the maximum (Fm) fluorescence were recorded. Fv was obtained by subtracting F₀ from FM, and Fv/Fm was calculated.

**Transmission electron microscopy (TEM)**

Chloroplasts isolated from each of the three species in HEPES buffer were fixed in 3% (v/v) glutaraldehyde at 4°C for 16 h. The material was post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series and infiltrated with epoxy resin (Spurr, 1969). After polymerisation for 16 h at 80°C, the samples were sectioned at a gold interference (95 nm) with a Reichert Ultracut-S microtome. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1965) and viewed with a Jeol 200 CX TEM.

Leaf segments (5 mm²) from fully hydrated (100% RWC) leaves from the three species and also dry (5% RWC) leaves (5 different leaves per treatment) of *M. flabellicolus* were prepared for TEM using the method previously described for these species (Sherwin and Farrant, 1996). Tissues were sectioned, stained and viewed as described above.

**Lipid characterisation**

Chloroplasts extracted from hydrated leaves were boiled for 5 min in isopropanol after which lipids were extracted as described by Sgherri et al. (1993). Analysis was done by gas-liquid chromatography.

**Protein studies**

Protein extraction from isolated chloroplasts and thylakoids was carried out as described by Russouw et al. (1995) except that soluble PVP (twice the weight of plant material) was added to the ground
tissue prior to extraction. Extracted proteins were electrophoresed on 20% SDS-PAGE gels containing 0.1% (w/v) N,N'-methylene bis-acrylamide (Laemmlli, 1970). Gels were routinely silver stained (Wray et al., 1981).

Western blotting onto nitrocellulose membranes was carried out as described (Harlow and Lane, 1988) and was developed using a chemiluminescent detection system (Amersham). Membranes were routinely checked by staining a duplicate blot with 1% (w/v) amido black. Antibodies to the p11 Group I LEA protein were prepared as described (Russow et al., 1995, 1997). Antibodies to Group II LEA proteins and C. plantagineum dsP 21 and dsP 34 proteins were the kind gifts of Dr. T. J. Close (Department of Botany and Plant Sciences, University of California, Riverside, USA) and Prof. D. Bartels (Fur Zuchthuftsforchung, Max-Planck Institute, Koln, Germany) respectively.

**RNA isolation and slot blot analysis**

Total RNA from dehydrated and hydrated leaves of M. flabellifolius was prepared using a combination of several protocols (Ainsworth, 1994; Bahloul and Burkard, 1993; Bruce and West, 1989; Soni and Murray, 1994). In the modified method, 100 mg fresh weight of leaf tissue was frozen in liquid N2 and ground to a fine powder in a pre-cooled mortar. The powder was extracted with 0.5 M extraction buffer, 100 mMol/L Tris-HCl (pH 8.6) containing 100 mMol/L NaCl, 1% (w/v) SDS, 1% (v/v) mercaptoethanol, 2% PVP (w/v) and 100 μg/ml proteinase K. Following centrifugation at 4°C for 2 min at 10,000 g, the supernatant was extracted twice with an equal volume of phenol : chloroform : isoamyl alcohol (24:24:1), and RNA was precipitated at -20°C after the addition of isopropanol supplemented with 0.8 mol/L sodium citrate and 1.2 mol/L NaCl. The RNA pellet was washed with 70% (v/v) ethanol, then dissolved in DEPC-water.

Equal amounts of total RNA (1 μg) from hydrated and dry leaves were blotted onto a Hybond-N membrane (Amersham). Following UV cross-linking, the blots were hybridised and hybridised in DIG Easy Hybridization solution (Boehringer Mannheim). The different oligo probes were DIG labelled, and detection (chemiluminescent) and washes were performed as outlined in the DIG manual (Boehringer Mannheim). Denstometric analysis was performed using a Macbeth TD 901 densitometer. The oligo probes for rbcS (rubisco small subunit) and cab (chlorophyll a/b binding protein) were kind gifts from Dr. S. Mackerness (Horticulture Research International, Warwick, UK) while the rbcL (rubisco large subunit) and 18S RNA were kind gifts from Dr. V. Abratt and Mr W. Mpoloka (Department of Microbiology, University of Cape Town, South Africa).

**Polyphenolic content**

Total (alcohol insoluble) phenolic compounds from isolated chloroplasts (not purified on a gradient in order to prevent loss of material during purification) and whole leaves were extracted and assayed after thioglycolic acid derivatisation. Acid-soluble derivatives were qualitatively determined using the procedure described by Schneiderbauer et al. (1991); alkali-soluble derivatives were determined spectrophotometrically at 280 nm (Bruce and West, 1989). A commercial polyphenolic extract from green tea (Sigma) was used as a control to quantitate the amount of polyphenolics in the different plant species.

Total anthocyanin content from dry and hydrated leaves was extracted as described (Sherwin and Farrant, 1998). Results are presented as (A) per gram dry weight. The cyanidin and delphinidin (proanthocyanidins) contents of chloroplasts were determined spectrophotometrically at 542 and 558 nm, respectively, using the protocol of Bate-Smith (1981). Gallotannins were assayed by adding potassium iodate to the aqueous chloroplast extract. In the presence of gallo-tannins, the samples turn red (pers. comm., Prof. T. Yoshida, University of Okayama, Japan). Each assay was performed in triplicate on two separately isolated chloroplast extracts.

**Sugar content**

Glucose, fructose and sucrose contents of chloroplasts (not purified on a gradient so as to avoid loss of material) from hydrated and dry leaves were determined using the Boehringer Mannheim sugar food kit (based on the methodology described by Bergmeyer and Bern, 1974). Analysis was performed according to the manufacturer's instructions, except that 1% (w/v) (final concentration) PVP was added to the reaction mixture prior to addition of the various enzymes and spectrophotometry. Extractions were performed in triplicate and internal replicates were performed.

**Metal content**

The metal content of chloroplasts (not purified on a gradient so as to avoid loss of material) from hydrated and dry leaves was determined using a modified protocol described by Malan and Farrant (1998). Samples were digested in 0.1 mol/L HCl at 180°C for 3 h, made up to a final volume of 10 ml in 0.1 mol/L HCl and passed through a 0.45 μmol/L microcap filter. Samples were analysed for Mn²⁺, Mg²⁺, Cu²⁺, Ca²⁺ and Fe²⁺ using a Jobin Yvon JY 138 Ultratrace ICP-AES (inductively coupled plasma-atomic emission spectrophotometer). Extractions were performed in triplicate and internal replicates were performed.

**Results**

**Isolation and purification of chloroplasts**

When chloroplast preparations from M. flabellifolius, P. sativum and C. wilmisii were purified on a discontinuous 30% (w/v)/52% (w/v) sucrose gradient, those from P. sativum and C. wilmisii banded at the gradient interface, whereas those from M. flabellifolius sedimented at the bottom of the tube. Interfacial banding of chloroplasts from that species occurred on a 52% (w/v)/80% (w/v) sucrose gradient (not shown). This would imply that either M. flabellifolius chloroplasts have a higher buoyant density than those from P. sativum and C. wilmisii or that artificial aggregation occurred during their isolation.

The ultrastructure of M. flabellifolius chloroplasts, which banded at the 52%/80% interface, and those from P. sativum and C. wilmisii, which banded at the 30%/52% interface, were examined using TEM. Isolated chloroplasts of P. sativum appeared identical to those present in intact leaves (not shown). The outer envelope was intact and the thylakoid arrangement was typical of photosynthetically active tissue. In contrast, chloroplasts isolated from both resurrection plants showed considerable membrane damage (Fig. 1). Outer membranes were not present and thylakoid membranes were disrupted. This damage may have been caused by release of polyphenolic compounds (e.g. from vacuoles) during the extraction process.

Despite the use of various concentrations of PVP and caffeine, substances known to ameliorate the effects of polyphenolics (Anderson, 1968; Loomis, 1974) during tissue extraction, chloroplast membranes of both resurrection species re-
mainly damaged (not shown). A Percoll (polyvinylpyrrolidone coated silica beads) gradient was used in place of sucrose in order to eliminate the possibility that the high osmolarity of the sucrose gradient solutions caused membrane damage. However, membrane damage was still evident in both resurrection plants (not shown). Trehalose, which naturally occurs in dry M. flabellifolius plants (Bianchi et al., 1993; Drennan et al., 1993; Saau et al., 1991), has been reported to afford better protection than sucrose to membranes during desiccation (Crowe et al., 1984, 1985; Gadd et al., 1987). Use of a 30–100% linear gradient of trehalose resulted in chloroplasts from M. flabellifolius banding at approximately 75% and those from P. sativum and C. wilmisia at 45%, and there was less damage to the chloroplasts from the resurrection plants (Fig. 2A, C). Although the outer membranes were not well defined, thylakoid membranes were intact and had similar arrangements to those from intact, hydrated leaves (Fig. 2B, D). Chlorophyll fluorescence was used to determine the physiological integrity of isolated chloroplasts. The parameter Fv/Fm, which gives an indication of the quantum efficiency of PS II, was calculated as 0.63 for hydrated chloroplasts. Although this value was slightly below the normal range of 0.7–0.8, indicative of an intact photosynthetic machinery (Lichtenthaler and Miehe, 1997), we consider the chloroplasts from M. flabellifolius to be essentially intact. It is known that isolated chloroplasts are very susceptible to degradation (Demmig-Adams and Adams, 1992), possibly accounting for the slightly lower value of Fv/Fm. Since chloroplasts from M. flabellifolius have both an unusual buoyant density and ultrastructure we tested their biochemical properties.

**Lipid composition of chloroplasts**

Since an altered membrane lipid composition might alter the buoyant density of isolated chloroplasts, we compared the lipid content of chloroplasts isolated from M. flabellifolius with those isolated from P. sativum and C. wilmisia (Table 1). The lipid compositions of all the plants were similar with unsaturated fatty acids accounting for between 76% and 85% of the total fatty acids present. There is a correlation between the density and the number of unsaturated bonds in fatty acids and thus the buoyant density of chloroplasts from P. sativum would be expected to be the highest. We would therefore postulate that the increased buoyant density of chloroplasts isolated from M. flabellifolius was not due to differences in the lipid composition.

**Characterisation of chloroplastic proteins**

Total protein from chloroplasts of M. flabellifolius, C. wilmisia and P. sativum were separated by SDS-PAGE (Fig. 3). A wide variety of proteins ranging in size from approximately 10 to 97 kDa were present in all the chloroplast preparations. The banding pattern of the proteins extracted from C. wilmisia and P. sativum chloroplasts were similar and resembled those reported to occur in other angiosperm species (Kieselbach et al., 1998). The separation from chloroplasts of M. flabellifolius was different from the other species. Prominent polypeptide bands at 55 and 16 kDa (probably representing the large and small subunits of rubisco, respectively (Keegstra and Yousif, 1986)) and several less prominent bands with molecular weights between 30 and 43 kDa, present in the former two species, were absent or present in very low concentrations (thus not easily detected) in extracts from M. flabellifolius chloroplasts. A number of unique proteins, especially in the range of 10 to 15 kDa, were present in M. flabellifolius, but not in C. wilmisia and P. sativum.

**Table 1:** Lipid composition (% w/w) of chloroplasts isolated from M. flabellifolius, P. sativum and C. wilmisia.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>M. flabellifolius</th>
<th>C. wilmisia</th>
<th>P. sativum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (16:0)</td>
<td>17.3</td>
<td>14.7</td>
<td>11.6</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>6.0</td>
<td>3.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>14.6</td>
<td>17.4</td>
<td>4.8</td>
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<tr>
<td>Linoleic (18:2)</td>
<td>26.4</td>
<td>26.0</td>
<td>18.2</td>
</tr>
<tr>
<td>Linolenic (18:3)</td>
<td>35.7</td>
<td>38.0</td>
<td>62.3</td>
</tr>
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</table>
Thylakoid membranes, isolated by lysis of chloroplasts from the three species, were centrifuged on linear 52% to 100% sucrose gradients. Membranes from _M. flabellifolius_ were significantly more dense, banding at 83% sucrose, than those from _P. sativum_ and _C. wilmii_, which banded at 40%. SDS-PAGE separations of thylakoid membrane proteins showed that the only difference among the species was the absence, or considerable reduction in the quantity of proteins with molecular masses 30–43 kD in _M. flabellifolius_ (not shown). Since many of the PSII proteins have molecular weights of between 32 and 47 kD (Kieselbach et al., 1998), we assume that it was those proteins that were present in low concentrations. The 10–15 kDa proteins uniquely present in _M. flabellifolius_ chloroplast preparations were not present in thylakoid isolations, suggesting that they were stromal proteins. The increased density of the thylakoids was thus unlikely to be due to the nature of the thylakoid membrane proteins.

Because proteins such as rubisco and cab are known to be ubiquitously present and yet could not be detected by SDS-PAGE (appeared to be missing or present in low concentrations), we tested for the presence of their respective transcripts using slot blot analysis. It was found that the _rbcS_ and _rbcL_ transcripts (corresponding to the small and large subunits of Rubisco) as well as _cab_ transcripts were present in both hydrated and dry chloroplasts (Fig. 4). Densitometry measurements showed that the accumulation of _rbcS_ and _rbcL_ remained constant, but _cab_ declined by 2-fold during drying. Equal loading of the RNA was confirmed by probing the blots with 18 S rRNA. This would therefore imply that rubisco as well as cab proteins were present in low concentrations or were labile (and possibly were degraded during the
Fig. 3: Silver-stained SDS-PAGE of chloroplastic proteins. Lanes 2, 3, and 4 are samples from *C. wulmsii*, *M. flabellifolius* and *P. sativum*, respectively. Lanes 1 and 5 are molecular weight markers.

Fig. 4: Chloroplastic gene expression in the dry (Panel A) and the rehydrated leaf (Panel B) of *M. flabellifolius*. Slot blot analysis was prepared using 10 µg total RNA per lane. 18S RNA was used to show that equal amounts of total RNA were loaded per lane.

It has been well documented that chloroplastic proteins, especially cab and rubisco are prone to proteolytic degradation during the extraction procedure (reviewed in Adam, 1996). Although very little is known about the mechanisms of degradation, this might explain the protein profile we obtained for *M. flabellifolius*. Although a more comprehensive study is required to test this, it is unlikely that *M. flabellifolius* differs markedly from other C3 plants in the nature of the key photosynthetic proteins.

LEA proteins and desiccation stress proteins (dsp) have been reported to accumulate in response to dehydration in leaf tissues of several species (Bartels et al., 1990; Close et al., 1993; Mundy and Chua, 1988; Schneider et al., 1993). To investigate whether LEA and/or dsp proteins were present in chloroplasts isolated from *M. flabellifolius*, Western blot analysis using a variety of antibodies specific to LEA and dsp proteins was performed. Although antibodies against LEA group I and II proteins failed to detect any immunopositive bands, an antibody against *C. plantagineum* dsp 21 protein (Bartels...
Polyphenolic and sugar content of isolated chloroplasts

Sugars and amphipathic substances are thought to protect membranes of DT organisms. The former are believed to interact with phosphate of the phospholipid headgroups, replacing water in the dry state (Crowe et al., 1984, 1985; Hoekstra et al., 1991, 1997) while the latter partition readily into membranes (Terao et al., 1994; Golovina et al., 1998; Hoekstra et al., 1997), facilitating maintenance of fluidity and possibly preventing elimination of sugars from membranes during drying (Golovina et al., 1998; Hoekstra et al., 1997). The sugar and polyphenolic contents of chloroplasts from hydrated and dry leaves are given in Table 2. The crude chloroplast preparations were used in order to prevent loss of material and hence allow better comparison between the two different samples. We could not determine sucrose concentration, possibly because invertase is sensitive to contaminating substances such as polyphenolics (Koonjul et al., 1999). Glucose and fructose contents increased significantly (from 39 to 347.2 nmol/L g dw⁻¹ and 153 to 298 nmol/L g dw⁻¹, respectively) during dehydration. The increase in glucose may be a consequence of starch hydrolysis, which occurs during drying of most resurrection plants (Dalla Vecchia et al., 1998; Farrant et al., 1999; Gaff, 1989). The source for increase in fructose is less clear. It could be a consequence of hydrolysis of oligosaccharides or import from the cytoplasm. Glucose has been shown to protect chloroplasts from the chaotropic effects of high ion concentration (Heber and Santarius, 1964),

Table 2: Content of various polyphenolics, soluble sugars and cations in chloroplasts from hydrated and dry leaves of M. flabelifolius. See Materials and Methods for details on extraction and calculation of results. The hydrated : dry ratio used in the calculations was 1:2.625 (Kruger, 1998). At least three extracts and several internal replicates were performed per assay. Cnd: could not be determined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Unit of measure</th>
<th>Hydrated</th>
<th>Dry</th>
</tr>
</thead>
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<tr>
<td>Polyphenolics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaloid-soluble</td>
<td>µg g⁻¹ dw⁻¹</td>
<td>0.429±0.028</td>
<td>0.730±0.037</td>
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<tr>
<td>Acid-soluble</td>
<td></td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>µg g⁻¹ dw⁻¹</td>
<td>59±7.540</td>
<td>133.38±14.79</td>
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<td>Cyanidin</td>
<td>µg g⁻¹ dw⁻¹</td>
<td>13.78±1.080</td>
<td>30.81±3.006</td>
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<tr>
<td>Delphinidin</td>
<td>µg g⁻¹ dw⁻¹</td>
<td>25.89±2.008</td>
<td>61.01±5.002</td>
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<td>Galloctonin</td>
<td>Colour intensity</td>
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<td></td>
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<td>Cnd</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Fructose</td>
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<td>153*</td>
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<tr>
<td>Ca²⁺</td>
<td>µg g⁻¹ dw⁻¹</td>
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<td>Fe²⁺</td>
<td>µg g⁻¹ dw⁻¹</td>
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<td>µg g⁻¹ dw⁻¹</td>
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<td>118.65±8.636</td>
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<td></td>
<td></td>
<td>99.06±8.613</td>
<td>29.65±1.818</td>
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</table>

Fig. 5: Western blot of chloroplastic proteins with an antibody raised against the Group III LEA protein (dip 21) from C. plantagineum. Samples are proteins extracted from chloroplasts of P. sativum (lane 1); C. wilmisii (lane 2) and M. flabelifolius (lane 3).
a likely consequence of desiccation; hence an increase in glucose level upon drying may serve such a function in resurrection plants. On their own, monosaccharides are not as effective as di- and oligosaccharides, in water replacement or vitrification (Hoeckstra et al., 1997). If they serve such a protective role, it is likely that they do so in combination with other (e.g. amphipathic) compounds (Golovina et al., 1998).

There was an increase in alkali-soluble polyphenolic content (from 0.429 to 0.730 μg·mg dw⁻¹) in total anthocyanin content (59 to 133.8 mg·g dw⁻¹), in the level of all proanthocyanidins as well as in galloantin content of chloroplasts during drying (Table 2). Such increases suggest that these polyphenolics may facilitate increased membrane fluidity and, together with sugars, afford protection to membranes during drying (Golovina et al., 1998; Hoeckstra et al., 1997). Polyphenolics, particularly anthocyanins, have been proposed to mask chlorophyll and prevent excess light absorption under water limiting conditions (Hopkins, 1992; Sherwin and Farrant, 1998). They also act as antioxidants (Larson, 1988; Smirnoff, 1993; Terao et al., 1994). Both functions help prevent photo-oxidation during desiccation.

**Cation content**

During drying, thylakoid membranes from *M. flabellifolius* became blistered (Fig. 6). This was due to the aequous fixation used in this study as vapour fixation gave similar ultrastructural detail (Goldsworthy and Drennan, 1991). The chloroplasts became rounded and the appressed membranes of the thylakoids appeared to have pulled apart. Since it is known that removal of divalent cations from isolated thylakoid membranes causes unfolding of the appressed membranes (Anderson and Aro, 1994), we tested whether metal depletion occurred in chloroplasts of *M. flabellifolius* during drying (Table 2). Crude samples of chloroplasts were used so as to prevent loss of material and hence allow better comparison between the different tissues used. All of the cations measured declined in the chloroplast during desiccation, with Cu²⁺ declining by 95.5 %, Fe²⁺ by 81 %, Mn²⁺ by 70 %, Ca²⁺ by 65 % and Mg²⁺ by 46 %. Presumably, these metal cations were exported to other compartments, possibly the vacuoles (which are reduced and filled with non-aqueous substances in dry leaves) (Farrant and Sherwin, 1998) or to the cell wall.

**Discussion**

Our results suggest that the increased buoyant density observed for chloroplasts from *M. flabellifolius* could be due to the unique ultrastructure of the thylakoid membranes. This ultrastructure was observed both in the intact leaf and upon isolation of the organelle, suggesting that the increased buoyant density was not artifactual. Moreover, the buoyant density of both isolated chloroplasts and isolated thylakoid membranes was found to be increased relative to other species studied. Indeed, all other resurrection plants studied to date, for example *Borya nitida* (Hetherington et al., 1982), *Craterostigma* spp. (Sherwin and Farrant, 1996, 1998), *Sporobolus stapfianus* (Dalla Vecchia et al., 1998) and *Xerophyta* spp. (Farrant et al., 1999; Sherwin and Farrant, 1996, 1998; Tuba et al., 1993), have a chloroplastic ultrastructure and buoyant density similar to DS plants. No correlation was found between the lipid content of chloroplasts isolated from *M. flabellifolius* and the higher buoyant density observed. The lipid compositions of these chloroplasts and those isolated from *P. sattivum* and *C. wilmsii* were very similar. Since the specific densities of palmitic acid and linolenic acid are 0.85 and 0.91 g·mL⁻¹ respectively, one would anticipate a significant increase in the content of the latter to account for the higher buoyant density. In fact, the linolenic acid content of chloroplasts isolated from *P. sattivum* was almost double that of chloroplasts isolated from *M. flabellifolius*. It is unlikely that a different protein content from chloroplast of *M. flabellifolius* compared with *C. wilmsii* and *P. sattivum* would greatly affect the buoyant density as, in general, proteins have a similar buoyant density of approximately 0.75 g·mL⁻¹.

Use of conventional protocols to isolate chloroplasts resulted in ultrastructural damage to the chloroplasts from *C. wilmsii* and *M. flabellifolius* but not to those from the DS *P. sattivum*. In order to obtain intact chloroplasts from the former species, various protectants of protein and membrane structure were required during extraction. Isolation of intact organelles was effective only in the presence of trehalose. Thus, chloroplasts from these DT plants appear to be less robust than those from DS plants such as *P. sattivum*. This result appears anomalous as one might expect that organelles from the former are inherently more tolerant of variations in the cytoplasmic milieu as would occur during desiccation. It is interesting that sucrose (the principle sugar present in dry *Craterostigma* spp. (Bianchi et al., 1991; Ghasempour et al., 1998), which is believed to play an important role in protection against desiccation in plants (Ghasempour et al., 1998; Hoeckstra et al., 1991, 1997), did not enable stabilisation of chloroplast membranes in either resurrection plant. Trehalose has been shown to be particularly effective in stabilising biological membranes against desiccation stress (Crowe et al.,

![Fig 6: Electron micrograph showing a chloroplast from a dry (5% RWC) intact leaf of *M. flabellifolius* (×16,500). Note the separation of the thylakoid membranes.](image)
1984, 1985; Gadd et al., 1987) and was effective in maintaining ultrastructural integrity in the resurrection plants used in this study. This sugar occurs in low concentrations (30 mg g\(^{-1}\) dw\(^{-1}\)) in *M. flabellicolius* (Bianchi et al., 1991; Drennan et al., 1993) and it is unlikely that alone it serves to stabilise the chloroplast membranes in *vivo* during dessication. It has been shown that trehalose can substitute for HSP 12 in membrane stabilisation of yeast (Sales et al., 1999) and it is possible that the sugar served a similar role here, substituting for a component(s) that was either removed during isolation, or not present in sufficient quantities in hydrated chloroplasts.

Despite the apparent fragility of chloroplasts from *M. flabellicolius*, this study has revealed several means by which such chloroplasts might achieve protection against the stresses associated with water loss. A 66 kDa protein recognised by the antibody to the d2p 21 protein from *C. plantagineum* was present in isolated chloroplasts from both resurrection plants studied, but not *P. sativum*. This protein belongs to the LEA family and is likely to have some role in their ability to withstand water stress. There were also a number of unique 10–15 kDa stromal proteins present in chloroplasts from *M. flabellicolius*, which might have a role in protection of organelar structure during dessication.

The leaves and chloroplast of *M. flabellicolius* have high levels of polyphenolics and concentrations increase in the chloroplast during drying. These compounds can be implicated in dessication tolerance of this species in several ways. Firstly, they could protect membranes, as proposed by Golovina et al. (1998), by partitioning into them and preventing removal of sugar protectants. Although we did not measure total sugar content, glucose and fructose concentrations did increase in the chloroplasts during drying, and it is possible that such a protective function was fulfilled in *M. flabellicolius*. Glucose may have an additional function in the protection against the chaotropic effects of high ion concentration during drying (Heber and Santarius, 1964). The increase in polyphenolics, particularly anthocyanins, could act as sunscreens to minimise light-chlorophyll interaction and, being antioxidants, could quench free radical activity (Larson, 1988; Sherwin and Farrant, 1998; Smirnoff, 1993).

The separation of thylakoid membranes during drying (Fig. 6) may also play a role in the prevention of photo-oxidation. Separation of membranes will significantly reduce efficiency of light capture and the transfer of electrons through the photosynthetic pathway. This in turn could reduce the potential for production of superoxide radicals at PS I and PS II. The reduction in metal concentration in chloroplasts during drying might facilitate this membrane separation, since a reduction in cations has been shown to cause segregation of thylakoid membranes (Anderson and Aro, 1994). Furthermore, cations such as Cu\(^{2+}\), Fe\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) are integral components and/or cofactors to proteins and enzymes of the photosynthetic pathway; metal removal would therefore suspend their activity and also their involvement in free radical formation. Indeed, this could be a mechanism to switch off photosynthesis before water becomes limiting. Farrant et al. (1999) have shown that PS II activity (as measured by chlorophyll fluorescence) declines at relatively high water contents (1.25 g H\(_2\)O g dw\(^{-1}\) or 55 % RWC), above which metabolism would be water limited (Vertucci and Farrant, 1995). We propose that the staircase arrangement of thylakoid membranes in *M. flabellicolius* may more easily accommodate their regular separation and reaggregation during drying and rehydration.

In conclusion, it was found that *M. flabellicolius* uses unique strategies for coping with excess light under water limiting conditions.

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