Thylakoid-Dense Chloroplasts of *Callisia fragrans*

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Descriptions and micrographs of chloroplast ultrastructure report 40-60 grana per chloroplast with 2-100 thylakoids per granum (*Spinacia*, Granick and Porter 1947, Leyon 1953). Although higher plant chloroplasts average 4-6 μm in diameter (Kirk and Tilney-Bassett 1967), no work has been reported on their density or sedimentation response due to different g forces. Their shape has been described as being plano-convex (Hall *et al.* 1974) although it is somewhat variable. Valanne (1977) and Descomps and Deroche (1973) found that under continuous light, the grana size increased per chloroplast in the protonemata of *Ceratodon* and those of tomato. However, Descomps and Deroche and (1973) Bronchart (1967) found no such increase in the total number of thylakoids per plastid in *Spinacia*. Exceptionally large grana stacks due to mineral deficiencies have also been reported (Hall *et al.* 1972). In none of these cases does the density of thylakoids per chloroplast approach that which has been found in *Callisia* and which is reported here.

In this study sedimentation properties, shape, and ultrastructure of the chloroplasts of *Callisia fragrans* (Lindl). Woods (syn. *Tradescantia dracaenoides*) were investigated and compared to those of several species of higher plants which have been commonly described in the literature. *Callisia* is a monocot in the Commelinaceae.

Materials and methods

All plants were grown under Plant-Gro lighting and greenhouse conditions for at least six months prior to utilization.

Fractional centrifugation: Strips of leaf tissue were homogenized in a Sorvall Omni-Mixer in a sucrose-ficol-dextran (SFD) medium (Honda *et al.* 1966) at a high speed for 30 seconds or one minute depending on the species. The brei was filtered through 4-ply cheesecloth and centrifuged at 100 g for 5 minutes in a Sorvall RC2-B centrifuge equipped with an HB-4 rotor. A fresh mount was made of the pellet and approximately 50 chloroplasts were randomly measured using a Zeiss 2278 microscope and an Olympus filar micrometer. The supernatant was centrifuged at 250 g, a fresh mount was made, and again approximately 50 chloroplasts were randomly measured as above. This procedure was repeated at 500, 1000, 1500, 2000, 2500, 3000, and 3500 g for all species except *Callisia*

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where it was not continued past 1500 g.

After each fraction was sedimented, the volume of the pellet was recorded and the supernatant was centrifuged at the next higher g force. The percentage of the total volume of pellets it represented was determined and this determined the distribution of chloroplasts among different g forces. This procedure was not continued past 1500 g for *Callisia*.

Sucrose molarity test: Strips of leaf tissue were homogenized in SFD medium (Honda *et al.* 1966) with 0.20 M sucrose, filtered as above, then centrifuged at 9150 g for 5 minutes after a 10-minute response time. This procedure was repeated with the same pellet in sucrose concentrations of 0.25, 0.30, 0.35, and 0.40 M sucrose.

Bovine serum albumin (BSA) and native leaf protein test: Leaf strips of *Callisia* were homogenized and filtered as above. The brei was placed in four tubes and centrifuged at 9150 g force for 10 minutes. Tube 1 received no further treatment. (Honda *et al.* 1966, considered that this homogenate was protective of organelle integrity due to its content of native leaf protein). The supernatant in tube 2 was discarded and replaced with fresh SFD medium (Honda *et al.* 1966). The supernatant in tube 3 was discarded and replaced with SFD medium and 1200 mg BSA (100 mg/ml). The supernatant in tube 4 was retained and treated with 1200 mg BSA (100 mg/ml). All four pellets were resuspended in their new media and recentrifuged. A fresh mount was made of each pellet and approximately 50 chloroplasts were randomly measured as above. Slides were stored at 0–2°C for 24 hours and again 50 chloroplasts were randomly measured.

Scanning electron microscopy: One-cm squares from *Callisia* leaves were fixed in 4% osmium tetroxide vapor for 30 minutes at room temperature. Specimens were dehydrated in a graded acetone series, dried with CO₂ in a Samdri PVT-3 critical point drier, and coated with 75–100Å carbon and 275–300 Å gold-palladium. Tissue was examined in a JEOL JSM U-3 scanning electron microscope at 15–25 KV.

Transmission electron microscopy: One-cm squares of *Callisia* leaf tissue were vacuum infiltrated with 3% glutaraldehyde and 3% acrolein in 0.02 M cacodylate buffer at 0–3°C and a pH of 7.2. Following two hours of fixation, the samples were rinsed in 0.02 M cacodylate buffer for two hours with a change of buffer every 10 minutes. Post-fixation with 2% osmium tetroxide in 0.02 M cacodylate buffer at 0–3°C and a pH of 7.2 was continued for two hours and followed by several distilled water rinses. The leaf squares were then homogenized in a Sorvall Omni-Mixer, filtered, and centrifuged as above in distilled water. The pellet was resuspended in 1% agar at 60–70°C and again sedimented. The solidified agar pellet of chloroplasts was trimmed into 1 mm cubes, dehydrated in a graded series of cold (0–3°C) ethanol, rinsed twice in 100% acetone, and embedded using the materials and methods of Spurr (1969). Ultrathin sections with refractive index of gray to silver were obtained with a Sorvall MT2–B ultramicrotome and a glass or DuPont diamond knife. Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS-8F-1 transmission electron microscope at 50 KV.

Results and discussion

In order to determine their uniqueness, chloroplasts of *Callisia* were compared with those from three vascular plants found to be often used in research, *Pelargonium*, *Lycopersicon*, and *Cyphomandra*. Although the study species was a monocot and the reference species were dicots, no distinctive differences have been reported between their plastids and they have been compared before (Laetsch 1971). Previous ultrastructural studies of *Cyphomandra* (Harris 1979), *Pelargonium* (Kirk and Tilney-Bassett 1967), and *Lycopersicon* (Arnott *et al.* 1969) have been reported.

Fig. 1. Size distribution of *Callisia*, *Cyphomandra*, *Pelargonium* and *Lycopersicon* chloroplasts. The total sample (300–400 chloroplasts) for each species was divided into 10 classes of $0.6\ \mu\text{m}$ increments ranging from 1.8 to $8.4\ \mu\text{m}$. The percent of chloroplasts falling in each class was computed.

Kirk and Tilney-Bassett (1967) report the average size of higher plant chloroplasts to be $4\text{--}6\ \mu\text{m}$ in diameter. *Callisia* and the three reference species have chloroplasts falling precisely in this range (Fig. 1). *Callisia*'s and *Cyphomandra*'s bulk are in the $5\ \mu\text{m}$ class while most of those from *Pelargonium* and *Lycopersicon* are in the $4\ \mu\text{m}$ range. There is considerable overlap between all 4 species. Most chloroplasts of *Callisia* are quite normal as far as size is concerned but the few which occur in the $9\ \mu\text{m}$ range are somewhat spectacular.

When chloroplasts from all four species were subjected to various sedimentation forces, an interesting characteristic of *Callisia* chloroplasts became apparent

(Fig. 2). A force of 1000 g was sufficient to sediment all of the chloroplasts from *Callisia*, but *Lycopersicon* had a few small chloroplasts which sedimented with forces of 2500 and 3000 g, and both *Pelargonium* and *Cyphomandra* had a measurable amount in the 3000 g fraction. The chloroplasts of *Callisia* were sedimented with much less force than those of the reference species.

Fig. 2. Percent of chloroplasts sedimented by different centrifugal forces. The three reference species had chloroplasts in the 2000–3000 g range, but all *Callisia* chloroplasts were sedimented by 1000 g.

Because sedimentation rates are a function of size, density, and shape, the latter was investigated for *Callisia* with scanning electron microscopy (Figs. 3–5). They show the typical plano-convex form (Hall *et al.* 1974) common to chloroplasts of higher plants. Thus, other explanations for their unusual sedimentation property were sought. To verify that the *Callisia* chloroplasts were heavier due to their unique characteristics, and not to experimental techniques, two tests were undertaken.

The sucrose molarity test subjected the chloroplasts of all four species under investigation to five different molar environments: 0.20 M, 0.25 M, 0.30 M, 0.35 M, and 0.40 M sucrose in the SFD medium (Honda *et al.* 1966). If any of the chloroplasts were sensitive to osmotic pressure effects then the volume of the centrifuged pellet should have decreased with the increase in sucrose molarity. After an analysis of the data, it was found that this had little or no effect on the volume of the chloroplast pellet of any of the four species. In the bovine serum albumin (BSA) test, the chloroplasts of only *Callisia* were subjected to four different environments with different combinations of introduced and native proteins to look for non-osmotic pressure effects. The four sets of conditions were: 1) native leaf proteins (those released by homogenizing the leaf) present but BSA

Figs. 3-5. Scanning electron micrographs of *Callisia* chloroplasts. Plano-convex shape and 4-6 μm size demonstrated. 3 shows relative abundance of plastids per cell. 4 and 5 show surface features and irregular edges.

Figs. 6-9. *Callisia* chloroplasts sedimented with different g forces. 6 is from the 100 g fraction, 7 from 250 g, 8 from 500 g, and 9 from the 1000 g fraction. Although a size decrease seemed apparent with increasing g force statistical evaluation showed no difference, and no other differences exist between the fractions. Note grana (G) and starch granules (S).

Figs. 10-15. Late stages in chloroplast ontogeny in *Callisia*. 10 is identified as representative of young chloroplasts by prolamellar body (PB), small starch granule (S), and sparse grana (G). 11-14 demonstrate the increase in grana (G), starch granule (S) size, and stromal thylakoids (ST), with a decrease in stroma (SA). 15 is identified as aging by the disassociation of thylakoids and the black, osmiophilic plastoglobuli. Mitochondria (M) appear in 12 and 15.

absent, 2) both native leaf proteins and BSA absent, 3) native leaf proteins absent but BSA present, and 4) both native leaf proteins and BSA present. When the data were analyzed, no swelling responses were noted. Small volume changes due to illumination intensities have been reported for some species (Zurzycki and Metzner 1977, Godziemba-Czyz 1975), but this was not investigated in this study.

Callisia chloroplasts sediment at relatively low g forces (Fig. 2), and it appears

Fig. 16. The dense thylakoid system of *Callisia* chloroplasts. Bifurcating thylakoids (arrow), starch granule (S), and the peristarch matrix (PM) were observed.

that whatever property or properties contribute to this phenomenon are shared by a very high percentage of the plastids. Light microscopy investigations (Figs. 6–9) showed no significant difference in size (see Table 1) or shape between individual plastids at the four sedimentation forces applied. Table 1 shows that the size averages at the same sedimentation forces and although there is decrease in size with increasing g force the differences are not enough to explain the results shown in Fig. 2. Sedimentation of different numbers of chloroplasts with different forces might be explained by variations in internal structure as indicated in Figs. 10–15.

Table 1: Relationship between *Callisia* chloroplast and force required for sedimentation

	Sedimentation force (g)			
	100	250	500	1000
Size (μm)	5.36 ± 1.5	$5.08 \pm .80$	4.80 ± 1.1	4.65 ± 0.6

Several unique ultrastructural features were found in the chloroplasts of *Callisia* that have not been reported elsewhere. Relatively young ones appear like other developing chloroplasts (Fig. 10), but the increase of both granal and stromal thylakoids continues during development until the plastids are entirely filled with them (Figs. 11–15). This abundance of internal structure might explain why they were sedimented at low g forces: they were more dense because there was so much more to their thylakoid systems than the reference species (Arnott 1969, Harris 1979, Kirk and Tilney-Bassett 1967).

The development of the thylakoids seemed to continue to the complete exclusion of the stroma (Fig. 16). At this point, it was somewhat difficult to ascertain if the thylakoids were granal or stromal in nature. This suggests that functional distinctions may then be of little value. Ultrastructure work on *Tradescantia* spp. chloroplasts (Gyurjan *et al.* 1977, Keresztes 1971, Keresztes and Faludi-Daniel 1973) does not show this abundance or complexity of the thylakoid system except in relatively rare cases. A peculiar thylakoid/starch grain association seemed to show a distinct peristarch matrix (Figs. 11, 13, 21, and 23). This was not observed in all cases. Figures 14, 16, and 18 show an electron-transparent mass surrounding the starch grains, as is typical for other species.

At the perpendicular junctions of two grana, the interrupted thylakoids appeared to end in blind sacs and were not continuous to the other grana (Fig. 17, arrow). Other thylakoids that intersect at lesser angles may come to lie parallel with intersected grana (Fig. 17, left of arrow).

The branching of the thylakoids of *Callisia* deserves special attention. Multiple branches and forks have been found (Fig. 19) between separate grana. Although bifurcations are not uncommon (Hall *et al.* 1974, Gregory 1971, Wehrmeyer 1964) and have been included in explanations of chloroplast ultrastructure, branching to this degree has not been previously reported. Also present are what may be either a twisting or a multiple branching of thylakoids (Fig. 20). In either case, the activity is another of *Callisia* chloroplast's peculiar-

Figs. 17–18. 17, abundant thylakoids and their perpendicular junctions (arrow). At the junction they appeared to end in blind sacs, thus seemed discontinuous. Starch granules (S) and a mitochondrion (M) are indicated. 18, broadening and branching of thylakoids more clearly observed in older chloroplasts, either surrounding a starch granule or free in the stroma (arrows). Also apparent are grana (G) and stromal thylakoids (ST) and the close association between the stromal thylakoids.

Figs. 19–21. 19 a and b, high magnification view ($117,000\times$) of a thylakoid junction complex. 19b is an interpretation of 19a to emphasize the system's branching. 20 a and b, diagonal arrangement of thylakoids to grana (arrows). 21, densely packed grana (G), stroma (S), and a granular stroma (SA). This stroma is seldom found in *Callisia* chloroplasts where it is usually excluded by the extensive grana systems.

Figs. 22–23. 22, numerous thylakoids and grana typical of *Callisia* chloroplasts. Of particular interest is the plastosome-thylakoid relationship (arrow), where the body appears to be continuous with three thylakoids and is bound by the same membrane that defines them. A second plastosome was sectioned tangentially (below arrow). 23, connections between adjacent grana via numerous stromal thylakoids. This is a mature plastid as evidenced by the plastoglobuli (P) and the abundance of thylakoids. The arrows indicate thylakoid branching around starch granules.

rities.

Although few of the chloroplasts contained extensive stroma (it all having been replaced by the thylakoid system), when it was found it was highly granular (Figs. 11–13, 21). If the granularity is mostly due to ribosomes, since they function in protein synthesis, and since the stroma is the site of Calvin carbon-fixation reactions, how the typical *Callisia* chloroplasts function with so little stroma and so few ribosomes would seem to deserve investigation. Carbon fixation studies and studies of RNA and DNA content were not undertaken and certainly seem to be needed.

Membrane-bound plastosomes have been reported in chloroplasts (Harris 1978), and a peculiar plastosome/thylakoid relationship appeared in *Callisia*. In one specimen (Fig. 22) three parallel thylakoids are seen to be continuous with a plastosome. The thylakoid membrane appears to be the same one that bounds the plastosome. The role of this relationship is not known.

Counting the numbers of thylakoids per chloroplast in published electron micrographs shows that by comparison with the chloroplasts of *Cyphomandra*, *Lycopersicon*, and *Spinacia* the thylakoids in *Callisia* are approximately twice as numerous. *Callisia* has an average of 39.1 thylakoids/ μm , while *Cyphomandra* (Harris 1979) has 15.1 thylakoids/ μm , *Lycopersicon* (Arnott *et al.* 1969) has 26.9 thylakoids/ μm , and *Spinacia* (Sprey and Laetsch 1975 and 1976) has 17.3 thylakoids/ μm .

Summary

Using the techniques of fractional centrifugation, light and electron microscopy, unique features of the chloroplasts of *Callisia fragrans* (Lindl.) Woods were observed. In size, shape, and in their different stages of development, these chloroplasts were comparable to those described for *Lycopersicon*, *Pelargonium*, and *Cyphomandra*. *Callisia* chloroplasts differ from others in sedimentation properties and in the relatively high volume of the chloroplast occupied by the thylakoid system. There was almost no stroma. All *Callisia* chloroplasts were sedimented by a force of 1000 g. There were very few fragments. Starch-associated stroma was highly granular and dense in most cases. Plastosomes were not numerous, yet showed unique relationships with thylakoids. Plastoglobuli were observed.

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