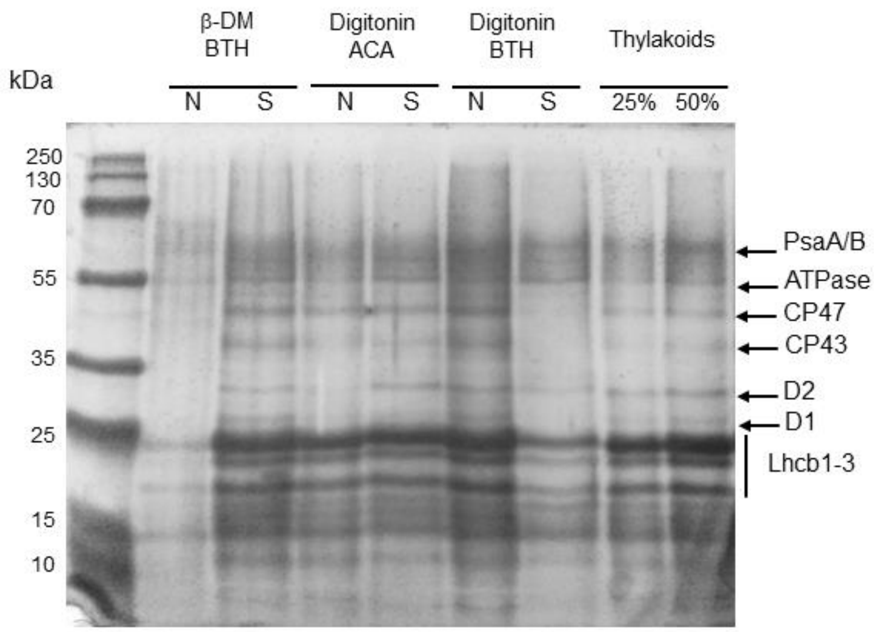
Thylakoid membrane appression in the giant chloroplast of *Selaginella martensii* Spring: a lycophyte challenges grana paradigms in shade-adapted species

Andrea Colpo, Alessandra Molinari, Paola Boldrini, Marek Živčak, Marian Brestič, Sara Demaria, Costanza Baldisserotto, Simonetta Pancaldi, Lorenzo Ferroni

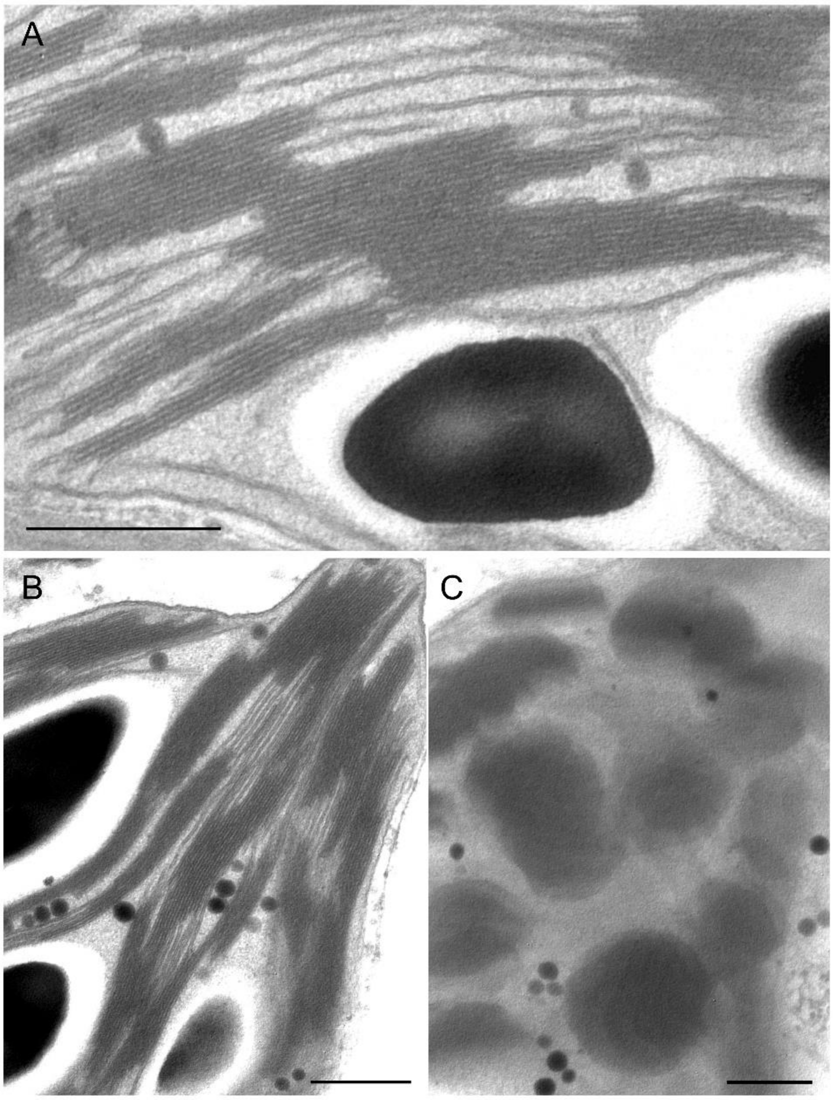
SUPPLEMENTARY MATERIAL



Supplementary Figure S1. Proteins of the thylakoid membranes of *Selaginella martensii* solubilized with different detergents and subsequently separated on denaturing SDS-PAGE.

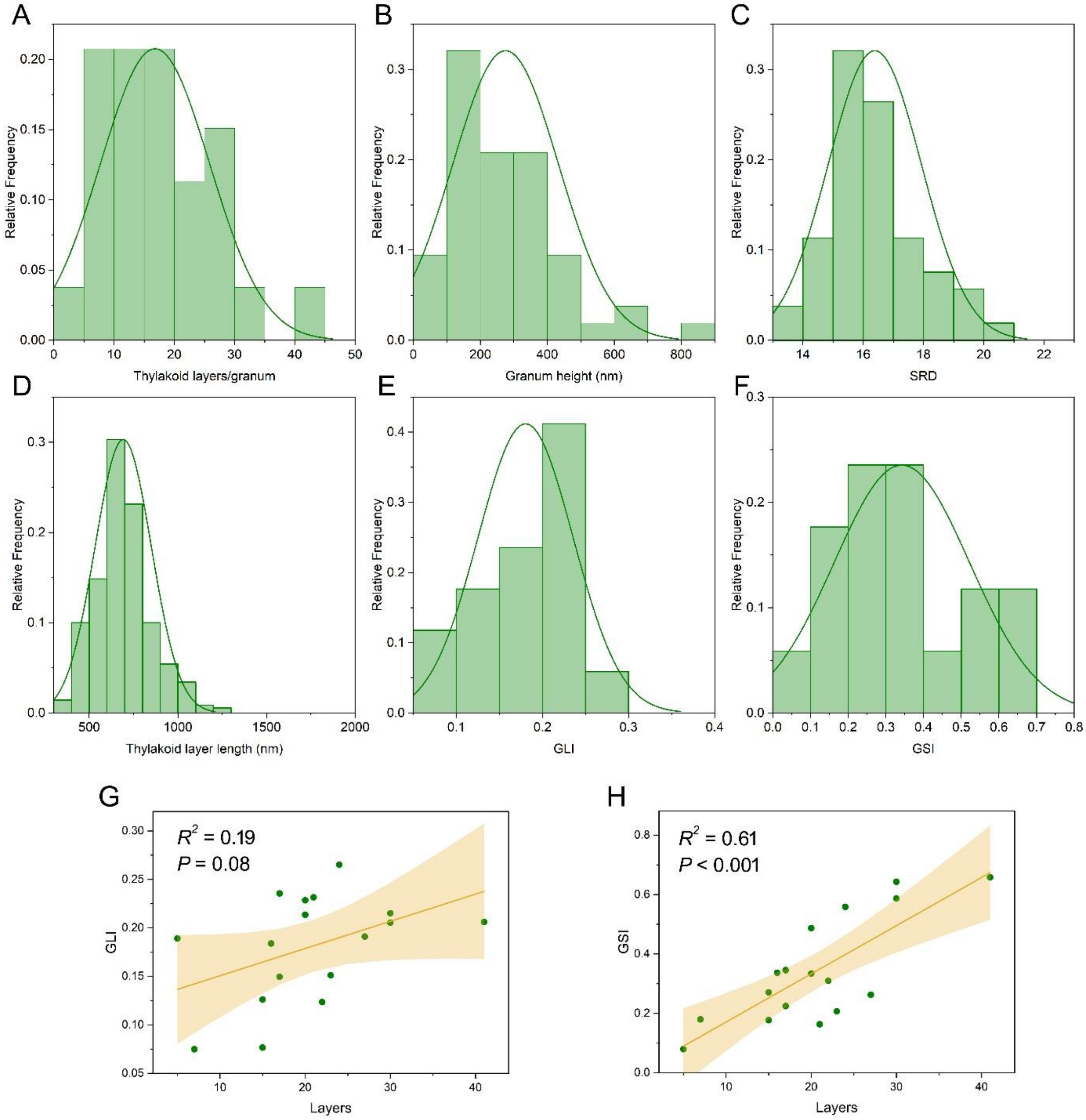
Thylakoid membranes corresponding to 8 μg chlorophyll were solubilized with 1.5% β-dodecyl maltoside (β-DM) in bis-tris-HCl (BTH) buffer, or 1.5% digitonin in BTH, or 1.5% digitonin in aminocaproic acid buffer (ACA) and, after centrifugation, the solubilized (supernatant) and non-solubilized (pellet) were separated (see paragraph 2.4 of the main text). Subsequently, 15 μL of 2× Laemmli buffer (Laemmli 1970) was added to all samples with the necessary volume of deionized water to reach a total sample volume of 30 μL. After vigorous vortexing, the samples were incubated for 15 min at 60°C to promote protein denaturation. After centrifugation at 18.000 *g* for 5 min, the supernatant was recovered and loaded into the gel for the electrophoretic run according to routine protocols. For reference, entire thylakoids were denatured using the same protocol and loaded in the same gel. Bands were silver stained, and the proteins were assigned based on Ferroni et al. (2014, 2016): PsaA/B, core proteins of PSI reaction centre; ATPase, ATP-β subunit of the ATP synthase; CP47 and CP43, proteins PsbC and PsbB of the inner antenna complexes of PSII core, respectively; D1 and D2, core proteins of the PSII reaction centre; Lhcb1-3, subunits of the major light-harvesting complex of PSII, LHCII. N, detergent-insoluble fraction; S, detergent-soluble fraction.

Note that the insoluble fraction after digitonin-BTH treatment still contains PsaA/B and ATP-β, which are typically excluded from the grana cores because their steric hindrance at the stromatic side, meaning that the insoluble fraction also includes some stroma-exposed membranes. After digitonin-ACA treatment, the evident D2 enrichment in the soluble fraction, without a similar enrichment in the other PSII subunits, is indicative of an artifact occurred during the solubilization.



Supplementary Figure S2. Electron micrographs of the thylakoid system in the chloroplasts of the mesophyll and lower epidermis cells in *Selaginella martensii* leaf at the end of the night.

(A) Thylakoid system in a chloroplast hosted in a mesophyll cell. Note the co-existence of small grana formed by 4-6 layers and a large granum with a high degree of layer and cross-sectional irregularity. (B) The thylakoid system in a chloroplast of the lower epidermis exemplifies the occurrence of very wide granum layers. (C) In a chloroplast of the lower epidermal cell, a section tangent to the grana stacks shows individual disks with a large diameter and partly overlapping each other. Scale bars: 0.5 µm.



Parameter	Upper epidermis	Mesophyll	*P* (*df*)	Weighted mean
Thylakoid layers/granum	15.1 ± 0.7	16.8 ± 1.2	0.21 (*185*)	15.3
Granum height (nm)	252.4 ± 10.5	278.7 ± 21.7	0.28 (*185*)	255.6
SRD (nm)	16.9 ± 0.14	16.4 ± 0.2	**0.04** (*185*)	16.8
Thylakoid layer length (nm)	732.6 ± 7.0	691.6 ± 8.5	**0.0011** (*1280*)	727.6
GLI	0.213 ± 0.013	0.180 ± 0.013	0.14 (*60*)	0.209
GSI	0.318 ± 0.027	0.343 ± 0.044	0.63 (*60*)	0.321

Supplementary Figure S3. Granum morphometrics of the mesophyll chloroplasts of *Selaginella martensii* leaf.

(A) Height of the grana stacks. (B) Number of thylakoid layers per granum. (C) Stacking repeat distance, *SRD*. (D) Length of the thylakoid layers. (E) Granum lateral irregularity, *GLI*. (F) Granum cross-sectional irregularity, *GSI*. Histograms of the parameters, each reported with the corresponding normal distribution (for A, B, C, *N*=53 grana; for D, *N*=350 layers; for E and F, *N*=17 chloroplasts. Morphometry was performed on 10 micrographs taken from 4 independent plants). (G-H) Co-variation of the number of thylakoid layers per granum with GLI or GSI. The regression lines with 95% confidence bands, R^2 and corresponding *P* values are reported. For the definitions of parameters, see Mazur et al. (2020) and the main text of this paper, section 2.6.

The table reports grana morphometric parameters in comparison between the upper epidermis and mesophyll chloroplasts, with *P* values obtained using a Student's *t*-test. Degrees of freedom for each test (*df*) are reported for each comparison. The weighted mean of the parameters is based on the estimates of the leaf area section covered by plastids belonging to the two tissues. The contribution by the lower epidermis chloroplasts is assumed to be negligible.

Supplementary Table S1

Comparative values of the granum diameters in species of vascular plant. In some cases, the values are average diameters explicitly reported by the referenced papers, in others they were reckoned from published micrographs.

The reported values are affected by different methods and equipment used by laboratories for 50 years and are intended to give a rough idea about variation in granum width among vascular plants.

Plant species	Granum diameter (nm)	Reference
Angiosperms Eudicots		
Arabidopsis thaliana	410-470	Fristed et al. 2009 Armbruster et al. 2013
Raphanus sativus	536	Meier and Lichtenthaler 1981
Lactuca sativa	450-500	Kaftan et al. 2002
Urtica dioica	400-420	Pfeiffer and Krupinska 2005a
Spinacia oleracea	350-600	Daum et al. 2010 Kouril et al. 2011 Wood et al. 2019
Fagus sylvatica	339-399	Lichtenthaler et al. 1981
Solanum lycopersicum	390-430	Moriwaki et al. 2019
Glechoma longituba (shade)	430	Zhang et al. 2015
Primulina tabacum (shade)	550	Liang et al. 2011
Panax quinquefolium (deep shade)	400	Lee et al. 2017
Angiosperms Monocots		
Hordeum vulgare	480	Pfeiffer and Krupinska 2005b
Hydrocharis morsus-ranae	340	Kordyum et al. 2022
Arum italicum (shade)	470	Pancaldi et al. 1998
Tradescantia albiflora (deep shade)	530	Adamson et al. 1991
Alocasia macrorrhiza (deep shade)	483	Anderson et al. 1973 Chow et al. 1988
Monstera deliciosa (deep shade)	430	Demmig-Adams et al. 2015
Anoectochilus roxburghii (deep shade)	507-580	Shao et al. 2014
Cycadophyte		
Lepidozamia peroffskyana (shade)	450	Medeghini Bonatti and Fornasiero Baroni 1990
Monilophytes (ferns)		
Acrostichum danaeifolium	430	Fonini et al. 2017
Asplenium australasicum (deep shade)	726	Leong et al. 1985
Trichomanes speciosum (deep shade)	560	Makgomol and Sheffield 2001
Teratophyllum rotundifoliatum (deep shade)	1290	Nasrulhaq-Boyce and Duckett 1991
Lycophytes		
Selaginella martensii Giant chloroplast Mesophyll chloroplast	 733 692	 This report
Selaginella erythropus Giant chloroplast Mesophyll chloroplast	 594 - 680 662	 Sheue et al. 2007, Ghaffar et al. 2018 Sheue et al. 2007
Selaginella apoda	660	Jagels 1970
Isoetes sinensis	860	Ding et al. 2015

Supplementary Table S2

Thylakoid layers per granum *N* and granum regularity indexes in shade or deep-shade vascular plants (Granum lateral irregularity, *GLI*, and Granum cross-sectional irregularity, *GSI*). *N* values are either declared in the referenced papers, or counted from the published micrographs or, when impossible to count, estimated by dividing the granum height by a postulated stacking repeat distance of 17 nm. *GLI* and *GLI* are estimated from micrographs published in the referenced papers. GI_{TOT} is the sum of *GLI* and *GSI*.

The reported values are affected by different methods and equipment used by laboratories for 50 years and are intended to give a rough idea about variation in granum regularity in vascular plants. Note that GSI is *negative* when the granum shape is convex (e.g., oval-like as in *Anoectochilus roxburghii*): in such cases, the granum is considered free of lateral sliding and a value of 0 *GSI* was assigned.

Plant species	*N*	*GLI*	*GSI*	GI_{TOT}	Reference
Angiosperms Eudicots					
Glechoma longituba	19	0.17	0.19	0.36	Zhang et al. 2015
Primulina tabacum	17	0.15	0.05	0.20	Liang et al. 2011
Panax quinquefolium	21	0.18	0.11	0.29	Lee et al. 2017
Angiosperms Monocots					
Arum italicum	12	0.16	0.17	0.33	Pancaldi et al. 1998
Tradescantia albiflora	14	0.18	0.13	0.31	Adamson et al. 1991
Alocasia macrorrhiza	43 (max >100)	0.19	0.02	0.21	Anderson et al. 1973 Chow et al. 1988
Monstera deliciosa	53	0.09	0.33	0.42	Demmig-Adams et al. 2015
Anoectochilus roxburghii 20% sunlight 5% sunlight	 18 110	 0.15 0.18	 0.20 0	 0.35 0.18	Shao et al. 2014
Cycadophyte					
Lepidozamia peroffskyana (shade)	34 (max 120)	0.11	0.08	0.19	Medeghini Bonatti and Fornasiero Baroni 1990
Monilophyta (ferns)					
Acrosticum danaeifolium	9	0.16	0.10	0.26	Fonini et al. 2017
Asplenium australasicum (deep shade)	16	0.14	0.18	0.32	Leong et al. 1985
Trichomanes speciosum (deep shade)	18	0.16	0.18	0.34	Makgomol and Sheffield 2001
Teratophyllum rotundifoliatum (deep shade)	86 (max 280)	0.20	0.10	0.30	Nasrulhaq-Boyce and Duckett 1991
Lycophytes					
Selaginella martensii Giant chloroplast Mesophyll chloroplast	 15 (max 48) 17 (max 41)	 0.18 0.21	 0.32 0.34	 0.50 0.55	 This report
Selaginella erythropus	18 (max 44)	0.27	0.31	0.58	Sheue et al. 2007 Ghaffar et al. 2018
Selaginella apoda	13 (max 25)	0.19	0.22	0.41	Jagels 1970
Isoetes sinensis	8 (max 23)	0.17	0.06	0.23	Ding et al. 2015

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