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THYLAKOID MEMBRANE REORGANIZATION DURING *ZANTEDESCHIA AETHIOPICA* SPATHE REGREENING: CONSEQUENCE OF THE ABSENCE OF Δ^3-*TRANS*-HEXADECENOIC ACID IN PHOTOCHEMICAL ACTIVITY

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Key Word Index—*Zantedeschia aethiopica*; Araceae; white calla lily; photosystem I and II; photophosphorylation; LHCII, granal stacking; Δ^3-*trans*-hexadecenoic acid; regreening.

Abstract—Soon after its formation the fruiting spathe of *Zantedeschia aethiopica* undergoes senescence which is characterized by the loss of its photosynthetic activity. During fruiting, spathe senescence is inhibited and regreening takes place. The amyloplasts present in the white spathe turn to functional chloroplasts and the spathe acquires photosynthetic capacity. In this work, thylakoid membranes were isolated from three distinct stages of spathe development (floral bud, white and regreened spathes) and used for the study of pigment-protein complexes, as well as for the determination of PSI and PSII activities and photophosphorylation rates. Our results indicate that, besides their ultrastructural similarity to leaf chloroplasts, regreened spathe chloroplasts showed values of photochemical and photophosphorylation rates lower than those found in leaf chloroplasts. These results are discussed in relation to the absence of the fatty acid Δ^3-*trans*-hexadecenoic acid from regreened spathe thylakoid membranes, and we suggest that this fatty acid could be an important factor for optimal photochemical and photophosforylation activities. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The spathe of the white calla lily (*Zantedeschia aethiopica* Spreng., Araceae) exhibits a natural regreening process [1]. During its ontogeny, the first stage of spathe development (floral bud spathe) develops into a white foliar organ, referred to as white spathe. When fruiting occurs the white spathe, which envelopes the spadix, changes into a green leaf-like structure (regreened spathe), as the differentiation of amyloplasts into chloroplasts takes place. If fruiting does not occur, the white spathe dies.

Evidence for a possible participation of endogenous cytokinins in *Z. aethiopica* spathe senescence inhibition and regreening has been reported. Two major cytokinins were isolated from *Z. aethiopica* immature fruits and identified as 6-(-*o*-hydroxybenzylamino)-purine and 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine [2, 3]. After inflorescence ablation, the exogenous application of these substances induced regreening in a similar way to the natural process [4]. It was then postulated that this self-inhibition of senescence and subsequent spathe regreening is related to the presence of cytokinin-like substances in the fruits, which could be translocated to the spathe. Moreover, in previous work the lipid composition of thylakoid membranes from both regreened spathe and leaf chloroplasts was studied [5]. The major difference found in fatty acid composition of both membranes was the absence of trans-Δ^3-hexadecenoic (C16:1-*tr*) acid from phosphatidylglycerol (PG) in regreened spathe thylakoids. PG-containing C16:1-*tr* has been implicated in supramolecular organization of LHCII and in granal stacking [6–8].

The aim of this work is to study chloroplast differentiation during *Z. aethiopica* spathe development in what concerns chloroplast reorganization and function, especially during the regreening process, considering the possible consequences of the absence of C16:1-*tr* in regreened spathe thylakoid membranes.

RESULTS

Photosynthetic and photochemical activities

During the whitening of the floral bud spathe, there was an evident reduction of chlorophyll content per

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Table 1. Chlorophyll density and photosynthetic activity of leaf and spathes. Data represent means of three independent experiments ± SD. FS, floral bud spathe; WS, white spathe; RS, regreened spathe; L, leaf (nd—not detectable).

Parameter	FS	WS	RS	L
Phytosynthetic activity (μmol O_2 m^{-2} s^{-1})	5.1 ± 0.4	nd	12.0 ± 0.5	22.1 ± 1.3
Chlorophyll/area (μg cm^{-2})	86.4 ± 5.2	10.3 ± 0.7	285 ± 21	704 ± 62

Table 2. PSI and PSII activities in leaf and spathes thylakoid membranes. The values are means of three independent experiments ± SD. FS, floral bud spathe; WS, white spathe; RS, regreened spathe; L, leaf (nd—not detectable).

Activity	FS	WS	RS	L
PSI				
(μmol O_2 mg^{-1} Chl h^{-1})	143.5 ± 3.8	97.8 ± 2.2	119.6 ± 3.8	354.0 ± 2.8
(μmol O_2 cm^{-2} h^{-1})	12.4 ± 0.02	1.0 ± 0.002	34.0 ± 0.08	249.2 ± 0.2
PSII				
(μmol O_2 mg^{-1} Chl h^{-1})	41.1 ± 1.0	nd	64.3 ± 0.7	99.0 ± 3.4
(μmol O_2 cm^{-2} h^{-1})	12.4 ± 0.005	nd	18.3 ± 0.01	69.7 ± 0.2

area unit (12% of leaf Chl content in floral bud spathe was reduced to 1.46% of leaf chlorophyll content, in white spathe). Only when fruits were produced was a white-regreened transition observed in spathes involving such fruits. This regreened stage was characterized by an increase in chlorophyll content (from 1.46 to 40% of leaf Chl content) (Table 1).

Photosynthetic activity, measured as O_2 evolution rate, was low in floral bud spathe (23% of that found in leaf) and was not detected in white spathe discs. In regreened spathe an increase of photosynthetic activity was observed, reaching a value which was 54% of that obtained for leaves (Table 1).

PSI and PSII activities were studied by *in vitro* assays and were expressed relatively to Chl content or area units (Table 2). Rates of electron transport through PSI decreased as the transition from floral bud to white spathe took place, while during the regreening process an increase of PSI activity was observed. However, in regreened spathe thylakoids, PSI activity was lower than in leaf thylakoids (34%, when expressed per Chl unit and 14%, when expressed per foliar area, of the values found in leaf). An identical pattern was observed for PSII-dependent O_2 evolution activity, which was not detected in white spathe. Regreened spathe thylakoid membranes exhibited a PSII activity value 65% (expressed in terms of Chl content) and only 26% (expressed in terms of foliar area) of the values obtained for leaves.

Pigment-protein complexes

Electrophoretic analysis of thylakoid membranes isolated from floral bud spathes, regreened spathes and leaves exhibit a similar pattern of four pigment-protein complexes and a free pigment band (Fig. 1). These complexes were designated according to ref. [9] as CPI, $LHCP^1$, CPa and $LHCP^3$, on account of their spectral properties and previous separation [10]. $LHCP^2$ was not detected following the mentioned protocol, probably due to its labile properties [9]. In floral bud spathe thylakoid electrophoretic pattern, the bands corresponding to $LHCP^1$ and CPa were easily perceived, when compared to those obtained for leaf and regreened spathe.

The absorption spectrum of the gel slice containing CP1 was similar to that of CPa, both having red maxima at 663–665 nm and peak in blue at 432 nm. However, CP1 showed a Chl a/b ratio of 7.2 and CPa a Chl a/b ratio of 4.8. $LHCP^1$ and $LHCP^3$ absorption spectra were identical, both having red maxima at 663 nm and peaks in blue at 432 and 459 nm. These two PPCs exhibited an identical Chl a/b ratio of 1.3.

Photophosphorylation rates

The rate of photophosphorylation was determined by measuring proton uptake due to light-dependent scalar consumption of protons in the presence of the

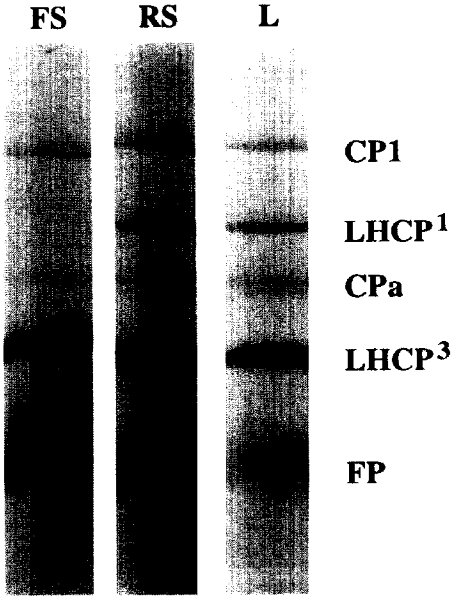

Fig. 1 Electrophoretic patterns of OG/LDS-solubilized thylakoid membranes from floral bud spathe (FS; lane 1), regreened spathe (RS, lane 2) and leaf (L, lane 3), after 7.5–15% acrylamide gradient native PAGE. Floral bud spathe thylakoid membranes exhibited a reduced amount of both LHCP^1 and CPa, while regreened spathe and leaf thylakoids showed identical patterns.

electron acceptor methyl viologen (MV) and assuming a stoichiometry of 1.0 (i.e. scalar consumption of 1 H^+ per ATP) [11]. Illuminated regreened spathe thylakoids exhibited a lower rate of chloroplastidic H^+-ATPase-catalyzed ATP synthesis than in leaf thylakoids (13.6% of the value obtained for leaf thylakoids, when expressed per Chl unit and 5.5%, when expressed per foliar area) (Table 3).

Ultrastructural aspects of plastids

The floral bud spathe exhibited chloroplasts with few grana regions (Fig. 2A). During floral bud-white spathe transition, chloroplasts gradually lose their thylakoid membranes and accumulate starch granules. In the white spathe, amyloplasts exhibited large starch granules where only very few stromal thylakoids were observed (Fig. 2B). In palisade cells from regreened spathe well structured chloroplasts, with very well organized grana regions similar to those of leaf chloroplasts, were observed (Fig. 2C and D).

Table 3. Specific rate of ATP synthesis expressed by chlorophyll and area, in regreened spathe and leaf thylakoid membranes. Data represent means of three independent experiments ± SD. RS, regreened spathe; L, leaf.

ATP synthesis	RS	L
(μmol ATP mg^{-1} Chl h^{-1})	10.8 ± 0.8	79.4 ± 6.0
(μmol ATP cm^{-2} h^{-1})	3.07 ± 0.02	56.0 ± 0.4

DISCUSSION

The results from chlorophyll content and photosynthetic activity measurements indicate that the spathe of *Z. aethiopica* undergoes senescence, soon after its formation. Floral bud spathe, the early stage of spathe development, exhibits chloroplasts characterized by the presence of reduced grana regions, which suggests a low photochemical capacity. This was confirmed by both PSI and PSII activity measurements, carried out on thylakoid membranes isolated from floral bud spathes. Our results agree with those reported by other authors [12–15], which inferred that the declining functional capacity of senescing leaves could be interpreted in terms of progressive changes in the electron transport system of the plastids. The electrophoretic pattern analysis of PPCs from floral bud thylakoids showed a low amount of the oligomeric form of LHCII (LHCP^1) and CPa, which could explain the reduced extension of stacking, since grana stacks were observed to be related to the presence of the main light-harvesting chlorophyll a/b protein complex [16].

In the white spathe, no photosynthetic activity was measured and only PSI activity was detected, which could be correlated to the presence of some remaining stromal thylakoids in amyloplasts, as revealed by electron microscopy observations. In plants where fruiting does not occur, senescence of the spathe will reach its maximum expression, resulting in death of white spathe. When fruiting occurs, a self-inhibition process of spathe senescence takes place, which corresponds to the transition from white to regreened stage. We presume that the regreening of spathe is correlated to the action of endogenous cytokinins possibly produced by immature fruits [4], and translocated to the white spathe at time of fruiting. Accordingly, acquisition of photosynthetic capacity observed in regreened spathe, due to cytokinin action, is in agreement with that reported by several authors [17, 18].

According to our results, no significant differences were found concerning ultrastructural organization between regreened spathe and leaf chloroplasts. Our results suggest that the major difference between regreened spathe and leaf chloroplasts lies at the functional level. Regreened spathe chloroplasts are functional, but exhibit lower values of PSI and PSII activities. Data indicate that regreened spathe exhibit a lower photosynthetic capacity, probably because it is limited by a less efficient photosynthetic electron chain, which could also affect photophosphorylation and explain the differences found in ATP synthesis rates between regreened spathe and leaf thylakoids. Photosynthetic activity can also be limited by ribulose 1,5-bisphosphate carboxylase/oxygenase activity,

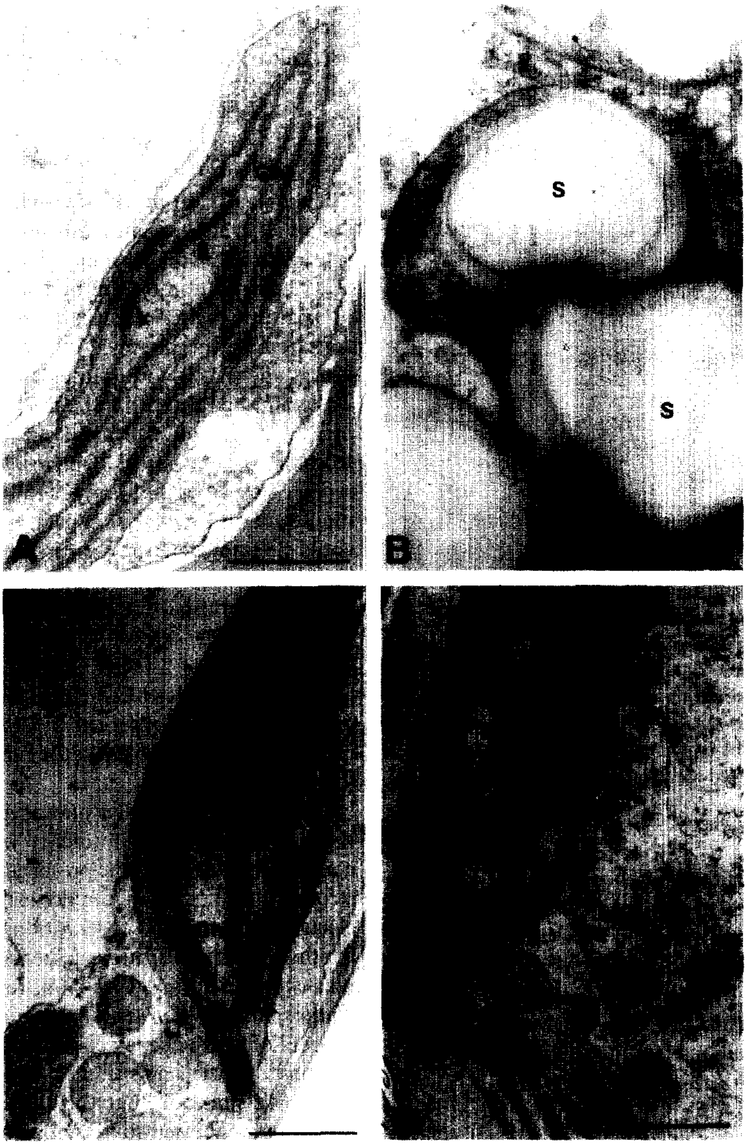

Fig. 2 Electron microscopic aspects of plastids during the senescence and regreening of *Z. aethiopica* spathe. A, Floral bud spathe chloroplasts showing reduced grana regions (arrows). B, Portion of a palisade cell from white spathe exhibiting an amyloplast with large starch granules and reduced stromal thylakoids. C, Portion of a palisade cell from regreened spathe showing a chloroplast with well organized grana regions (arrows). D, Leaf chloroplast, showing grana regions (arrows) similar to those observed in C. Amy, amyloplast; Chl, chloroplast; S, starch. Bar = 1 μm.

which is, in regreened spathe, ca. 50% of that found in leaf (unpublished results).

In previous work the glycerolipid and fatty acid composition of regreened spathe thylakoid membranes was studied [5]. The major difference found in lipid composition between regreened spathe and leaf thylakoids was the absence of the unusual fatty acid C16:1-*tr* in PG from regreened spathe thylakoid membranes. This fact strongly suggested the possibility of marked differences between regreened spathe and leaf chloroplasts, either at ultrastructural or at functional levels. Although the role of this fatty acid has not yet been clarified, it has been correlated with granal stacking and with the supramolecular organization of LHCII [6–8]. The results presented here do not support this hypothesis since no significant differences between the extension of granal stacking in regreened spathe chloroplasts, lacking C16:1-*tr*, compared to leaf chloroplasts, were observed. In addition, electrophoretic analysis of PPCs from regreened spathe and leaf thylakoids showed an identical pattern, both exhibiting the LHCII oligomeric form, corroborating the hypothesis that in *Z. aethiopica*, oligomeric LHCII establishment and grana stacking are independent of the presence of C16:1-*tr* [5]. Our results agree with those of other workers [19, 20] that reported a mutant (*fad* A) of *Arabidopsis thaliana* lacking C16:1-*tr*, that did not exhibit apparent changes in chloroplast structure or changes in properties of LHCII. However, when comparing the electrophoretic pattern of pigment-protein complexes from the wild-type and the mutant, these authors observed that the mutant lacked LHCP^1, concluding that the reported changes of LHCP^1: LHCP^3 could be artefacts of the detergent solubilization.

Rather than having an important role in the supramolecular organization of LHCII and in grana stacking, the presence of C16:1-*tr* could be important for the functional stability of LHCII oligomeric form, resulting in a more efficient energy transfer from LHCII to PSII reaction center. This hypothesis could explain the lower PSI and PSII activities and, consequently, the lower photophosphorylation capacity observed in regreened spathe thylakoid membranes when compared to those found in leaf thylakoid membranes.

We also presume that regreened spathes' contribution to overall photosynthesis in *Z. aethiopica* plants is reduced. Probably the cytokinin-induced senescence inhibition of the white spathe, and the corresponding regreening, occurs as a way of sustaining spathe form and function during a longer period, which could be important if the regreened spathe has a major role in the protection of the fruits during growth and maturation.

EXPERIMENTAL

Plant material. Plants of *Zantedeschia aetiopica* Spreng. grown under natural conditions were used for all the experiments. Spathes in different stages of development (floral bud, white and regreened spathe) were harvested but only portions between 2 and 4 cm from the floral peduncle were used. Leaves were used after mid-rib removal.

Thylakoid membrane preparation. Thylakoid isolation was performed using a modification of the method described by ref. [21]. All steps were carried out at 4°. Freshly harvested and de-veined leaves and spathes were homogenized in a Waring blender homogenizer with 30 mM Hepes-KOH pH 7.6 containing 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol and 1% (w/v) PVP [ratio of plant material/homogenization buffer was 1:4 (w/v)]. The crude homogenate was filtered through 8 layers of cheesecloth and one layer of nylon cloth (20 μm pore size) and centrifuged at 1000 *g* for 2 min. The pellet was re-suspended and washed twice in homogenization buffer for 10 min. at 1000 *g*. The washed pellet was resuspended in 1 mM Hepes-KOH pH 7.6 containing 5 mM and 5 mM $MgCl_2$. After. incubation for 5 min, the suspension was centrifuged for 10 min at 1000 *g*. The pellet was finally re-suspended in a small volume of the same osmotic shock medium. The purity of thylakoid membranes was determined by transmission electron microscopy, according to ref. [5].

Pigment-chlorophyll complexes analysis. Samples for electrophoresis were prepared by solubilization of isolated thylakoid membranes in 50 mM Na_2CO_3, 50 mM DTT, 12% (w/v) sucrose, 0.45% (w/v) lithium dodecyl sulfate (LDS), 1.8% (w/v) octyl glucoside (OG) to a final chlorophyll concentration of 1 mg ml^{-1} and a Chl/LSD/OG (w/w/w) ratio of 1:5:20. Samples were incubated in solubilization medium for 5 min, at 4° in dark. Polyacrylamide gel electrophoresis under non-denaturating conditions was carried out according to ref. [22]. The solubilized material was loaded on a 7.5–15% acrylamide gradient (50 μg Chl per well). LDS and EDTA were added to the upper reservoir buffer to a final concentration of 0.1% (w/v) and 1 mM, respectively. Electrophoresis was performed at a constant voltage setting of 100 V, in the dark at 4° for about 12 hr.

Absorption spectra. Absorption spectra and Chl a/b ratios of the pigment bands were obtained after excision, homogenization in 80% (v/v) acetone and centrifugation of the gel strips.

PSI and PSII activities. PSII-dependent O_2 evolution and PSI-dependent O_2 uptake were measured polarographically with a Clark-type oxygen electrode (Hansatech. Ltd.) as described by ref. [23]. The reaction mixture for O_2 evolution contained 50 mM Hepes-KOH pH 7.6, 5 mM $MgCl_2$, 5 mM NaCl, 5 mM NH_4Cl and 2.5 mM $K_3Fe(CN)_6$. O_2 uptake was assayed in a medium containing 50 mM Hepes-KOH pH 7.6, 5 mM $MgCl_2$, 5 mM NH_4Cl, 0.1 mM methyl viologen (MV), 0.1 mM DCPIP, 5 mM sodium ascorbate, 0.1 mM DCMU and 10 mM KCN. Thylakoid membrane preparations were used at a concentration of 10–30 μg Chl ml^{-1} at 25°, under white light obtained from a 100 W LS2 (Hansatech, Ltd.) halogen lamp.

Photosynthetic activity. CO_2-dependent O_2 evolution was measured in a leaf disc electrode LD2 (Hansatech, Ltd.) according to ref. [24]. Sections of leaves and spathes (10 cm^2) were cut and assayed immediately. Saturating CO_2 conditions, approximately 5% (v/v), were maintained by adding 1 ml of 1 M bicarbonate/carbonate buffer pH 9.0 to the capillary matting. White light was obtained from a 100 W LS2 (Hansatech, Ltd.) halogen lamp and the rate of oxygen evolution was measured at 25°.

Measurement of steady-state ATP synthesis. ADP phosphorylation rate was measured by the quantification of light-induced proton uptake in thylakoids from regreened spathe and leaf, according to ref. [25]. Thylakoid membranes were obtained as described above, except that 2-mercaptoethanol was omitted from homogenization medium. Thylakoids were re-suspended in 2 mM Tricine-KOH pH 8.0 containing 6 mM $MgCl_2$, 25 mM KCl. The assay mixture contained 2 mM Tricine-KOH pH 8.0, 6 mM $MgCl_2$, 25 mM KCl, 0.1 mM MV, 1 mM NaH_2PO_4 and 1 mM ADP. White light was obtained from a 100 W LS2 (Hansatech, Ltd.) halogen lamp. Initial rates of proton uptake were measured at 25° on a standard pH meter (PHM82 Radiometer, Copenhagen) connected to a flat recorder.

Chlorophyll determination. Chlorophyll content of isolated thylakoids and foliar organs samples were determined in 80% (v/v) acetone according to ref. [26]

Electron microscopy. Samples of leaf and spathes palisade parenchyma were prepared for electron microscopy according to ref. [5].

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