

# Photosynthetic characteristics of light-sensitive, chlorophyll-deficient leaves from sectorially chimeric stinging-nettle

Fernando S. HENRIQUES\*

*Plant Biology Unit, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal*

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**ABSTRACT.** Mutated leaves of sectorially chimeric stinging nettle (*Urtica dioica* L.) showed decreased pigment content and reduced photosynthetic activity. Relative to their wild type siblings, the mutated leaves were not only largely depleted in total chl and car, suggestive of a significant loss of photosynthetic units, they also exhibited much higher chl a/b ratios, indicative of a major reduction in the antenna size of the remaining photosynthetic units. Light and electron microscopy confirmed a notable decrease in chloroplast number in mutated leaves, and analysis of polypeptide composition revealed a large depletion of the apoprotein of PSII antenna in these chloroplasts. The photosynthetic units present in mutated leaves showed an intrinsic photochemical efficiency, measured as the variable to maximum fluorescence ratio of dark-adapted leaves, only slightly lower than controls, and their concentration correlated strongly with the leaves's net CO<sub>2</sub> uptake capacity. It is concluded that mutated leaves of stinging nettle underwent a gradual chloroplast loss during development, but the remaining organelles preserved much of their photosynthetic competence. This chloroplast loss most likely arises from an accumulation of chl precursors that repress the synthesis of photosynthetic essential proteins and act as photosensitizers for chloroplast degradation.

**Keywords:** Chimerism; Chlorophyll fluorescence; Chloroplast peptides; Photosynthetic net CO<sub>2</sub> uptake; *Urtica dioica* L.

**Abbreviations:** Car, Carotenoids; Chl, Chlorophyll;  $F_0$ ,  $F_m$ ,  $F_v$ , Minimum, maximum and variable fluorescence of dark-adapted leaves, respectively; LHC-II, Light-harvesting chl a,b-protein complex of PSII; PQ, Plastoquinone; PSI, PSII, Photosystems I and II, respectively; PSU, Photosynthetic unit; Q<sub>A</sub>, PSII primary electron acceptor.

## INTRODUCTION

Many leaf chimeras arise from mutations that disrupt chl synthesis or accumulation and are phenotypically detectable by their lighter green color. It is well established that most chl in chloroplasts serve only as antenna pigments in photosynthesis, capturing incident photons and transferring the resulting excitation energy to reaction centers, where a minor, photochemically active chl fraction uses it to drive a charge separation process. Thus, mutations that cause only loss of chl, even if to a relatively large extent, should not necessarily result in the leaf's inability to carry on photosynthesis. However, it has been reported that pronounced chl deficiency affects the synthesis and/or assembly of other chloroplast components with which the chl interacts *in vivo* (Henriques and Park, 1975; Cumming and Bonnet, 1983; Green et al., 1988; Klein et al., 1988). This is the case, for instance, of the

chl b-less mutant of barley, which is not only deprived of chl b, but also of several major chloroplast polypeptides that act as scaffolds for the assembly of the chl a, b light-harvesting complex in the thylakoid membrane (Henriques and Park, 1975; Burke et al., 1979). Over the past two decades, it has been shown that a block in pigment biosynthesis, either of chl or car, causes the accumulation of intermediates that act as signals regulating the expression of nuclear-encoded plastid-destined gene products (Kropat et al., 1997; Rodermel, 2001; Strand et al., 2003; Nott et al., 2006; Reinbothe et al., 2006). As a consequence, leaf chimeras with largely reduced pigment content often possess additional chloroplast alterations and show more or less extensive photosynthetic impairments. The functional characterization of these pigment chimeras, thus, frequently proves to be rather complex, and attempts to identify the specific change(s) responsible for the photosynthetic disturbance(s) turn out immensely challenging (Sommerville, 1986).

We report here data on pigment content and certain photosynthetic characteristics of mutated leaves from

\*Corresponding author: E-mail: ffh@fct.unl.pt; Tel: +351-21-2948500; Fax: +351-21-2954461.

sectorially chimeric stinging-nettle at two defined developmental stages. Relative to controls, mutated leaves are largely depleted in both chl and car and possess fewer chloroplasts; the photosynthetic units (PSUs) of these remaining chloroplasts are of a smaller size due to extensive loss of their antennae subunits, but are only slightly disabled in their photochemical competence, and their concentration correlates highly with the leaf's net CO<sub>2</sub> uptake capacity. It is proposed that the smaller size of the PSUs present in the mutated leaves results from a block in chl biosynthesis that represses the expression of the light-harvesting chl a, b apoprotein genes. It is further proposed that the build up of chl precursors in mutated leaves impairs the normal turnover of proteins that make up the photosynthetic apparatus and causes photooxidative damage to the thylakoid membranes, ultimately accounting for the chloroplast loss.

## MATERIALS AND METHODS

### Plant material and growth conditions

This study was carried out with normal and sectorially chimeric stinging-nettle (*Urtica dioica* L.) plants grown in open air, under natural sunlight. The chimeric plant initially presented a typical, full green appearance, but it later developed a mutated lateral shoot of a uniformly lighter green color. All axillary buds originating from this shoot propagated the mutation, and the adult plant thus possessed branches with normal (control) leaves and branches with mutated leaves. Plants were watered and fertilized regularly, and measurements were carried out on leaves tagged on appearance and followed throughout their developmental course.

### Chloroplast isolation and pigment determination

Three grams of deveined normal leaves (or 10 g of chimeric leaves) kept in liquid nitrogen were finely broken with a mortar, 25 ml of sucrose-phosphate buffer [0.4 M sucrose, 0.15 M KCl, 2% sodium ascorbate, 1% polyvinylpyrrolidone, and 0.05 M potassium phosphate buffer (pH 6.8)] were added, and the slurry was homogenized by 2×5-second bursts in a Waring blender. The homogenate was strained through 8 layers of cheesecloth and centrifuged at 1,000 xg for 10 min to pellet down the chloroplasts (Henriques, 2004). Chl and car were extracted with 80% acetone and quantified spectrophotometrically using the equations of Porra et al. (Porra et al., 1984) and Lichtenthaler (Lichtenthaler, 1987), respectively.

### Gas exchange and chlorophyll fluorescence measurements

Measurements of leaf net CO<sub>2</sub> uptake were performed with an open, flow-through gas exchange system (LCi, Hansatech, King's Lynn, Norfolk, England) at ambient CO<sub>2</sub> levels, as described before (Henriques, 2003). *In vivo* chlorophyll fluorescence parameters were recorded using

a direct portable fluorometer (PEA, Hansatech Ltd, King's Lynn, Norfolk, England). Leaves were dark-adapted for 30 min before measurements were taken. Initial ( $F_0$ ), maximum ( $F_m$ ) and variable ( $F_v = F_m - F_0$ ) fluorescence yields as well as the  $F_v/F_m$  ratio were recorded. The area over the fluorescence induction curve measured in DCMU treated leaves (Srivastava et al., 1997) was also recorded. This area is directly related to the number of Q<sub>A</sub> molecules and is taken to indicate the number of active PSII units per illuminated leaf area volume, hereafter referred to as PSII concentration. Given the small differences in  $F_v/F_m$  found between the control and mutated leaves, this approach was used to compare PSII concentrations between the two genotypes.

### Protein preparation and SDS-polyacrylamide gel electrophoresis

After isolation, chloroplasts were twice washed with a 0.05 M phosphate buffer (pH 7.4) containing 0.15 M KCl, incubated for 30 min in 1 mM EDTA (pH 8.0), and centrifuged at 15,000 xg for 10 min. EDTA-washed chloroplasts were lipid-extracted several times with a chloroform:methanol mixture (1:2, v:v), followed by 3 washes with anhydrous methanol, and the resulting protein was dried under vacuum (Henriques and Park, 1975). The dried protein was dissolved in 0.0625 M Tris-HCl (pH 6.8), 5% glycerol, 5% β-mercaptoethanol and 2% SDS at a concentration of 1 mg protein/ml, incubated overnight at 37°C and heated in boiling water for 2 min. For electrophoresis, a 1.5 cm long, 3% acrylamide stacking gel (pH 6.8) and a 9.0 cm long, 9% acrylamide separating gel were used. The discontinuous SDS-PAGE system of Laemmli was used (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R overnight and destained in methanol:water:acetic acid (50:875:75, v:v:v) as described before (Henriques and Park, 1975). Molecular mass estimates were determined using Bioscience low-range protein standards (Amersham, Malmo, Sweden).

### Microscopy studies

Leaf segments were fixed in chilled 2% glutaraldehyde in 50 mM Na-phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide and dehydrated in a graded series of ethanol solutions. For light microscopy, the slices were embedded in methyl methacrylate and sectioned; thick sections were mounted on clear glass slides, stained with 1% toluidine blue and observed with a Leitz optical microscope. For scanning electron microscopy, the leaf slices were dried to the critical point, mounted on a specimen stub, coated with gold, and examined in a Jeol JSM 35 CF scanning electron microscope.

### Data presentation and statistical analyses

Data presented are the mean±SD of three independent experiments each with three to five replications. Comparisons between means were carried out by one-way

ANOVA (F-ratio test). Different letters indicate significant differences at  $P \leq 0.05$ .

## RESULTS

The sectorially chimeric stinging-nettle plant here described developed axillary branches with mutated leaves of identical genotype and uniform color. The mutated leaves were initially of a light-green color (hereafter referred to as young expanded leaves), turning to a yellow-greenish color after full expansion (hereafter referred to as mature expanded leaves) and whitening subsequently. Mean pigment contents and net  $\text{CO}_2$  uptake of mutated and control leaves are presented in Table 1. Data on pigments show that during the development of mutated leaves, the contents of both chl and car decrease progressively, reaching ca. 20% and 25%, respectively, of control values in mature expanded leaves. This loss of total pigment is accompanied by a significant increase in chl a/b and decrease in chl/car ratios, indicating that there occurs a preferential loss of chl b and a relative enrichment in car during the ontogeny of mutated leaves. These changes in pigment levels and ratios reflect major alterations in the number and composition of PSUs in mutated leaves, as will be discussed below.

Table 1 also compares net photosynthetic rates of control and mutated leaves. The net  $\text{CO}_2$  uptake rate of control leaves is within the range reported for many herbaceous  $\text{C}_3$  species and is significantly higher than those of mutated leaves on an area basis. Photosynthetic rates of young and mature expanded mutated leaves decreased to ca. 64 and 24% of control, respectively. The extent of this reduction in the photosynthetic rates of mutated leaves is significantly smaller than the extent of their losses in chl content, thus indicating that some of the lost chl served only as antenna pigment and was not essential for  $\text{CO}_2$  assimilation in the conditions under which measurements were carried out. This becomes immediately apparent when photosynthetic rates are expressed on a chl weight basis, which reveals that mutated leaves display higher photosynthetic rates than the controls (Table 1).

Control leaves showed an  $F_v/F_m$  ratio of  $0.824 \pm 0.006$  (Table 2), identical to those reported previously for leaves of higher plants (Björkman and Demming, 1987). This  $F_v/F_m$  ratio underwent a small, though statistically significant, decrease in young expanded mutated leaves and fell slightly further in older mutated leaves. Identical  $F_v/F_m$  values were found when the measurements were carried out at pre-dawn and after a 30-min dark adaptation. Mutated leaves also showed rather extensive decreases in their PSU concentrations, amounting to about 30 and 70% for young and mature expanded leaves, respectively (Table 2). The concentration of the remaining PSUs was found to correlate strongly with the measured photosynthetic rates ( $y = 0.0523x + 0.031$ ,  $R^2 = 0.9832$ ), thus confirming the PSUs present in mutated leaves retain much of their full functional competence.

**Table 1.** Pigment content and photosynthetic net  $\text{CO}_2$  uptake rate of control (mature leaf) and mutated leaves of chimeric stinging-nettle.

Variable	Treatment		
	Control	Young mutated	Mature mutated
Total chl ( $\mu\text{g}\cdot\text{cm}^{-2}$ )	$33.0 \pm 1^a$	$13.4 \pm 1^b$	$6.8 \pm 1^c$
Chl a/b	3.3 <sup>a</sup>	4.6 <sup>b</sup>	7.5 <sup>c</sup>
Total car ( $\mu\text{g}\cdot\text{cm}^{-2}$ )	$8.0 \pm 0.5^a$	$3.7 \pm 0.5^b$	$2.1 \pm 0.5^c$
Chl/car (molar ratio)	2.6 <sup>a</sup>	2.1 <sup>b</sup>	1.9 <sup>c</sup>
Net $\text{CO}_2$ uptake ( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$17.6 \pm 1^a$	$13.4 \pm 1^b$	$4.2 \pm 1^c$
Net $\text{CO}_2$ uptake ( $\mu\text{mol}\cdot\text{mg}^{-1}\text{chl}\cdot\text{s}^{-1}$ )	0.053	0.1	0.062

\*Results are the mean of three values  $\pm$  SD of three independent experiments. Different letters indicate statistically significant differences at  $P \leq 0.05$ .

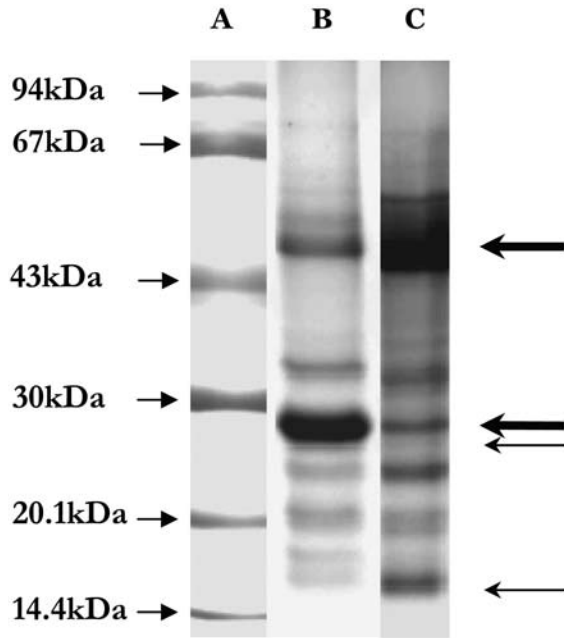
**Table 2.** Variable to maximum chl fluorescence ratio and concentration of PSII units in control (mature leaf) and mutated stinging nettle leaves.\*

	Control	Young mutated	Mature mutated
$F_v/F_m$	$0.824 \pm 0.006^a$	$0.799 \pm 0.008^b$	$0.766 \pm 0.009^c$
PSU conc.	100%	68%	27%

\*Results are the mean  $\pm$  SD of at least ten measurements. Different letters mean statistically significant differences at  $P \leq 0.05$ .

Figure 1 shows that the polypeptide composition of chloroplasts from normal and mutated leaves is qualitatively identical although some gross quantitative differences are immediately apparent, particularly in the 55 and 27 kDa regions. Most conspicuous in chloroplasts from mutated leaves is the large depletion of a protein band in the 27 kDa region of the gel, but the smaller peak running ahead of the 27 kDa band also showed a large decrease. The peak at 55 kDa corresponds to the large subunit of Rubisco (Henriques and Park, 1976), and its increase in the mutant profile results mostly from the relative decrease in the 27 kDa component for an equivalent amount of protein loaded onto the gels, but also from some unstacking that occurs in the chloroplasts of mutated leaves and that provides additional area for Rubisco adsorption. Note that the peak at 15 kDa, corresponding to the small subunit of Rubisco, is likewise enriched in the polypeptide profile of chloroplasts from mutated leaves.

Figure 2 shows scanning electron micrographs of normal (A) and expanded mutated (B) leaves from stinging-nettle. The transverse sections of mutated leaves showed no obvious morphological alteration relative to controls, exhibiting a characteristic mesophyll arrangement



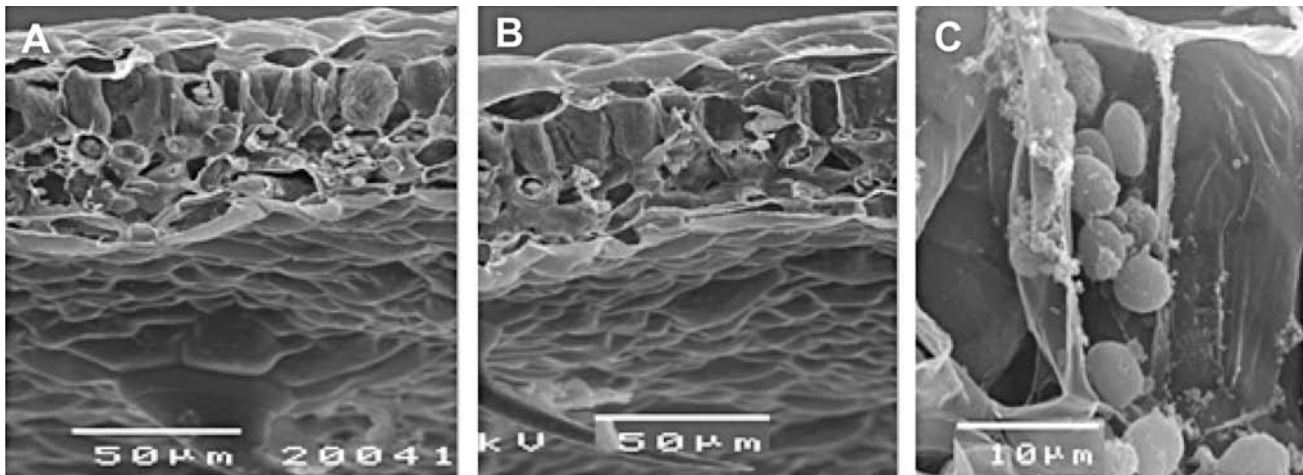
**Figure 1.** Polypeptide profiles of EDTA-washed, lipid-extracted chloroplast membranes from normal (B) and mutated (C) leaves of stinging-nettle; approximate equal amounts of total protein were loaded in the two lanes. Lane A shows the mol wt markers. Arrows on the right indicate major differences found between the mutant and wild type.

with an upper palisade and a lower spongy parenchyma. A large hair, with a multicellular basis, and a smaller one were visible in the lower epidermis of control and mutated leaves, respectively; stomata were restricted to the lower epidermis. Control cells that were fractured during leaf sectioning revealed the presence of a large number of chloroplasts (Figure 2C); however, far fewer chloroplasts were observed in mutated leaves, where cells were often collapsed. In an attempt to place this difference into a more quantitative perspective, leaf sections of control and mutated leaves were also examined by light microscopy.

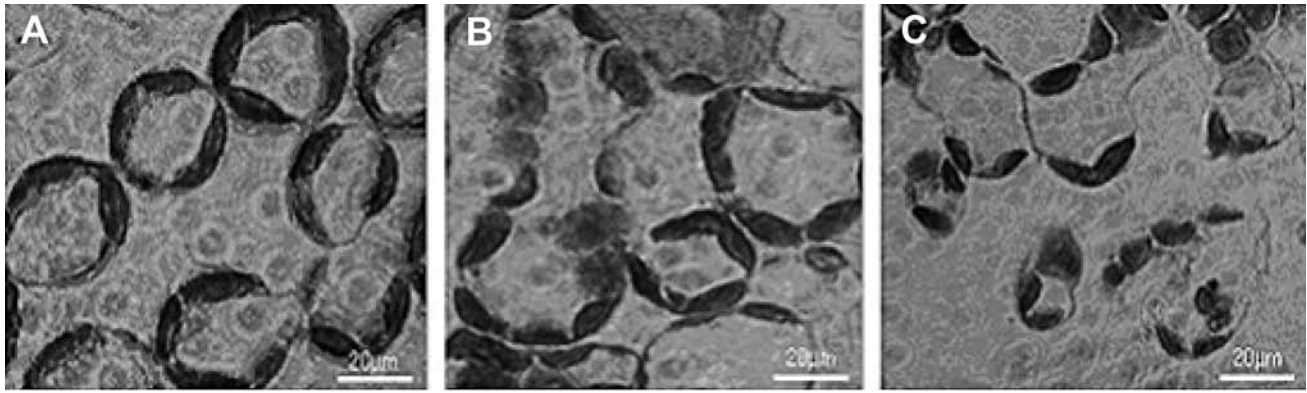
Figure 3 shows that control cells (Figure 3A) possessed a large number of chloroplasts arranged in a compact manner in the cell periphery while young expanded (Figure 3B) and mature expanded (Figure 3C) mutated leaves contained progressively fewer organelles. Given some variability among examined leaf samples, it can be only concluded that mature expanded leaves contained somewhere between one-third and one-fourth as many chloroplasts as controls.

## DISCUSSION

The results presented show that, on an area basis, the mutated leaves of chimeric stinging-nettle have a decreased photosynthetic capacity compared to their wild type siblings and that the magnitude of this decrease augments from young expanded to mature expanded leaves. It is further shown that this photosynthetic decline is highly correlated with the loss of PSUs per unit leaf area, but only indirectly related to the reduction in leaf chl content. This indicates that part of the lost chl served only as antenna pigment and is consistent with the much higher chl a/b ratios observed in mutated leaves, which reveal a preferential loss of chl b relative to chl a. In PSII, chl b is exclusively located in the LHC-II antenna [note that the term LHC-II is used here to refer to the whole chl-a, b light-harvesting chl-protein complex associated with PSII, comprising both the minor, tightly-bound LH-CIIa (CP29), LH-CIIc (CP26), LH-CII d (CP24), and PsbS (CP22) complexes, as well as the major, reversibly-bound LHC-IIb containing the Lhcb1/2 gene products], a family of closely related, nuclear-encoded chl a, b-binding polypeptides organized around the PSII core (Jansson, 1994; Green and Durnford, 1996). Recently, high resolution structural studies of the Lhcb1 and Lhcb2 subunits of the complex revealed the presence of 14 chls per polypeptide, 8 chls a and 6 chls b, as well as 4 carotenoids (Liu et al., 2004). There are no such detailed studies for most of the other LHC-II subunits, but biochemical analyses suggest that,



**Figure 2.** Scanning electron micrographs of cross-sections from normal (A) and mutated (B) leaves. C shows a magnified view of a ruptured control mesophyll cell with several chloroplasts.



**Figure 3.** Light microscopy of cross sections of normal (A), young (B) and mature (C) expanded mutated stinging-nettle leaves. Note the difference in chloroplast number of mesophyll cells between normal and mutated leaves.

in spite of some variability in their chl/protein ratios, their chl a/b ratios are not markedly different from that of the Lhcb1/2 monomers (Jansson, 1994; Green and Durnford, 1996), which would yield an overall chl a/b ratio of near 1.5 for the bulk LHC-II complex against an overall 3.3 for whole chloroplasts of stinging-nettle. Thus, removal of the chl b-enriched LHC-II subunits would decrease chl content and raise chloroplast chl a/b ratios, but should have no direct negative effect on PSII photochemical activity, as was found here. In fact, the  $F_v/F_m$  ratio in younger mutated leaves is rather close to that of controls, and this ratio decreases only slightly in older mutated leaves. The  $F_v/F_m$  ratio of dark-adapted leaves estimates the intrinsic quantum efficiency of PSII photochemistry, and the values measured in mutated leaves indicate that the remaining PSII units have undergone only minor impairments in their photochemical capacity. We cannot ascertain unequivocally the causes for this  $F_v/F_m$  ratio decrease in mutated leaves, but the fact that it is also observed at pre-dawn strongly indicates that it arises from the presence of a number of damaged PSII units concurrent with normal, fully-functioning ones. These damaged photosynthetic units absorb light energy but are unable to perform photochemistry, and thus lower the  $F_v/F_m$  ratio of the mutated leaves. In any case, it is clear that the PSII units remaining in the chloroplasts of mutated leaves retain most of their photochemical capacity, in spite of an extensive loss of their antenna complex. This conclusion is supported by comparative analysis of chloroplast polypeptide profiles from normal and mutated cells that revealed major differences between the two in the 25 to 27-kDa region. It is known that the polypeptides in this range comprise the protein moiety of the LHC-II complex (Henriques and Park, 1975; Burke et al., 1979), and their depletion in the chloroplasts from mutated cells further attests to the loss of a significant part of this complex. In particular, the major LHCII-b component appears to be largely missing. This PSII antenna component is relatively depleted in carotenoids, displaying chl/car ratios of 4.0 to 5.5 (Lichtenthaler et al., 1981; Lichtenthaler and Babani, 2004), and its preferential loss is revealed by the lower cha/b ratio found in mutated leaves.

The results just discussed suggest two immediate alternative explanations for the losses in the PSII-antenna components observed in mutated leaves. The first is that the primary effect of the mutation was to partially block chl biosynthesis, causing an accumulation of its precursors that, in turn, negatively controlled the expression of the apoprotein of LHC-II. The second is that the mutation originally affected the synthesis of the LHC-II polypeptides, thereby resulting in the absence of the protein scaffold to which the pigments would have anchored, which turned off their biosynthesis. One cannot select between these two alternatives based on the present experimental data but numerous genetic and biochemical studies, namely with a number of chl-deficient mutants (Cumming and Bonnet, 1983; Green et al., 1988; Klein et al., 1988), have shown that chl is necessary for both the synthesis and proper assembly of the LHC-II, thus favoring the first alternative. Recently, Reinbothe et al. (2006) showed that a lack of capacity to convert chlorophyllide a into chlorophyllide b in the chloroplast inhibits the import and stabilization of chl-binding light-harvesting proteins, further supporting the first alternative. Future work will provide a more solid basis from which to judge between the two alternatives.

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# 一種刺人的蕁麻之變種葉子（含斑點，缺葉綠素，對光敏感），及其光合作用之特性

Fernando S. HENRIQUES

Plant Biology Unit, Faculdade de Ciências e Tecnologia,  
Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

刺人蕁麻 (*Urtica dioica* L.) 部位鑲嵌之突變葉子顯示降低之色素含量及下降的光合作用活用。相對於野生型而言，變種葉不但大量缺少總葉綠素及胡蘿菠素（暗示喪失了大部份之光合作用單位），而言表現出非常高之葉綠素 a/b 比率（暗示剩餘之光合作用單位其天線大小已減小）。光學顯微鏡及電子顯微鏡証實變種葉中葉綠體數目明顯減少；多肽成分之分析顯示在剩餘之葉綠體中光合作用系統 II 之天線脫輔基蛋白大為減少。在變種葉之光合作用單位之內在光化學效率（以測得之螢光為分子，以暗適應之最大螢光為分母所得之比率表示）只比控制組略低，而且其數值和葉子之淨二氧化碳吸取能力呈強度相關。本文結論：所觀察之變種葉在發育過程，逐漸喪失葉綠體，但是殘存之葉綠體保存它們大多數之光合作用能力。葉綠體數目減少之原因很可能源自葉綠素前驅體之累積，進而抑制光合作用必需蛋白質之生合成，並且做為光敏劑導致葉綠素之分解。

**關鍵詞：**鑲嵌作用；葉綠素螢光；葉綠素胜肽；光合作用之二氧化碳淨吸取；蕁麻。

