

Photosynthetic properties and photosystem stoichiometry of *in vitro*-grown juvenile, adult, and rejuvenated *Sequoia sempervirens* (D. Don) Endl.

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ABSTRACT. Photosynthetic properties and photosystem stoichiometry of *in vitro*-grown juvenile, adult, and rejuvenated *Sequoia sempervirens* shoots were characterized. 77K fluorescence analysis indicated that photosystem II/photosystem I ratios were highest for chloroplasts in adult shoots. Photosynthetic oxygen evolution rates (on the same chlorophyll bases) were also slightly higher for chloroplasts in adult shoots. Our results suggested a significant alteration of photosystem stoichiometry in chloroplasts of *Sequoia sempervirens* shoots during phase change. In addition, chlorophyll *a* fluorescence analysis showed that juvenile, adult, and rejuvenated shoots showed virtually identical maximal quantum efficiencies of photosystem II (F_v/F_M). Nonphotochemical quenching (NPQ) during actinic light illumination, however, was significantly enhanced for AS76 adult shoots. The differences in photosynthetic properties and photosystem stoichiometry among juvenile, adult, and rejuvenated shoots may reflect adjustments in the photosynthetic apparatus to acclimate to distinct physiological states of *Sequoia sempervirens* during phase changes.

Keywords: Nonphotochemical quenching; Phase change; Photosystem stoichiometry; Photosynthesis; *Sequoia sempervirens*.

INTRODUCTION

Plants undergo sequential and distinct developmental stages, or phase changes, during their life cycle. The process normally begins with a strictly vegetative juvenile phase and culminates in the sexually reproductive mature adult phase. In trees, this phase change process can take several years. *Sequoia sempervirens* (D. Don) Endl, a coastal redwood, can be rejuvenated through repeatedly grafting its adult-phase shoot tips onto juvenile rootstocks *in vitro* (Huang et al., 1992). The rejuvenated shoots completely restored rooting competence and renewed vigor of roots and shoots (Huang et al., 1992). Apparent changes in esterase and peroxidase isozymes (Huang et al., 1996), different tyrosine phosphorylation patterns, and higher total protein phosphorylation were also observed in juvenile and rejuvenated shoots (Kuo et al., 1995 and Huang et al., 2003a). Juvenile and adult phase shoots also showed restriction fragment length polymorphism of mitochondrial DNA (Huang et al., 1995).

Growth rate, shoot elongation, and rates for photosynthesis and respiration (determined by fresh weight) were all higher for the juvenile and rejuvenated *S. sempervirens* shoots (Huang et al., 2003b). The photosynthetic rates were associated with more chlorophyll, especially chlorophyll *a*, in the juvenile and rejuvenated shoots. However, almost identical maximal quantum efficiency of PSII photochemistry (F_v/F_M) in juvenile, adult, and rejuvenated tissues indicated that the photosystems were operating with equal efficiency (Huang et al., 2003b).

In this work, we characterized photosynthetic activities and photosystem stoichiometry of intact chloroplasts from juvenile, adult, and rejuvenated *S. sempervirens* shoots. We also monitored PSII photochemistry and thermal energy dissipation on these shoots using chlorophyll *a* fluorescence analysis. The differences in photosynthetic properties and photosystem stoichiometry of juvenile, adult, and rejuvenated *S. sempervirens* shoots and their physiological significances are addressed.

MATERIALS AND METHODS

Tissues analyzed

Sequoia shoots at different developmental stages or

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phases were cultured as previously described (Huang et al., 1992). The adult shoot stocks (AS76 and AS94) were established by culturing shoot tips excised in 1976 and 1994, respectively. Cultures of juvenile shoot cuttings maintained as shoot stocks were established using fresh *in vitro*-germinated seeding. Rejuvenated shoots (RS76 and RS94) were obtained by repeatedly grafting the shoot tips five times from the two adult *Sequoia* shoots (AS76 and AS94) by grafting the adult shoot tips onto juvenile rootstocks. After each grafting, shoots were severed and maintained in stocks by sub-culturing 2-3 cm terminal sections every 2 months in MS-based medium (Murashige and Skoog, 1992). *Sequoia* shoots were grown under 60-65 $\mu\text{mol quanta}/(\text{m}^2\cdot\text{s})$ light intensity.

Isolation of intact chloroplasts

40 g shoot tissues (stem with leaves) were homogenized in 500 ml chloroplast (CP) buffer (50 mM HEPES-KOH, 1 mM MgCl_2 , 1 mM MnCl_2 , 2 mM EDTA, 0.5 M sucrose, pH 8.0) with a pre-cooled blender. Homogenate was filtered through two layers of miracloth and centrifuged at 200 g for 10 min. Supernatant was collected and centrifuged at 9870 g, and stop when reached at 9870 g. Supernatant was decanted and the pellet was re-suspended in 5 ml CP buffer. The suspension was loaded on a Percoll gradient (20-40-60%) and centrifuged in a swing bucket rotor at 4776 g for 15 min. Dark green intact chloroplast bands were collected at 20/40 and 40/60% interface. The intact chloroplast fractions were diluted with 4-6 volumes of CP buffer and centrifuged at 4471 g for 5 min. The pellet was re-suspended with a small volume of CP buffer.

Measurement of photosynthetic oxygen evolution

Steady state rates of oxygen evolution were measured with a Clark-type oxygen electrode (YSI model 5331 oxygen probe) fitted with a water-jacketed cell at 25°C (Hung et al., 2010). The oxygen evolution of isolated chloroplasts (32 μg of Chl ml^{-1}) was measured in CP Buffer and in the presence of 2 mM potassium ferricyanide and 2 mM 2, 6-dichloro-*p*-benzoquinone (DCBQ) as electron acceptors. Saturating illumination [about 2000 $\mu\text{mol quanta}/(\text{m}^2\cdot\text{s})$] was provided from both sides of the water-jacketed cells by two fiber-optics (Dolan-Jenner model MI 150) illuminators.

Measurement of chlorophyll a fluorescence

Chlorophyll *a* fluorescence measurements were performed with a dual pulse-amplitude-modulation fluorometer (Walz) at room temperature. Just prior to fluorescence measurements, the shoots were placed in darkness for 5 min. The dark fluorescence yield (F_0) in the dark-adapted state was measured by exciting with the pulse-modulated measuring light with sufficient low intensity not to induce significant variable fluorescence. Each shoot tip was then given one flash (0.6 s) of saturated pulse light to obtain the maximal (F_m) fluorescence value. The dark-adapted F_v/F_m

ratio is a measure of the maximal quantum efficiency of PSII photochemistry (Baker, 2008). In illuminated samples, the maximum fluorescence yield, F_m' is observed. Non-photochemical quenching, $\text{NPQ} = (F_m - F_m')/F_m'$, estimates changes in the apparent rate constant for excitation decay by heat loss induced by light relative to this rate constant in the dark (Baker, 2008). A moderate actinic light intensity [about 80 $\mu\text{mol quanta}/(\text{m}^2\cdot\text{s})$] was applied during NPQ measurement in order to assure light limitation at steady state illumination.

Measurement of fluorescence at 77 K

Fluorescence emission spectra were recorded with a fluorescence spectrometer (Jasco model FP-6500). All the measurements were carried out at 77 K using chloroplasts at a chlorophyll concentration of 50 $\mu\text{g}/\text{ml}$. The excitation wavelength used to excite chlorophyll was 435 nm (excitation bandwidth 5 nm, emission bandwidth 1 nm).

RESULTS

Isolation of intact chloroplasts

Figure 1 shows the separation of intact chloroplasts (dark green bands) from *S. sempervirens* shoots on a Percoll gradient (20-40-60%). Intact chloroplasts of juvenile, adult, and rejuvenated *S. sempervirens* shoots were located at 20/40 and 40/60% interface. The intactness of isolated chloroplasts was routinely checked by the microscope. Figure 2 shows a microscopy photograph of isolated intact chloroplasts from *S. sempervirens* shoots. Red auto-fluorescence of chlorophyll signified the images of chloroplasts. Isolated chloroplasts were mostly intact and their sizes were in the range of 5-10 μm .

Photosynthetic properties of intact chloroplasts

Table 1 shows photosynthetic O_2 evolution of intact chloroplasts from juvenile, adult, and rejuvenated *S. sem-*

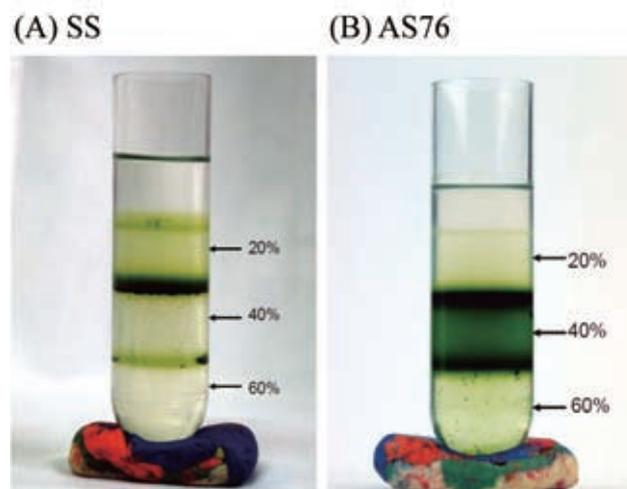


Figure 1. Separation of intact chloroplast (dark green bands) of *Sequoia sempervirens* shoots by Percoll gradients. (A) from juvenile shoots (SS); (B) from adult shoots (AS76).

sempervirens shoots. Photosynthetic O₂ evolution rates (on the same amount of chlorophyll basis) were significantly higher for chloroplasts from adult (AS76 and AS94) shoots than from juvenile (SS) and rejuvenated (RS76 and RS94) ones. Photosynthetic O₂ evolution rates for chloroplasts from adult (AS76 and AS94) shoots ranged from 143 to 155 μmoles of O₂ per mg of chlorophyll per hour. The O₂ evolution rates for chloroplasts from juvenile (SS) and rejuvenated (RS76 and RS94) ones were 138, 128 and 120 μmoles of O₂ per mg of chlorophyll per hour, respectively.

Fluorescence emission spectroscopy at 77K

Figure 3 shows 77 K fluorescence emission spectra of chloroplasts from *in vitro*-grown juvenile (SS), adult (AS76 and AS94), and rejuvenated (RS76 and RS94) *S. sempervirens* shoots. The emission peak at ~727 nm originated from photosystem I (Demming and Björkman, 1987). The emission peak at ~685 nm may originate from CP43 and light harvesting complexes, whereas the origin of the emission peak at ~693 nm originated from functional photosystem II (Hung et al., 2010). The relative contents of photosystems II and I were estimated from the amplitudes of fluorescence peaks at ~693 and ~727 nm, respectively. Our results in Figure 3 indicate that photosystem II/ photosystem I ratios were significantly higher in the adult (AS76 and AS94) shoots than in the juvenile (SS) and rejuvenated (RS76 and RS94) ones.

Room temperature chlorophyll fluorescence analysis

Table 2 shows the maximal quantum efficiency of PSII photochemistry (F_v/F_m) in *in vitro*-grown juvenile, adult, and rejuvenated *S. sempervirens* shoots. The F_v/F_m values were almost identical among juvenile (SS), adult (AS76 and AS94) and rejuvenated (RS76 and RS94) shoots. This result confirmed our previous work (Huang et al., 2003b). In addition, this parameter was found to have value around 0.83 under optimal physiological conditions (Demming and Björkman, 1987). The F_v/F_m values for *in vitro*-grown *S. sempervirens* shoots (Table 1) were very close to this reported optimal value, 0.83, suggesting that all

Table 1. Photosynthetic oxygen evolution rates (μmoles O₂/mg of Chl x hour) of chloroplasts in juvenile (SS), adult (AS76 and AS94), and rejuvenated (RS and RS94) *S. sempervirens* shoots.

Type	Oxygen evolution rates	%
SS	138 ± 15	100
RS94	120 ± 8	87 ± 5
RS76	128 ± 9	93 ± 7
AS94	155 ± 13	113 ± 10
AS76	143 ± 15	104 ± 11

Values are the mean±SD (n=6 from 3 independent experiments).

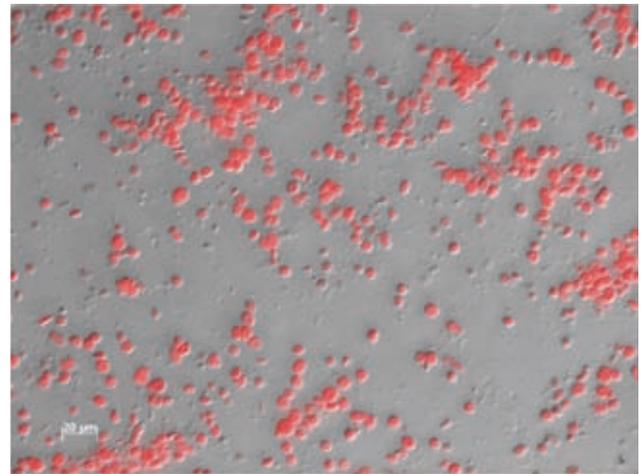


Figure 2. Photograph of isolated chloroplasts of *Sequoia sempervirens* shoots by using a ZEISS Axio Imager.Z1 Microscope and PvCam. Red auto-fluorescence of chlorophylls signified the images of chloroplasts.

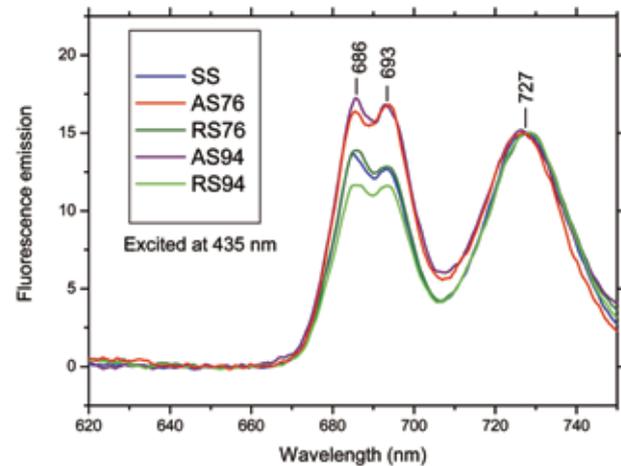


Figure 3. 77K-fluorescence emission spectra of chloroplasts from juvenile, adult, and rejuvenated *Sequoia sempervirens* shoots by exciting chlorophyll at 435 nm (Excitation bandwidth 5 nm; Emission bandwidth 1 nm). Spectra were normalized at 727 nm. All measurements were carried out at 77K, using chloroplast suspensions at a chlorophyll concentration of 50 μg/mL.

Table 2. Maximal quantum efficiency of photosystem II for juvenile (SS), adult (AS76 and AS94), and rejuvenated (RS76 and RS94) *S. sempervirens* shoots.

Type	F_v/F_m
SS	0.82 ± 0.06
AS76	0.81 ± 0.03
RS76	0.81 ± 0.01
AS94	0.78 ± 0.04
RS94	0.82 ± 0.02

Values are the mean±SD (n=6 from 3 independent experiments).

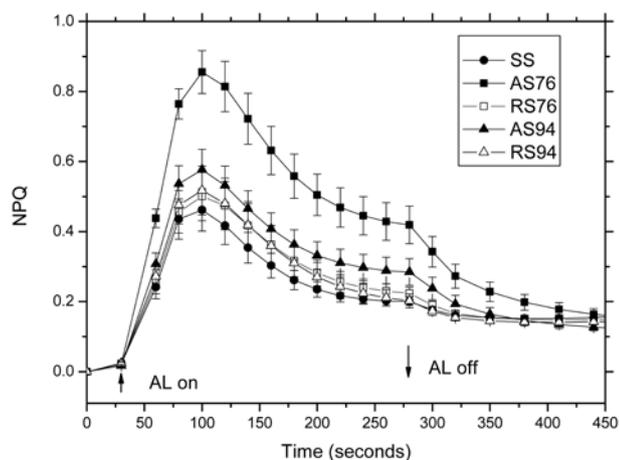


Figure 4. Time-dependent nonphotochemical quenching (NPQ) of juvenile, adult, and rejuvenated *Sequoia sempervirens* shoots in the presence and absence of CW actinic light (AL). Conditions: Samples were incubated in darkness for 5 min. The intensity of the actinic light was about $80 \mu\text{mol quanta}/(\text{m}^2\cdot\text{s})$. Values are the mean \pm SE ($n=12-15$).

these tissues were in good physiological condition. Figure 4 shows time-dependent non-photochemical quenching (NPQ) of *in vitro*-grown juvenile, adult, and rejuvenated *S. sempervirens* shoots in the presence and absence of CW actinic light (AL). NPQ values under actinic light (AL) were apparently higher for AS76 adult tissues compared to juvenile (SS), and rejuvenated (RS76 and RS94) ones. It requires further study to determine whether NPQ of AS94 is significantly higher than that of rejuvenated (RS76 and RS94) ones. Our results suggested that thermal dissipation of exciting energy during photosynthesis was significantly enhanced in AS76 adult *S. sempervirens* shoots.

DISCUSSION

Our results revealed significant differences in the photosynthetic properties and photosystem stoichiometry of *S. sempervirens* shoots during phase change. Our 77K fluorescence analysis in Figure 3 suggested that photosystem II/photosystem I ratios were significantly higher in adult *S. sempervirens* shoots (AS76 and AS94). Our results indicated significant alterations of photosystem stoichiometry in chloroplasts of *S. sempervirens* shoots during phase change. Our previous results showed that photosynthesis rates and overall chlorophyll contents (determined by fresh weight) were lower in adult *S. sempervirens* shoots (AS76 and AS94) (Huang et al., 2003b). Taken together, we proposed that slightly higher photosynthetic O_2 evolution rates in chloroplasts (on the same chlorophyll basis) from adult *S. sempervirens* shoots (see Table 1) might be due to significantly higher photosystem II/photosystem I ratios in adult *S. sempervirens* shoots. It still requires future experiments to verify our proposal.

The higher photosynthetic rates, nitrogen content, and respiration rates (determined by fresh weight) observed

in the juvenile and the rejuvenated *S. sempervirens* shoots are correlated with their more vigorous and rapid growth (Huang et al., 2003b). The increased nitrogen enables enhanced protein synthesis; photosynthesis and respiration provide the required energy for rapid growth. In addition, there was no significant difference in maximal quantum efficiency of PSII (F_v/F_m) in shoot tissues of *in vitro*-grown juvenile, adult, and rejuvenated *S. sempervirens* (Huang et al., 2003b and Table 2). However, non-photochemical quenching (NPQ) results suggest that a significantly larger fraction of exciting energy was dissipated as heat during the photosynthesis process in AS76 adult *S. sempervirens* shoots. Because AS76 adult *S. sempervirens* shoots have significantly lower photosynthetic rates (determined by fresh weight) but are accompanied by normal photosystem II photochemistry (F_v/F_m), the AS76 adult shoots may employ non-photochemical quenching (NPQ) to dissipate excess excitation energy and protect their photosynthetic apparatus from photo-inhibition (Horton and Ruban, 2005). Therefore, these differences in photosynthetic properties and photosystem stoichiometry of juvenile, adult, and rejuvenated shoots may reflect the adjustments of photosynthetic apparatus to acclimate the distinct physiological states of *S. sempervirens* during phase changes.

In our recent study, the protein abundance and transcript abundance of a phosphoprotein, SsOEE2, were consistently lower in *Sequoia* shoots during the adult stage but increased with repeated grafting (Chang et al., 2011). SsOEE2 is one of the extrinsic polypeptides on the lumen side of photosystem II. This correlation between rejuvenation and protein and transcript abundance of the gene suggests that SsOEE2 is associated with rejuvenation (Chang et al., 2011). Further study is required to determine the effect of SsOEE2 phosphorylation on photosystem II function and its role in *S. sempervirens* rejuvenation.

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紅杉試管幼年、老齡、及其復幼植莖光合作用特性和光系統比例改變之研究

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本研究主要探討紅杉不同的生長相（幼年期、成齡期、及返老還輕微後）的植莖光合作用特性和光系統比例的變化情形。發現成齡老株的葉綠體中的光系統二對應光系統一的比例和光合作用釋放氧氣的速率明顯的高於幼年莖和復幼莖。顯示紅杉的葉綠體中光系統的比例在不同的生長相有顯著的變化。雖然幼年期或成齡期的光合系統的電子傳遞效率 $(F_m - F_0)/F_m$ 並無明顯的差異，但是成齡老株 AS76 的光合作用有明顯較高的熱消散 (Nonphotochemical quenching; NPQ) 現象。這些光合作用特性和光系統比例的變化可能反映了紅杉不同的生長相間的生理狀態的差異。

關鍵詞：紅杉；生長相；光合作用；光系統比例；熱消散。

