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BRIEF COMMUNICATION

Enhanced antioxidant protection at the early stages of leaf expansion in ginkgo under natural environmental conditions

X.-S. YANG[1,2], G.-X. CHEN[1], X.-D. WEI[1] and K.-B. XIE[1]

College of Life Science, Nanjing Normal University, Nanjing 210046, P.R. China[1]
Department of Food and Bioengineering, Bengbu College, Bengbu, Anhui Province 233030, P.R. China[2]

Abstract

Photosynthetic pigments, gas exchange, chlorophyll (Chl) *a* fluorescence kinetics, antioxidant enzymes and chloroplast ultrastructure were investigated in ginkgo (*Ginkgo biloba* L.) leaves from emergence to full size. Under natural conditions, the net photosynthetic rate (P_N), contents of Chl *a*, Chl *b* and total soluble proteins and fresh and dry leaf mass gradually increased during leaf expansion. The maximum photochemical efficiency of photosystem (PS) 2 (variable to maximum fluorescence ratio, F_v/F_m) was considerably higher at the early stages of leaf development than in fully expanded leaves. During daily course, only reversible decrease in F_v/F_m was distinguished at various stages, implying that no photo-damage occurred. Absorption flux per cross section (CS) and trapped energy flux per CS were significantly lower in newly expanding leaves compared with fully expanded ones, however, dissipated energy flux per CS was only slightly lower in expanding leaves. The ratio of carotenoids (Car)/Chl decreased gradually during leaf expansion due to increasing Chl content. Moreover, activities of the antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, catalase and peroxidase, increased at the early stages of leaf expansion. The appearance of osmiophilic granules in fully expanded leaves further proves that photo-protection is significantly strengthened at the early stages of leaf expansion.

Additional key words: ascorbate peroxidase, carotenoids, catalase, chlorophyll fluorescence, chloroplast ultrastructure, *Ginkgo biloba*, net photosynthetic rate, peroxidase, superoxide dismutase.

Leaf expansion is a genetically controlled process (Sugiyama 2005). Morphological, anatomical and physiological traits significantly vary during leaf expansion (Marchi *et al.* 2008). The newly initiating leaves are often exposed to full sunlight at the topmost canopy, indicating that young leaves have to endure extremely high irradiance. However, during leaf ontogenesis, the structure and activity of the photosynthetic apparatus are altered and the capacity of carbon assimilation is rather low until the leaf fully expanded (Greer and Halligan 2001). These will inevitably result in excessive excitation energy in young leaves. It is well known that high irradiance can lead to increased production of reactive oxygen species (ROS) as by-products of photosynthesis (Smirnoff 1993, Müller *et al.* 2001). Therefore, it is a great challenge for young leaves to be subjected to strong irradiance. Nevertheless, plants have developed a number of strategies to balance the captured light energy, thereby protecting photosynthetic apparatus against photo-damage (Anderson *et al.* 1997). Among many of the photo-protective mechanisms, one of the most important mechanisms is the release of excessive excitation energy by thermal dissipation relying on xanthophyll cycle (Müller *et al.* 2001). In addition, plants have developed highly efficient antioxidant enzyme system, including superoxide

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Abbreviations: ABS/CS - absorption flux per CS; APX - ascorbate peroxidase; Car - carotenoids; CAT - catalase; Chl - chlorophyll; CS - cross section; DI_0/CS - dissipated energy flux per CS; F_v/F_m - maximum quantum yield of photosystem (PS) 2 photochemistry; P_N - net photosynthetic rate; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TR_0/CS - trapped energy flux per CS.

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* Author for correspondence; fax: (+86) 25 85898223; e-mail: gxchen@njnu.edu.cn

dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD), to protect them from the toxicity of ROS (Loggini *et al.* 1999, Jiang and Zhang 2002, Procházková and Wilhelmová 2009, Díaz-Vivancos *et al.* 2010, Hu *et al.* 2010). However, only a few papers have focused on the changes of the antioxidant enzyme defense system in young leaves, especially, at the early stages of leaf expansion (Jiang *et al.* 2005).

The most important milestone in the history of photosynthesis research is the discovery of the close correlation between light reactions in photosynthetic tissues and chlorophyll *a* fluorescence (Joly *et al.* 2005, Cai *et al.* 2010, Robredo *et al.* 2010). Introduction of user-friendly portable fluorometers further widened the scope of photosynthesis research, especially in *in situ* conditions. The most frequently utilized parameter is variable to maximum fluorescence ratio, F_v/F_m as a reliable indicator of the maximum photochemical quantum efficiency of photosystem (PS) 2 (Nayaka *et al.* 2009).

Ginkgo biloba L. is an ancient living gymnosperm. The species dates back to more than 170 million years ago (Zhou and Zheng 2003**)** and was referred by Charles Darwin as "a living fossil" (Major 1967). Ginkgo has been used in traditional Chinese as well as in modern pharmacology (Ahlemeyer and Krieglstein 2003). Meng *et al.* (1999) and Pandey *et al.* (2003) studied the response of ginkgo trees to irradiance and temperature under controlled conditions. Although changes in the activity of APX, SOD, CAT and POD during senescence have been reported (Kukavica and Jovanovic 2004), as yet there have been no studies focusing on the analysis of leaf photosynthetic and physiological traits of ginkgo during early stages of leaf expansion under natural environmental conditions. Therefore, in the present study, the changes in the gas exchange, chlorophyll *a* fluorescence, photosynthetic pigments and antioxidant enzymes as well as chloroplast ultrastructure have been investigated during leaf expansion in ginkgo grown in nature.

Ten-year-old male ginkgo (*Ginkgo biloba* L. cv. Dafozhi) trees were grown in field situated at Jiangsu Jiangdu, China. In the experiments all leaves were kept in their natural positions. Nutrition and water were supplied sufficiently throughout, thus potential nutrients and drought stresses were avoided. Measurements were performed from mid-April to early-June. Leaf area during development was determined by a leaf area meter (*LI-3000A*, *Li-Cor*, Lincoln, NE, USA). The area of fully expanded leaf was taken as 100 %, the area of unexpanded leaves was expressed as percentage of fully expanded area. Measurements were performed with several single leaves of different development stages simultaneously. All leaves used in this experiment were not shaded by other leaves during their development. During the experiment, the weather with many sunny days was typical for spring and summer in Eastern China.

Relative water content (RWC) was determined gravimetrically as described by Georgieva (2009). Total soluble protein was determined according to Bradford (1976). Net photosynthesis rate (P_N) was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, Hitchin, UK) at ambient temperature about 25 °C, CO_2 concentration 350 μmol mol^{-1} and the photosynthetic photon flux density (PPFD) 1000 μmol m^{-2} s^{-1}.

Leaf samples were collected at 08:00, immediately frozen in liquid nitrogen, and stored at -80 ºC until analysis. The fresh leaves were washed with distilled water and the petioles removed. The samples were extracted in ice-cold 80 % acetone, and the extract placed in a *TGLL-18G* centrifuge (6000 *g* for 10 min). After collecting the top solution, the precipitate was supplemented with ice-cold 80 % acetone, and centrifuged again for another 10 min. The supernatant was measured with a *UV-754* spectrophotometer at 470, 645 and 663 nm. Chl contents were calculated as described by Arnon (1949), and total carotenoids (Car) according to Lichtenthaler (1987).

In vivo chlorophyll fluorescence was measured using a *Handy-PEA* chlorophyll fluorometer (*Hansatech Instruments*, King's Lynn, Norfolk, UK). The transient red radiation was about 3 000 μmol m^{-2} s^{-1} provided by an array of three light-emitting diodes (peak 650 nm). The maximum PS 2 quantum yield (F_v/F_m) was determined in dark-adapted (20 min) leaves at 08:00, 10:00, 12:00, 14:00 and 16:00 according to Strasser *et al.* (1995). OJIP transient was analyzed according to the JIP-test. From OJIP transient, the extracted parameters (F_m, $F_{20\mu s}$, $F_{50\mu s}$, $F_{100\mu s}$, $F_{300\mu s}$, F_J, F_I *etc.*) led to the calculation and derivation of a range of new parameters according to Strasser *et al.* (1995, 2004).

Ginkgo leaves at different expanding stages (0.5 g fresh mass) were homogenized under ice-cold condition with 5 cm^3 of 50 mM phosphate buffer (pH 7.0), 10 mM ascorbic acid (AsA) and 1.0 % (m/v) polyvinyl-pyrrolidone. The homogenate was centrifuged at 20 000 *g* for 30 min, and the supernatant was collected for enzyme assays. Total SOD activity was assayed by monitoring inhibition of photochemical reduction of nitroblue tetrazolium according to Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of nitroblue tetrazolium reduction as monitored at 560 nm. APX activity was measured spectrophotometrically according to Nakano and Asada (1987). The assay was carried out in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM H_2O_2 and 0.1 cm^3 enzyme extract. The changes in A_{290} were recorded for 1 min after the addition of H_2O_2. CAT activity was determined according to Aebi (1984) by monitoring the disappearance of H_2O_2 at 240 nm in homogenization buffer contained 50 mM potassium phosphate buffer (pH 7.0), initially containing approximately 10 mM H_2O_2 and 0.2 cm^3 of enzyme extract. The activity of POD was determined in a reaction solution composed of 50 mM potassium phosphate buffer (pH7.0), 2 mM H_2O_2, 2.7 mM guaiacol and 0.05 cm^3 enzyme extract by monitoring the increase in absorbance at 470 nm due to guaiacol oxidation (Polle *et al.* 1994). The reaction mixture contained 100 mM, 16 mM guaiacol or 50 mM coniferyl alcohol, and 0.01 cm^3 of 10 % H_2O_2. The reaction was initiated by adding plant extract and followed for 10 min. All the activities of enzyme were measured using *UV-754* spectrophotometer.

The middle part of leaves was used and cut into small pieces (about 0.1 × 0.5 cm^2). These small pieces were fixed in a bottle for 2 h in 10 cm^3 of 4 % (v/v) glutaraldehyde in 0.3 M sodium phosphate buffer (pH 7.5) and the air was pumped out of the bottle with a syringe. The samples were then rinsed and post-fixed for 24 h at room temperature in 10 cm^3 of 1.0 % (v/v) osmium tetroxide with the same buffer. The post-fixed samples were dehydrated in a graded series of acetone solutions (30, 50, 70, 80 and 90 %; 15 min each) and in 100 % alcohol (three times by 7 - 8 min), and embedded in epoxy resin mixture. Ultra-thin sections (80 nm) were obtained using a *LKB-V* ultramicrotome (*LKB*, Bromma, Sweden) and were collected on copper grids (300 mesh), then stained with 1.0 % (m/v) uranyl acetate followed by 5.0 % (m/v) lead citrate. Sections were observed at 80 kV using a *H7650* (*Hitachi*, Tokyo, Japan) transmission electron microscope.

Table 1. The changes in fresh mass, dry mass, relative water content (RWC), total soluble protein content, net photosynthetic rate (P_N), Chl and Car contents and ratios, maximum quantum yield of PS 2 photochemistry (F_v/F_m), absorption flux per CS (ABS/CS), trapped energy flux per CS (TR_0/CS), dissipated energy flux per CS (DI_0/CS), and activities of SOD, APX, CAT and POD in leaves of ginkgo during their expansion (characterized as % of fully expanded leaf area). Each value represents the mean of at least five replicates ± SE. Different letters indicate statistically significant differences ($P < 0.05$).

Parameters	Daytime	32 % of fully expanded leaves	71 % of fully expanded leaves	81 % of fully expanded leaves	Fully expanded leaves
Leaf fresh mass [g leaf^{-1}]	08:00	0.19 ± 0.03c	0.30 ± 0.01b	0.37 ± 0.01a	0.35 ± 0.02a
Leaf dry mass [g leaf^{-1}]	08:00	0.04 ± 0.00c	0.08 ± 0.00b	0.11 ± 0.00a	0.11 ± 0.01a
RWC [%]	08:00	84.46 ± 3.83a	83.33 ± 1.50a	80.60 ± 4.77b	79.46 ± 2.28b
Soluble protein [mg g^{-1}(f.m.)]	08:00	0.01 ± 0.00c	0.03 ± 0.00b	0.09 ± 0.01a	0.09 ± 0.00a
P_N [μmol m^{-2} s^{-1}]	08:00	5.58 ± 0.19d	5.94 ± 0.24c	6.35 ± 0.19b	8.12 ± 0.21a
Chl a [mg g^{-1}(f.m.)]	08:00	1.18 ± 0.04d	1.34 ± 0.17c	1.55 ± 0.36b	1.99 ± 0.21a
Chl b [mg g^{-1}(f.m.)]	08:00	0.41 ± 0.06d	0.50 ± 0.11c	0.65 ± 0.09b	0.85 ± 0.15a
Chl (a + b) [mg g^{-1}(f.m.)]	08:00	1.59 ± 0.05d	1.84 ± 0.19c	2.20 ± 0.36b	2.84 ± 0.21a
Car [mg g^{-1}(f.m.)]	08:00	0.24 ± 0.03a	0.19 ± 0.02b	0.26 ± 0.04a	0.23 ± 0.02a
Chl *a*/*b*	08:00	2.88 ± 0.04a	2.68 ± 0.04ab	2.38 ± 0.06b	2.34 ± 0.06b
Car/Chl (*a*+*b*)	08:00	0.15 ± 0.01a	0.10 ± 0.02bc	0.12 ± 0.01b	0.08 ± 0.01c
F_v/F_m	08:00	0.76 ± 0.01b	0.78 ± 0.03b	0.78 ± 0.02b	0.82 ± 0.01a
	10:00	0.73 ± 0.03b	0.75 ± 0.03b	0.75 ± 0.03b	0.80 ± 0.02a
	12:00	0.72 ± 0.03b	0.73 ± 0.03b	0.73 ± 0.04b	0.76 ± 0.03a
	14:00	0.72 ± 0.03b	0.73 ± 0.03b	0.74 ± 0.03b	0.78 ± 0.02a
	16:00	0.75 ± 0.02b	0.75 ± 0.04b	0.75 ± 0.03b	0.79 ± 0.02a
ABS/CS	08:00	275.43 ± 20.40b	278.00 ± 12.97b	291.14 ± 16.96b	435.08 ± 19.90a
	10:00	278.25 ± 18.26c	290.42 ± 10.30bc	314.31 ± 14.73b	450.31 ± 20.20a
	12:00	284.21 ± 13.98c	301.85 ± 16.06bc	325.00 ± 7.63b	482.71 ± 28.35a
	14:00	286.85 ± 19.54c	299.38 ± 16.68bc	330.36 ± 19.81b	463.09 ± 25.70a
	16:00	269.00 ± 18.38c	285.46 ± 18.37c	322.36 ± 23.93b	448.23 ± 26.96a
TR_0/CS	08:00	207.63 ± 19.58c	208.26 ± 10.44c	227.14 ± 10.71b	354.04 ± 15.70a
	10:00	213.81 ± 13.18c	211.35 ± 10.00c	232.64 ± 11.18b	358.04 ± 17.36a
	12:00	215.10 ± 16.59c	214.09 ± 11.86c	235.77 ± 11.82b	359.50 ± 25.57a
	14:00	210.87 ± 12.46c	218.21 ± 10.68c	241.50 ± 15.14b	366.21 ± 18.09a
	16:00	206.14 ± 11.77c	214.79 ± 15.71c	233.95 ± 8.10b	359.50 ± 20.16a
DI_0/CS	08:00	59.72 ± 7.12c	66.32 ± 4.29b	64.01 ± 9.89b	79.73 ± 5.34a
	10:00	70.50 ± 12.35c	75.66 ± 10.94b	78.54 ± 12.66b	92.58 ± 11.84a
	12:00	70.35 ± 9.45c	83.63 ± 14.17b	86.60 ± 14.25b	116.51 ± 16.33a
	14:00	75.98 ± 11.75c	85.29 ± 11.84b	88.85 ± 10.76b	101.37 ± 12.21a
	16:00	68.81 ± 13.40c	70.67 ± 6.87c	78.89 ± 17.20b	97.67 ± 12.71a
SOD activity [U g^{-1}(f.m.)]	08:00	65.56 ± 5.05b	42.67 ± 1.59c	34.71 ± 3.39d	91.10 ± 3.54a
APX activity [μmol g^{-1}(f.m.) min^{-1}]	08:00	212.50 ± 19.24b	153.00 ± 31.18c	129.00 ± 18.73d	286.67 ± 24.28a
CAT activity [U g^{-1}(f.m.)]	08:00	281.60 ± 9.60b	209.33 ± 6.11c	153.33 ± 10.07d	405.33 ± 20.13a
POD activity [U g^{-1}(f.m.)]	08:00	106.67 ± 12.22b	80.67 ± 5.13c	68.00 ± 4.00d	144.60 ± 9.91a

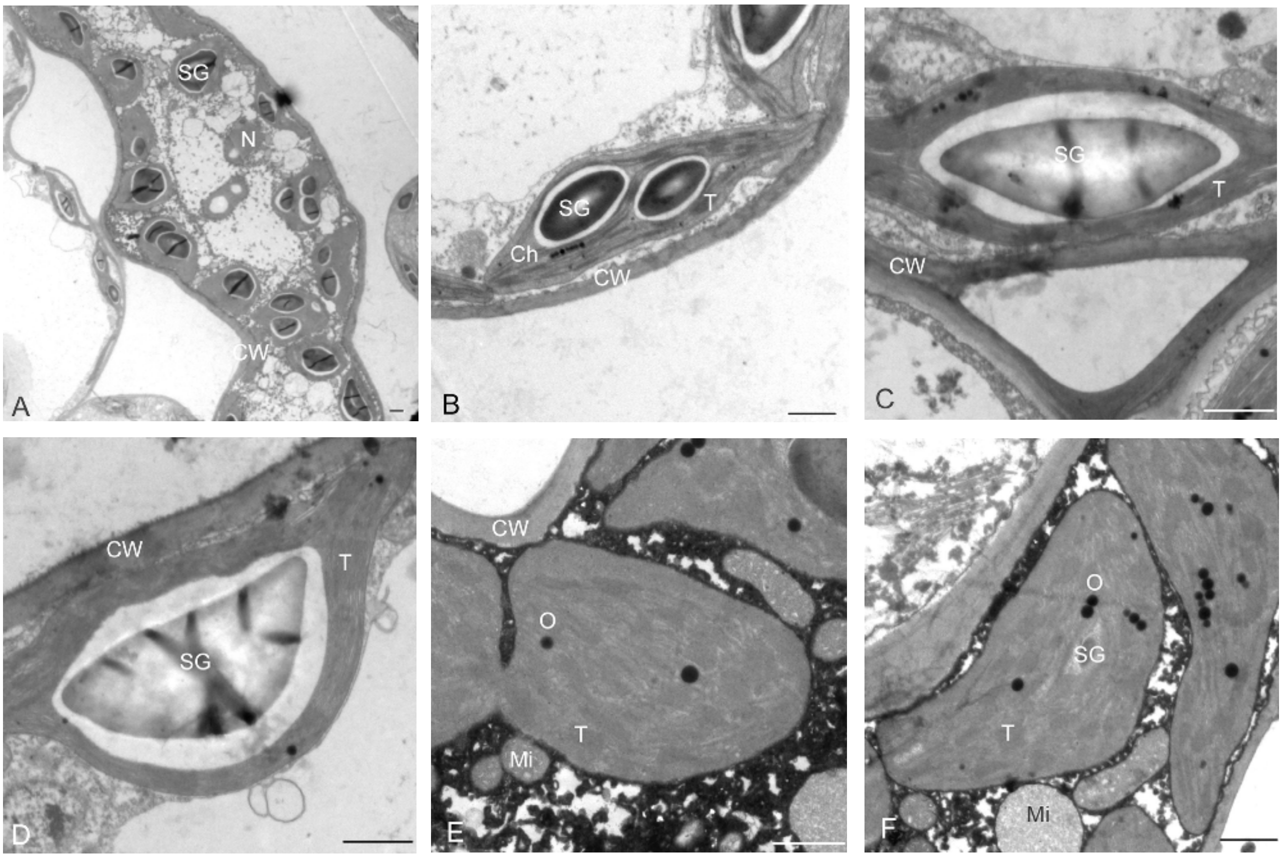

Fig. 1. Changes in the ultrastructure of chloroplasts in ginkgo leaf during expansion: *A*,*B* - the newly expanding leaves with an areas of approximately 32 % of fully expanded leaves; *C*,*D* - near fully expanded leaves with an areas of approximately 71 and 81 % of fully expanded leaves, respectively; *E*,*F* - fully expanded leaves. CW - cell wall, N - nucleus, Ch - chloroplast, Mi - mitochondria, SG - starch grain, T - thylakoid, O - osmiophilic granule. *Bars* = 1 μm.

The results were tested with *SPSS 17.0* for *Windows (SPSS Inc*., Chicago, IL, USA*)* by one-way analysis of variance (*ANOVA*) using Tukey's test calculating at *P < 0.05*.

The increase in Chl *a* and Chl *b* contents and total soluble protein content as well as the fresh and dry masses during leaf expansion was accompanied by a gradual development of photosynthetic apparatus, indicated by the increase of P_N (Table 1). The content of Chl *a* and *b* per leaf fresh mass increased significantly while the changes of total Car content were not significant. The ratio of Chl *a*/*b* and Car/Chl decreased gradually during leaf expansion. The decline of Chl *a*/*b* was owing to the fact that the increase of Chl *b* was much higher than that of Chl *a*. The decrease of Car/Chl during leaf expansion was owing to the fact that the Chl content increased while Car content not. The fact that Car/Chl in newly expanding leaves was significantly higher than in fully expanded leaves probably indicated a higher capacity of thermal dissipation in young leaves.

During leaf expansion, more excited energy would be utilized in CO_2 assimilation rather than dissipated, which was certified by the significant increase of absorption flux per CS (ABS/CS) and trapped energy flux per CS (TR_0/CS) but slight increase of dissipated energy flux per CS (DI_0/CS) (Table 1).

The daily courses of F_v/F_m showed that, not only fully expanded leaves, but also young leaves were not seriously photoinhibited by strong irradiance under field condition. These results proved that effective photoprotective mechanisms were accomplished in young leaves, especially, at the early stages of leaf expansion.

The activity of SOD was markedly enhanced at the initial stages of leaf development, which indicated the improvement of the ability to remove $O_2^{\bullet -}$. It is interesting to note that the activities of APX, CAT and POD that consist of H_2O_2 scavenging systems in plant cells were all elevated synchronously with the SOD in young leaves. It is reasonable to deduce that with the improvement of activities in both SOD and H_2O_2 scavenging systems, newly expanding leaves are able to protect themselves against photo-oxidation (Table 1).

Data in this paper revealed that photo-protective ability in young leaves was the same high as that in fully expanded leaves (Table 1), showing that photo-protection is significantly strengthened at the early stages of leaf expansion. The data presented above also demonstrates that the photo-protective mechanisms depending on antioxidant enzymes can be developed as soon as the ginkgo leaves initiate.

Chloroplast structure determines photosynthetic capacity of leaf cells. Chloroplasts are the sites of photosynthesis, and the increase in photosynthesis is expected to correspond with ultrastructural alterations in the chloroplast during leaf expansion (Fig. 1). Large osmiophilic granules within the chloroplast are the most conspicuous indicator of leaf senescence (Bulter and Simon 1971). The appearance of osmiophilic granules only in fully expanded leaves (Fig. 1 *E,F*) further proves that photo-protection is significantly strengthened at the early stages of leaf expansion.

In conclusion, the results show that the early development of the photo-protective mechanisms in young leaves is the main reason of preventing their photosynthetic apparatus against photodamage.

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