Changes in Photosynthetic Characteristics and Antioxidative Protection in Male and Female Ginkgo during Natural Senescence

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ABSTRACT. *Ginkgo* (*Ginkgo biloba*), a dioecious tree species, is widely distributed throughout the world, yet little is known about sex-related responses to autumnal senescence in ginkgo. The aim of this study was to investigate changes in photosynthetic activities, concentration of oxidative stress parameters [malondialdehyde (MDA) and H₂O₂] and antioxidant systems, and ultrastructure of chloroplasts in the naturally senescing leaves of two ginkgo sexes and to examine whether progression of senescence is sex-specific in ginkgo. Photosynthesis in ginkgo leaves of both sexes was not limited by stomatal factors, but rather non-stomatal factors such as decreased photosynthetic pigments and photochemical activities that became more important during autumnal senescence. The responses of antioxidative enzymes were different from those of antioxidants to leaf senescence. Correlation analysis revealed that autumnal leaf senescence was significantly correlated to antioxidative enzymes changes but not to antioxidants such as ascorbate (ASA) and glutathione (GSH). Guaiacol peroxidase (POD) became more important in senescing leaves and played a major protective role, especially at the late stage of senescence. The shape of chloroplasts of both sexes changed from oblong to round, and there was an increase in the number and size of osmiophilic granules during senescence; swollen thylakoid membranes in the stroma and grana with a significant increase in MDA content were also observed. During autumnal senescence, female ginkgo plants showed smaller decreases in net photosynthetic rates, photosynthetic pigments, photochemical activities, superoxide dismutase, ascorbate peroxidase and catalase activities, higher POD activity, ASA and GSH contents, and smaller increases in H₂O₂ and MDA contents than did males. In addition, female plants had a later senescence of chloroplasts, a smaller accumulation of osmiophilic granules, and a slower rate of membrane damage. These results show that female ginkgo exhibit slower leaf senescence, which may be related to increased reproductive costs.

Leaf senescence is characterized by programmed degradation of cellular constituents such as proteins, nucleic acids, and lipids, together with organelles and structures of leaf cell, resulting in a significant photosynthetic decline. Photosynthesis is a vital physiological factor for the biomass and grain yield. However, initiation of grain filling coincides with the onset of leaf senescence, offsetting the grain yield. So the onset and rate of senescence are important factors for determining grain yield

(Subhan and Murthy, 2001; Zhang et al., 2006). In addition, the different responses of *Populus cathayana* to photoperiod transitions may be related to its different leaf senescence speed under changing environments (Zhao et al., 2009). Therefore, early or rapid senescence can lead to productivity losses or affect the growth of plants.

Ginkgo is a long-lived, deciduous gymnosperm species that is resistant to pests and pollutants. The extracts of ginkgo leaves contain flavonoid glycosides and terpenoids and have been used pharmaceutically (Cao, 2002). Ginkgo leaves are usually harvested in the fall, because the contents of these pharmaceutical constituents are highest in fall and then decline with senescence in ginkgo leaves (Chen et al., 1997). In addition, this harvest date coincides with the onset of autumnal leaf senescence (Cheng et al., 2009). Therefore, modification of cultural practices may help to delay senescence and result in economic benefits to cultivating this plant.

Chloroplasts are the sites of photosynthesis, and a decline in photosynthesis corresponds with ultrastructural alterations in

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the chloroplast as a leaf progresses toward senescence (Quirino et al., 2000). One of the major events in leaf senescence is the increase of reactive oxygen species (ROS) levels, which can result in a decline in antioxidant protection, including both enzymatic and non-enzymatic factors (Zimmermann and Zentgraf, 2005). Superoxide dismutases (SOD), catalases (CAT), and various peroxidases such as guaiacol peroxidase and ascorbate peroxidase (APX) are the primary antioxidant enzymes. In conjunction with these enzymes, antioxidant compounds such as ascorbate and glutathione also play important roles in maintaining the integrity of photosynthetic membranes under oxidative stress (Kuk et al., 2003).

Many morphological, physiological, and ecological differences between males and females have been observed in a number of dioecious species in relation to environmental stresses (Rozas et al., 2009; Stehlik et al., 2008). Because of the differences in reproductive resource requirements, females are more common in high-resource microsites, whereas males are more tolerant to environmental stresses (Zhang et al., 2010b, 2011). Although it has been reported that male and female ginkgo have similar responses to light (Jin et al., 2008), little is known about the sex-specific progress to senescence.

It has been well documented that in ginkgo and other dioecious plants, males senesce earlier and faster than females because male plants are more susceptible to infection (Molisch, 1929). Changes in the antioxidant status of ginkgo leaves during senescence have been reported (Kukavica and Veljovic-Jovanovic, 2004). However, this study was primarily focused on a single sex. In this study, we used natural conditions of leaf senescence to investigate whether progress of senescence is sex-specific in ginkgo leaves (Zhang et al., 2010a). The aim of the present work is to compare a series of changes in photosynthetic and antioxidative activity and chloroplast ultrastructure in male and female plants during autumnal senescence to assess which sex senesces earlier or more rapidly. We believe that this comparison between sexes could be used to develop strategies to increase the growth and productivity of ginkgo.

Materials and Methods

PLANT MATERIAL. The experiments were performed using the leaves of 30-year-old ginkgo trees (female and male) standing in pairs on the campus of Nanjing Normal University in Nanjing, China (lat. 32°03' N, long. 118°45' E). Nanjing is located in the monsoon climate area of the north subtropical zone with four distinctive seasons (Fan and Chen, 1997). Weather conditions are given in Figure 1. From 1 Sept. (16 d before the first sampling) to 13 Nov. (the last sampling date), the mean maximum and minimum temperature was (mean $\pm$ SE) 24.9 ± 0.4 °C and 16.2 ± 0.5 °C, respectively. The mean ($\pm$ SE) daily precipitation and relative humidity was 1.4 ± 0.5 mm and $75.8\% \pm 1.2\%$, respectively.

The ginkgo leaves emerge in early April, then expand from April to June, and senesce in late September in male and female trees. Three trees of each sex with similar age ($\approx$ 30 years old) were sampled from 16 Sept. 2006 (close to the time of leaf yellowing, Day 259) through 13 Nov. 2006 (close to the time of leaf drop, Day 317) on sunny mornings (0730 to 1130 HR) every 6 to 16 d, depending on the weather. The leaves used for analyses were fully expanded and from lateral branches of the outer part of the crowns with the same exposure to light.

Fig. 1. Precipitation, relative humidity, and maximum/minimum temperatures during the study period (September to November).

PIGMENT DETERMINATIONS. Leaves ($n = 5$) of each tree were detached at each sampling point, immediately frozen in liquid nitrogen, and stored at -80 °C until further analysis. Each leaf sample was extracted in ice-cold 80% (v/v) acetone. The extract was centrifuged at $3000 g_n$ for 5 min. The absorbance of the supernatant was measured with a spectrophotometer (GENESYS 10UV; Thermo Fisher Scientific, Waltham, MA) at 470, 645, and 663 nm. The levels of chlorophyll (Chl) and carotenoids were calculated as described by Lichtenthaler and Wellburn (1983).

PHOTOSYNTHETIC ACTIVITY MEASUREMENTS. The net photosynthetic rates (P_n), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured in the field on sun-exposed leaves ($n = 10$ leaves per tree) using a portable photosynthesis system (CIRAS-2; PP Systems, Amesbury, MA) in the morning between 0730 and 1130 HR. To avoid potential photoinhibition from high light stress, the measurements were conducted under saturating photosynthetic photon flux densities ($1000 \mu mol \cdot m^{-2} \cdot s^{-1}$) from a light-emitting diode (red and white) light source. The leaves in the cuvette were exposed to ambient CO_2 concentration ($\approx 360 \mu mol \cdot mol^{-1}$), relative humidity, and temperature.

To further elucidate changes in the primary photochemical reactions of photosynthesis during senescence, photosynthetic electron transport and photophosphorylation were investigated using thylakoids isolated from ginkgo leaves. Approximately 2 g of leaves, without petioles ($n = 5$), were taken from each tree and stored at 4 °C for 30 min at each sampling time until further analysis. The membranes were isolated as described by Zhang et al. (2006). Photophosphorylation activity was measured with a luminescent photometer (FG-300; Shanghai Institute of Plant Physiology, Shanghai, China) as described by Ketcham et al. (1984). The Ca^{2+} -ATPase activity was measured according to Vallejos et al. (1983). The adenosine-5'-triphosphate (ATP) content was measured using the bioluminescence method described by Zhu et al. (2001). The electron transport

activities [photosystem I (PSI), photosystem II (PSII), and whole-chain activities] were measured polarographically with a Clark-type liquid-phase electrode (Chlorolab-2; Hansatech, Norfolk, U.K.) fitted with a circulating water jacket at 25 ± 0.5 °C.

H₂O₂ DETERMINATION. For the H₂O₂ determination, 1 g of leaves ($n = 5$) was harvested from each tree and homogenized in 5% (v/v) HClO₄. The suspension was centrifuged at 14,000 g_n for 10 min at 4 °C. The supernatant was neutralized with 5 M K₂CO₃ to reach pH 4, then centrifuged at 14,000 g_n for 2 min at 4 °C, and immediately used to evaluate the H₂O₂ content according to Veljovic-Jovanovic et al. (2002).

LIPID PEROXIDATION AND ANTIOXIDANT ENZYMES ANALYSIS. For this analysis, 1 g of leaves ($n = 5$) was harvested from each tree, pulverized in liquid nitrogen using a mortar and pestle, and extracted with 0.05 M phosphate buffer (pH 7.8) containing 5 mM EDTA. The suspension was centrifuged at 10,000 g_n for 20 min at 4 °C. The supernatant was used to assay for SOD, POD, CAT, and malondialdehyde. For the APX assay, the extraction buffer also contained 2 mM ascorbate.

The level of MDA production was assayed to estimate lipid peroxidation according to the method described by Zhao and Li (1999). The MDA in the supernatant was considered to be a thiobarbituric acid-reactive substance. The absorbance was recorded at 532, 600, and 450 nm. The SOD activity was determined using the procedure described by Giannopolitis and Ries (1977). The activity was assayed by the capacity of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT) by xanthine oxidase-generated O₂⁻, producing an increase in the absorbance at 560 nm of ≈ 0.02 units per minute at 25 °C in the absence of enzyme. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the initial rate of NBT reduction. The CAT activity was assayed according to the method described by Mishra et al. (1993). The enzyme activity was determined by monitoring the change in optical density (OD) at 240 nm and was defined as the decrease of 0.1 Δ OD per minute. The APX activity was determined by monitoring the decline in absorbance at 290 nm as ascorbate is oxidized, according to the method described by Nakano and Asada (1981). One unit of APX activity was defined as the decrease of 0.1 Δ OD per minute. The POD activity was assayed according to the method described by Kochba et al. (1977). The change of OD was recorded at 470 nm and the enzyme activity was defined as the increase of 0.1 Δ OD per minute.

ASCORBATE AND GLUTATHIONE DETERMINATIONS. The ginkgo leaves (0.5 g, $n = 5$ per tree) were ground with a mortar and pestle in trichloroacetic acid at 4 °C. The homogenate was centrifuged at 13,000 g_n for 15 min, and the supernatant was collected for ASA and GSH analyses. The ASA and GSH pools were assayed according to Tanaka et al. (1985) and Ellman (1959), respectively. The concentrations of ASA and GSH were calculated from a standard curve prepared from known concentrations of ASA and GSH.

TRANSMISSION ELECTRON MICROSCOPY. To avoid differential structure in different parts of leaves, the midsection of the leaves without the midrib was used and cut into small pieces (2 × 20 mm). The pieces were fixed at room temperature in 4% (v/v) glutaraldehyde dissolved in 0.3 M sodium phosphate buffer (pH 7.5) for 2 h. After washing in buffer, the samples were postfixed with 1% osmium tetroxide in the same buffer. After 2 h, the samples were dehydrated in graded acetone solutions and embedded in Epon 812 resin. The resin was polymerized at 60 °C. Ultrathin sections (70 nm) were cut with

a diamond knife on a microtome (LKB-V ultramicrotome; LKB, Bromma, Sweden). The sections were stained with uranyl acetate, followed by lead citrate, and observed using a transmission electron microscope (TEM 600A-2; Hitachi, Tokyo, Japan). To attain sufficient accuracy, two sections per leaf piece, three pieces per leaf, and three leaves per tree were observed at different stages of senescence.

STATISTICAL ANALYSES. Analysis of variance and correlation were based on the general linear model procedure. Statistical differences between sexes and between different dates with respect to photosynthetic pigments and activities, H₂O₂ content, lipid peroxidation, and antioxidant systems were analyzed by performing an analysis of variance using SPSS (Version 16.0; IBM Corp., Armonk, NY). Differences were considered significant at the $P < 0.05$ level.

Results

The photosynthetic pigments and photosynthetic rates were determined in sun-exposed leaves of ginkgo plants over 58 d as biomarkers of autumnal leaf senescence (Garcia-Plazaola et al., 2003).

Both chlorophyll and carotenoid levels were highest by Day 266 and subsequently decreased in both sexes (Fig. 2). After Day 282, a decrease in the Chl a/b ratio was also observed, indicating that Chl a content was more affected by leaf aging. The decrease in the carotenoid content was less than that of Chl content. Males showed greater decreases in Chl, carotenoids, and Chl a/b ratio than females under natural conditions. Females possessed significantly higher Chl than males after Day 282 ($P = 0.001$). There were no sex-specific differences in the carotenoid content and Chl a/b ratio during most of leaf growth. However, males had lower values at the late stage of leaf senescence.

The P_n and g_s were negatively affected by aging in both sexes. As shown in Figure 3, marked decreases in these two parameters were observed after Day 266. However, the C_i remained stable throughout the process of senescence. The change in Chl content correlated with the decreased P_n during leaf senescence. Females possessed higher P_n , g_s , and C_i than males during leaf senescence; however, males showed greater decreases in P_n .

Taken together, the data on photosynthetic pigment contents and photosynthetic rates indicated that the ginkgo leaves at Day 266 (September) were mature, and the leaves after this time showed progressive stages of senescence.

As shown in Figure 4, the decrease in the PSII electron transport activity was larger than that of the PSI and the whole electron chain, indicating that PSII was more sensitive during the process of senescence of ginkgo leaves. Although males had higher electron transport values during the early stage of leaf senescence, males showed larger decreases in electron transport than females, especially at the late stage of senescence.

As shown in Figure 5, photophosphorylation, Ca²⁺-ATPase activity and ATP content demonstrated a similar trend during leaf yellowing, reaching the highest levels at Day 266 and then decreasing significantly. During senescence of ginkgo leaves (especially during the middle and late stages), the higher ATP content in combination with the higher Ca²⁺-ATPase and photophosphorylation activities in females suggests that the turnover of ATP was higher in females than in males. Before leaf senescence, males displayed higher Ca²⁺-ATPase activity

Fig. 2. Changes in chlorophyll (A) and carotenoid (B) contents and chlorophyll a to b ratio [Chl a/b (C)] in female (filled symbols) and male (open symbols) ginkgo leaves during autumnal senescence. Values represent the average of 15 replicates $\pm$ SE. Single asterisks indicate statistically significant differences ($P \leq 0.05$) between female and male ginkgo during the same period; Day 1 = 1 Jan.

and ATP content, which might be the reason for their higher P_n at this stage (Miginiac-Maslow and Lancelin, 2002).

The hydrogen peroxide content and the level of lipid peroxidation, measured in terms of MDA content, increased with leaf age in both sexes, reaching the highest values in the late stage of senescence at Day 317 (Fig. 6). Moreover, the sex-specific differences were significant with males showing higher H_2O_2 and MDA contents than females after Day 282 (H_2O_2 , $P = 0.014$; MDA, $P = 0.007$). Significant sex-specific differences

Fig. 3. Changes in net photosynthetic rate [P_n (A)], stomatal conductance [g_s (B)], and internal CO_2 concentration [C_i (C)] in female (filled symbols) and male (open symbols) ginkgo leaves during autumnal senescence. Values represent the average of 30 replicates $\pm$ SE. Single asterisks indicate statistically significant differences ($P \leq 0.05$) between female and male ginkgo during the same period; Day 1 = 1 Jan.

were also observed in ASA and GSH contents during leaf yellowing (ASA, $P = 0.001$; GSH, $P = 0.001$). However, ASA and GSH contents remained at a relatively stable level in both sexes until the late stage of leaf senescence. Females showed higher ASA and GSH contents during leaf senescence.

The changes in the activities of primary antioxidative enzymes (SOD, APX, CAT, and POD) are shown in Figure 7.

Fig. 4. Changes in photosystem I [PSI (A)], photosystem II [PSII (B)], and whole-chain electron transport activities (C) in female (filled symbols) and male (open symbols) ginkgo leaves during autumnal senescence. Values represent the average of 15 replicates $\pm$ SE. Single asterisks indicate statistically significant differences ($P \leq 0.05$) between female and male ginkgo during the same period; Day 1 = 1 Jan. Chl = chlorophyll.

The activities of SOD, CAT, and APX increased, reaching their highest values at Day 266 and then decreasing significantly, which is probably the result of increased oxidative stress during the process of leaf senescence. Males exhibited greater decreases in the activities of these three enzymes than females. In contrast, POD activity increased significantly in the senescent leaves of both sexes, but was much higher in females than in males ($P = 0.001$).

Correlations showed that g_s was positively correlated to senescence parameters (P_n , Chl, and carotenoid contents), whereas C_i had no significant correlations with these parameters in both sexes (Tables 1 and 2). There were significant positive correlations of PSI, PSII, and whole-chain electron

Fig. 5. Changes in photophosphorylation (A), Ca²⁺-ATPase activity (B), and adenosine triphosphate (ATP) content (C) in female (filled symbols) and male (open symbols) ginkgo leaves during autumnal senescence. Values represent the average of 15 replicates $\pm$ SE. Single asterisks indicate statistically significant differences ($P \leq 0.05$) between female and male ginkgo during the same period; Day 1 = 1 Jan. Chl = chlorophyll.

transport activities, photophosphorylation, Ca²⁺-ATPase activity, ATP content, SOD, CAT, and APX activities with all senescence parameters for both sexes. POD activity, H₂O₂, and MDA contents also correlated with these parameters, but negatively. No significant correlations were detected between ASA, GSH contents, and senescence parameters.

Fig. 6. Changes in hydrogen peroxide [H₂O₂ (A)], malondialdehyde [MDA (B)], ascorbic acid [ASA (C)], and glutathione [GSH (D)] contents in female (filled symbols) and male (open symbols) ginkgo leaves during autumnal senescence. Values represent the average of 15 replicates $\pm$ SE. Single asterisks indicate statistically significant differences ($P \leq 0.05$) between female and male ginkgo during the same period; Day 1 = 1 Jan.

As shown in Figures 8 and 9, at the early stage of leaf senescence, the chloroplasts from both sexes exhibited a normal ultrastructural organization; most were lens-like oblong-shaped with a typical arrangement of grana and stroma thylakoids; the appearance of osmiophilic granules was observed at this stage. Chloroplasts from females and males changed in their shapes from Day 295 and Day 289, respectively. They were usually lens-shaped but they were more rounded than chloroplasts at the early stage of leaf senescence. The number and size of the osmiophilic granules changed slightly in females, whereas more and large osmiophilic granules were observed in males. In addition, the thylakoids of grana and stroma were swollen and reduced. At Day 310, close to the time of leaf drop, the ultrastructure of the chloroplasts from both sexes changed significantly. The peripheral double membrane of the chloroplasts and the internal system of grana and stroma became disorganized in both males and females; the stratiform structure of the chloroplasts became inconspicuous, and the parallel membrane was disrupted. The osmiophilic granules became more and larger in the chloroplast of females. Moreover, the thylakoids became swollen and the intrathylakoid space was increased. In comparison, the osmiophilic granules were

developed further in males, and the number and the size increased significantly. The thylakoids in the chloroplasts from males were severely damaged, and the membrane structure was almost lost.

Discussion

A decline in photosynthesis has been reported in many plants during leaf senescence (Cabello et al., 2006; Zhang et al., 2007). In this study, marked decreases in P_n were observed in both sexes after Day 266 in ginkgo leaves, and females exhibited higher values than males. An increase of photorespiration was observed with a decrease of g_s as a result of stomatal closure in ginkgo leaves exposed to high light, which inhibited photosynthesis (Meng et al., 1999). In the present study, the decrease of g_s was attributed to stomatal closure in senescent ginkgo leaves. However, the C_i remained constant in both sexes during senescence, which indicated that the decrease in P_n was not the result of a decrease in the available CO₂ as a result of decreased g_s but possibly the result of non-stomatal factors such as a decline in Chl content, decreased biochemical activities, and damage to the photosynthetic system (Whitehead

Fig. 7. Changes in superoxide dismutase [SOD (A)], catalase [CAT (B)], ascorbate peroxidase [APX (C)], and peroxidase [POD (D)] activities in female (filled symbols) and male (open symbols) ginkgo leaves during autumnal senescence. Values represent the average of 15 replicates $\pm$ SE. Single asterisks indicate statistically significant differences ($P \leq 0.05$) between female and male ginkgo during the same period; Day 1 = 1 Jan.

et al., 2011). This result is consistent with the report by He et al. (2007). Actually, the Chl content was significantly decreased in both sexes, and females exhibited higher Chl than males during leaf senescence. Leaf yellowing results from chlorophyll degradation and is widely used as a phenotype marker of plant senescence together with a series of other biochemical and physiological changes (Ougham et al., 2008). Higher Chl content indicated that the process of leaf senescence was delayed in female ginkgo plants. The Chl a/b ratio might play an important role in controlling leaf senescence, and the decrease in this ratio may be in favor for the plants before late senescence (Zhang et al., 2006). The Chl a/b ratio was greater in females during late senescence after Day 303, showing that the females had a slower rate of leaf senescence. During ginkgo leaf senescence, the Chls were more susceptible to degradation than carotenoids. Carotenoids have been reported to protect chloroplasts against blue light during autumnal senescence (Garcia-Plazaola et al., 2003). The decrease of carotenoid content was greater in males, which indicated that the senescence of chloroplasts might be faster in males than in females.

Leaf senescence generally induces an alteration in the structure and function of the chloroplasts concomitant with a decrease in photosynthetic activity. Previous studies have shown that leaf senescence also induces a decrease in the photochemical activities of both PSII and PSI, and in most cases, PSII is more responsive to senescence than PSI (Zhu et al., 2001). Similar results were also observed in this study. Females showed smaller decreases in the activities of PSII, PSI, and whole-chain electron transport during autumnal senescence. However, the photosystem activities were higher in males at the early stage of leaf senescence. The change pattern was similar to that of male and female cottonwood (Letts et al., 2008) or spinach (Sklensky and Davies, 2011) plants. This may be the result of higher leaf maturity in males than in females, because males showed increased Chl and P_n (Liu et al., 2011). Photophosphorylation activity is the capacity of chloroplasts to produce ATP under light conditions, indicating the light energy conversion ability of chloroplasts. Similarly, after Day 289 (middle and late senescence), the photophosphorylation, Ca^{2+} -ATPase activity, and ATP content were higher in females than in males. These results suggest that higher photochemical

Table 1. Correlations of photosynthetic characteristics, oxidative stress, and antioxidative responses with senescence in male ginkgo.^z

	Carotenoid	Chl a/b	P _n	g _s	Ci	PSI	PSII	Whole chain	Photophosphorylation	Ca ²⁺ -ATPase	ATP	H ₂ O ₂	MDA	ASA	GSH	SOD	CAT	POD	APX
Chlorophyll	0.97***	0.62 NS	0.99***	0.97***	0.15 NS	0.98***	0.98***	0.97***	0.98***	0.97***	0.97***	-0.96***	-0.92**	0.33 NS	0.22 NS	0.97***	0.99***	-0.77*	0.97***
Carotenoid		0.68 NS	0.99***	0.99***	0.30 NS	0.98***	0.98***	0.98***	0.98***	0.97***	0.97***	-0.96***	-0.94**	0.40 NS	0.32 NS	0.99***	0.98***	-0.79*	0.98***
Chl a/b			0.69 NS	0.64 NS	0.74*	0.53 NS	0.67 NS	0.57 NS	0.63 NS	0.55 NS	0.52 NS	-0.79*	-0.80*	0.72*	0.66 NS	0.67 NS	0.68 NS	-0.87**	0.57 NS
P _n				0.98***	0.27 NS	0.98***	0.98***	0.98***	0.97***	0.95***	0.97***	-0.97***	-0.94***	0.36 NS	0.28 NS	0.99***	0.99***	-0.78*	0.97***
g _s					0.22 NS	0.98***	0.99***	0.96***	0.98***	0.96**	0.96***	-0.95***	-0.94***	0.44 NS	0.35 NS	0.98***	0.98***	-0.79*	0.99***
Ci						0.11 NS	0.22 NS	0.23 NS	0.20 NS	0.16 NS	0.13 NS	-0.36 NS	-0.37 NS	0.45 NS	0.37 NS	0.33 NS	0.26 NS	-0.40 NS	0.15 NS
PSI							0.97***	0.98***	0.98***	0.98***	0.99***	-0.92**	-0.89*	0.28 NS	0.19 NS	0.97***	0.98***	-0.71*	0.99***
PSII								0.97***	0.99***	0.97***	0.96***	-0.98***	-0.96***	0.48 NS	0.37 NS	0.97***	0.99***	-0.84**	0.98***
Whole chain									0.97***	0.97***	0.99***	-0.92**	-0.88*	0.24 NS	0.14 NS	0.98***	0.97***	-0.68 NS	0.97***
Photophos-										0.99***	0.98***	-0.96***	-0.93***	0.42 NS	0.30 NS	0.97***	0.98***	-0.80*	0.98***
phorylation																			
Ca ²⁺ -ATPase											0.98***	-0.93***	-0.90**	0.36 NS	0.23 NS	0.95***	0.97***	-0.76*	0.97***
ATP												-0.91**	-0.86**	0.25 NS	0.15 NS	0.97***	0.96***	-0.68 NS	0.98***
H ₂ O ₂													0.98***	-0.54 NS	-0.42 NS	-0.95***	-0.98***	0.90**	-0.92**
MDA														-0.61 NS	-0.51 NS	-0.92**	-0.95***	0.92**	-0.91**
ASA															0.93***	0.35 NS	0.40 NS	-0.84**	0.37 NS
GSH																0.25 NS	0.28 NS	-0.74*	0.29 NS
SOD																	0.98***	-0.74*	0.97***
CAT																		-0.80*	0.97***
POD																			-0.74*

^zNS, **, ***, **** correlation coefficients non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Chl = chlorophyll; P_n = net photosynthetic rate; g_s = stomatal conductance; Ci = internal CO₂ concentration; PSI = photosystem I; PSII = photosystem II; ATP = adenosine triphosphate; H₂O₂ = hydrogen peroxide; MDA = malondialdehyde; ASA = ascorbic acid; GSH = glutathione; SOD = superoxide dismutase; CAT = catalase; POD = peroxidase; APX = ascorbate peroxidase.

activities may be responsible for the higher photosynthetic rate in females.

Plant aging increases oxidative stress and ROS levels (Zimmermann and Zentgraf, 2005). Chloroplasts are the primary source of ROS and are the main target of age-associated oxidative stress in plants (Asada, 2006). In the thylakoids, Chl is complexed with carotenoids, which prevents the occurrence of activated oxygen by either quenching triplet states of Chl or scavenging singlet oxygen and thereby dissipating excessive energy (Matile, 2001). Therefore, the lower levels of Chl and carotenoids resulted in the accumulation of more ROS in males, which might be supported by an increase of hydrogen peroxide. MDA is a common product of membrane lipid peroxidation. Lipid peroxidation is an integral feature of membrane deterioration leading to cell death and is correlated with oxidative stress during leaf senescence (Zimmermann and Zentgraf, 2005). In this study, MDA content significantly increased in both sexes and males exhibited a higher value than females during the process of senescence. Higher hydrogen peroxide and MDA suggest that males suffered more oxidative stress and greater damage to the cellular membrane or chloroplast structure. This result is consistent with the report on *P. cathayana* by Zhao et al. (2009).

The antioxidant protection generally decreased during senescence, and H₂O₂ regulation may play a critical role in the onset and dynamics of senescence. The SODs are metalloenzymes that catalyze the dismutation of a superoxide radical to molecular oxygen and H₂O₂. The decrease in SOD activity that we observed during leaf senescence suggested that a decrease in H₂O₂ levels might occur; however, this was not the case. Indeed, there was an increase of H₂O₂ with ginkgo leaf aging. This increase might be the result of the decreased scavenging activity of H₂O₂ in both sexes. Three important antioxidant enzymes (APX, CAT, and POD) are responsible for H₂O₂ detoxification in cell compartments, and there are some reports about the increase of POD activity and the decrease of APX and CAT activities during leaf senescence (Hodges and Forney, 2001; Kar and Mishra, 1976; Veljovic-Jovanovic et al., 2006). The ASA–GSH cycle is also an important antioxidant protection system against H₂O₂, and its occurrence has been considered to be associated with leaf senescence (Foyer and Shigeoka, 2011; Palma et al., 2006). In ginkgo leaves, the ASA and GSH levels stayed relatively constant until Day 303, when a significant increase in H₂O₂ content was detected. This and the decreased APX and CAT activities, with increased POD activity, indicated that in senescent plants, ASA could not perhaps be used to scavenge H₂O₂ by the ASA–GSH cycle and declines in the APX and CAT activities may contribute to the increased H₂O₂ during the early stage of senescence. In the late stage of senescence, the decreases in ASA and GSH were accompanied by continuous POD activity increase and progressive inactivation of APX and CAT. These results suggested that POD could still participate in removing H₂O₂ when the photosynthetic apparatus has been severely damaged, which were consistent with those reported by Kukavica and Veljovic-Jovanovic (2004). In addition, sex-specific responses in antioxidative defenses were observed in ginkgo during the process of senescence. Females showed smaller decreases in SOD, APX, and CAT activities, higher POD activity and antioxidant contents, and slower increases in H₂O₂ and lipid peroxidation levels than males. These results suggest that females have a better protection mechanism than

Table 2. Correlations of photosynthetic characteristics, oxidative stress and antioxidative responses with senescence in female ginkgo.^z

	Carotenoid	Chl a/b	P _n	g _s	Ci	PSI	PSII	Whole chain	Photophos-phorylation	Ca ²⁺ -ATPase	H ₂ O ₂	MDA	ASA	GSH	SOD	CAT	POD	APX
Chlorophyll	0.96***																	
Carotenoid		0.37 NS	0.98***	0.98***	0.11 NS	0.76*	0.98***	0.86**	0.97***	0.89***	0.95***	-0.87**	0.50 NS	0.43 NS	0.98***	0.99***	-0.89**	0.96***
Chl a/b		0.30 NS	0.95***	0.98***	0.13 NS	0.82*	0.97***	0.90**	0.99***	0.95***	0.98***	-0.88**	0.46 NS	0.54 NS	0.97***	0.98***	-0.85**	0.99***
P _n			0.50 NS	0.43 NS	0.87**	0.25 NS	0.47 NS	0.39 NS	0.33 NS	0.07 NS	0.25 NS	-0.59 NS	0.72*	0.48 NS	0.39 NS	0.38 NS	-0.57 NS	0.27 NS
g _s				0.99***	0.29 NS	0.83*	0.99***	0.91**	0.96***	0.83*	0.92**	-0.96***	0.60 NS	0.52 NS	0.99***	0.97***	-0.91**	0.96***
Ci					0.24 NS	0.79*	0.99***	0.87**	0.98***	0.88**	0.94***	-0.98***	0.59 NS	0.56 NS	0.99***	0.99***	-0.93***	0.98***
PSI						0.13 NS	0.26 NS	0.23 NS	0.15 NS	-0.11 NS	0.07 NS	-0.29 NS	0.59 NS	0.54 NS	0.21 NS	0.16 NS	-0.44 NS	0.11 NS
PSII							0.80*	0.87**	0.78*	0.67 NS	0.75*	-0.69 NS	0.49 NS	0.43 NS	0.85**	0.75*	-0.60 NS	0.83*
Whole chain								0.92**	0.99***	0.88**	0.96***	-0.91**	0.52 NS	0.49 NS	0.99***	0.98***	-0.89**	0.96***
Photophos-phorylation									0.90**	0.81*	0.92**	-0.79*	0.34 NS	0.34 NS	0.92**	0.87**	-0.68 NS	0.86*
Ca ²⁺ -ATPase										0.94***	0.98***	-0.95***	0.43 NS	0.47 NS	0.98***	0.98***	-0.86**	0.98***
ATP											0.96***	-0.86**	0.24 NS	0.39 NS	0.86**	0.92**	-0.70 NS	0.91**
H ₂ O ₂												-0.9**	0.31 NS	0.39 NS	0.95***	0.97***	-0.78*	0.94***
MDA													-0.64 NS	-0.63 NS	-0.94***	-0.97***	0.95***	-0.95***
ASA													-0.75*	-0.73*	-0.87**	-0.90**	0.94***	-0.88**
GSH														0.82*	0.52 NS	0.50 NS	-0.73*	0.50 NS
SOD															0.48 NS	0.50 NS	-0.69 NS	0.53 NS
CAT																0.98***	-0.88**	0.98***
POD																	-0.90**	0.97***
																		-0.88**

^zNS, **, *** correlation coefficients non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Chl = chlorophyll; P_n = net photosynthetic rate; g_s = stomatal conductance; Ci = internal CO₂ concentration; PSI = photosystem I; PSII = photosystem II; ATP = adenosine triphosphate; H₂O₂ = hydrogen peroxide; MDA = malondialdehyde; ASA = ascorbic acid; GSH = glutathione; SOD = superoxide dismutase; CAT = catalase; POD = peroxidase; APX = ascorbate peroxidase.

Fig. 8. Changes in the ultrastructure of chloroplasts from female ginkgo leaves during autumnal senescence: (A) Day 266, (B) Day 282, (C) Day 289, (D) Day 295, (E) Day 303, (F) Day 310. G = granum thylakoid; ST = stroma thylakoid; O = osmiophilic granule; SG = starch grain. Bars = 1 μm; Day 1 = 1 Jan.

males for ROS detoxification to delay leaf senescence to meet their greater resource demands.

Data from correlation analysis demonstrated that photochemical activity decreases, antioxidative enzymes inactivation or activation, H₂O₂, and lipid peroxidation accumulation were significantly correlated natural senescence; however, the correlation with stomatal closure and antioxidants were not significant. Moreover, photochemical activities were positively correlated with photosynthesis, whereas stomatal closure was not. POD activity was positively correlated and another three

Fig. 9. Changes in the ultrastructure of chloroplasts from male ginkgo leaves during autumnal senescence: (A) Day 266, (B) Day 282, (C) Day 289, (D) Day 295, (E) Day 303, (F) Day 310. G = granum thylakoid; ST = stroma thylakoid; O = osmiophilic granule; SG = starch grain. Bars = 1 μ m; Day 1 = 1 Jan.

antioxidative enzymes were negatively correlated with oxidative stress, whereas the correlations with antioxidants were not significant. Thus, approaches that can increase photochemical activities or activate antioxidative enzymes may be useful to delay senescence of ginkgo leaves.

The ultrastructural changes were apparent in ginkgo leaves during senescence. Chloroplasts are one of the first organelles targeted for breakdown during leaf senescence (Quirino et al., 2000). In this study, we found that the chloroplasts senesced similarly, but the dynamics of senescence were different in females and males. After Day 266 (the beginning of senescence),

the shape of chloroplasts changed from oblong to round in both sexes (earlier in males at Day 289 than in females at Day 295). The osmiophilic granules increased remarkably in size and number (larger in males than in females), which is a typical feature of chloroplast aging (Spundova et al., 2003). The membrane system of grana and stroma thylakoids became reduced and swollen, which resulted in the disorganization of the parallel pattern of the lamellae and a considerable change in the orientation of the grana. During the last stage of senescence, the membrane structure was almost lost filled with osmiophilic granules in the chloroplasts of males, but not in females. The senescence of chloroplasts in aspen (*Populus tremula*) was different from that of female ginkgo, but similar to that of male ginkgo, which may be attributed to earlier fall leaf senescence (Keskitalo et al., 2005). An increase in the number and size of osmiophilic granules resulted from a gradual thylakoid membrane degradation (Papadakis et al., 2004). These results revealed that females exhibited a slower rate of chloroplast breakdown under natural conditions than males. Moreover, the delayed Chl breakdown and maintenance of photosynthesis were accompanied by a less rapid breakdown of chloroplasts in female ginkgo.

In conclusion, we observed more rapid leaf senescence of males was related to sex-specific differences in photosynthesis and antioxidative protection. In both sexes, the decrease in photosynthesis was caused by non-stomatal limitation of ginkgo leaves. Males showed a greater decrease in photosynthetic pigments, reduced photochemical activities, a lower antioxidative capacity to defend against oxidative stress and a more rapid degradation of chloroplast membrane structure than did females.

Females of woody dioecious usually expend proportionally more of their resources on reproduction and less on maintenance and growth when compared with males (Obeso, 2002). However, high reproductive costs or sink strength (e.g., fruit or seeds) can be accompanied with increased leaf photosynthesis and antioxidative activities, which delays leaf senescence (Kaschuk et al., 2010; Sklensky and Davies, 2011; Zhao et al., 2009). In some female plants such as *Silene latifolia*, fruit may be contributed to the differences on photosynthetic rates throughout the flowering period among those individuals (Laporte and Delph, 1996). Therefore, it is hypothesized that female ginkgo may respond to greater cost of reproduction through a higher capacity to delay the decline in photosynthesis and better protection against oxidative stress, which could offset the respiratory costs and maintain its reproductive organs, whereas male ginkgo may not (Case and Ashman, 2005).

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