Leaf photosynthesis, fluorescence response to salinity and the relevance to chloroplast salt compartmentation and anti-oxidative stress in two poplars

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Abstract In a 16-day study, the effect of increasing soil NaCl on leaf photosynthesis, chlorophyll *a* fluorescence, chloroplast ion compartmentation, variations of SOD (superoxide dismutase) and POD (peroxidase) isoenzymes and the relevance to salt resistance were investigated in seedlings of *Populus euphratica* Oliv. (*P. euphratica*) (salt-resistant) and rooted cuttings of *P. “popularis 35–44”* (*P. popularis*) (salt-sensitive). Initial salinity caused a rapid decline of net photosynthetic rate (Pn) and unit transpiration rate (TRN) in *P. euphratica*, resulting from the NaCl-induced stomatal closure. In a longer-term of salinity, CO₂ assimilation in *P. popularis* was severely reduced whereas stressed *P. euphratica* maintained a relatively higher and constant level of Pn. Pn–Ci curves showed that salt stress (12 days) reduced CO₂ saturation point (CSP), CO₂ saturated Pn (CSP_n), and carboxylation efficiency (CE), but increased CO₂ compensation point (CCP) in the two genotypes. Similarly, salinity lowered light saturation point (LSP), light saturated Pn (LSP_n), and apparent quantum yield (AQY) in both genotypes but the inhibitory effect of NaCl on light reaction was more pronounced in *P. popu-*

laris, as compared to *P. euphratica*. Chlorophyll *a* fluorescence data indicated that a longer-term of salt stress (12 days) exhibited a marked influence on fluorescence parameters of *P. popularis* in both dark- and light-adapted states: (a) NaCl inhibited the maximal efficiency of PSII photochemistry (Fv/Fm) due to the salt-induced increase of Fo (the minimal fluorescence) and the marked decline of Fm (the maximal fluorescence); (b) salinity decreased coefficient of photochemical quenching (qP) but markedly elevated coefficient of nonphotochemical quenching (qN) in the light-adapted state. In contrast, there were no corresponding changes of chlorophyll *a* fluorescence in salinised *P. euphratica*. X-ray microanalysis results showed that salinity caused salt accumulation in the chloroplasts of *P. popularis* in which Na⁺ and Cl[−] increased up to 42 and 221 mmol dm^{−3}, respectively. Great buildup of Na⁺ and Cl[−] in chloroplasts of *P. popularis* may exhibit direct and indirect restrictions on dark and light reactions. The activity of SOD isoenzymes (CuZn-SOD I and CuZn-SOD II) and POD isoenzymes in *P. popularis* decreased with increasing exposure period, and leaf malondialdehyde (MDA) content and membrane permeability (MP) increased correspondingly. In contrast to *P. popularis*, stressed *P. euphratica* maintained activity of SOD and POD isoenzymes and there was no significant increase of MDA and MP during the period of salt stress. In conclusion, *P. euphratica* plants exhibited a higher capacity to maintain the activity of anti-oxidant enzymes and restrict salt accumulation in the chloroplasts, the photosynthesis processes were less restricted consequently.

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Introduction

Populus spp. are one of the most important tree species for afforestation in north China, but the productivity of fast-growing poplars is usually restricted by soil salinity. Comparative studies have been carried out to determine the salt tolerance of several poplar genotypes (Ma et al. 1997; Fung et al. 1998). Results showed that salt stress reduced photosynthesis of poplar leaves, but there was much variation in salt tolerance among genotypes of *Populus*. Net photosynthetic rate (Pn) of highly salinised *P. euphratica* Oliv. (*P. euphratica*) was less inhibited as compared to the hybrid, *P. talassica* Kom $\times$ (*P. euphratica* + *Salix alba* L.) (Ma et al. 1997). In general, the Pn reduction in response to an initial osmotic shock caused by NaCl was mainly the result of decreased stomatal conductance (Chen et al. 2002a), while in a longer-term of severe salt stress, the declined Pn was largely due to non-stomatal limitations (Ma et al. 1997).

Salinised poplar trees transport Na^+ and Cl^- from roots to the shoots under NaCl stress, causing a great buildup of salt in leaves (Chen et al. 2001, 2002b, 2003). A recent study revealed that Na^+ preferential accumulated in the apoplast relative to vacuole in succulent *P. euphratica* leaf cells (Ottow et al. 2005a). Salt accumulation in leaves may cause physiological disturbances if mesophyll cells could not restrict their entry into the cytoplasm (Greenway and Munns 1980). Ma et al. (1997) found that the reduction of photosynthesis under high salt treatment was not due to damage of the photosynthetic apparatus, but more likely due to the inhibition of dark reaction since chlorophyll *a* fluorescence was not reduced by salinity. However, the ion compartmentation in chloroplasts and the effects of salts on light and dark reaction needs to be investigated.

Apart from salt-induced osmotic stress and ion toxicity, an increasing body of evidence suggests that high salinity also induces oxidative stress (Hernández et al. 1993, 1995, 1999, 2000; Gosset et al. 1996; Gómez et al. 1999; Savouré et al. 1999). It is suggested that plant salt resistance may partly depend on anti-oxidant capacities under saline conditions. Anti-oxidant enzymes, e.g. superoxide dismutase (SOD) and peroxidase (POD) play a key role in plant anti-oxidative stress (Takahashi and Asada 1983; Fridovich 1986; Asada and Takahashi 1987; Elstner 1987; Hassan and Scandalios 1990; Bowler et al. 1992; Asada 1994; Hernández et al. 1995; Alscher et al. 2002; Shigeoka et al. 2002). Tsugane et al. (1999) suggested that salt-induced activities of anti-oxidant enzymes increased plant salinity resistance. In accordance, increasing expression of SOD in *Arabidopsis* and tobacco enhanced salt tolerance of transgenic plants (Badawi et al. 2004; Wang et al. 2004).

There are drastic differences of salt tolerance in *P. euphratica*, *P. $\times$ euramericana* cv. I-214 (*P. cv. Italica*) and *P. "popularis 35–44"* (*P. popularis*) in terms of timing that

leaf damage occurred (Chen et al. 2002b). Under the same saline conditions, *P. popularis* and *P. cv. I-214* exhibited a clear symptom of leaf injury whereas there was no leaf necrosis in *P. euphratica* (Chen et al. 2002b; Wang et al. 2006). We found that the increased membrane permeability in salt-sensitive *P. popularis* leaves was associated with salt-induced lipid peroxidation, which was likely due to the drastic decline of SOD and POD activity (Wang et al. 2006). It seemed that stressed *P. popularis* was unable to scavenge stress-induced reactive oxygen species (ROS), thus leading to an uncontrolled oxidation in a longer-term of salt stress. In comparison, salt-tolerant *P. euphratica* maintained activity of SOD and POD during the period of salt stress and limited lipid peroxidation, which contributing to membrane integrity maintenance (Wang et al. 2006). However, how *P. euphratica* retained the activity of anti-oxidant enzymes during the period of salt stress has not been investigated. Salt-induced changes in isoenzymes of SOD and POD are helpful to elucidate the anti-oxidant capacity in *P. euphratica*.

Collectively, the objective of this study was to evaluate leaf photosynthesis response to salinity and the relevance to NaCl-induced anti-oxidative stress in two poplar genotypes, salt-tolerant *P. euphratica* and salt-sensitive *P. popularis*. We attempted to investigate: (a) salt compartmentation in chloroplasts and the inhibitory effects of salt ions on light and dark reaction in CO_2 assimilation; (b) genotypic differences in salt-induced changes in isoenzymes of SOD and POD and the relevance to anti-oxidative stress.

Materials and methods

Plant materials

One-year-old seedlings of *Populus euphratica* Oliv. (*P. euphratica*) and 1-year-old rooted cuttings of *P. "popularis 35–44"* (*P. popularis*) were used in this experiment. Hardwood cuttings of *P. popularis* were obtained from nursery of Beijing Forestry University (China) and seedlings of *P. euphratica* were from Xinjiang Uygur Autonomous Region of China. On 6th April 2004, plants were planted in 10 L individual pots containing nursery soil and sand (soil:sand = 2:1). Potted plants were kept well watered and received 1 L of full strength Hoagland's nutrient solution every 2 weeks. Potted plants were placed in a greenhouse at nursery of Beijing Forestry University prior to the initiation of salt treatment (July).

Salt treatment and soil analysis

Plants were subjected to 16 days of increasing NaCl stress. Saline treatment was imposed by top watering of 1 L of 50,

100, 150, and 200 mM NaCl (in full strength Hoagland's nutrient solution) on day 1, day 4, day 8 and day 12, respectively. Control plants were kept well watered and weekly fertilized by watering 1 L of full strength Hoagland's nutrient solution with no addition of NaCl. Visible symptoms of salt damage were observed in aged leaves of *P. popularis* after 12 days of exposure to salinity. Leaves exhibited chlorosis or necrosis at the edge of leaves, then leaves developed necrosis areas prior to abscission. In contrast, no visible damage and leaf abscission were seen in *P. euphratica* plants during the period of salt treatment.

Soil NaCl increment was examined during the period of increasing salt stress. Soil sample were taken at 20–30 cm depth in pots and soil water content was determined. Extracts of soil samples (dried soil:deionised water = 1:5, weight/volume) were used for cations and Cl^- analysis. Na^+ , K^+ , Ca^{2+} and Mg^{2+} were measured by an atomic absorption spectrophotometer (Perkin-Elmer 2280) and Cl^- by silver titration (Chen et al. 2001). Ion concentrations (mmol/L) were calculated as: ion content (ion mmol/dried soil weight kg)/soil water content (water kg/dried soil weight kg) (Chen et al. 2001). Soil analysis showed that NaCl concentration in the soil reached 326 mM after 16 days of salt treatment, whereas soil NaCl level in the control treatment was maintained at approximately 20 mM throughout the experiment. K^+ , Ca^{2+} and Mg^{2+} concentrations in the soil were not significantly changed by NaCl treatment.

Gas exchange

Gas exchange of upper mature leaves (leaf index number 6–8 from shoot tip, in general these leaves have higher gas exchange values) was measured on day 1 (1, 4, 8 and 24 h after initiation of salt stress), day 8, day 12 and day 16, respectively. Net photosynthetic rate (P_n), stomatal conductance (G_s) and transpiration rate (TRN) were measured at 8:30–10:00 a.m. using a Ciras-2 portable photosynthesis system (PP-systems, UK) under natural conditions where the air temperature (T_{air}) was 31.3–36.2°C and photosynthetically active radiation (PAR) was 974–1,004 $\mu\text{mol m}^{-2} \text{s}^{-1}$, supplied by cool white fluorescent lamps supplemented with dysprosium lamps. Leaf temperature (T_{leaf}) ranged from 31.1 to 36.3°C during the period of gas exchange measurement. After day 12, green parts of *P. popularis* leaves were used for gas exchange measurements since salt-stressed plants exhibited chlorosis or necrosis at the edge of leaves. Three individual plants per treatment were examined at each sampling time.

P_n versus C_i relationships

On day 12, P_n – C_i curves of upper mature leaves (leaf index number 6–8 from shoot apex) were measured over a

range of intercellular CO_2 concentrations (C_i) (0, 30, 60, 100, 200, 300, 400, 500, 600, 800, 1,000 $\mu\text{mol mol}^{-1}$) controlled by a mini CO_2 cartridge. PAR was 1,081–1,238 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by cool white fluorescent lamps supplemented with dysprosium lamps. T_{air} and T_{leaf} was 33.5–36.7 and 33.0–37.1°C, respectively. Leaves were allowed at least 5–6 min to adjust to the stepwise increased CO_2 concentration in the cuvette. The averaging time period for gas exchange measurement was ca. 2 min at each CO_2 concentration after the steady-state value of gas exchange was reached. CO_2 saturation point and CO_2 saturated P_n were calculated by linear quadratic equation regression from data measured over a range of C_i (0 → 950 $\mu\text{mol mol}^{-1}$). The initial slope of the linear portion of the P_n – C_i curve represents the carboxylation efficiency (CE) and the intercept of the C_i axis represents the CO_2 compensation point. Three individual plants per treatment were examined in both genotypes.

P_n versus PAR relationships

On day 13, P_n –PAR curves of upper mature leaves (leaf index number 6–8 from the shoot tip) were measured over a range of PAR (1,800, 1,600, 1,400, 1,200, 1,000, 800, 600, 500, 400, 300, 200, 100, 80, 60, 40, 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$) that controlled by a fan-cooled white Halogen light source. T_{air} and T_{leaf} was 29.6–41.3 and 28.7–39.5°C during the observation period (supplemented irradiance, >1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ caused transitory higher temperature in leaf cuvette although a fan-cooled light source was used). Light saturation point and light saturated P_n were calculated by natural logarithm regression from data measured over a range of PAR (20–1,800 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The initial slope of the linear portion of the P_n –PAR curve represents the apparent quantum yield (AQY) and the intercept of the PAR axis represents the light compensation point. Three individual plants per treatment were examined in both genotypes.

Chlorophyll *a* fluorescence

On day 2 and day 12, chlorophyll *a* fluorescence of 0.5-h dark-adapted leaves was measured using the same leaf for gas exchange measurement (leaf index number 6–8 from shoot apex, green parts were used for fluorescence measurement in salt-injured *P. popularis* leaves on day 12). Chlorophyll *a* fluorescence was measured using a PAM-2100 Fluorometer (Heinz Walz GmbH, Germany) at 8:30–10:00 a.m. and air temperature was 32.2–36.2°C. The minimal fluorescence level (F_0) was determined by a modulated light, which was lower enough (<0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) not to induce any significant variable fluorescence and the maximal fluorescence level (F_m) was determined by a 0.8 s

saturating pulse at $15,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ on dark-adapted leaves (30 min). Then the leaf was continuously illuminated with a white actinic light at a light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, the steady-state value of fluorescence (F_s) was reached within about 5–6 min and thereafter was recorded and a second saturating pulse at $15,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was imposed to determine the maximal fluorescence level in the light-adapted state (F_m'). The modulation frequency of measuring light source was 20 kHz. Following fluorescence parameters were calculated based on the above measurements:

1. Maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m);
2. Photochemical quenching coefficient $qP = (F_m' - F_s)/(F_m' - F_o)$;
3. Nonphotochemical quenching coefficient $qN = (F_m - F_m')/(F_m - F_o)$.

Plant harvests

Destructive harvests were carried out after 2, 8, 12 and 16 days of exposure to the saline treatment and three replicated plants per treatment were harvested at each sampling time. Leaves in upper shoot, leaf index number 4–10 from shoot apex were sampled for biochemical analysis, including soluble protein content, malondialdehyde (MDA) content, membrane permeability and isoforms of SOD and POD. Green parts in salt-injured *P. popularis* leaves were collected for biochemical analysis since visible symptoms of salt damage (leaves exhibited chlorosis or necrosis at the edge of leaves) were observed after 12 days of exposure to salinity.

MDA content

MDA content was measured according to Heath and Packer (1968). Approximately 0.5 g (fresh weight) leaf tissue was homogenized in 1.5 mL of 5% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at $1,500g$ for 10 min, and then the supernatant was diluted to 10 mL. A 2 mL of the diluted extract was mixed with 2 mL 0.67% 2-thiobarbituric acid (TBA) (weight/volume). The mixture was incubated in boiled water ($95\text{--}100^\circ\text{C}$) for 30 min, then centrifuged at $1,500g$ for 10 min. Absorbencies of the aqueous phase at 450, 532 and 600 nm were measured, respectively. MDA content in the aqueous phase was calculated based on the following formula:

$$C (\mu\text{mol/L}) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Membrane permeability

Thirty fresh leaf discs, 0.2 cm in diameter, were immersed in 10 mL distilled water and a subsequent 30 min vacuum was applied. Electrical conductivity (E_1) was then measured with a DDS-307 conductivity meter (Shanghai Precision & Scientific Instrument) at room temperature. Afterwards, leaf discs were incubated in boiled water ($95\text{--}100^\circ\text{C}$) for 30 min and electrical conductivity (E_2) was measured at room temperature. Leaf membrane permeability was calculated as: $E_1/E_2 \times 100\%$.

Analysis SOD and POD isoenzymes

Extracting SOD and POD were followed as per Dhindsa et al. (1981). Approximately 0.5 g (fresh weight) leaf tissue was homogenized on ice in 2 mL cold 0.05 M sodium phosphate buffer (pH 7.8) containing 1% PVPP-40. The homogenate was centrifuged at $10,000g$ for 20 min at 4°C and the supernatant was diluted to 5 mL. Then total protein content was determined according to Bradford (1976) using bovine serum albumin as standard. The supernatant was used as crude extracts for determination of SOD and POD isoenzymes. In brief, 30 μg of soluble protein were applied to native polyacrylamide gel electrophoresis at 4°C using a stacking gel (4% w/v) and a separating gel (10% w/v), respectively (Wang et al. 2004). Thereafter, SOD activity was detected by staining with riboflavin and nitroblue tetrazolium, as described by Beauchamp and Fridovich (1971). POD activity was detected by staining with acetic acid and benzidine, as described by Ros Barceló et al. (1987).

Different types of SOD were identified by adding inhibitors to the staining solution, such as 5 mmol/L KCN to inhibit CuZn-SOD activity, and 10 mmol/L H_2O_2 to inhibit CuZn-SOD and Fe-SOD activities (Bridges and Salin 1981; Almansa et al. 1989).

X-ray microanalysis of Na^+ and Cl^- in chloroplast

At the final harvest time (day 16), leaves (leaf index number 6–8 from shoot tip) were sampled from control and salt stressed plants of *P. euphratica* and *P. popularis*, respectively. Green parts were collected in salt-injured *P. popularis* leaves. Standard procedures required for sample preparation were followed as per Fritz (1989). A brief description is given.

Sampling and freeze-drying

Leaf segments, 2–3 mm long and 1–2 mm wide were cut along the branch veins adjacent to the central vein. Then

samples were immediately placed into aluminium sample holders and rapidly frozen in a 2:1 mixture of propane:isopentane at the temperature of liquid nitrogen. Samples were vacuum freeze-dried at -60°C for 72 h and then slowly allowed to equilibrate to room temperature (ca 22°C) over a period of 24 h. Then samples were stored over silica gel until infiltration in plastic.

Infiltration and polymerization

Freeze-dried leaves were transferred into vacuum-pressure-chambers, and infiltrated in ether at 27°C overnight before infiltrating with plastic. Plastic used was a 1:1 mixture of styrene and butyl methacrylate containing 1% benzoyl-peroxide stabilized with 50% phthalate. Infiltration with plastic was carried out in following steps: 1:1 ether:plastic for 24 h, 1:3 ether:plastic for 24 h, and finally 100% plastic for 24 h. Following infiltration, samples were transferred into gelatine capsules and polymerised at 60°C for 12 h, then transferred into 30°C oven and polymerised for at least 7 days.

Cutting

After polymerisation, samples were cut into $1\text{ }\mu\text{m}$ thick sections using dry glass knife with an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria). The slices were mounted in copper grids (mesh 50), coated with carbon and stored over silica gel until analysis.

X-ray microanalysis

Leaf sections were analysed in a Phillips EM 420 electron microscope equipped with the energy dispersive system EDAX DX-4 (EDAX International, Mahwah, NJ 07430, USA). The operating parameters were as follows: accelerating voltage, 120 kv; take-off angle, 25° and the time for collecting X-rays was 60 live seconds. Probe measurements were made on chloroplasts in spongy and palisade mesophyll cells and the magnification was at $\times 6350$. Ten to twenty measurements were taken from each leaf section. The X-ray spectra were processed with EDAX DX-4 software after manual fitting of the background. Concentrations of the elements at the analysed sites were determined by analytical calibration standard (Fritz and Jentschke 1994).

Data analysis

All data were subjected to oneway ANOVA and significant differences between means were determined by Duncan's multiple-range test. Unless otherwise stated, differences were considered statistically significant when $P < 0.05$.

Results

Photosynthesis response to salinity

Gas exchange of the two genotypes differed greatly in response to the initial salinity (50 mM NaCl). Net photosynthetic rate (P_n), stomatal conductance (G_s) and unit transpiration rate (TRN) in *P. euphratica* rapidly declined after 1–4 h of salt stress, then returned to control levels at 24 h, whereas there were no corresponding changes in stressed *P. popularis* plants (Fig. 1). In a longer-term of increasing salinity, gas exchange of salinised *P. popularis* markedly decreased after 8 days of stress, and continuously dropped to the lowest level on day 16 (Fig. 1). In comparison, NaCl-stressed *P. euphratica* plants maintained a relatively higher and constant level of P_n , G_s and TRN although salinity significantly lowered the gas exchange (Fig. 1).

P_n versus PAR under salt stress

Compared with control *P. popularis* plants, control *P. euphratica* plants had typically lower light compensation point (LCP), light saturation point (LSP), light saturated P_n (LSP_n), and apparent quantum yield (AQY) (Table 1). Generally, salinity increased LCP but lowered LSP, LSP_n and AQY in the two genotypes (Table 1). However, the effects of NaCl on these parameters were much less pronounced in *P. euphratica* as compared to *P. popularis* (Table 1).

P_n versus C_i under salt stress

Data from P_n – C_i curves showed that salinity reduced CO_2 saturation point (CSP), CO_2 saturated P_n (CSP_n), carboxylation efficiency (CE) but increased CO_2 compensation point (CCP) in both genotypes (Table 2). Compared with *P. euphratica*, the salt-induced increase of CCP was more pronounced in *P. popularis* (Table 2).

Chlorophyll *a* fluorescence response to salt stress

Initial salt stress (day 2) did not influence the minimal fluorescence level (F_o) and the maximal fluorescence level (F_m) in dark-adapted leaves of both genotypes although *P. euphratica* plants had typically lower F_o and F_m (Table 3). In a longer-term of salinity (12 days), visible change of chlorophyll *a* fluorescence was only observed in *P. popularis* leaves: (a) the maximal efficiency of PSII photochemistry (F_v/F_m) in the dark-adapted state significantly decreased due to the salt-induced increase of F_o and the marked decline of F_m (Table 3); (b) salt stress significantly decreased coefficient of photochemical quenching (qP) in

Fig. 1 Effects of NaCl on leaf net photosynthetic rate (P_n), stomatal conductance (G_s) and unit transpiration rate (TRN) of *P. euphratica* and *P. popularis* plants. Gas exchange of upper mature leaves (leaf index number 6–8 from the shoot apex) were measured at 8:30–10:00 a.m. under natural conditions where the air temperature (T_{air}) was 31.3–36.2°C and photosynthetically active radiation (PAR) was $974\text{--}1,004\ \mu\text{mol m}^{-2}\text{ s}^{-1}$, which was supplied by cool white fluorescent lamps supplemented with dysprosium lamps. Leaf temperature (T_{leaf}) ranged from 31.1 to 36.3°C during the period of gas exchange measurement. Each point is the mean of three plants and bars represent the standard error of the mean. Asterisk (*) represents significant difference at $P < 0.05$ between control and NaCl treatment

Table 1 Effects of NaCl on light compensation point (LCP), light saturation point (LSP), light saturated P_n (LSP_n) and apparent quantum yield (AQY) in leaves of *P. euphratica* and *P. popularis*

Species	Treatments	LCP $\mu\text{mol m}^{-2}\text{ s}^{-1}$	LSP $\mu\text{mol m}^{-2}\text{ s}^{-1}$	LSP_n $\mu\text{mol CO}_2\text{ m}^{-2}\text{ s}^{-1}$	AQY $\mu\text{mol CO}_2/\mu\text{mol photon}$
<i>P. euphratica</i>	Control	$37 \pm 4.23\text{ b}$	$900 \pm 37.2\text{ a}$	$7.5 \pm 0.40\text{ a}$	$0.0166 \pm 0.0018\text{ a}$
	NaCl	$79 \pm 6.16\text{ a}$	$500 \pm 5.21\text{ b}$	$2.5 \pm 0.52\text{ b}$	$0.0093 \pm 0.0013\text{ b}$
<i>P. popularis</i>	Control	$41 \pm 3.13\text{ b}$	$1,000 \pm 50.4\text{ a}$	$10.3 \pm 1.27\text{ a}$	$0.0354 \pm 0.0074\text{ a}$
	NaCl	$216 \pm 28.3\text{ a}$	$300 \pm 61.7\text{ b}$	$0.7 \pm 0.23\text{ b}$	$0.0073 \pm 0.0013\text{ b}$

P_n –PAR curves of mature leaves in upper shoot (leaf index number 6–8 from the shoot tip) were measured on day 13 at 8:30–10:00 a.m. over a range of PAR ($1,800 \rightarrow 20\ \mu\text{mol m}^{-2}\text{ s}^{-1}$). Irradiance was controlled by a fan-cooled white Halogen light source, T_{air} and T_{leaf} was 29.6–41.3 and 28.7–39.5°C during the observation period. Each value ($\pm$ SE) is the mean of three individual plants and values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment

Table 2 Effects of NaCl on CO_2 compensation point (CCP), CO_2 saturation point (CSP), CO_2 saturated P_n (CSP_n), and carboxylation efficiency (CE) in leaves of *P. euphratica* and *P. popularis*

Species	Treatments	CCP $\mu\text{mol mol}^{-1}$	CSP $\mu\text{mol mol}^{-1}$	CSP_n $\mu\text{mol m}^{-2}\text{ s}^{-1}$	CE $\text{mol m}^{-2}\text{ s}^{-1}$
<i>P. euphratica</i>	Control	$83 \pm 3.04\text{ b}$	$703 \pm 62.36\text{ a}$	$9.5 \pm 1.32\text{ a}$	$0.0366 \pm 0.0049\text{ a}$
	NaCl	$109 \pm 4.15\text{ a}$	$467 \pm 42.36\text{ b}$	$3.8 \pm 0.30\text{ b}$	$0.0167 \pm 0.0062\text{ b}$
<i>P. popularis</i>	Control	$89 \pm 8.36\text{ b}$	$827 \pm 147.50\text{ a}$	$16.6 \pm 2.50\text{ a}$	$0.0452 \pm 0.0029\text{ a}$
	NaCl	$146 \pm 17.21\text{ a}$	$522 \pm 27.68\text{ b}$	$4.2 \pm 0.81\text{ b}$	$0.0226 \pm 0.0061\text{ b}$

P_n – C_i curves of mature leaves in upper shoot (leaf index number 6–8 from the shoot tip) were measured on day 12 at 8:30–10:00 a.m. over a range of intercellular CO_2 concentrations (C_i) ($0 \rightarrow 1,000\ \mu\text{mol mol}^{-1}$) controlled by a mini CO_2 cartridge. PAR was $1,081\text{--}1,238\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ supplied by cool white fluorescent lamps supplemented with dysprosium lamps. T_{air} and T_{leaf} was 33.5–36.7 and 33.0–37.1°C during the observation period. Each value ($\pm$ SE) is the mean of three individual plants and values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment

Table 3 Effects of NaCl on leaf chlorophyll *a* fluorescence in *P. euphratica* and *P. popularis*

Days after treatment	Species	Treatments	Chlorophyll <i>a</i> fluorescence parameters				
			qP	qN	Fo	Fm	Fv/Fm
2	<i>P. euphratica</i>	Control	0.945 ± 0.011 a	0.693 ± 0.002 a	0.271 ± 0.005 a	1.342 ± 0.035 a	0.796 ± 0.024 a
		NaCl	0.938 ± 0.023 a	0.702 ± 0.003 a	0.279 ± 0.012 a	1.378 ± 0.043 a	0.801 ± 0.028 a
	<i>P. popularis</i>	Control	0.992 ± 0.007 a	0.846 ± 0.006 a	0.369 ± 0.034 a	2.050 ± 0.014 a	0.818 ± 0.021 a
		NaCl	0.994 ± 0.004 a	0.792 ± 0.002 a	0.371 ± 0.010 a	2.055 ± 0.062 a	0.820 ± 0.042 a
12	<i>P. euphratica</i>	Control	0.819 ± 0.014 a	0.831 ± 0.022 a	0.240 ± 0.026 a	1.258 ± 0.051 a	0.811 ± 0.027 a
		NaCl	0.792 ± 0.024 a	0.852 ± 0.001 a	0.264 ± 0.032 a	1.525 ± 0.067 a	0.825 ± 0.026 a
	<i>P. popularis</i>	Control	0.901 ± 0.016 a	0.781 ± 0.006 b	0.334 ± 0.008 b	1.910 ± 0.017 a	0.823 ± 0.006 a
		NaCl	0.868 ± 0.007 b	0.918 ± 0.001 a	0.377 ± 0.036 a	1.643 ± 0.032 b	0.771 ± 0.029 b

On day 2 and day 12, chlorophyll *a* fluorescence was measured using the same leaf for gas exchange measurement (leaf index number 6–8 from the shoot tip) with a PAM-2100 Fluorometer (Heinz Walz GmbH, Germany) at 8:30–10:00 a.m. Half-hour dark-adapted leaves were used in this experiment and air temperature was 32.2–36.2°C. The minimal fluorescence level (F_o) was determined by measuring modulated light ($<0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the maximal fluorescence level (F_m) by a 0.8 s saturating pulse at $15,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Then the leaf was continuously illuminated with a white actinic light at a light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, the steady-state value of fluorescence (F_s) was reached within about 5–6 min and thereafter was recorded and a second saturating pulse at $15,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was imposed to determine the maximal fluorescence level in the light-adapted state (F_m'). Based on the above mentioned measurements, following fluorescence parameters were calculated: (1) the maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), (2) the photochemical quenching coefficient $qP = (F_m' - F_s)/(F_m' - F_o)$, and (3) nonphotochemical quenching coefficient $qN = (F_m - F_m')/(F_m - F_o)$. Each value ($\pm$ SE) is the mean of three plants and values in the same column followed by different letters are significantly different ($P < 0.05$) between control and NaCl treatment

the light-adapted state but markedly elevated coefficient of nonphotochemical quenching (qN) after 12 days of salt treatment, although they were not altered by the initial salinity (day 2) (Table 3). In contrast, there were no significant effects of salinity on chlorophyll *a* fluorescence in *P. euphratica* during the period of salt stress (Table 3).

Na^+ and Cl^- concentrations in the chloroplasts

X-ray microanalysis showed that control *P. euphratica* plants had typically higher Cl^- concentration (58 mmol dm^{-3}) in the chloroplasts, as compared to control *P. popularis* plants (32 mmol dm^{-3}) (Fig. 2). Chloroplast Na^+ was not detectable in control plants of both genotypes (Fig. 2). Significant accumulation of Na^+ in the chlorop-

lasts was observed in the two poplars after 16 days of salinity, but *P. popularis* had 1.9-fold higher Na^+ than *P. euphratica* (Fig. 2). Chloroplast Cl^- in salinised *P. popularis* markedly increased to 221 mmol dm^{-3} , which were 2.7 times of that in stressed *P. euphratica* plants (Fig. 2).

MDA content and membrane permeability

Compared with non-stressed plants, malondialdehyde (MDA) content and membrane permeability (MP) in *P. popularis* leaves remained unchanged in the first 12 days of salt stress, but increased by 120% (MDA) and 130% (MP), respectively on day 16 (Fig. 3). In contrast, leaf MDA and MP in *P. euphratica* did not significantly increase during the period of salt stress (Fig. 3).

Fig. 2 Effects of NaCl on chloroplast Na^+ and Cl^- concentration in *P. euphratica* and *P. popularis* leaves. Leaves in upper shoot (leaf index number 6–8 from shoot apex) were sampled on day 16. Leaf segments were vacuum freeze-dried, infiltrated in ether and polymerised with plastic. Samples were cut into $1 \mu\text{m}$ thick sections for X-ray microanalysis. Probe measurements were made on chloroplasts

in spongy and palisade mesophyll cells and the magnification was at $\times 6350$. Ten to twenty measurements were taken from each leaf section. Each column is the mean of three plants and bars represent the standard error of the mean. Asterisk (*) means significant difference at $P < 0.05$

Fig. 3 Effects of NaCl on MDA contents and membrane permeability (MP) (%) in leaves of *P. euphratica* and *P. popularis*. Leaves in upper shoot (leaf index number 4–10 from shoot tip) were sampled in both genotypes. Each point is the mean of three plants and bars represent the standard error of the mean. Asterisk (*) represents significant difference at $P < 0.05$

Isoenzyme patterns of SOD and POD under salinity

SOD isoenzymes

After gradient PAGE of crude leaf extracts, two SOD isoenzymes were detected in control plants of both genotypes although the pattern—with regard to migration and activities of isoenzymes was different (Fig. 4). KCN or H_2O_2 both inhibited activity of the two dominant SOD isoenzymes in both genotypes, indicating that these SOD isoenzymes were CuZn-SOD isoforms (Fig. 5). The activity of CuZn-SOD I and CuZn-SOD II in *P. popularis* was not detectable after 16 days of salt stress although they were not reduced on day 12 (Fig. 4). In comparison, there were no marked inhibitory effects of NaCl on the two SOD isoenzymes in *P. euphratica* during the period of observation (Fig. 4).

POD isoenzymes

Similarly, there were genotypic differences in the pattern of POD isoenzymes under control conditions although four distinct POD isoenzymes were observed in both genotypes (Fig. 4). Control *P. popularis* exhibited typically higher Pod2 and Pod3 than control *P. euphratica* plants (Fig. 4). However, Pod1 and Pod2 were not detected in stressed *P. popularis* and the activity of Pod3 and Pod4 diminished with increasing the duration of salt stress (Fig. 4). In contrast, activities of POD isoforms in *P. euphratica* were remained almost unaltered during the period of salt treatment (Fig. 4).

Fig. 4 Effects of NaCl on isoenzyme patterns of SOD and POD in leaves of *P. euphratica* and *P. popularis*. Leaves in upper shoot (leaf index number 4–10 from shoot apex) were sampled in both genotypes. In each track 30 μ g of soluble protein were applied to native polyacrylamide gel electrophoresis at 4°C using a stacking gel (4% w/v) and a separating gel (10% w/v)

Fig. 5 Densitometric analysis of non-denaturing activities gels of SOD isoforms from *P. euphratica* (E) and *P. popularis* (P) leaves. Leaves in upper shoot (leaf index number 4–10 from shoot apex) were sampled in both genotypes. In each track 30 μ g of soluble protein were applied to native polyacrylamide gel electrophoresis at 4°C using a stacking gel (4% w/v) and a separating gel (10% w/v). Identification of SOD isoforms was performed by pre-incubation of gels with inhibitors (5 mmol/L KCN and 10 mmol/L H_2O_2)

Discussion

Photosynthetic response to salinity

Unlike *P. popularis*, *P. euphratica* plants exhibited a rapid decline of gas exchange in response to the initiation of salt stress (Fig. 1). The decline of Pn and TRN was mainly resulted from the stomatal closure (Fig. 1). It can be inferred that salt-induced inhibition of photosynthesis was not due to ion toxicity caused by a lower soil salt; rather it presumably was a function of signal from the roots. Chen and colleagues reported that xylem abscisic acid (ABA) concentration increased more rapidly in *P. euphratica* than other salt-sensitive poplar genotypes, e.g. a hybrid of *P. euphratica*, *P. talassica* Kom $\times$ (*P. euphratica* + *Salix alba* L.) and *P. popularis* (Chen et al. 2001, 2002a). Recently, we found that the stomatal closure in *P. euphratica* was associated with a rapid increase of endogenous ABA and calmodulin (CaM) (Chang et al. 2006). Therefore, the salt-induced elevation of stress signals, e.g. ABA, Ca^{2+} /CAM, caused the stomatal closure and reduced the gas exchange. The reduced water flow would limit the amount of root-to-shoot salt transport (Chen et al. 2002a). The insensitivity of *P. popularis* to salt stress implies that this genotype was not able to trigger the initial adjustments that conferring salt tolerance after the onset of salt stress (Chen et al. 2001, 2002a).

In a longer-term of salinity, *P. popularis* plants exhibited a great reduction of photosynthesis, whereas stressed *P. euphratica* retained a relatively higher capacity for CO_2 assimilation (Fig. 1). Drastic decline of Gs in salt-stressed *P. popularis* indicated that the reduced Pn was mainly the limited diffusion of CO_2 in the leaves through a decrease of stomatal and mesophyll conductances (Flexas et al. 2004;

Grassi and Magnani 2005). Pn–Ci curves exhibited lower values of CO_2 saturation point (CSP), CO_2 saturated Pn (CSP_n) and carboxylation efficiency (CE) in control plants of both genotypes as compared to what is usual in poplars (Table 2), mainly resulting from the higher air temperature (33.5–36.7°C) and leaf temperature (33.0–37.1°C) during the observation period. Salt stress reduced the carboxylation efficiency by a similar magnitude (50–54%) in *P. popularis* and *P. euphratica* (Table 2), however, the inhibitory effect of NaCl on light reaction was significantly different in the two tested genotypes (Table 1). NaCl caused a higher restriction on apparent quantum yield (AQY), light saturation point (LSP), and light saturated Pn (LSP_n) in *P. popularis*, as compared to *P. euphratica* (Table 1). Chlorophyll *a* fluorescence data indicated that a longer-term of salt stress (12 days) decreased the maximal efficiency of PSII photochemistry (Fv/Fm) in the dark-adapted state and photochemical quenching (qP) in the light-adapted state (Table 3). The salt-induced increase of Fo and the marked decline of Fm in *P. popularis* imply the inhibitory effects of chloroplast salt ions on PSII reaction centre although the elevated coefficient of nonphotochemical quenching (qN) may serve to dissipate excess excitation energy (Table 3).

Salt accumulation in chloroplasts, SOD and POD isoenzymes and lipid peroxidation under salt stress

The restriction of Pn in *P. popularis* appears to be a consequence (at least in part) of salt entry into the chloroplasts. Salt ions transport from root to shoot via transpiration stream and accumulate in leaf apoplast after water evaporates from leaf. High Na^+ and Cl^- in the mesophyll cell walls of *P. popularis* (data not shown) brought about an osmotic shock (Oertli 1968; Greenway and Munns 1980; Flowers and Yeo 1986). Moreover, salinity markedly increased chloroplast Na^+ and Cl^- in *P. popularis*, especially Cl^- (Fig. 2). Great buildup of Na^+ and Cl^- in chloroplasts may directly cause ion toxicity and induce the subsequent oxidative stress (Hernández et al. 1993, 1995, 1999; Gosset et al. 1996; Gómez et al. 1999; Savouré et al. 1999). The activity of CuZn-SOD isoenzymes in *P. popularis* was not detectable at the end of experiment and the reduction of POD isoenzymes increased with increasing exposure period (Fig. 4). Salt-induced decline of SOD and POD isoenzymes suggests the reduced ability in reactive oxygen species (ROS) elimination. ROS may initiate membrane lipid peroxidation, leading to reduced membrane lipid unsaturation and membrane protein polymerization, thus, results in an increase of membrane permeability (Chen 1991). In this study, the membrane permeability in *P. popularis* increased with MDA under salt stress (Fig. 3). We conclude the decline of SOD and POD isoenzymes accelerated lipid

peroxidation and membrane permeability, which in turns resulted in more salt entry into the cytoplasm and chloroplast. The buildup of salt ions in chloroplasts may exhibit negative effects on light and dark reactions, resulting in a photosynthesis drop in *P. popularis* (Fig. 1).

In contrast to *P. popularis*, *P. euphratica* maintained activity of SOD and POD isoenzymes during the period of salt stress (Fig. 4), correspondingly, there was no significant increase of MDA and membrane permeability in stressed plants (Fig. 3). Compared with control plants, chloroplast Na^+ increased to 15 mmol dm^{-3} and Cl^- increased by 41% in NaCl-stressed plants, which were much lower than salinised *P. popularis* (Fig. 2). The lower salt buildup in chloroplasts may partly result from the effective Na^+/H^+ antiporters in *P. euphratica*, *PeNhaD1*. Under saline conditions, the transcript levels of *PeNhaD1* were maintained in *P. euphratica* but collapsed in a salt-sensitive genotype, *P. canescens* (Ottow et al. 2005b). Moreover, salinity increased the activity of plasma membrane H^+ -ATPase— H^+ pumps in *P. euphratica* cells (our unpublished data). Accordingly, *P. euphratica* was able to drive toxic Na^+ ions out of the cytoplasm via Na^+/H^+ antiporter systems. Therefore, less salt ions accumulated in the chloroplasts of mesophyll cells, the photosynthesis of *P. euphratica* was less restricted.

In conclusion, salinity restricted CO_2 assimilation in the two genotypes, but a more pronounced inhibitory effects was observed in the salt-sensitive genotype, *P. popularis* as compared to the salt-tolerant genotype, *P. euphratica*. The reduced Pn in both poplars was mainly the result of decreased stomatal and mesophyll conductances, which causing a lower CO_2 concentration in chloroplasts of stressed leaves. Furthermore, great buildup of Na^+ and Cl^- in chloroplasts of *P. popularis* may exhibit direct and indirect restrictions on dark and light reactions. Salt-induced decline of SOD and POD isoenzymes in *P. popularis* would reduce the free radical scavenging ability of the cell, leading to a variety of toxic responses to uncontrolled oxidation, thus resulted in lipid peroxidation and ion entry into the cytoplasm. In comparison, stressed *P. euphratica* plants restricted the salt influx into the chloroplasts and maintained activity of SOD and POD isoenzymes, the photosynthetic capacity of *P. euphratica* was consequently less restricted.

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