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ORIGINAL ARTICLE

Changes in photosynthesis of alpine plant *Saussurea superba* during leaf expansion

Sheng-Bo Shi[1] · Rui Shi[2] · Miao Li[1]

Abstract The native alpine plant *Saussurea superba* is widely distributed in Qinghai–Tibetan Plateau regions. The leaves of *S. superba* grow in whorled rosettes, and are horizontally oriented to maximize sunlight exposure. Experiments were conducted in an alpine *Kobresia humilis* meadow near Haibei Alpine Meadow Ecosystem Research Station (37°29′–37°45′N, 101°12′–101°33′E; alt. 3200 m). Leaf growth, photosynthetic pigments and chlorophyll fluorescence parameters were measured in expanding leaves of *S. superba*. The results indicate that leaf area increased progressively from inner younger leaves to outside fully expanded ones, and then slightly decreased in nearly senescent leaves, due to early unfavorable environmental conditions, deviating from the ordinary growth pattern. The specific leaf area decreased before leaves were fully expanded, and the leaf thickness was largest in mature leaves. There were no significant changes in the content of chlorophylls (Chl) and carotenoids (Car), but the ratios of Chl a/b and Car/Chl declined after full expansion of the leaves. The variation of Chl a/b coincided well with changes in photochemical quenching (q_P) and the fraction of open PSII reaction centers (q_L). The maximum quantum efficiency of PSII photochemistry after 5 min dark relaxation ($F_{(v)}/F_{(m)}$) continuously increased from younger leaves to fully mature leaves, suggesting that mature leaves could recover more quickly from photoinhibition than younger leaves. The light-harvesting capacity was relatively steady during leaf expansion, as indicated by the maximum quantum efficiency of open PSII centers (F_v'/F_m'). UV-absorbing compounds could effectively screen harmful solar radiation, and are a main protection way on the photosynthetic apparatus. The decline of q_P and q_L during maturation, together with limitation of quantum efficiency of PSII reaction centers ($L_{(PFD)}$), shows a decrease of oxidation state of Q_A in PSII reaction centers under natural sunlight. Furthermore, light-induced (Φ_{NPQ}) and non-light-induced quenching (Φ_{NO}) were consistent with variation of $L_{(PFD)}$. It is concluded that the leaves of *S. superba* could be classified into four functional groups: young, fully expanded, mature, and senescent. Quick recovery from photoinhibition was correlated with protection by screening pigments, and high level of light energy trapping was correlated with preservation of photosynthetic pigments. Increasing of Φ_{NPQ} and Φ_{NO} during leaves maturation indicates that both thermal dissipation of excessive excitation energy in safety and potential threat to photosynthetic apparatus were strengthened due to the declination of q_P and q_L, and enhancement of $L_{(PFD)}$.

Keywords Alpine plant · Chlorophyll fluorescence parameters · Leaf growth · Photosynthetic pigments · Qinghai–Tibetan Plateau · *Saussurea superba*

Abbreviations
SLA	Specific leaf area
LWR	Ratio of dry to fresh weight of leaves
Chl	Chlorophylls
Car	Carotenoids

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Chl a/b	Ratio of chlorophyll a to chlorophyll b
Car/Chl	Ratio of carotenoids to chlorophylls
q_P	Photochemical quenching coefficient
NPQ	Non-photochemical quenching coefficient
q_L	Fraction of opened PSII reaction centers
$F_{(v)}/F_{(m)}$	Maximum quantum efficiency of PSII photochemistry after 5 min dark relaxation
F'_v/F'_m	Maximum quantum efficiency of PSII photochemistry at given light
$L_{(PFD)}$	Relative limitation of quantum efficiency of PSII reaction centers
Φ_{NPQ}	Quantum yield of light-induced PSII regulatory energy dissipation
Φ_{NO}	Quantum yield of non-light-induced PSII non-regulatory energy dissipation
Φ_{PSII}	Actual quantum yield of PSII photochemistry
Q_A	Primary quinone electron acceptor of PSII

Introduction

Plants absorb sunlight to drive photochemical reactions of photosynthesis, and absorbed sunlight may also lead to deleterious effects on photosynthetic performance. Under natural conditions, plants often absorb sunlight energy exceeding what the photosynthetic apparatus can utilize, and onset of photoinhibition is strongly correlated with the absorption of excess excitation energy for photosynthesis (Murchie and Niyogi 2011). The term of photoinhibition has been characterized as depression of net photosynthetic rates and reduction of photochemical efficiency of PSII (Tikkanen et al. 2014), which could be indicative of either an injurious effect, or a regulatory process of rearrangement PSII and consequently an expression of a photoprotective energy dissipation (Demmig-Adams and Adams 2006; Sáez et al. 2013).

Because only part of the absorbed light energy can be utilized in photochemical reactions, excess energy needs to be harmlessly dissipated. Excess excitation energy causes production of reactive oxygen species (ROS), and results in damage on photosynthetic apparatus (Demmig-Adams and Adams 1992; Müller et al. 2001; Jiang et al. 2005; Asada 2006). Careful regulation of the transition between two alternative states of the photosynthetic machinery is considered essential to both utilize and dissipate energy in continuously changing environments (Demmig-Adams et al. 2012). Usually, when the whole plant is exposed to excess solar irradiation, differently developed leaves may exhibit different photosynthetic activity even irradiated by nearly similar actinic light intensity. Till now, few studies have focused on photosynthetic performance during leaf expansion (Sperdouli and Moustakas 2012), especially regarding the relationship between growth and leaf photosynthetic pigments content in natural environments.

Alpine plants often experience extreme variations in temperature, water availability and sunlight. Although alpine plants are well adapted for living in such harsh environmental conditions, photoinhibition can still be a significant phenomenon affecting the photosynthetic activity. Thick blade and heavy pigmentation have been considered as main photoprotective mechanism, and heavy pigmentation might have compensated the protective effects of a stronger leaf thickness (Rikkinen 1995).

The Qinghai–Tibetan Plateau is the highest and youngest plateau in the world, and is known as the third pole due to its fragile and sensitive characters during present global environmental changes (Yu and Lu 2011). *Saussurea superba* plants mainly grow in alpine meadow grassland, and are widely distributed on the Qinghai–Tibetan Plateau. Rosette whorled phyllotaxy of *S. superba* exhibits a series of developed leaves from inner younger to outside senescent ones. Expanding leaves of most dicotyledonous plants show increases in leaf area, thickness, chlorophyll contents, and photosynthesis rates, along with a decrease in dark respiration (Catský and Šesták 1996). Maximum photosynthesis rates and photochemical activity are typically reached before leaves fully expand (Choinski et al. 2003). But few studies have focused on the differences of harmless dissipation of excess solar energy during leaf ontogeny, and its physiological significance of non-radiative thermal dissipation.

The present study is aimed at characterizing phototolerance and susceptibility among differently developed leaves which mainly based on the analysis of chlorophyll fluorescence parameters. The objectives of this study are: (1) to examine differences of PSII photochemistry during leaf expansion; (2) to determine the development of photosynthetic activity and its acclimation to harsh environmental factors; and (3) to investigate light-induced and non-light-induced non-photochemical quenching. Based on our findings, the potential eco-physiological roles of leaf thickness and UV-absorbing substances are discussed.

Materials and methods

### Field sites and growth conditions

The experiments were conducted in the research and observation field site of the Haibei Alpine Meadow Ecosystem Research Station, the Chinese Academy of Sciences. The research area is located at the northeast of Qinghai–Tibetan Plateau, and south hillside of the Leng-Long-Ling mountain which is at the east side of the Qilian Mountains, 37°29′–

37°45′N, 101°12′–101°33′E; altitude 3200 m. The research region is situated in the inland with a typical plateau continental climate. As influenced by higher elevation, the daily variation of air temperature is very large, and climate can only be separated into cold and warm seasons. The annual average air temperature is -1.7 °C, with the warmest month at 9.8 °C in August, and the coldest month at -14.8 °C in January. The annual precipitation is about 600 mm with nearly 80 % in the plant growing season from May to September. The air is thin with annual average of the barometric pressure at 691.4 hPa.

Alpine *Kobresia humilis* meadow community is one of the main vegetation types in this area, containing various perennial grasses and forbs that mainly depend on grazing intensity. From July to August, it is the luxuriant growth period of alpine *K. humilis* meadow. The canopy structure of this plant community is simple, and plant height is low, so the primary productivity is small (Zhao and Zhou 1999). *S.*

superba is one of the main accompanied native alpine plants which is widely distributed in Qinghai–Tibetan Plateau meadow regions (Fig. 1). Its rosette whorled phyllotaxis exhibits continuously developing leaves from inner younger to outside senescence, successively grown in extremely short stem. *S. superba* is a perennial herb, its short stem is usually sturdy and buried under the soil of 2–4 cm depth during the early developing stage before flowering, its long petiole grows up nearly vertically to soil surface and then bents into horizontal direction, especially for mature leaves (Fig. 1a–d). Horizontally oriented leaves are fully exposed to sunlight, so it is easy and convenient to identify the changes of leaf growth property and its regulation of photosynthetic light harvesting in terms of developed leaf stages.

Soil type is mainly alpine meadow soil. Soil thin, usually with depth 60 cm and rich in organic matter (refs). Root grows densely and tight turf is formed from live and dead roots.
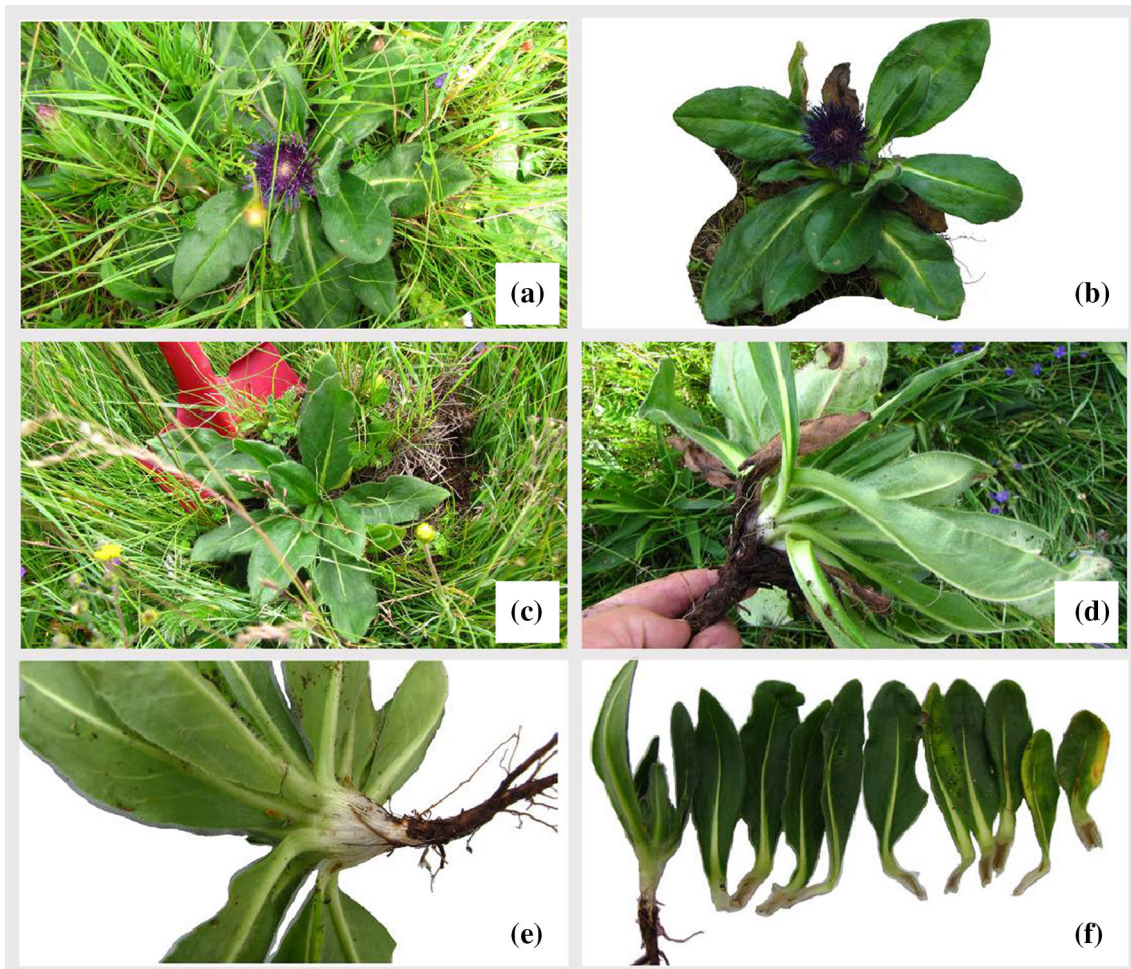
Fig. 1 Alpine plant *S. superba* and its separation of whole leaves. **a** the flowering *S. superba* plant in the field site; **b** an representative plant separated from other accompanying plants; **c** excavation from soil; **d** separated *S. superba* plant; **e** an representative plant with root appendages; **f** separation of all leaves in a sequential manner

Experimental setup

The experiments were conducted mainly from the middle of July to early August. S. superba plants were selected from the flat and uniform meadow area. For the most mature and healthy S. superb plants, the leaf number was at least 10–14 even if the youngest leaves were not counted. Each measuring plant was chosen with nearly the same leaf number and size. The leaf development stages were determined by leaf size, leaf color and luster along with the order and marked with capital letters from A to L to classify leaves from younger to older. Very small leaves marked A01 and A02 were included only in growth analysis. When measuring photosynthetic pigments and UV-absorbing compounds, the leaves were combined into AB, CD, EF, GH, IJ, KL groups because of shortage of leaf material for leaf disc punching. The combined capital letters of AB, CD, EF, GH, IJ, and KL approximately represented the development stages from younger, expanding, fully expanded, mature, fully mature and senescent. The senescent leaves marked KL were not suitable for chlorophyll fluorescence because of irregular distribution of brown patches.

S. superba plants were picked up in the morning. For determining the photosynthetic pigments and UV-absorbing compounds, the plant materials were collected before 9:00 in the morning in a clear day. When collecting, each plant was cleaned with soft brush in the field and some damaged older leaves were removed. The plants were sealed in plastic bags and immediately stored in an ice chest and then returned to the laboratory for leaf measurements. Chlorophyll fluorescence parameters were measured on attached leave in the field. To avoid chronic photosynthetic photoinhibition, only data measured in the morning from 9:00 to 11:30 were used for chlorophyll fluorescence analysis.

Leaf growth analysis

In the cold room with subdued light, individual leaves were separated and marked (Fig. 1e, f). After removing the petiole, leaf area was determined with Li-COR 3000 area meter, and weighed on an electronic scale. Plants were dried in an electric oven at the temperature of 100 °C for 30 min and then kept at 75 °C for nearly 24 h till steady weight. The specific leaf area (SLA) was expressed as the leaf area based on dry weight unit, and the leaf weight ratio (LWR) was expressed as the ratio of dry to fresh weight.

Leaf thickness measurement

Leaf thickness was measured with an electronic digital thickness caliper in the cold room using another group of S.

superba. Individual leaves were separated from outside one by one, and then measured on both right and left sides of blade in middle parts avoiding the main vein. For younger and very small leaves, the measurement position was around middle of whole leaf. Total 3–6 data were collected in each blade and average value was calculated for further statistical analysis.
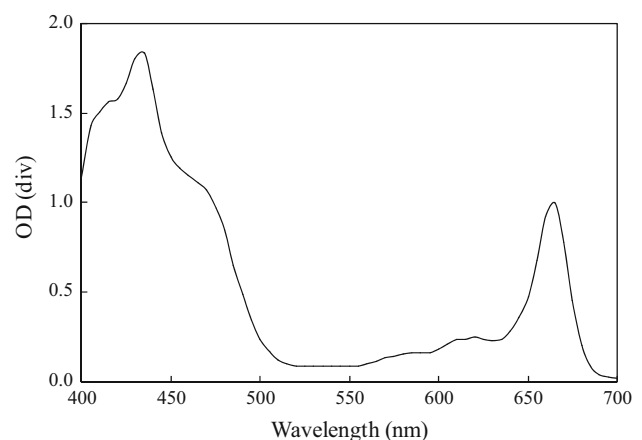
Photosynthetic pigments measurement

The content of photosynthetic pigments was expressed on leaf area basis. Differently developed leaves were identified from outside mature to inner younger leaves except for some older and damaged outside leaves. Leaf discs of 0.5 cm diameter were punched, and eight of them were immersed in a bottle containing 10 mL of acetone:ethanol:water (4.5:4.5:1 by volume) mixture. The bottle was sealed tightly and kept in a dark room with air temperature below 10 °C, and left them for pigment extraction for about 10 days until the leaf discs were colorless (Shi et al. 2004). Because of shortage of leaf material, in particular for younger leaves, two neighboring blades were combined together when punching leaf discs and usually A01 and A02 leaves were included in AB groups. The typical absorption spectrum of leaching solution is shown in Fig. 2 and chlorophyll and carotenoids contents were estimated spectrophotometrically by the absorbance and equation of Zhu (1990) and Wellburn (1994), as below:

$$\mathrm{Chl}\,a = 12.71D_{663} - 2.59D_{645}$$

$$\mathrm{Chl}\,b = 22.88D_{645} - 4.67D_{663}$$

$$\mathrm{Car} = (1000D_{470} - 2.86\mathrm{Chl}\,a - 129.8\mathrm{Chl}\,b)/245$$

Fig. 2 Absorption spectrum of photosynthetic pigment extracted from leaves of S. superba using acetone:ethanol:water (4.5:4.5:1 by volume) mixed liquor

UV-absorbing compounds measurement

The content of UV-absorbing compounds was analyzed in terms of leaf area units. Leaf discs of 0.5 cm diameter were punched and classified like measurement of photosynthetic pigments. Eight leaf discs were immersed in a bottle containing 10 mL of acidic methanol (MeOH:H$_2$O: HCl = 79:20:1 by volume). The lid was tightly sealed, kept and extracted in dark room with air temperature below 10 °C for about 10 days. UV-absorbing compounds were determined using spectrophotometer UV-1601 (Day et al. 1994; Shi et al. 2004), and the content was expressed as absorbance value at 305 nm and average absorbance value was calculated between 255 and 355 nm in 5 nm steps.

Chlorophyll fluorescence parameters analysis

Chlorophyll fluorescence was measured on leaves at various stages of expansion with FMS-2 portable pulse amplitude modulation fluorometer (Hansatech Instruments LTD, Norfolk, UK). Plants were chosen to make sure that all leaves were exposed to sunlight during most of daytime. A leaf clip holder was clamped in the middle of the left or right side of leaf, avoiding the midrib and main branch vein. Leaves were classified as for the measurement of photosynthetic pigments except senescent leaves KL.

The PSII maximal fluorescence yield (F'_m) and steady-state fluorescence yield (F_s) were measured at three consecutive 30 s intervals with the leaf spot fully exposed to natural sunlight, then adapter/fiber combination (opening type) of fiber optic probe was covered immediately with a dark adaptation sealer for accurately 5 min, the minimal fluorescence yield ($F_{(o)}$) and the maximum fluorescence yield ($F_{(m)}$) of PSII reaction centers were determined. The maximum quantum efficiency of PSII photochemistry after 5 min dark adaptation was expressed as $F_{(v)}/F_{(m)}$, here $F_{(v)} = F_{(m)} - F_{(o)}$.

Fully relaxed maximum quantum efficiency of PSII photochemistry F_v/F_m was measured on other healthy plants of *S. superba* in a nearby area in the afternoon before sunset. Mature leaves were darkened for about 4 h to determine the initial fluorescence yield (F_o) and the maximum fluorescence yield (F_m) of PSII reaction centers in the evening at about 21:00. Saturating flash light flashes of approximately 6500 µmol photons m^{-2} s^{-1} during a 0.7 s pulse were used for determination of F_m, $F_{(m)}$ and F'_m. The value of minimal level of fluorescence during illumination (F'_o) was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m + F_o/F'_m)$.

Maximum quantum efficiency of PSII photochemistry during illumination was expressed as F'_v/F'_m, here $F'_v = F'_m - F'_o$. Actual photochemical quantum yield of PSII electron transfer is $\Phi_{PSII} = (F'_m - F_s)/F'_m$ (Genty et al. 1989). Photochemical and non-photochemical quenching coefficients were calculated as $q_P = (F'_m - F_s)/(F'_m - F'_o)$ and NPQ $= F_m/F'_m - 1$ (Bilger and Björkman 1990). Relative limitation of quantum efficiency of PSII reaction centers was calculated as $L_{(PFD)} = 1 - (q_P \times F'_v/F'_m)/0.83$, 0.83 being the optimum quantum efficiency of PSII reaction centers. Relative photoinhibition was estimated according to the method of Dodd et al. (1998), $100 - [(F_{(v)}/F_{(m)}$ after 5 min darken)/(F_v/F_m at 2100 hours)] $\times$ 100. Fraction of open PSII reaction centers (q_L) was estimated according to the formula given by Baker (2008), $q_L = q_P \times (F'_o/F_s)$. Quantum yield of light-induced PSII regulatory energy dissipation (Φ_{NPQ}) and quantum yield of non-light-induced PSII non-regulatory energy dissipation (Φ_{NO}) were calculated according to the formula given by Kramer et al. (2004): $\Phi_{NO} = 1/[NPQ + 1 + q_L \times (F_m/F_o - 1)]$; $\Phi_{NPQ} = 1 - \Phi_{PSII} - 1/[NPQ + 1 + q_L \times (F_m/F_o - 1)]$.

Statistical analysis

The data were analyzed by one-way ANOVA. All analyses were performed with SPSS (version 16.0) software, and the results were expressed as average values and their standard error of mean (SE), least-significant difference (LSD) were used to compare the mean values among different developed leaf groups.

Results
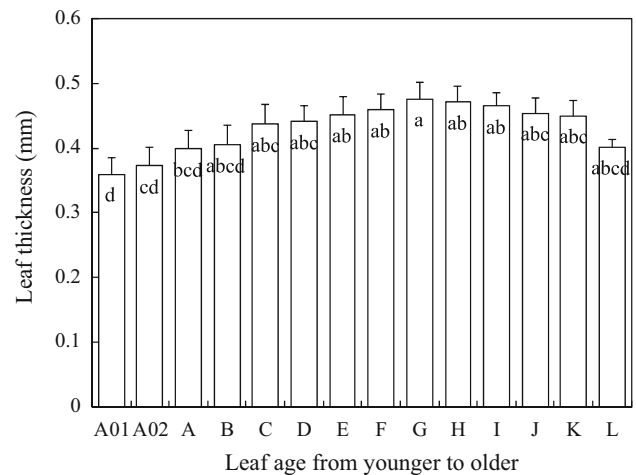
Leaf growth analysis

Rosette whorled leaves of *S. superba* showed significant difference in leaf size. Leaf area increased from younger to maturity, and slightly declined after full expansion (Fig. 3a). The leaves marked A01 and A02 were smaller than 5 cm^2, and these leaf blades were not suitable for chlorophyll fluorescence measurement and even not enough for photosynthetic pigment analysis. The SLA declined during leaf expansion. Although there were no significant changes when leaves gradually became senescent, a small increasing trend could be identified (Fig. 3b). There were no significant changes in the LWR among differently developed leaves (Fig. 3c). An opposite tendency in leaf area and SLA appeared through the entire period of leaf expansion until close to senescence.

Although statistical analysis was not all significant, leaf thickness increased gradually during leaf expansion until leaves fully maturation, and then a slight decrease took place (Fig. 4). Younger leaves of A01, A02 and A were
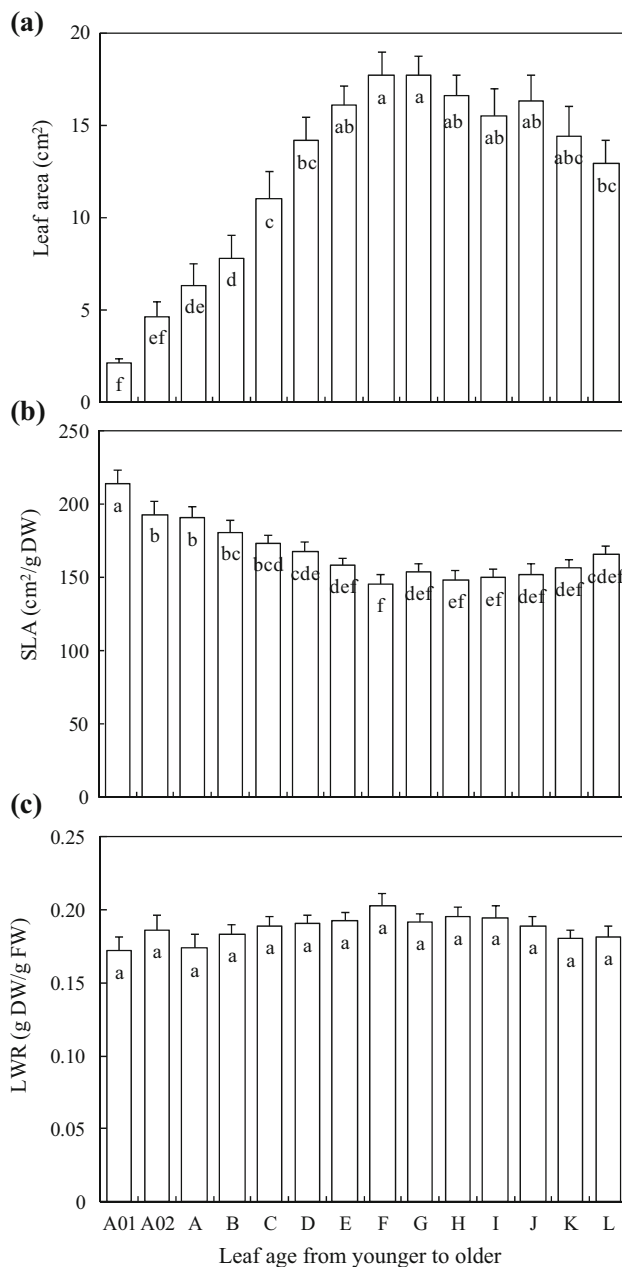
Fig. 3 The leaf properties of alpine plant *S. superba* at differently developed leaf stages. **a** leaf area; **b** specific leaf area; **c** leaf weight ratio of dry to fresh. *Capital letters* in *horizontal axial* from *A* to *L* represent leaf development whorled from inner younger to outside senescent leaves, and *A01* and *A02* are the leaves younger than leaf *A* and mostly do not fully flatten. Data are mean ± SE, sample number is 20 in leaf *A01*, 12 from leaf *A02* to *I*, and only 5–7 in leaf *J*, *K* and *L*. Values with *different letters* (presented in each *vertical bars*) are significantly different at $p = 0.05$ level

Fig. 4 Leaf thickness of alpine plant *S. superba* as a function of differently developed leaf stages. *Capital letters* in *horizontal axial* from *A* to *L* represent whirling developed leaves from inner younger to outside senescent leaves, and *A01* and *A02* are the leaves younger than leaf *A* and mostly do not fully flatten. Data are mean ± SE, sample number is 10 from leaf *A01* to *I*, and only 5–6 in leaf *J*, *K* and *L*. Values with *different letters* (presented in each *vertical bars*) are significantly different at $p = 0.05$ level
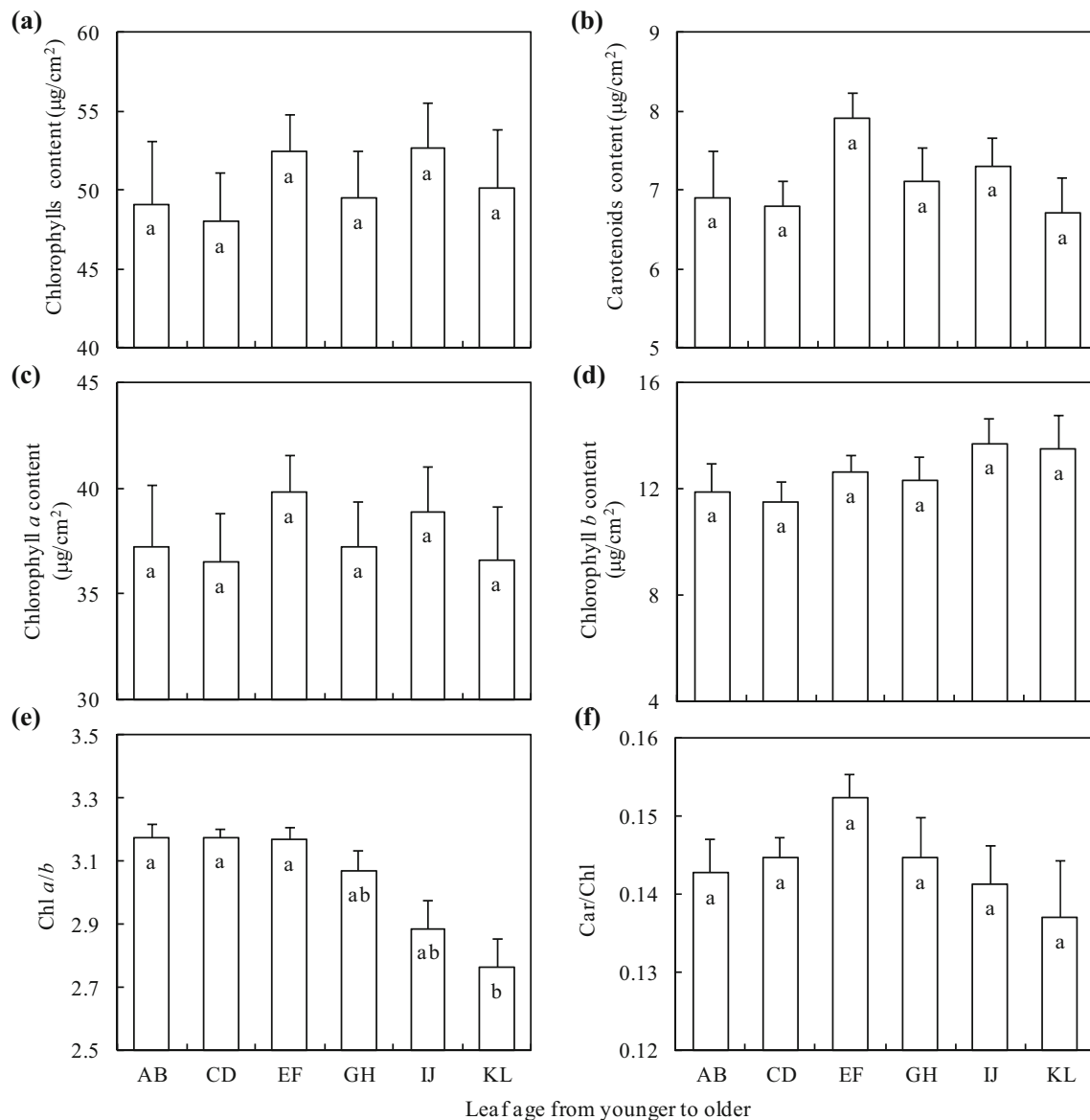
Leaf photosynthetic pigments and screening pigments

The content of photosynthetic pigments in relation to leaf area did not show any significant difference among differently developed leaves (Fig. 5a, b). Although the content of chlorophyll *a* and chlorophyll *b* also did not show significant variation (Fig. 5c, d), the ratio of chlorophyll *a* to chlorophyll *b* (Chl *a/b*) decreased after leaves fully expanded, and there was no difference from younger (AB) to fully expanded leaves (EF) (Fig. 5e). The ratio of carotenoids to chlorophylls (Car/Chl) did not show significant changes (Fig. 5f). It seems that the ratio of Car/Chl was at maximum in fully expanded leaves EF, and showed a declining trend when leaves tend to senescence.

The content of UV-absorbing compounds in the younger leaves was lower compared to that in expanded and mature leaves. When the leaves tend to senescence (KL), the content of UV-absorbing compounds exhibited a declined tendency in the 305 nm wavelength (Fig. 6a) and also a little decreased in average absorbance value determined in the range from 255 to 355 nm (Fig. 6b).

Maximum quantum efficiency of PSII photochemistry

The maximum quantum efficiency of PSII after 5 min dark adaptation ($F_{(v)}/F_{(m)}$) increased with leaf maturity (Fig. 7a). Referring to fully relaxed maximum quantum

significantly thinner than fully expanded leaves, and there was no significant difference between mature and older leaves. As seen in Figs. 3a and 4, the changes of leaf area and leaf thickness showed similar trend, and the correlation coefficient for linear correlation (R^2) was 0.893.

Fig. 5 Changes of photosynthetic pigments in alpine plant *S. superba* during leaf expansion. *Capital letters* in *horizontal axial* from *AB* to *KL* represent deve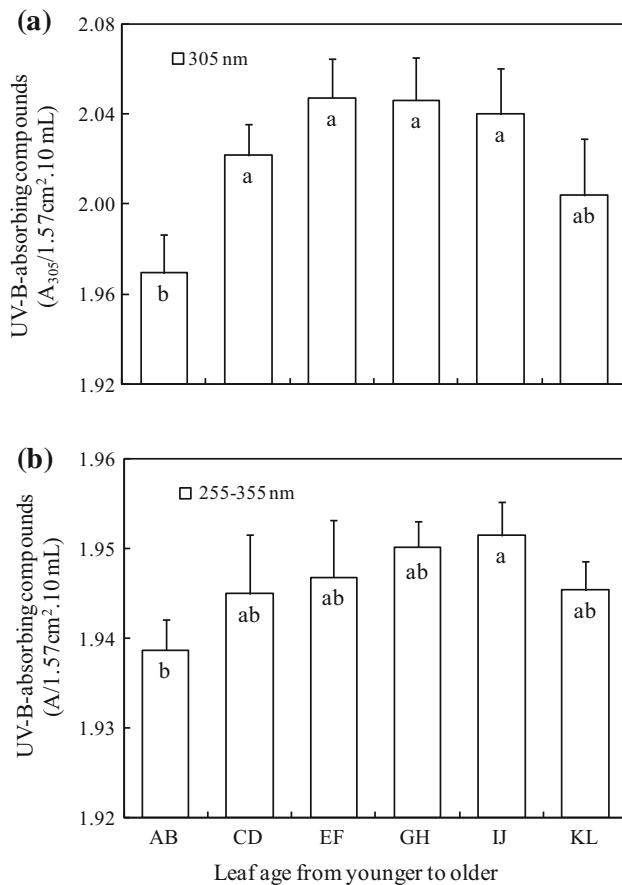loped leaf stages from young to senescence. Data are mean ± SE, sample number is 16–18 from leaf *AB* to *IJ*, and 12 in nearly senescent leaf *KL*. Values with *different letters* (presented in each *vertical bars*) are significantly different at $p = 0.05$ level

efficiency of PSII photochemistry F_v/F_m after nearly 4 h adaptation in the evening, the relative photoinhibition was estimated using data in Fig. 7a according to the method of Dodd et al. (1998). The results indicated that percentage of photoinhibition was at 10.00, 9.87, 8.00, 7.23 and 6.54 % in developed groups from AB to IJ, respectively, which were coincident with increases in $F_{(v)}/F_{(m)}$ during leaf expansion. Although there were no significant difference among differently developed leaves, the maximum efficiency of PSII reaction centers that were open under steady sunlight (F'_v/F'_m) was a little higher after full expansion of leaves, and giving only 2.4 % relative increase when compared with younger leaves (Fig. 7b).

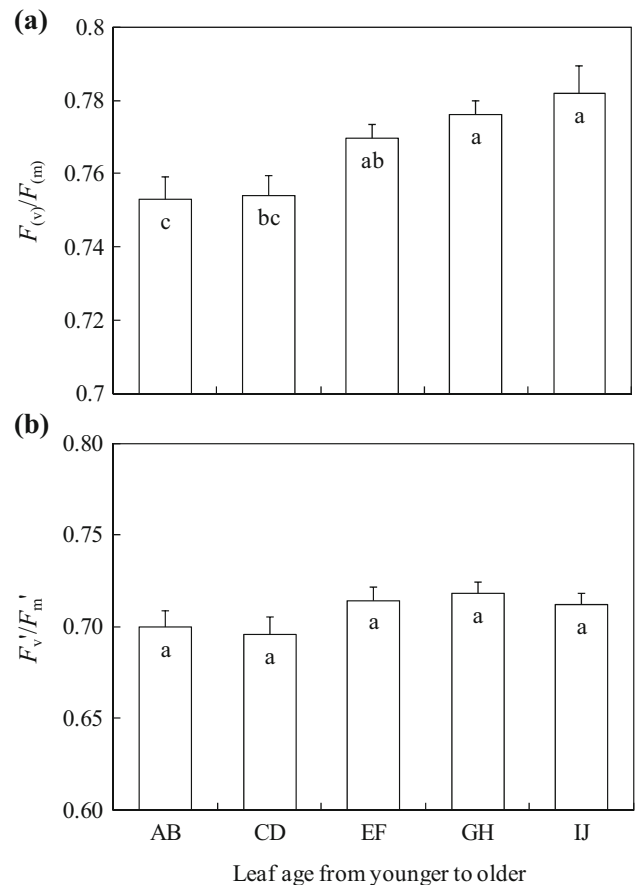## Distribution of excitation energy in PSII reaction centers and its limitation of photochemistry

The coefficient of photochemical quenching (q_P) gradually declined when leaves progressed from fully expanded EF to completely mature leaves IJ (Fig. 8a), but the non-photochemical quenching coefficient (NPQ) was not significantly dependent on leaf developmental stage (Fig. 8b). In most

Fig. 6 Changes of content of UV-absorbing compounds in alpine plant *S. superba* during leaf maturity. **a** absorbance values at the 305 nm waveband; **b** average absorbance values calculated from 255 to 355 nm in 5 nm steps. *Capital letters* in *horizontal axial* from *AB* to *KL* represent developed leaf stages from young to senescence. Data are mean ± SE, sample number is 10 from leaf *AB* to *IJ*, and only 5 in nearly senescent leaf *KL*. Values with *different letters* (presented in each *vertical bars*) are significantly different at $p = 0.05$ level

Fig. 7 Changes of the maximum quantum efficiency of PSII photochemistry at different light regimes as a function of differently developed leaf stages. **a** adapted after 5 min dark; **b** irradiated in solar light. *Capital letters* in *horizontal axial* from *AB* to *IJ* represent the leaves stages from young to fully matured group. Data are mean ± SE, sample number is 23 from leaf *AB* to *EF*, and 35 in *GH* and 25 in *IJ*, respectively. *Low-case letters* in each *vertical bar* represents significantly different means between leaf stages at $p = 0.05$ level

cases, NPQ was a little higher in younger and expanding leaves than in fully expanded leaves. In fully expanded leaves (EF), q_P was higher but NPQ was a little lower; and in fully mature leaves (IJ), both q_P and NPQ were lower.

There was an inverse relationship between the relative limitation of quantum efficiency of PSII reaction centers ($L_{(PFD)}$) and the fraction of open PSII reaction centers (q_L) (Fig. 9a, b). In fully expanded leaves EF, $L_{(PFD)}$ was lower but q_L was higher; conversely, in fully mature leaves IJ, $L_{(PFD)}$ was higher but q_L was lower. Clearly, contrasting tendencies could be observed when leaves developed from fully expanded stage to completely mature stage, but no difference in $L_{(PFD)}$ between younger and expanding leaves, and q_L showed a little increasing trend from younger leaves AB to fully expanded ones EF.

Light-induced and non-light-induced components of non-photochemical quenching

Excitation energy dissipation by non-photochemical pathways can be resolved into light-induced PSII regulatory energy dissipation and non-light-induced PSII non-regulatory energy dissipation (Baker 2008). The quantum yield of light-induced quenching (Φ_{NPQ}) was low in fully expanded leaves (EF), and increased with leaf maturity to completely mature stages (IJ) (Fig. 10a). The quantum yield of non-light-induced quenching (Φ_{NO}) also increased when leaves developed from fully expanded leaves (EF) to completely mature stages (IJ), but there was no difference among younger, expanding and fully expanded leaves (Fig. 10b).

(a)

(b)
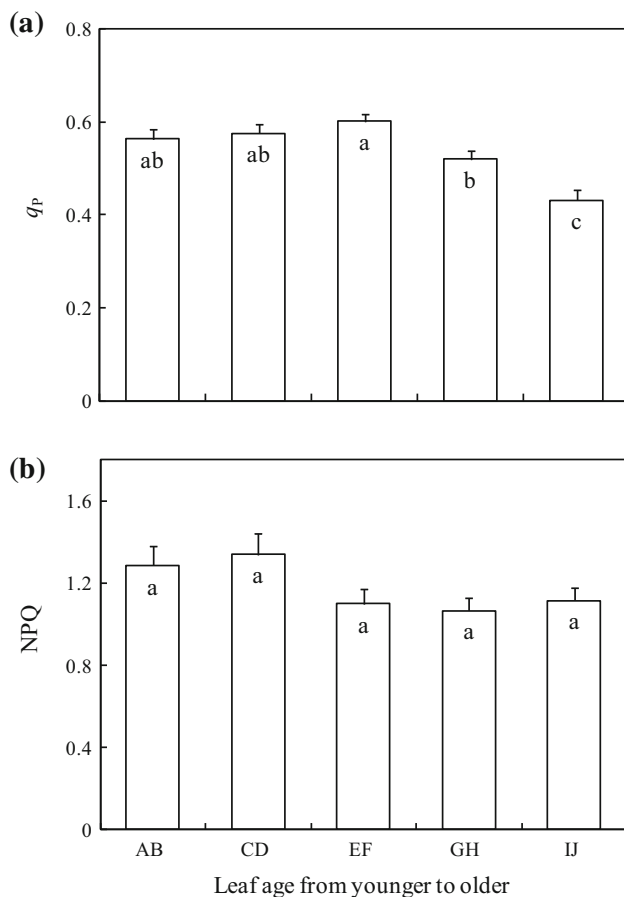
Fig. 8 Thermal energy dissipation in alpine plant *S. superba* during leaf maturity. **a** changes of photochemical quenching coefficient; **b** changes of non-photochemical quenching coefficient. *Capital letters in horizontal axial from AB to IJ* represent the leaves stages from young to fully matured group. Data are mean ± SE, sample number is 23 from leaf *AB* to *EF*, 35 in *GH* and 25 in *IJ*, respectively. *Low-case letters* in each *vertical bar* represents significantly different means between leaf stages at $p = 0.05$ level

Both Φ_{NPQ} and Φ_{NO} were significantly higher when leaf was fully mature (IJ), and their percentage increase relative to fully expanded stage was in 21.6 and 22.2 %, respectively.

Discussion

### Leaf growth, photosynthetic pigments and screening pigments with leaf maturity

Usually, growth includes not only the increase of cell number but also cell enlargement, and is considered as an irreversible process of increase in volume and weight, accompanied by the development and differentiation of individual cells. In *S. superba*, there simultaneously exist differently developed leaves, from inner younger to outside almost senescent ones. The leaf area enlarged significantly

(a)

(b)
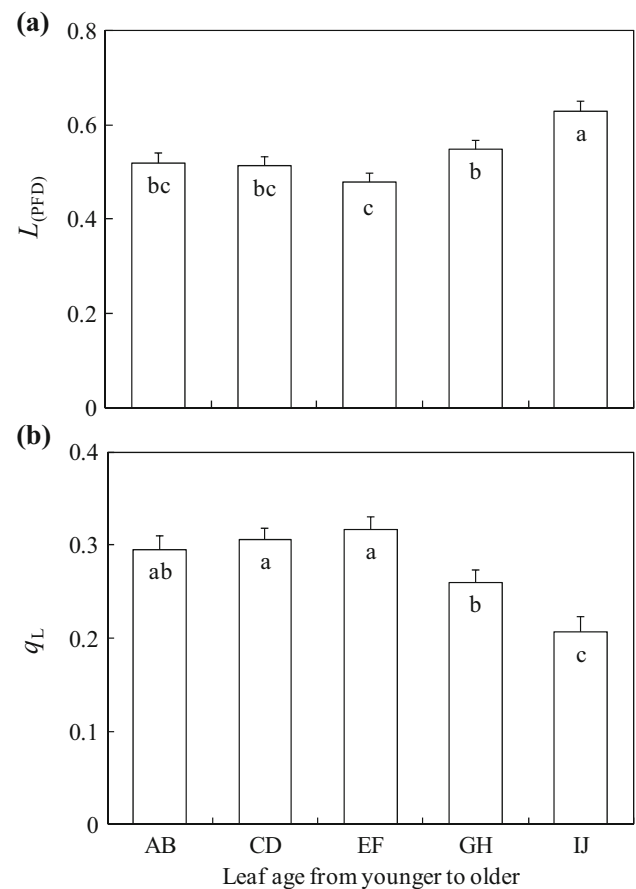
Fig. 9 Relative limitation of quantum efficiency and fraction of open PSII reaction centers in alpine plant *S. superba* during leaf maturity. **a** changes of the relative limitation of quantum efficiency; **b** changes of the fraction of open PSII reaction centers. *Capital letters in horizontal axial from AB to IJ* represent the leaves stages from younger to fully matured group. Data are mean ± SE, sample number is 23 from leaf *AB* to *EF*, 35 in *GH* and 25 in *IJ*, respectively. *Low-case letters* in each *vertical bar* represents significantly different means between leaf stages at $p = 0.05$ level

from younger leaves (2.1 cm^2) to fully expanded leaves (17.7 cm^2), and then showed small continuous tendency to decrease to nearly senescent leaves (12.9 cm^2). The leaf life-span of *S. superba* is longer as compared with other alpine plants, especially for species growing during a short luxurious period from early July to the end of August. The slow late reduction of leaf area results mainly from low precipitation and low temperature prior to the luxuriant growth period (Shi et al. 2001a). The development of leaf thickness follows the same course, a little delayed in comparison to leaf area expansion.

The SLA and the LWR also varied continuously and correlated well with leaf area, although their increases were not pronounced from newly expanding green juvenile leaves to fully expanded adult ones. The SLA is considered as a given amount of biomass which can be spread over a
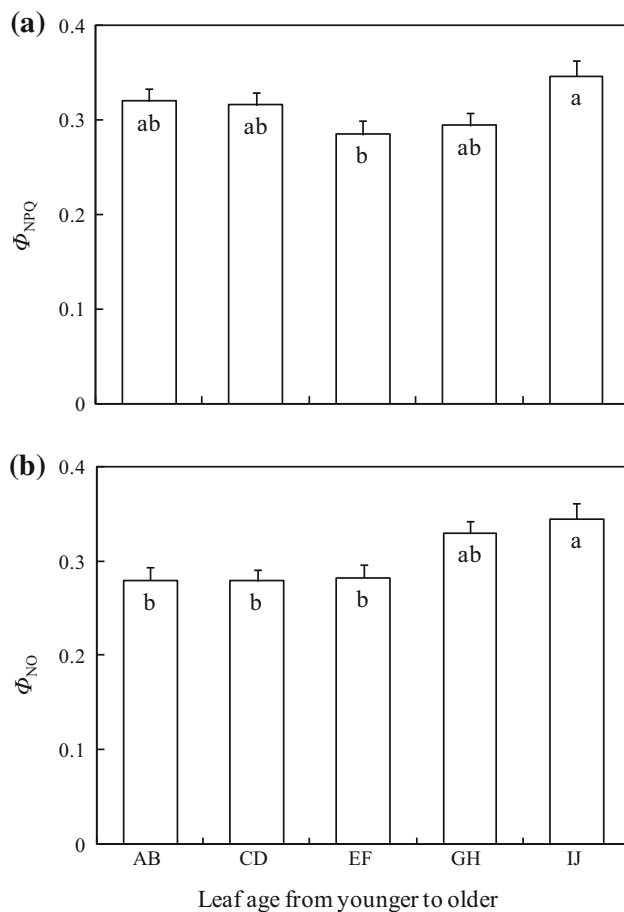
Fig. 10 Light-induced PSII regulatory and non-light-induced PSII non-regulatory energy dissipation in quantum yield in alpine plant *S. superba* during leaf maturity. **a** changes in light-induced PSII regulatory energy dissipation in quantum yield; **b** changes in non-light-induced PSII non-regulatory energy dissipation in quantum yield. *Capital letters* in *horizontal axial* from *AB* to *IJ* represent the leaves stages from young to fully matured group. Data are mean ± SE, sample number is 23 from leaf *AB* to *EF*, 35 in *GH* and 25 in *IJ*, respectively. *Low-case letters* in each *vertical bar* represents significantly different means between leaf stages at $p = 0.05$ level

small or a large area. A thick leaf has low SLA, and a change in SLA alters the amount of light that can be intercepted per unit leaf dry mass (Evans and Poorter 2001). The decrease of SLA during leaf expansion is consistent with the result from *Corymbia gummifera* measured in sandstone plateau woodland communities (Choinski et al. 2003). In fully expanded and mature leaves, reduction of SLA suggested thicker leaves which were verified by direct measurement with electronic digital thickness caliper. The constancy of the LWR points to stable water content, providing an advantage for metabolism and physiological function (Xu 2002).

Photosynthetic pigments play a key role in determining the light absorption of a leaf (Evans and Poorter 2001). *S.*

superba did not show noticeable enhancement in photosynthetic pigment content during leaf expansion, in contrast to the results reported for *C. gummifera* (Choinski et al. 2003). Note that a previous study also shown that photosynthetic pigment content increases rapidly in younger leaves under induction of strong sunlight intensity, whereas there is no pronounced increase during later leaf growth (Catský and Šesták 1996). This property seen in our study may be due to the fast development of photosynthetic apparatus in *S. superba* leaves. In contrast to SLA, the content of Chl and Car per unit leaf area does not change much during leaf maturation. Although low SLA means increases in the number of chloroplasts and the amount of photosynthetic enzymes and thereby enhances the photosynthetic capacity per unit leaf area, it is confirmed that SLA does not affect the whole leaf efficiency of light capture by the pigments (Evans and Poorter 2001). The Chl *a/b* was 3.17 before leaf full expansion and then declined to 2.76 in nearly senescent leaves. The decline in Chl *a/b* after leaf full expansion was consistent with the result in *Nicotiana tabacum* L. (Dhindsa et al. 1980). The value of Chl *a/b* in common healthy leaves is about 3, the relative content depends on its relative rate of synthesis and decomposition under different light, temperature, drought and salt stress, and so on (Xu 2002).

Carotenoids perform many important roles in the photosynthetic process. Besides acting as essential components in the light-harvesting complexes of PSII (LHCII) structure, they also function as accessory pigments in trapping solar energy, and scavenge active oxygen molecules in photosynthetic apparatus and carry out the vital role of protecting membrane system from strong light damage and other stress factors (Middleton and Teramura 1993; Solovchenko et al. 2014). It was proposed that the retention or accumulation of carotenoids could be used as a quantitative measure of leaf senescence (Evans and Poorter 2001). Furthermore, Holleboom and Walla pointed out that interactions of carotenoids and chlorophylls could contribute the regulation of photosynthetic light harvesting of plants, and switch photosynthetic apparatus between light harvesting and excess energy dissipation (Holleboom and Walla 2014). As a result, reduction of Car/Chl, along with decreasing in Chl *a/b*, could provide a possibility to assess the excess of excited light energy in photosynthetic apparatus.

UV-absorbing compounds provide effective protection on photosynthetic apparatus against accompanied strong ultraviolet radiation (Day et al. 1994; Day and Neale 2002). UV-absorbing compounds known as screening pigments mainly exist in the epidermis tissue and have strong absorption in ultraviolet range, especially in ultraviolet-B range (UV-B, 280–315 nm). Despite slight difference in the content of UV-absorbing compounds between the two

methods for estimating them, the present investigation shows that younger leaves (AB) and nearly senescent leaves (KL) were susceptible to solar UV radiation when leaves of *S. superba* were fully exposed to strong sunlight, and in fully expanded and mature leaves, the photosynthetic apparatus in particular thylakoid membrane system was to be protected by high content of UV-absorbing compounds. Therefore, UV-absorbing compounds along with leaf thickness contribute to sunlight defense.

Changes of chlorophyll fluorescence parameters among different developed leaf stages and analysis on non-photochemical quenching precess

Plants cannot avoid biotic and abiotic stressful factors through movement, so it is common to be influenced by lots of environmental stresses, especially strong sunlight irradiation. The responses of light sensitivity are quite different among plant species, and these responses may also differ among differently developed leaves. Light capture ability develops earlier than CO_2 assimilation capacity in newly expanding green leaves (Dillenburg et al. 1995), and juvenile leaves have lower chlorophyll content and lower photochemical activity than more matured ones (Choinski et al. 2003). Early studies have proved that *K. humilis* meadow community and some of alpine plants such as *Gentiana straminea* are susceptible to strong sunlight intensity in Qinghai–Tibetan Plateau regions and exhibit a midday depression in photosynthesis. If strong sunlight is accompanied by other unfavorable environmental factors, chronic photoinhibition is frequently induced (Shi et al. 1997, 2001b). The field investigation shows that rosette whorled *S. superba* leaves are nearly horizontally oriented and are fully exposed to sunlight during most of the daytime. Consequently, the extent of photosynthetic photoinhibition will change among differently developed leaves.

The maximum quantum efficiency of PSII photochemistry after 5 min dark relaxation, $F_{(v)}/F_{(m)}$, provides an estimate of the relative degree of inactivity or/and damage of open PSII centers. It is generally considered that the fast component of non-photochemical quenching can completely relax in such short time, and differences in $F_{(v)}/F_{(m)}$ are mainly due to the slow component of photoinhibition (Quick and Stitt 1989; Galvez-Valdivieso et al. 2009). Significant increase of $F_{(v)}/F_{(m)}$ from rosette whorled inner younger leaves (AB) to outside fully mature leaves (IJ) indicates that the ability of photoprotection and photorepair all gradually improved, and leaves can recover quickly from photoinhibitory status with leaf maturity. At the same time, a small increase of F'_v/F'_m provides a confirmation that the maximum of open PSII reaction centers is nearly

similar at all leaf stages and its maximum efficiency of excitation energy capture in the antennae shows small increasing at nature sunlight condition. These phenomena partly result from the protection of screening pigments in epidermis especially higher content existed in fully expanded leaves and the retention of photosynthetic pigments levels in differently developed leaves (Cha-um et al. 2010; Perez et al. 2014). The reduction of Car/Chl and Chl a/b might lead to excess of excited light energy in photosynthetic apparatus, but these did not cause negative influence on PSII photochemistry in *S. superba*. Estimated relative photoinhibition confirms a chronic photoinhibition in newly expanding juvenile leaves, and this phenomenon is caused by seriously slow relaxing quenching processes (Baker and Rosenqvist 2004). The relative limitation of quantum efficiency of PSII reaction centers, $L_{(PDF)}$, provides an estimate of the limitation on actual quantum yield of PSII photochemistry when compared with the optimum quantum efficiency of PSII reaction centers (Xu 2002). A study on *C. gummifera* showed more variable on actual quantum yield of PSII electron transport in older and larger leaves (Choinski et al. 2003), which differs a little from our study in *S. superba*. The present investigation shows that the quantum yield of PSII photochemistry decreased after leaves were fully expanded, suggesting a decrease in quantum yield of linear electron flow through PSII centers (Baker 2008).

Photochemical and non-photochemical quenching are the two fates of light energy absorbed by PSII complexes besides small quality of light reemission as chlorophyll fluorescence which is usually less than 1–2 % of total absorbed light (Maxwell and Johnson 2000). Frequently, photochemical quenching coefficient q_P is used to estimate the redox state of the primary quinone acceptor of PSII centers (Q_A). Unfortunately, in most cases, q_P is not linear with the fraction of open PSII centers, excepting negligible excitation energy transfer among individual PSII centers and associated antennae (Baker 2008). Based on the Stern–Volmer approach using Lake Model, a modified new fluorescence parameter q_L was used (Kramer et al. 2004). q_L provides a linear estimation of the redox state of Q_A and therefore can give an accurate assessment of the fraction of open PSII centers (Baker 2008). The variation tendency of q_L and q_P was consistent with variation of Chl a/b during leaf expansion, whether this phenomenon is correlated with the relative reduction of chlorophyll a molecule (Chl a) in PSII centers still needs further study.

Thermal energy dissipation is one of the mechanisms for photoprotection, and more photoprotective thermal dissipation leads to less photodamage. Non-photochemical quenching NPQ can be used for estimating changes in the apparent rate constant for excitation delay by heat loss

induced by light relative to this rate constant in the dark, and is often used to determine the level of thermal energy dissipation (Baker 2008; Demmig-Adams et al. 2012). NPQ did not show evident difference among differently developed leaves of *S. superba* under natural sunlight, which confirms that the capacity of NPQ to regulate light capture is saturated at very high light (Kramer et al. 2004). A small variation of NPQ mirrors the small variation of PSII maximum efficiency F'_v/F'_m. It was confirmed that changes of F'_v/F'_m mainly resulted from the variation of non-radiated heat loss (Maxwell and Johnson 2000; Demmig-Adams et al. 2012), and calculated parameter $1 - F'_v/F'_m$ had once been used to assess the fraction of light absorbed in PSII antenna that dissipated thermally (Demmig-Adams et al. 1996). Since the fraction of PSII centers in the open status tends to decrease with increasing incident actinic light, this parameter has been considered that it cannot provide an accurate estimation for the yield of non-radiative decay processes at PSII in light adapted state (Baker 2008). In other words, the present finding indicates that changes of F'_v/F'_m reflect the change of thermal dissipation of excess excitation energy before it reaches the PSII reaction centers, because measurements at all leaf stages were conducted under nearly constant sunlight.

Non-photochemical dissipated excitation energy flux can be resolved into that corresponding to down regulation and other energy losses (Kramer et al. 2004). These are expressed as Φ_{NPQ} and Φ_{NO}, which are defined as light-induced quenching process and non-light-induced quenching process (Baker 2008). The sum of the quantum yield of PSII photochemistry Φ_{PSII} with Φ_{NPQ} and Φ_{NO} equals 1. The increase of Φ_{NPQ} and Φ_{NO} from fully expanded leaves (EF) to fully mature ones (IJ) is accompanied with the decrease of q_P and q_L. This suggests that PSII photochemistry tends to decline after leaves have fully expanded, and at the same time the dissipation by down-regulatory and other non-photochemical losses are both enhanced in *S. superba*. PSII regulatory energy dissipation Φ_{NPQ} reflects all light-induced energy-dependent quenching q_E, photoinhibitory quenching q_I, ^3Car, ^3Chl, etc., or only the rapidly reversible processes (Kramer et al. 2004), and Φ_{NO} point to a proportion of inactive and/or damaged PSII complexes and it may also have a positive effect by providing effective energy dissipation and photoprotection to the remaining functional PSII centers (Osório et al. 2013). The enhancement of Φ_{NPQ} and Φ_{NO} demonstrates that both thermal dissipation of excessive excitation energy in safety and potential threat to photosynthetic apparatus approach each other because of noticeable declination of PSII operating efficiency Φ_{PSII} (Sáez et al. 2013). Furthermore, coincident variation of $L_{(PFD)}$ and Φ_{NPQ} proposed that PSII regulatory energy dissipation in quantum yield was the main reason of limitation on PSII photochemistry in alpine plant *S. superba*, and after leaves were

fully expanded, both Φ_{NPQ} and Φ_{NO} could evidently affect PSII photochemistry.

Comparison of differently developed leaf stages shows that fully expanded leaves EF can utilize more absorbed light energy in PSII photochemistry because of higher of q_P and q_L, and lower of NPQ, $L_{(PFD)}$ and Φ_{NPQ}; for mature leaves GH and IJ, both q_L and NPQ are lower, although $F_{(v)}/F_{(m)}$ and F'_v/F'_m remain a little higher. The decrease of q_L and enhancement of $L_{(PFD)}$ suggest that PSII photochemistry is gradually reduced; leaf stages AB and CD are identified as young leaves, which exhibit a fast increasing in leaf area and relative steady in PSII photochemistry and in two competed non-productive processes, Φ_{NPQ} and Φ_{NO}.

Conclusion

Based on the analysis of leaf growth parameters and chlorophyll fluorescence parameters, the rosette whorled leaves of *S. superba* can be resolved into four functional groups: young (from A to D), fully expanded (from E to F), mature (from G to J), and senescent (from K to L). At the fully expanded stage, leaf area and q_P and q_L reached their highest value, and lower in $L_{(PFD)}$. Young leaves showed significant changes in leaf growing, but relative steady in most of fluorescence parameters. In contrast, mature group identified as small changes in leaf growth parameters and significant variations in most of chlorophyll fluorescence parameters. Both young and mature groups contained more number of leaves and showed some dynamic changes in leaf growth parameters. In senescent group, brown patch casually appeared in leaf blade especially in leaf edge, and that is not suitable for chlorophyll fluorescence measurements.

Small enhancement of F'_v/F'_m after full expansion, and quick recovery from photoinhibition at leaf maturity resulted from the protection by high content of screening pigments and retention of photosynthetic pigments level. The Chl *a/b* coincided well with q_P and q_L throughout the entire period of leaf expansion until fully mature. After leaves were fully expanded, significantly increasing of Φ_{NPQ} and Φ_{NO} indicated that both thermal dissipation of excessive excitation energy in safety and potential threat to photosynthetic apparatus were strengthened, and variation of Φ_{NPQ} and Φ_{NO} was the reason affecting PSII photochemistry in alpine plant *S. superba*.

References

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 141:391–396

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Annu Rev Plant Biol 59:89–113

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J Exp Bot 55:1607–1621

Bilger W, Björkman O (1990) Role of the xanthophyll cycle protoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. Photosynth Res 25:173–185

Catský J, Šesták Z (1996) Photosynthesis during leaf development. In: Pessarakli M (ed) Handbook of photosynthesis. Marcel Dekker, New York, pp 633–660

Cha-um S, Takabe T, Kirdmanee C (2010) Osmotic potential, photosynthetic abilities and growth characters of oil palm (*Elaeis guineensis* Jacq.) seedlings in responses to polyethylene glycol-induced water deficit. Afr J Biotechnol 9:6509–6516

Choinski JS Jr, Ralph P, Eamus D (2003) Changes in photosynthesis during leaf expansion in *Corymbia gummifera*. Aust J Bot 51:111–118

Day TA, Neale PJ (2002) Effects of UV-B radiation on terrestrial and aquatic primary producers. Annu Rev Ecol Syst 33:371–396

Day TA, Howells BW, Rice WJ (1994) Ultraviolet absorption and epidermal-transmittance spectra in foliage. Physiol Plant 92:207–218

Demmig-Adams B, Adams WW (1992) Photoprotection and other responses of plants to high light stress. Annu Rev Plant Physiol Plant Mol Biol 43:599–626

Demmig-Adams B, Adams WW (2006) Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. New Phytol 172:11–21

Demmig-Adams B, Adams WW, Barke DH, Logan BA, Bowling DR, Verhoeven AS (1996) Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiol Plant 98:254–264

Demmig-Adams B, Cohu CM, Muller O, Adams WW (2012) Modulation of photosynthetic energy conversion efficiency in nature: from seconds to season. Photosynth Res 113:75–88

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1980) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93–101

Dillenburg LR, Sullivan JH, Teramura AH (1995) Leaf expansion and development of photosynthetic capacity and pigments in *Liquidambar styraciflua* (Hammamelidaceae). Am J Bot 82:433–440

Dodd IC, Critchley C, Woodall GS, Stewart GR (1998) Photoinhibition in differently coloured juvenile leaves of *Syzygium* species. J Exp Bot 49:1437–1445

Evans JR, Poorter H (2001) Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area and nitrogen partitioning in maximizing carbon gain. Plant Cell Environ 24:755–767

Galvez-Valdivieso G, Fryer MJ, Lawson T, Slattery K, Truman W, Smimoff N, Asami T, Davies WJ, Jones AM, Baker NR, Mullineaux PM (2009) The high light response in *Arabidopsis* involves ABA signaling between vascular and bundle sheath cells. Plant Cell 21:2143–2162

Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990:87–92

Holleboom C-P, Walla PJ (2014) The back and forth of energy transfer between carotenoids and chlorophylls and its role in the regulation of light harvesting. Photosynth Res 119:215–221

Jiang CD, Li PM, Gao HY, Zou Q, Jiang GM, Li LH (2005) Enhanced photoprotection at the early stages of leaf expansion in field-grown soybean plants. Plant Sci 168:911–919

Kramer DM, Johnson G, Kiirats O, Edwards GE (2004) New fluorescence parameters for the determination of Q_A redox state and excitation energy fluxes. Photosynth Res 79:209–218

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. J Exp Bot 51:659–668

Middleton EM, Teramura AH (1993) The role of flavonol glycoside and carotenoids in protecting soybean from ultraviolet-B damage. Plant Physiol 103:475–481

Müller P, Li XP, Niyogi KK (2001) Non-photochemical quenching: a response to excess light energy. Plant Physiol 125:1558–1566

Murchie EH, Niyogi KK (2011) Manipulation of photoprotection to improve plant photosynthesis. Plant Physiol 155:86–92

Osório ML, Osório J, Romano A (2013) Photosynthesis, energy partitioning, and metabolic adjustments of the endangered Cistaceae species *Tuberaria* major under high temperature and drought. Photosynthetica 51:75–84

Oxborough K, Baker NR (1997) Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components: calculation of q_P and F_v'/F_m' without measuring F_o'. Photosynth Res 54:135–142

Perez CEA, Rodrigues FÁ, Moreira WR, DaMatta FM (2014) Leaf gas exchange and chlorophyll *a* fluorescence in wheat plants supplied with silicon and infected with *Pyricularia oryzae*. Biochem Cell Biol 104:143–149

Quick WP, Stitt M (1989) An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves. Biochim Biophys Acta 977:287–296

Rikkinen J (1995) What's behind the pretty colours? A study on the photobiology of lichen. Bryobothera 4:231–239

Sáez PL, Bravo LA, Latsague MI, Toneatti MJ, Sánchez-Olate M, Ríos DG (2013) Light energy management in micropropagated plants of *Castanea sativa*, effects of photoinhibition. Plant Sci 201:12–24

Shi SB, Han F, Ben GY (1997) Midday depression in net photosynthetic rate of plant community in alpine *Kobresia humilis* meadow. Acta Phytoecol Sin 23:405–409 **(in Chinese with English abstract)**

Shi SB, Ben GY, Han F, Li YN, Shen ZX (2001a) Plant growth analysis of *Kobresia humilis* meadow community in Qing-Zang plateau regions. Acta Ecol Sin 21:871–876 **(in Chinese with English abstract)**

Shi SB, Han F, Li HY (2001b) Midday depression of photosynthesis of *Gentiana straminea* and *Saussurea superba* in alpine *Kobresia humilis* meadow. Acta Phytophysiol Sin 27:123–128 **(in Chinese with English abstract)**

Shi SB, Zhu WY, Li HM, Zhou DW, Han F, Zhao XQ, Tang YH (2004) Photosynthesis of *Saussurea superba* and *Gentiana*

straminea is not reduced after long-term enhancement of UV-B radiation. Environ Exp Bot 51:75–83

Solovchenko A, Lukyanov A, Solovchenko O, Didi-Cohen S, Boussiba S, Khozin-Goldberg I (2014) Interactive effects of salinity, high light, and nitrogen starvation on fatty acid and carotenoid profiles in *Nannochloropsis oceanica* CCALA 804. Eur J Lipid Sci Technol 116:1–10

Sperdouli I, Moustakas M (2012) Differential response of photosystem II photochemistry in young and mature leaves of *Arabidopsis thaliana* to the onset of drought stress. Acta Physiol Plant 34:1267–1276

Tikkanen M, Mekala NR, Aro EM (2014) Photosystem II photoinhibition-repair cycle protects photosystem I from irreversible damage. Biochim Biophys Acta 1837:210–215

Wellburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Plant Physiol 144:307–313

Xu DQ (2002) Photosynthetic efficiency. Shanghai Scientific and Technical Press, Shanghai, pp 29–37 **(in Chinese)**

Yu BH, Lu CH (2011) Assessment of ecological vulnerability on the Tibetan Plateau. Geogr Res 30:2289–2294 **(in Chinese with English abstract)**

Zhao XQ, Zhou XM (1999) Ecological basis of Alpine meadow ecosystem management in Tibet: Haibei Alpine Meadow Ecosystem Research Station. Ambio 28:642–647

Zhu GL (1990) Plant physiological experiment. Peking University Press, Beijing, pp 51–54 **(in Chinese)**