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The higher resistance to chilling stress in adaxial side of *Rumex* K-1 leaves is accompanied with higher photochemical and non-photochemical quenching

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Abstract

Responses of two sides of *Rumex* K-1 leaves to chilling stress (5 °C, photon flux density of 100 μmol m^{-2} s^{-1}) were studied by using gas exchange, chlorophyll (Chl) fluorescence, and spectrum reflectance techniques. The Chl and carotenoid contents in the two sides were not affected by chilling treatment, and both were higher in the adaxial side. The maximum quantum yield of photosystem (PS) 2 and fraction of functional PS1 in the abaxial side decreased more markedly than those in the adaxial side during the chilling treatment, indicating that the abaxial side was damaged more significantly than the adaxial side. Before chilling, there were no obvious differences in actual photochemical efficiency of PS2, photosynthesis, and photorespiration between two sides of the leaves. Under chilling stress, the actual photochemical efficiency of PS2, photosynthesis, and photorespiration all declined more significantly in the abaxial side, which was partly attributed to lower carboxylation efficiency in the abaxial side than that in the adaxial side. Non-photochemical quenching was higher in the adaxial side, though the de-epoxidation of xanthophyll cycle pigments' pool on basis of Chl was higher in the abaxial side. Both the slower decrease in the photochemical quenching and the higher non-photochemical quenching may account for the higher resistance to chilling stress in the adaxial side of *Rumex* K-1 leaves.

Additional key words: carotenoids; chlorophyll; photochemical and non-photochemical quenching; oxygen evolution; xanthophyll cycle.

Introduction

Bifacial leaf has a densely packed palisade mesophyll layer of cells on the adaxial side, and a more open layer of spongy mesophyll cells on the abaxial side. This structural differentiation relates to the differences in physiological function between the two cell types within the leaf (Vogelmann *et al*. 1996). Gas exchange studies show that when bifacial leaves are irradiated either on adaxial or abaxial side, photosynthetic rate is usually higher in the adaxial side than that in the abaxial side because of different microenvironment and protein and pigment distribution (Terashima and Saeki 1983, Terashima 1986, Cui *et al.* 1991, Evans *et al.* 1993, Nishio *et al.* 1993, Vogelmann 1993, Sun *et al.* 1996, Nishio 2000, Sun and Nishio 2001).

The photosynthetic performance of chloroplasts near the leaf surface is probably more susceptible to environmental stresses (Vogelmann 1993, Han and Vogelmann 1999, Han *et al.* 1999). The abaxial sides of leaves are more susceptible to high radiation damage in contrast to the adaxial side when the leaves are irradiated on either adaxial or abaxial side, which may be related to higher photosynthetic carbon fixation rate (Terashima 1986, Evans *et al.* 1993, Sun *et al.* 1996, Sun and Nishio 2001), abundant carotenoids such as xanthophyll cycle pigments in adaxial than abaxial side (Lu *et al.* 1993), or related to the differences of other possible energy dissipative mechanisms between the two sides.

With evolution, plants have possessed some

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Abbreviations: F_V/F_M – maximum quantum yield of photosystem 2; F_V'/F_M' – the capture efficiency of excitation energy; NPQ – non-photochemical quenching; PFD – photon flux density; PS2 – photosystem 2; q_E – high energy state quenching; q_P – photochemical quenching coefficient; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; $\Delta I/I_0$ – fraction of functional PS1; Φ_{PS2} – actual photochemical efficiency of PS2.
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protective mechanisms to alleviate stress damage such as non-photochemical quenching dependent on xanthophyll cycle (Demmig-Adams and Adams 1992, 1996, Dai *et al.* 2004, Jiang *et al.* 2005), photorespiration (Foyer and Noctor 2000, Jiang *et al.* 2006a), water-water cycle (Asada 1999, Chen *et al.* 2004), and so on. The high extinction of blue and red radiation at the adaxial side of leaves suggests the possibility that the adaxial side of leaves has greater capacity of non-photochemical quenching than the abaxial side (Sun *et al.* 1998). However, up to now, information about different responses, especially xanthophyll cycle to environmental stresses between the two sides *in vivo* is still lacking, the reason of which is partly the lacking efficient method to distinguish the differences in xanthophyll cycle, chlorophyll (Chl) content, energy dissipation, *etc*. *in vivo* between the two sides.

Recently, a new technique of using spectral reflectance to assess contents of leaf pigments and xanthophyll cycle *in vivo* has been developed (Gamon and Surfus 1999, Sims and Gamon 2002, Evain *et al.* 2004, Weng *et al.* 2006). Besides, technique of Chl fluorescence has become a powerful tool to investigate photosynthetic performance including photoinhibition and photoprotection mechanisms in plant leaves. All these techniques make it possible to distinguish the differences in pigments, xanthophyll cycle, and photosynthetic performance between the two leaf sides *in vivo*.

This study is focused on photosynthetic performance and mechanisms of protecting leaf cells against chilling stress under weak irradiance between the adaxial and abaxial side of *Rumex* leaves.

Materials and methods

Plants: *Rumex* K-1 plants (*Rumex patientia* × *R. tianschaious*) were grown from seed under a 14-h photoperiod at 22/18 °C (day/night) in a pot containing soil. The plants were thinned to one plant per pot 2 weeks after sowing. Nutrients and water were supplied sufficiently throughout to avoid any potential nutrient and drought stresses. The photon flux density (PFD) during growth was about 100 μmol m^{-2} s^{-1}. Fully expanded leaves of 4-week-old plants were used in the experiments.

Before chilling treatment, leaves were excised at the petiole from the whole plants. Then, the petioles of excised leaves were quickly dipped into water in triangular bottles with the second excision in the water. Leaves and the triangular bottles were transferred into a growth chamber in which the temperature was controlled at 5 °C for chilling treatment. During chilling treatment, leaves were put upright with both the sides facing radiation source identically and directly under 100 μmol m^{-2} s^{-1} PFD. Before and after different time of chilling treatment, measurements were carried out. All measurements were performed on 0 (namely before chilling treatment), 1, 2, 3, and 4 d of chilling treatment, respectively.

Gas exchange was measured at 20 °C after *Rumex* leaves had been recovered for 1 h at 20 °C under 100 μmol m^{-2} s^{-1} PFD. Compensation irradiances of the two sides of *Rumex* leaves were about 25 and 15 μmol m^{-2} s^{-1} at 20 °C and 360 μmol mol^{-1} CO_2. When each side of the *Rumex* leaves was irradiated with a "white light" unit with PFD of 100 μmol m^{-2} s^{-1}, the transmission was about 8–12 %, which indicated that the transmitted radiation could not initiate photosynthesis in the other side effectively when exposed to 100 μmol m^{-2} s^{-1} PFD for gas exchange measurements.

O_2 exchange measurement was performed with a *LEAFLAB-2* gas-phase oxygen electrode (*Hansatech*, UK). In the electrode chamber the *Rumex* leaf is supported by a sandwich of materials. Beneath the leaf there are a stainless steel grid, a non-perforated centre, layer of capillary matting, stainless steel grid with open centre, sponge spacer, and a stainless steel washer which locates nearest the electrode disc. Three drops of a 1 M bicarbonate solution was added to the capillary matting in the reaction chamber to yield saturating CO_2 for photosynthesis in the reaction chamber.

100 μmol m^{-2} s^{-1} PFD offered by a "white light" source of the *LEAFLAB-2* was used to irradiate one side of the *Rumex* leaf disc from top of it during the measurement. When the photosynthetic oxygen evolution measurement was finished, the radiation source was removed and respiratory rate was measured.

CO_2 exchange measurement was performed using a *CIRAS-2* portable photosynthesis system (*PP Systems*, UK). For all cases, the air temperature, air relative humidity, and PFD were maintained as 20 °C, 75 % relative humidity, and 100 μmol m^{-2} s^{-1}, respectively, with an automatic leaf cuvette of the *CIRAS-2*. CO_2 exchange measurements were performed under three conditions: (*1*) 360 μmol mol^{-1} CO_2/21 % O_2; (*2*) 360 μmol mol^{-1} CO_2/2 % O_2; (*3*) 2 000 μmol mol^{-1} CO_2/21 % O_2. The irradiation was offered by an integrated "white light" unit of the *CIRAS-2* from one side of the *Rumex* leaf. CO_2 assimilation rate (P_N) measured under different O_2 concentrations was used to calculate photorespiratory rate (R_P), $R_P = P_N$(at 2 % O_2/360 μmol mol^{-1} CO_2) – P_N(at 21 % O_2/360 μmol mol^{-1} CO_2). Carboxylation efficiency was estimated from the initial slope of the CO_2 response curve under 100 μmol m^{-2} s^{-1} PFD.

Chl fluorescence: PS2 Chl fluorescence measurements were performed at 20 °C after *Rumex* leaves had been recovered for 1 h at 20 °C, under 100 μmol m^{-2} s^{-1} PFD using an *FMS-2* portable pulse modulated fluorometer (*Hansatech*, UK). The dark-adapted minimum fluorescence (F_0) and maximum fluorescence (F_M) were measured in *Rumex* leaves that were dark adapted for

20 min at 20 °C. Next, the actinic radiation (100 μmol m^{-2} s^{-1} PFD) was turned on after the fluorescence signal levelled off, the steady-state fluorescence (F_S) and the light-adapted maximum fluorescence (F_M') were determined, then the actinic radiation was removed and the minimal fluorescence in the light-adapted state (F_0') was determined by irradiating the leaf segment for 2 s with infrared radiation. Maximum quantum yield of PS2 (F_V/F_M), the capture efficiency of excitation energy (F_V'/F_M'), photochemical quenching coefficient (q_P), and the actual PS2 efficiency under irradiance (Φ_{PS2}) were calculated according to Genty *et al.* (1989), $F_V/F_M = (F_M - F_0)/F_M$, $F_V'/F_M' = (F_M' - F_0')/F_M'$, $q_P = (F_M' - F_S)/(F_M' - F_0')$, $\Phi_{PS2} = (F_M' - F_S)/F_M'$. Non-photochemical quenching (NPQ) was calculated according to Demmig-Adams and Adams (1996), $NPQ = F_M/F_M' - 1$. High energy state quenching (q_E) was measured according to Johnson *et al.* (1993).

PS1 Chl transmission change at 820 nm was measured using a dual channel PEA senior instrument (*Hansatech*, UK). The far-red radiation source was a *QDDH73520* LED (*Quantum Devices*) filtered at 720±5 nm. The modulated (33.3 kHz) far-red measuring radiation was provided by an *OD820LED* (*Opto Diode Co*.) filtered at 830±20 nm. Irradiated with a far-red pulse (250 μmol m^{-2} s^{-1} PFD) for 2 s, the transmission at 820 nm in leaves decreases gradually, which is mainly caused by the oxidation of P_{700} (Schansker *et al.* 2003). Then, the changes in amplitude of the transmission at 820 nm could be used to estimate the fraction of functional PS1 (Jiang *et al.* 2006b). In this study, the fraction of functional PS1 was calculated as $\Delta I/I_0 = (I_{4\ ms} - I_{2\ s})/I_{0.4\ ms}$.

Reflectance spectrum was measured with a *Unispec* portable spectrometer combining with bifurcated fibre optics and a leaf clip (*PP Systems*, UK). The leaf clip held the fiber at a 60° angle to the adaxial and abaxial leaf sides. Leaf irradiation was provided through one side of the bifurcated fibre from a halogen lamp in the spectrometer.

Results

Reflectance spectra and pigment contents: The reflectance spectrum was different between the two sides, and the reflectance was higher in the abaxial than adaxial side (Fig. 1).

Many studies have shown that the simple ratio (mSR_{705}) calculated as $mSR_{705} = (R_{750} - R_{445})/(R_{705} - R_{445})$ is well correlated with the Chl content (Sims and Gamon 2002), and the index R_{800} ($1/R_{520} - 1/R_{700}$) is well correlated with carotenoid content (Merzlyak *et al.* 2003). Therefore, we used parameters mSR_{705} and R_{800} ($1/R_{520} - 1/R_{700}$) to compare the Chl and carotenoid contents between the two sides of *Rumex* leaves. The pigment contents were hardly influenced by chilling stress treatment and were both higher in the adaxial side (Fig. 2).

Two photosystems: Before chilling treatment, there were no obvious distinctions in the maximum quantum yield of PS2 (F_V/F_M) and the fraction of functional PS1 ($\Delta I/I_0$) between the two sides (Table 1). However, after chilling treatment both the F_V/F_M and the $\Delta I/I_0$ decreased more pronouncedly in the abaxial than adaxial side.

Photochemical reactions: Before chilling treatment, there were no obvious differences in P_N (Fig. 3), R_P (Fig. 4*A*), F_V'/F_M', q_P, the actual PS2 photochemical efficiency (Φ_{PS2}) (Fig. 5), and the O_2 evolution rate (Fig. 6*A*) between the two sides, whereas the

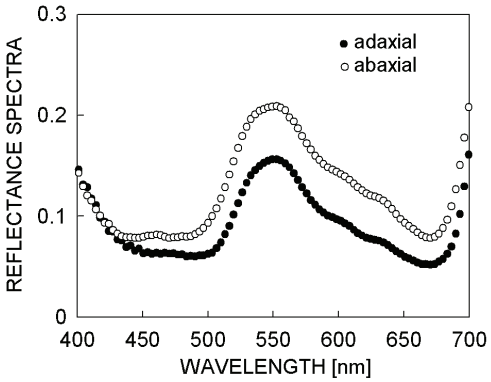

Fig. 1. Different reflectance spectra in two sides of *Rumex* leaves. Each curve represents the average of 5 independent measurements.

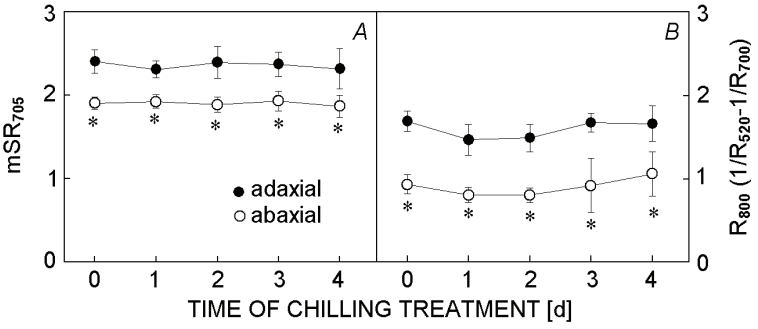

Fig. 2. Changes in index mSR_{705} (*A*) and R_{800} ($1/R_{520} - 1/R_{700}$) (*B*) derived from leaf reflectance of adaxial and abaxial sides of *Rumex* leaves under 5 °C, 100 μmol m^{-2} s^{-1} PFD, and 360 μmol mol^{-1} CO_2. Means ± SE (n = 5). *Single asterisks* indicate the significance of difference at $p<0.05$ between the two sides of leaves (F-test).

Table 1. Changes in maximum quantum yield of PS2 (F_V/F_M) and fraction of functional PS1 ($\Delta I/I_0$) in two sides of *Rumex* leaves under 5 °C, 100 μmol m^{-2} s^{-1} PDF, 360 μmol mol^{-1} CO_2. Means ± SE (n = 5). Single asterisks indicate the significance of difference at p<0.05 between two sides of leaves (F-test).

		Time of chilling [d] 0	1	2	3	4
F_V/F_M	adaxial	0.867±0.013	0.806±0.021	0.779±0.025	0.761±0.029	0.754±0.034
	abaxial	0.870±0.014	0.750±0.026*	0.717±0.031*	0.688±0.034*	0.667±0.035*
$\Delta I/I_0$	adaxial	0.441±0.006	0.360±0.013	0.298±0.014	0.261±0.018	0.247±0.020
	abaxial	0.433±0.005	0.313±0.012*	0.248±0.013*	0.200±0.025*	0.182±0.021*

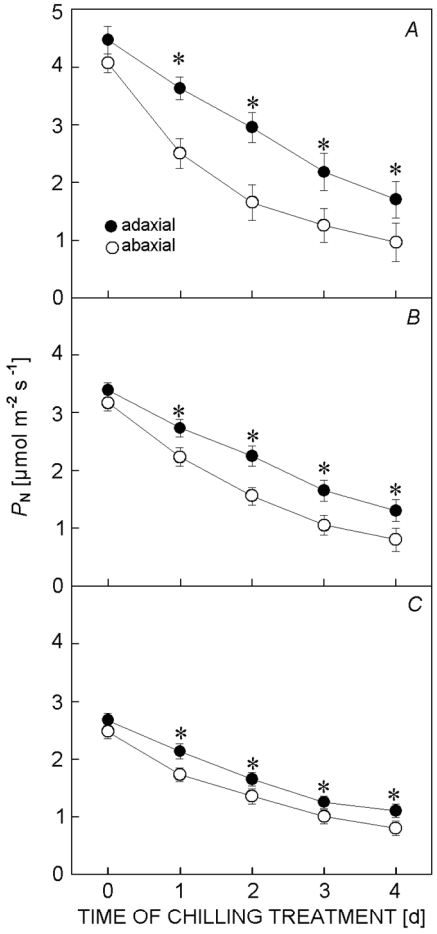

Fig. 3. Changes in net photosynthetic rate, P_N at 2 000 μmol mol^{-1} CO_2/21 % O_2 (*A*), 360 μmol mol^{-1} CO_2/2 % O_2 (*B*), and 360 μmol mol^{-1} CO_2/21 % O_2 (*C*) in two sides of *Rumex* leaves under 5 °C and 100 μmol m^{-2} s^{-1} PFD. Means ± SE (n = 5). *Single asterisks* indicate the significance of difference at p<0.05 between the two sides of leaves (F-test).

Discussion

NPQ is directly proportional to heat dissipation and increases as a result either of processes that protect the leaf from light induced damage or of the damage itself (Demmig-Adams 1990, Maxwell and Johnson 2000). Under most conditions, q_E is a major contributor to NPQ and is xanthophyll cycle-dependent (Demmig-Adams 1990). q_E is essential in protecting the leaf from irradiance-induced damage (Johnson *et al*. 1993, Horton *et al.* 1996). In this study, although the actual de-epoxidation of xanthophyll cycle pigments normalized to Chl increased more significantly in the abaxial side than that in the adaxial side during chilling treatment (Fig. 7*D*), NPQ, q_E,

carboxylation efficiency was higher in the adaxial side (Fig. 6*B*). Under chilling stress treatment, P_N, R_P, F_V'/F_M', q_P, Φ_{PS2}, and the O_2 evolution rate all decreased more drastically in the abaxial side in contrast to those in the adaxial side (Figs. 3, 4*A*, 5, and 6*A*), except that the difference in the O_2 evolution rate disappeared from 3 d after chilling stress. The chilling stress reduced R_P more drastically than P_N in both the sides, especially in the abaxial side (Fig. 4*B*). The carboxylation efficiency in chilled leaves also decreased in both the sides, and in the adaxial side was higher than in the abaxial side during the chilling treatment (Fig. 6*B*).

Non-photochemical quenching: Chilling stress increased NPQ more pronouncedly in the adaxial than abaxial side, however, after treatment for 3 d, the NPQ did not rise any more in both the sides (Fig. 7*A*). NPQ is composed of high energy state quenching (q_E) and photoinhibitory quenching (Johnson *et al.* 1993). The q_E varied almost consistently with NPQ (Fig. 7*B*). However, the ratio q_E/NPQ hardly varied in the adaxial side, whereas it decreased by about 20 % in the abaxial side 4 d after chilling treatment (Fig. 7*C*).

Some studies have shown that the variance of PRI [photochemical reflectance index, PRI = $(R_{531} - R_{570})/(R_{531} + R_{570})$] calculated from different values in saturating irradiance and in the dark (ΔPRI) can be used to estimate total xanthophyll pigment pool size normalized to Chl, whereas that calculated from different values in ambient light and in the dark (ΔPRI') can be used to estimate the actual de-epoxidation of xanthophyll cycle pigments normalized to Chl (Gamon and Surfus 1999, Stylinski *et al.* 2002). Although there was no difference in ΔPRI between the two sides before and after chilling treatment, the ΔPRI' was higher in the abaxial than adaxial side during the chilling treatment (Fig. 7*D*).

and q_E/NPQ were all higher in the adaxial side than those in the abaxial side (Fig. 7*A,B,C*), suggesting that perhaps it was the relatively smaller xanthophyll cycle pigment pool (Fig. 7*D*) that limited the contribution of q_E to NPQ in the abaxial side.

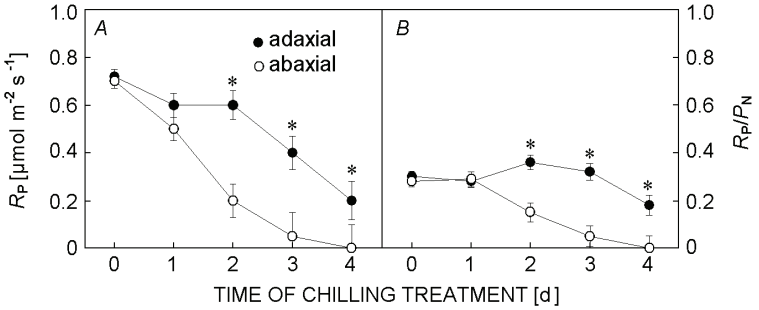

Fig. 4. Changes in photorespiratory rate, R_P (*A*) and ratio of R_P to net photosynthetic rate, P_N (*B*) in adaxial and abaxial sides of *Rumex* leaves under 5 °C, 100 μmol m^{-2} s^{-1} PFD, and 360 μmol mol^{-1} CO_2. Means ± SE (n = 5). *Single asterisks* indicate the significance of difference at p<0.05 between the two sides of leaves (F-test).

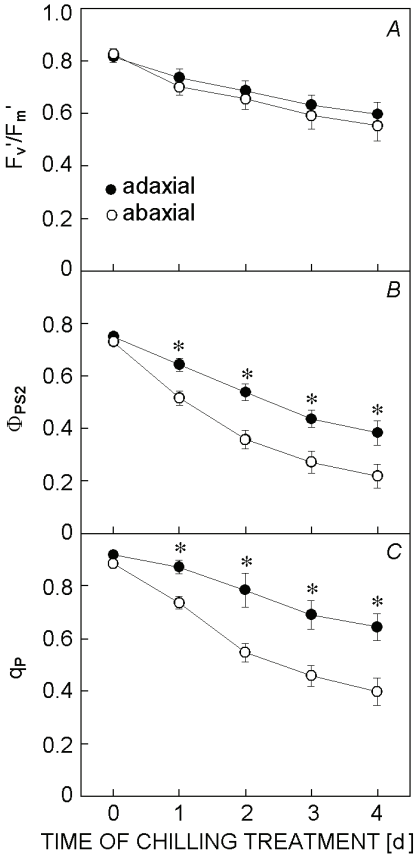

Fig. 5. Changes in capture efficiency of excitation energy (F_V'/F_M'; *A*), photochemical quenching coefficient (q_P; *C*), and the actual PS2 photochemical efficiency (Φ_{PS2}; *B*) in adaxial and abaxial sides of *Rumex* leaves under 5 °C, 100 μmol m^{-2} s^{-1} PFD, and 360 μmol mol^{-1} CO_2. Means ± SE (n = 5). *Single asterisks* indicate the significance of difference at p<0.05 between the two sides of leaves (F-test).

Factors that influence photosynthesis, such as Chl content, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) distribution, and light microenvironment are different in two sides of leaves (Terashima and Saeki 1983, Terashima and Inoue 1985, Cui *et al.* 1991, Lu *et al.* 1993). However, the oxygen evolution is relatively uniform from the upper to the lower surface of the leaf (Han and Vogelmann 1999, Han *et al.* 1999). In this study, the irradiance was 100 μmol m^{-2} s^{-1}, *i.e.* not strong enough to excite well the whole photosynthetic apparatus. Then it became the limiting factor of photosynthesis in both the sides of the *Rumex* leaves under non-stress condition. Therefore, there were no differences in the photochemical reactions between the two sides of *Rumex* leaves before chilling treatment, although the Chls and carboxylation efficiency were both higher in the adaxial side (Figs. 1 and 6*B*).

At low temperature, even weak radiation can do harm to plants, resulting in the inactivation of the two photosystems, PS1 and PS2 (Table 1). Several studies have shown that the photosynthesis at low temperature is often limited by triose-phosphate utilization (Sage *et al.* 1990, Huner *et al.* 1993). Hikosaka *et al.* (1999) argued that photosynthetic rate in low temperature grown leaves is determined solely by RuBP carboxylation, whereas Hendrickson *et al.* (2004) suggested that the decreased carboxylation efficiency was involved in the reduction of photosynthesis under low temperature. Under chilling stress, the different variations between P_N (Fig. 3) and the O_2 evolution rate (Fig. 6) implied that the more decreased P_N in the abaxial side was not correlated with the damaged oxygen evolution complex. The best correlation between carboxylation efficiency and photosynthetic rate (Fig. 6*B*) revealed that the decreased photosynthesis was partly correlated with the reduction of carboxylation efficiency in the two sides. We speculate that under chilling stress, maybe the carboxylation efficiency was seriously inhibited, then it became one of limiting factors to photosynthesis. Therefore, although it was weak, irradiance of 100 μmol m^{-2} s^{-1} no more limited photosynthesis as it did under normal conditions. As a result, the decline of carboxylation efficiency accounted for the reduction of the photosynthetic rate. The carboxylation efficiency is correlated with RuBPCO activity very well (Collatz 1977, Caemmerer and Farquhar 1981). The higher carboxylation efficiency in the adaxial side (Fig. 6*B*) indicated higher RuBPCO activity in the adaxial side. Nishio *et al.* (1993) and Sun and Nishio (2001) also

observed that the content and activity of RuBPCO were both higher in the adaxial than abaxial side in spinach leaves, and they suggested that the pattern of photosynthetic capacity across the leaf closely correlates with the RuBPCO activity profile across the leaf when irradiance is not limiting.

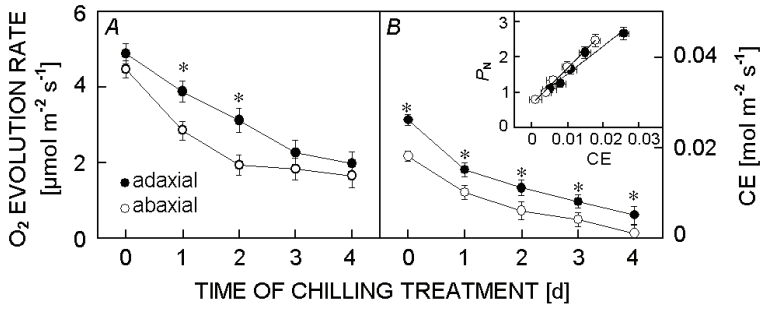

Fig. 6. Changes in O_2 evolution rate (*A*), carboxylation efficiency (*B*) and the relationship between net photosynthetic rate (P_N) and carboxylation efficiency (CE, *insert* in *B*) in two sides of *Rumex* leaves under 5 °C and 100 μmol m^{-2} s^{-1} PFD. Means ± SE (n = 5). *Single asterisks* indicate the significance of difference at p<0.05 between the two sides of leaves (F-test). The equation in the insert figure is y = 77.5 x + 0.752, r^2 = 0.956 for adaxial side and y = 100.3 x + 0.688, r^2 = 0.992 for abaxial side.

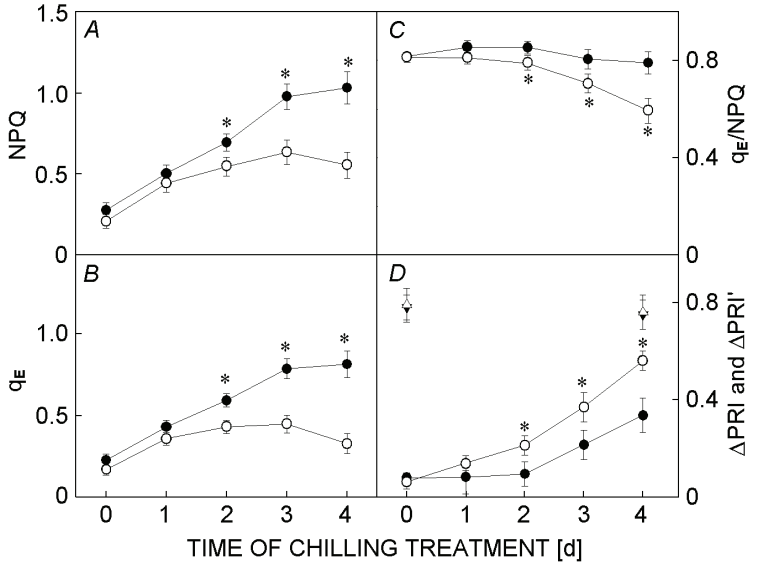

Fig. 7. Changes in non-photochemical quenching (NPQ; *A*), high energy state quenching (q_E; *B*), ratio q_E/NPQ (*C*), and variances of photochemical reflectance index (PRI) between the saturating irradiance and the dark (ΔPRI, *triangles*: ▼ adaxial, Δ abaxial, *D*) or between the ambient irradiance and the dark (ΔPRI', *circles*, *D*) in adaxial and abaxial sides of chilling-treated *Rumex* leaves under 5 °C, 100 μmol m^{-2} s^{-1} PFD, and 360 μmol mol^{-1} CO_2. Means ± SE (n = 5). *Single asterisks* indicate the significance of difference at p<0.05 between the two sides of leaves (F-test).

Photorespiration is more inhibited than photosynthesis by chilling temperature (Markhart *et al.* 1980). This study also exhibited that chilling stress reduced R_P more drastically than P_N in both the sides of *Rumex* leaves, especially in the abaxial side (Fig. 4*B*). Therefore, the relatively slower decrease in P_N and R_P accounted for the slower decrease in photochemical quenching in the adaxial side than those in the abaxial side (Fig. 5*C*).

In conclusion, the relatively higher resistance to chilling stress under low irradiance in the adaxial side of *Rumex* leaves was correlated with the slower decline in photochemical quenching and higher non-photochemical quenching.

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