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Physiological variations in chloroplasts of *Rhodiola coccinea* along an altitudinal gradient in Tianshan Mountain

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Abstract For centuries in Asia, *Rhodiola coccinea* has been used as a valuable adaptogen medicinal plant known for its remarkable resistance to various stress factors. Physiological tolerance in chloroplasts is believed to be an important factor, which affects the geographic distribution of plant species. However, there is currently no report on how the physiological tolerance in the chloroplasts of R. coccinea varies along an altitudinal gradient in a semiarid mountain region. In this work, we investigated the physiological variations of the chloroplasts of R. coccinea plants along an altitudinal gradient in the Tianshan Mountains, which are located in northwest China. It was found that the physiological parameters of chloroplasts in R. coccinea plants, including superoxide generation, fatty acid compositions, thylakoid membrane fluidity, chlorophyll a/b ratios and photosynthetic electron transport rates (ETRs), varied nonlinearly with altitude. Indeed, an inflection point was observed at approximately 3,800 m. For altitudes were higher or lower than 3,800 m, the ETR, index of unsaturated

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Agriculture College, Henan University of Science and Technology, 471003 Luoyang, China e-mail: zyfgau@163.com fatty acids in the thylakoid membrane and Chl *a/b* ratios decreased with increasing altitude, whereas the superoxide generation and DPH polarisation of thylakoid membranes increased. In addition, variations in the AsA and GSH contents of chloroplasts could be divided into two distinct phases along the altitudinal gradient. The AsA content slowly decreased with increasing altitudes up to 3,800 m and rapidly decreased above 3,800 m. However, there was a gradual increase in the GSH content between 3,570 and 3,750 m, followed by an abrupt rise to a plateau level. These results demonstrate that the physiological tolerance of chloroplasts widely varies with altitude and have a tight relationship with the range of growth altitude of *R. coccinea* in Tianshan Mountains.

Keywords *Rhodiola coccinea* · Chloroplast · Altitude · Physiological variations

Abbreviations

AsA	Ascorbic acid
Chl a/b	Chlorophyll <i>a/b</i>
DPH	Diphenyl hexatriene
ETR	Electron transport rates
GSH	Reduced glutathione
IUFA	Index of unsaturated fatty acid
PS I	Photosystem I
PS II	Photosystem II
UFA	Unsaturated fatty acid

Introduction

At high altitudes, alpine plants necessarily become adapted to very harsh habitats that result from the combined action of various extreme environmental stress agents, including a very short growing season during the annual cycle (no more than 2.5–3 months), drastic changes in ambient temperature between day and night, a highly increased luminosity, enhanced contribution from UV radiation, and a predominance of poor soils (Tsydendambaev et al. 2004). Such adaptations might lead to considerable changes in the membrane fatty acid pattern and the antioxidative properties of the plants. There is ample evidence supporting that both the antioxidative activity of alpine plants and the stability of their cellular membrane play an important role in their survival in adverse environments (Tsydendambaev et al. 2004; Zhang et al. 2009). However, it remains uncertain whether this is also the case for alpine plants located at different altitudes in semi-arid mountain regions.

Chloroplasts are the main sources of reactive oxygen species (ROS) in plants (Edreva 2005); they are also the source and target of cellular redox regulation (Baier and Dietz 2005). Isolated chloroplasts were used to measure superoxide anion generation to reflect the physiological features of leaves (McRae and Thompson 1983), which are the first and most severely impacted organelles under conditions of stress (Kratsch and Wise 2000). Thus, chloroplasts are important in the study of plant responses to various stress factors (Kutik et al. 2004). During the evolution of a plant, photosynthesis regulation is geared towards minimising the generation of ROS and intracellular antioxidants, such as ascorbic acid (AsA) and reduced glutathione (GSH), play a very important role in scavenging ROS (Foyer et al. 2002). Other enzymatic antioxidant systems also play important roles in scavenging ROS in chloroplasts. One example is the enzyme participating in the ascorbate-glutathione cycle and superoxide dismutase (SOD) (Asada 2006).

In addition, it is widely accepted that the transition of the lipids presented in the membrane of a plant from a sol phase to a gel phase can lead to the death of the plant (Lyons 1973). In cold-tolerant plants, this transition is prevented by an accumulation of lipids with a high content of unsaturated fatty acids (Novitskaya et al. 2000). Thus, regulation of a membrane lipid composition and adjustment of the unsaturation level of the membrane fatty acids are extremely important to maintain the fluidity and function of a membrane in high mountains. However, few studies have been performed with species from the whole distribution range of a certain species and it is unclear whether antioxidative capacities and membrane fatty acids contribute to the distribution limits of alpine plants.

For centuries in Asia, *Rhodiola coccinea*, which belongs to the Crassulaceae family, has been used as a valuable adaptogen medicinal plant that increases resistance to various stressors. Its distribution near Glacier No. 1 at the headwaters of the Urumqi River in Tianshan Mountains is confined to an altitude between 3,400 and 4,000 m. The aims of this study were to investigate the relationship between the physiological tolerance of chloroplasts and the local altitudinal range of *R. coccinea* in Tianshan Mountains and determine whether the better balance between the production and scavenging of superoxide anions in chloroplasts could be related to adaptation to altitude.

Materials and methods

Study area and plant materials

The sampling area of this study is close to Glacier No. 1 (43°05'N, 86°49'E) near the headwaters of the Urumgi River in the Tianshan Mountains, Xinjiang, China. With a typical continental climate, a westerly jet prevails across these high mountains. Near the surface, local valley winds prevail from March through September (Li et al. 2007). The annual rainfall recorded at the local Daxigou Weather Station (3,700 m above sea level) was 512 mm, and the mean annual temperature was -4° C in 2006. The subnival vegetation in this area was classified into two categories: altifrigetic meadow and altifrigetic cushion vegetation. The main vegetation formations are Carex sp., Kobresia sp., Poa sp., Sibbaldia tetrandra, Rhodiola coccinea, Thylacospermum caespitosum and Polygonum viviparum (An et al. 2000). Well-grown R. coccinea plants (too young, too old or flowering plants were omitted) from five to eight clusters of plants were mixed as a sample and collected from nine different altitudes (from 3,400 to 3,950 m above sea level) on 15 July 2006; the clusters at the same altitude were approximately the same light regime. Three samples were collected at each altitude. The samples were frozen in liquid nitrogen directly in the field and taken back to the laboratory.

Isolation of chloroplasts

Intact chloroplasts were prepared as described by (Reeves and Hall 1980). The samples were homogenised in a mortar in an ice-cold isolation medium (1:10 w/v). The medium (pH 6.5) contained 50 mM HEPES–NaOH, 0.4 M sucrose, 10 mM NaCl and 5 mM MgCl₂. The homogenate was filtered through four layers of cheesecloth and centrifuged at $1,000 \times g$ for 1 min. The supernatant was then again centrifuged at $6,000 \times g$ for 10 min, and the pellets were resuspended in the medium. Intactness of chloroplasts (72–85%) was determined by comparing the rates of 0.5 M potassium ferricyanide reduction before and after osmotically shocking the chloroplast suspension (Lilley et al. 1975). Freshly prepared chloroplasts were used for photosynthesis ETRs assays and superoxide anion generation assays. Aliquots of the remaining chloroplasts were frozen in liquid nitrogen and stored at -80° C until further use. The chlorophyll from the chloroplasts was extracted with an 80% water–acetone mixture, and the chlorophyll concentration was calculated according to the Arnon (1949) formula.

Determination of GSH and AsA contents

AsA was assayed with a modification of the method used previously (Zaharieva and Abadía 2003). Aliquots of chloroplasts (corresponding to 1.2 mg chlorophyll) were thawed and centrifuged at 4,000*g* for 2 min and resuspended in 0.5 ml of 5% phosphoric acid by vigorously shaking the mixture. After centrifugation at 10,000*g* for 10 min, 0.1 ml of the sample was mixed with 0.3 ml of 0.3 MK phosphate buffer (pH 7.5) with 5 mM EDTA. Colour was developed after the addition of 0.5 ml of trichloroacetic acid (10%), 0.4 ml of 42% (v/v) phosphoric acid, 0.4 ml of 4% (w/v) α , α' -dipyridyl (dissolved in 70%[v/v] ethanol), and 0.2 ml of 3% (w/v) FeCl₃. The mixture was then incubated in a water bath for 1 h at 37°C, and the A_{525} value was measured.

GSH was determined by a modification of the procedure described by Zaharieva and Abadía (2003). Chloroplasts were collected and resuspended as described above. The reaction mixture contained 0.7 ml of 0.3 mM NADPH prepared in 12.5 mM potassium phosphate buffer (pH 7.5) and 6.3 mM EDTA, 0.1 ml of 6 mM 5,5'-dithiobis(2-nitrobenzoic acid) prepared in the same buffer, and 0.2 ml of diluted extract. The reaction was initiated by adding 5 μ l (0.5 U) of glutathione reductase (GR; EC 1.6.4.2, type III) (Sigma). The change in A_{412} was monitored for 200 s.

EPR measurement of superoxide anion generation in illuminated chloroplasts

Freshly prepared chloroplasts were used for EPR measurements of superoxide anion generation as described in our previous study (Zhang et al. 2009). Superoxide production in chloroplasts was measured at room temperature with a Bruker EPR spectrometer (ER200D-SRC) based on the reaction of Tiron with a superoxide anion to form the Tiron semiquinone radical (Sgherri et al. 1996). Prior to sample measurements, the superoxide anions generated by the xanthine/xanthine oxidase system were used to locate the signals of Tiron semiquinone radicals. The spectra were recorded by scanning at microwave of 9.67 GHz and 10 mW, a mid-field range of 3,445G, and at a time constant of 0.5 s. For sample assays, the magnetic field intensity (3,441.3G) was held at the low field spectrum line of the Tiron semiquinone radical, and the signal amplitude was recorded as a function of time.

Chloroplast solutions corresponding to 5 µg of chloroplasts in 100 µl were mixed with 100 µl of 200 mM Tiron (in chloroplast suspending buffer) under a safe green light. The mixed sample was then introduced into a 100-µl quartz sample tube and inserted in the microwave cavity of the EPR instrument. After incubation at room temperature for 2 min in the dark, the sample was illuminated with white light (4,000 µmol m⁻² s⁻¹). Time scanning against the constant magnetic field intensity (3,441.3G) of the first EPR spectrum line was initiated before illumination and lasted approximately 5 min. The maximum signal amplitude was used to represent the generation rate of the superoxide anions of chloroplasts.

GC assays of fatty acid compositions of thylakoid membranes

Chloroplasts were collected as described above and resuspended in 0.4 ml of H₂O. The resuspended chloroplasts were extracted with 2 ml of CHCl₃–CH₃OH (1:1, v:v), and the lower phase was pooled and evaporated until dry to yield crude polar lipids. Saponification was carried out with 2 ml of 1.5% KOH in methanol at 60°C for 30 min. Fatty acid methyl esters were prepared with boron trifluoride/methanol (Morrison and Smith 1964). GC analysis was performed on a Varian model CP3800 gas chromatograph equipped with an FID detector and a CP-Wax 52CB column. The oven temperature was programmed from 150 to 250°C at 3°C min⁻¹, and held at 250°C for 5 min. The injector and detector temperatures were both at 260°C, and the carrier gas N₂ velocity was 300 cm s⁻¹.

Determination of diphenyl hexatriene fluorescent polarisation

The fluidity of thylakoid membranes was estimated using the diphenyl hexatriene (DPH) fluorescent polarisation method (Qiu and Su 1998). Chloroplasts were incubated at room temperature for 2 h in a mixture of 10 mM Tris– MES, pH 7.4, 250 mM sucrose and 10 μ M DPH. The polarisation values were determined by measuring the DPH fluorescence with a Hitachi F-4010 fluorescence spectrophotometer with a polarisation attachment at excitation and emission wavelengths of 369 and 430 nm, respectively.

Measurements of electron transport rates

Freshly prepared chloroplasts were used for electron transport rate (ETR) measurements. The ETRs of PS I and PS II of chloroplast suspensions (10 μ g chl mL⁻¹) were measured in a medium consisting of 10 μ M DCMU, 100 mM DCIP, 1 mM sodium ascorbate, 10 mM NaCl,

3 mM MgCl₂, 2 mM NH₄Cl, 0.5 mM methylviologen (pH 7.5) and 1.0 mM NaN₃ and in a medium consisting of 50 mM HEPES–NaOH buffer (pH 7.0), 10 mM NaCl, 3 mM MgCl₂ and 400 μ M DCIP, respectively. Oxygen uptake or evolution were monitored using a Clark-type oxygen electrode (model YS, Cole-Parmer Co.) connected to a recorder under white light at a saturating intensity filtered through H₂O (Tripathy and Mohanty 1980).

Statistical analysis

Linear regression and a one-way analysis of variance (ANOVA) were performed using the statistical tools of the Origin software (OriginLab Corp, version 8). The data in the tables and figures were expressed as the mean values \pm SD. Three replications were performed for all experiments.

Results

Variation in the antioxidant contents in chloroplasts from *R. coccinea* along an altitudinal gradient

The variations of AsA and GSH contents in the chloroplasts were divided into two distinct phases along the altitudinal gradient (Fig. 1a, b). The AsA content in chloroplasts slowly decreased with increasing altitudes below 3,800 m and rapidly decreased above 3,800 m. However, there was a fast increase in the GSH content between 3,570 and 3,750 m, followed by a gradual rise to a plateau level.

Variation in superoxide anion generation in chloroplasts from *R. coccinea* along an altitudinal gradient

In contrast to the antioxidants, the generation of superoxide anions in chloroplasts did not follow a monotonic trend with respect to altitude. The lowest superoxide anion generation was found in chloroplasts from *R. coccinea* at an altitude of 3,800 m above sea level (a.s.l.) (Fig. 2). There appears to be an inflection point for superoxide anion generation in chloroplasts. Indeed, the generation of superoxide anions decreased at altitudes below 3,800 m and increased at higher altitudes.

Variations in fatty acid composition, IUFA, membrane fluidity and Chl a/b molar ratio in chloroplasts from *R*. *coccinea* along an altitudinal gradient

The main fatty acids found in chloroplasts (almost completely localized in the thylakoid membranes) of *R. coccinea* were C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 (Table 1). With increasing altitudes, the contents of C14:0, C18:2 and C18:3 changed frequently. The lowest content (C14:0) and the highest contents (C18:2 and C18:3) were found in chloroplasts from plants collected at an altitude of approximately 3,800 m (3,800–3,840 m) a.s.l. These contents were found to increase (C14:0) or decrease (C18:2 and C18:3) in plants collected away from around 3,800 m a.s.l.

The index of unsaturated fatty acid (IUFA), an integrated index to show the degree of unsaturation of fatty acids in the thylakoid membrane, decreased at altitudes above 3,800 m and increased at altitudes below 3,800 m (Fig. 3a).

Contrary to the results obtained for the thylakoid membrane IUFA but similar to those obtained in the case of superoxide generation, DPH fluorescent polarisation increased at altitudes above 3,800 m a.s.l. and decreased at altitudes below 3,800 m a.s.l. (Fig. 3b). Because high DPH fluorescent polarisation indicates low membrane fluidity (Qiu and Su 1998), these results suggest that the fluidity of the thylakoid membrane decreased in *R. coccinea* plants collected in the vicinity of their upper or lower distribution limits.

Fig. 1 Glutathione (**a**) and ascorbate (**b**) contents in *R. coccinea* chloroplasts collected at different altitudes





Fig. 2 Superoxide generation in *R. coccinea* chloroplasts collected at different altitudes

The Chl *a/b* molar ratio decreased at altitudes below 3,800 m a.s.l. and increased at altitudes above 3,800 m a.s.l. (Fig. 3c). However, the Chl *a/b* molar ratio was more variable. An ANOVA analysis showed that the correlation between the Chl *a/b* molar ratio and the altitude was not significant (*P* value > 0.05) at either high or low altitudes.

R. coccinea plants collected at an altitude of approximately 3,800 m a.s.l. displayed the highest IUFA, fluidity (i.e., the lowest DPH fluorescent polarisation) and Chl *a/b* molar ratio in chloroplasts.

Variation in photosynthetic ETRs in chloroplasts from *R. coccinea* along an altitudinal gradient

The highest photosynthetic PS I and PS II ETRs were found in *R. coccinea* at 3,850 and 3,800 m a.s.l., respectively. The same slopes were found for the PSI and PSII activity in *R. coccinea* plants collected at altitudes other than 3,800 m a.s.l. However, there was only a slight difference in PS II ETR activities in *R. coccinea* plants collected at altitudes between 3,500 and 3,700 m a.s.l. (Fig. 4a, b).

Correlation among superoxide anion generation, GSH, AsA, IUFA, DPH, Chl *a/b* and PS I and PS II activities of *R. coccinea* chloroplasts

To determine the correlation among the measured physiological parameters, pairwise linear fits and ANOVA analyses were performed using the Origin 8 software. The results showed that there was no significant correlation between the GSH and AsA contents and the other physiological parameters examined. Superoxide anion generation was significantly correlated with IUFA, DPH, Chl alb molar ratio and the PS I ETR activity. In addition to being significantly correlated with the superoxide anion generation, the PS I ETR activity was also significantly correlated with IUFA. Chl a/b molar ratio and PS II ETR activity. IUFA and Chl a/b molar ratio displayed significant correlations with the three other parameters. However, DPH was only correlated with superoxide anion generation, and PS II ETR activity was only correlated with PS I ETR activity (Table 2).

Discussion

During a Mehler's reaction, molecular oxygen can serve as an NADPH-alternative acceptor of an unpaired electron, so as to create additional ATP and superoxide anion, which requires photosynthetic electron transport (Zhang et al. 2009). However, possible reasons of increased superoxide anion production under stress conditions may be due to leakage of electrons from an electron transport system (Quartacci and Navari-Izzo 1992). Superoxide anions are usually generated at PS I photosynthetic ETRs in chloroplasts (Asada 2006). In the present study, a close relationship between PSI photosynthetic ETRs and the amount of superoxide anions produced in chloroplasts was observed, suggesting that higher electron transport rates can reduce the susceptibility to leakage of electrons within the chloroplasts of alpine plants (Zhang et al. 2009).

Table 1 Fatty acid compositions of thylakoid membranes of R. coccinea chloroplasts collected at different altitudes

Altitude (m)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
3,570	13.56 ± 0.12	19.70 ± 0.24	16.07 ± 0.04	14.9 ± 0.05	4.12 ± 0.6	12.39 ± 0.02	19.26 ± 0.17
3,600	11.05 ± 0.09	18.28 ± 0.11	12.06 ± 0.52	12.22 ± 0.25	4.63 ± 0.24	13.77 ± 0.36	27.98 ± 0.13
3,700	9.58 ± 0.01	17.87 ± 0.21	9.75 ± 0.18	12.01 ± 0.02	4.54 ± 0.19	15.41 ± 0.09	30.85 ± 0.07
3,750	11.07 ± 0.2	18.49 ± 0.2	10.92 ± 0.53	12.34 ± 0.02	4.49 ± 0.01	13.23 ± 0.01	29.46 ± 0.11
3,800	7.23 ± 0.11	18.13 ± 0.19	9.68 ± 0.38	12.12 ± 0.06	4.25 ± 0.18	15.37 ± 0.08	33.22 ± 0.54
3,840	3.94 ± 0.06	20.01 ± 0.21	11.37 ± 0.45	13.97 ± 0.2	5.14 ± 0.26	16.59 ± 0.01	28.98 ± 0.24
3,870	10.21 ± 0.01	18.8 ± 0.04	9.30 ± 0.42	11.61 ± 0.13	5.02 ± 0.02	12.14 ± 0.06	32.91 ± 0.46
3,900	12.82 ± 0.28	18.8 ± 0.14	13.84 ± 0.17	13.81 ± 0.02	4.10 ± 0.09	11.17 ± 0.01	25.45 ± 0.15
3,930	15.16 ± 0.1	19.92 ± 0.11	16.00 ± 0.38	16.03 ± 0.31	4.92 ± 0.37	8.27 ± 0.07	19.70 ± 0.35



Fig. 3 IUFA (a), DPH fluorescent polarisation (b) and Chl *a/b* molar ratio (c) in chloroplasts of *R. coccinea* chloroplasts collected at different altitudes. IUFA was calculated with the data of Table 1. IUFA = $C16:1\% + C18:1\% + 2 \times C18:2\% + 3 \times C18:3\%$

Fig. 4 The PS I (**a**) and PS II (**b**) electron transport rates of *R. coccinea* chloroplasts collected at different altitudes



However, changes in superoxide anion production by thylakoids might not be strictly linked to electron transport. The superoxide anion level was found to be the lowest in *R. coccinea* plants collected at an altitude of approximately 3,800 m a.s.l.; the highest superoxide anion levels were found in plants collected at both ends of the distribution range (Fig. 2). These results suggest that, at these altitudes,

R. coccinea plants suffered from more severe oxidative stress conditions and photoinhibition in chloroplasts, which would occur in cold sunny days in high mountains (Streb et al. 2003a, b). This is consistent with the decrease in the Chl *a/b* molar ratio observed for plants collected near the altitudinal limits. In fact, under conditions of photoinhibition, Chl *a*, which is partly found in photosynthetic reaction

	*							
	Glutathione	Ascorbate	Superoxide	IUFA	DPH	Chl a/b	PS I	PS II
Glutathione		0.118	0.547	0.836	0.389	0.402	0.882	0.709
Ascorbate	0.118		0.116	0.368	0.605	0.452	0.791	0.654
Superoxide	0.547	0.116		0.003*	0.034*	0.027*	0.018*	0.074
IUFA	0.368	0.836	0.003*		0.247	0.001*	0.002*	0.081
DPH	0.605	0.389	0.034*	0.247		0.641	0.068	0.106
Chl a/b	0.452	0.402	0.027*	0.001*	0.641		0.048*	0.152
PS I	0.791	0.882	0.018*	0.002*	0.068	0.048*		0.012*
PS II	0.709	0.654	0.074	0.081	0.106	0.152	0.012*	

Table 2 Pairwise linear fit and ANOVA analysis the correlation among superoxide generation, glutathione, ascorbate, IUFA, DPH, Chl *a/b*, PS I and PS II of *R. coccinea* chloroplasts

P values were shown

* Significant linear correlation (P < 0.05) between the two physiological indices

centres, is more vulnerable to photobleaching than Chl b, which is only localised in light-harvesting antennae. A decrease of the Chl a/b ratio in stressed plants has already been reported (Moran et al.1994; Loggini et al.1999).

In the present study, R. coccinea plants collected at an altitude of approximately 3,800 m a.s.l. displayed the highest IUFA, fluidity (i.e., the lowest DPH fluorescent polarisation), Chl a/b molar ratio and photosynthetic PS I and PS II ETRs in chloroplasts (Figs. 3, 4), suggesting that 3,800 m a.s.l. may be an optimal altitude for photosynthesis in these plants. Similar results were reported in monkey flowers, whose greatest net photosynthetic rate and effective quantum yield of PS II were found in plants grown near an altitudinal range centre (Angert 2006). Qiang et al. (2003) also found that the environmental conditions near 3,000 m a.s.l. in the Oilian Mountains were the most suitable for the growth and development of Qinghai spruce, occurring from 2,300 m a.s.l. to more than 3,400 m a.s.l. The variations in photosynthesis might be due to changes in the thylakoid membrane composition (unsaturated fatty acid composition) and superoxide anion generation. A linear fit showed that the activity of PS I ETR was significantly correlated with the generation of superoxide anions and the IUFA of chloroplasts. In fact, some studies have shown that photosynthesis benefits from thylakoid unsaturation (Moon et al. 1995; Terrados and Lopez-Jimenez 1996; Routaboul and Fischer 2000; Ariizumi et al. 2002; Vijayan 2002) and is inhibited by superoxide anions (Herbert et al. 1992; Fryer et al. 2002; Zhao et al. 2007).

To increase the composition of unsaturated fatty acids in membrane systems is one of the basic responses of plants to cold temperatures. An increase in IUFA genetically improves the cold-tolerance of plants (Ariizumi et al. 2002; Yu et al. 2009). In the sampling region of this research project, the air temperature decreased by approximately 1° C with a 100 m higher altitude (data not shown). However, the IUFA of chloroplasts was not found to increase continuously with altitude. On the contrary, the IUFA decreased at altitudes above 3800 m a.s.l. (Fig. 3a). The decrease in IUFA is mainly due to an increase in C14:0 and decreases in C18:2 and C18:3 (Table 1). These changes, particularly the decrease in linolenic acid (C18:3), may lead to a higher sensitivity to low temperatures and photoinhibition. The results were further confirmed by data obtained for the DPH fluorescent polarisation of thylakoid membrane. These results suggest that *R. coccinea* plants failed to increase their stress tolerance by increasing the thylakoid unsaturation and membrane fluidity at altitudes above 3,800 m.

Several studies have reported that alpine plants adapt to their environments by increasing their antioxidative properties (Miszalski et al. 2005; Yin et al. 2009). AsA and GSH act as primary antioxidants by reacting nonenzymatically with ROS and are also important components of the ascorbate-glutathione cycle, which is involved in ROS scavenging (Kuzniak and Sklodowska 2001). However, in this study, both GSH and AsA were not significantly correlated with superoxide generation in chloroplasts (Table 2). Interestingly, the variation trend of the AsA content with increasing altitudes was opposite from that observed for the GSH content (Fig. 1a, b). A possible explanation for these results is that GSH and ASA respond to different factors in alpine environments but not to the combination of all adverse effects. The variations of the AsA and GSH contents could clearly be divided into two phases along the altitudinal gradient studied, with a peak at 3,800 m a.s.l. R. coccinea plants from the upper and lower altitudinal limits did not display consistent high or low antioxidant levels (Fig. 1a, b). These results suggest that the antioxidative property does not contribute directly to the altitudinal distribution limit of R. coccinea plants.

Taken together, our results show that the metabolic and componential characteristics of chloroplasts from *R. coccinea* plants along the altitudinal gradient studied in the Tianshan Mountains of China were highly variable. However, except for the antioxidant contents (GSH and AsA contents), the superoxide generation, fatty acid compositions, thylakoid membrane fluidity, chlorophyll *a/b* ratio and photosynthetic electron transport rates of chloroplasts varied nonlinearly with altitude, with a relationship reversal point at approximately 3,800 m a.s.l. These results suggest that the physiological tolerance of chloroplasts in *R. coccinea* plants is correlated with their altitudinal distribution in the Tianshan Mountains.

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