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Altitudinal changes in Rubisco and APX activities in *Aconogonum weyrichii* in the alpine region of Mt. Fuji

Tsuyoshi Sakata[1]*, Takashi Nakano[2] and Yota Yokoi[1]

[1]*Biological Laboratory, Center for Natural Sciences, College of Liberal Arts and Sciences, Kitasato University, Kitasato 1-chome, Sagamihara 228-8555*
[2]*Yamanashi Institute of Environmental Sciences, Kamiyoshida, Fuji-Yoshida 403-0005*
**Corresponding author. E-mail: sakata@clas.kitasato-u.ac.jp*

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Abstract: To identify the determinants of the altitudinal distribution of an alpine herbaceous plant (*Aconogonum weyrichii*) from an eco-physiological viewpoint, we investigated the leaf characteristics concerned with photosynthetic capacity and tolerance of oxidative stress throughout this species altitude distribution on Mt. Fuji. Larger amounts of leaf nitrogen and Rubisco were found in populations growing at higher altitudes; however, initial activity of Rubisco did not increase with altitude in the summer, indicating that inactivation of Rubisco occurred in the higher populations. This inactivation would lead to a decrease in photosynthetic nitrogen use efficiency. When the leaves of *A. weyrichii* began to turn yellow in autumn, amounts of leaf nitrogen and Rubisco remarkably decreased in all populations throughout the altitude distribution. However, Rubisco activity in the higher populations did not decline until immediately before defoliation, suggesting that recovery from Rubisco inactivation occurred in these populations. The higher populations had a higher activity of APX than lower populations, contributing to maintaining Rubisco activity and photosynthetic production until the end of the growing period, which, in turn, are necessary for survival at higher altitudes.

key words: Alpine plants, ribulose-1,5-bisphosphate carboxylase/oxygenase, ascorbate peroxidase, leaf nitrogen, photosynthetic nitrogen use efficiency

Introduction

Aconogonum weyrichii (Polygonaceae) is a perennial herb mainly distributed in alpine regions and believed to be of northern origin (Maruta, 1994). Restricted photosynthetic production as a result of an inherent short growing period (Maruta, 1994) would lead this species to a disadvantage compared with allied lowland species at lower altitudes. In addition, Maruta (1994) reported that most seedlings sown artificially beyond the upper altitudinal limit of distribution had a reduced growth rate and failed to attain the critical size for survival in winter due to the shortened growing period. This study showed that the growing period and photosynthetic production might be main factors in the altitudinal distribution pattern of *A. weyrichii.* However, the photosynthetic capacity during the growing period has not been studied along the

altitudinal distribution of this species.

In general, photosynthetic capacity and production strongly correspond to the content of leaf nitrogen. In line with this, a large amount of leaf nitrogen has been found in alpine plants (Körner and Diemer, 1987; Friend *et al*., 1989; Friend and Woodward, 1990; Vitousek *et al*., 1990; Westbeek *et al*., 1999; Körner, 1999). It has therefore been suggested that alpine plants compensate for photosynthetic restriction (*e.g.* as a result of low temperatures and low atmospheric pressure) with a large amount of leaf nitrogen (Körner and Diemer, 1987; Cordell *et al*., 1999). Photosynthetic nitrogen use efficiency (PNUE) is strongly affected by the allocation of leaf nitrogen to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Lloyd *et al*., 1992; Hikosaka *et al*., 1998; Poorter and Evans, 1998), the key enzyme for photosynthesis, and by the specific activity of Rubisco. Thus, in order to show whether a large amount of leaf nitrogen results in a high photosynthetic capacity in alpine plants, and to elucidate the altitudinal changes in leaf characteristics involved in the limitations of altitudinal distribution, it is necessary to determine the amount and activation state of Rubisco.

In addition to photosynthetic production, tolerance to environmental stress is also thought to be related to the upper altitudinal limit of distribution of alpine plants. Under chilling and high light conditions, light absorbed by leaves cannot be used efficiently for photosynthesis and, moreover, becomes potentially damaging because of active oxygen species (AOS) produced by the excess electrons (*cf.* Foyer *et al*., 1994; Breusegem *et al*., 2002). Thus, the activity of APX, a key enzyme in the hydrogen peroxide-scavenging system protecting chloroplasts and other cell constituents from AOS damage (Asada *et al*., 1973; Asada, 1992), is also thought to be related to the altitudinal distribution of alpine species growing in open and cold alpine habitats.

The main purpose of this study is to elucidate the altitudinal changes in leaf characteristics concerned with photosynthetic capacity and tolerance of oxidative stress in *A. weyrichii* in order to explain the altitude distribution of this species from an eco-physiological viewpoint.

Materials and methods

Aconogonum weyrichii (F. Schmidt) H. Hara var. *alpinum* (Maxim.) H. Hara occurs in Sakhalin, Chisima Retto, Hokkaido, and alpine regions of central Japan (Maruta, 1994). To investigate the leaf characteristics of this species throughout its altitude distribution in alpine regions, we compared four populations growing in open habitats on the northeast slope of Mt. Fuji, Japan (35°21′N, 138°44′E), at 2250, 2580, 2850, and 3130 m a.s.l., respectively,. All study sites were located on nutrient-poor scoria and were very sparsely vegetated.

All populations investigated rapidly expanded their leaves from early to late June and almost all individuals, in the lowest population, bloomed by 30 July. The percentage of flowering individuals gradually decreased to about 50% in the highest population on 30 July. In early September, defoliation began at all altitudes. The percentage of leaves turning yellow per individual gradually decreased from 70% in the lowest population to 40% in the highest population.

Leaf characteristics were determined as follows on 30 July and 3 September, 2002.

Leaf disks (*ca.* 0.5 cm^2 $disk^{-1}$) were randomly harvested from fully expanded sunlit leaves of five individuals in each population at almost 2 to 4 hours after sunrise. The leaf disks were then immediately frozen in liquid nitrogen and stored at −80°C until use. Leaf mass per leaf area (LMA) and nitrogen content were determined in three or four disks.

Rubisco activity was determined using the spectrophotometric method of Lilley and Walker (1974), partly modified by Sakata and Yokoi (2002). Two frozen leaf discs were rapidly homogenized in a chilled mortar with 1 m*l* of a CO_2-free extraction buffer (100 mM HEPES buffer containing 10 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 2% w/v PVP40, 1% v/v Triton X-100, and 0.2 mM leupeptin, pH 7.8). The homogenate was centrifuged at 17400×g for 2 min at 4°C then the supernatant was used for the assay of initial activity of Rubisco immediately after extraction. Total activity of Rubisco was also determined after activation of the enzyme, which was achieved by preincubation for more than 10 min at 0°C in the presence of 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ at pH 7.8. The protein content in the extract was assayed according to the method of Bradford (1976) using a BIO-RAD Protein Assay Kit.

The Rubisco content in the extract was determined with a TEFCO SDS-PAGE mini-system (TEF Corporation, Japan) using a gradient of 4–20% polyacrylamide slab gel stained with Coomassie Brilliant Blue R-250 and Tris-glycine buffer, pH 8.8. The gel images were photographed using a digital camera (COOLPIX 880, Nikon Ltd., Japan) and analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA) to determine the amount of Rubisco.

APX activity was assayed according to the partly modified method of Asada (1984). A frozen leaf disk was rapidly homogenized in a chilled mortar with 0.5 m*l* of an extraction buffer (50 mM phosphate buffer containing 2% w/v PVP40 and 1% v/v Triton X-100, pH 7.0). The homogenate was centrifuged at 17400×g for 20 min at 4°C then the supernatant (*ca.* 0.5 m*l*) was used for the assay of APX activity. A 50 μl supernatant was added to a cuvette with 1900 μl of reaction buffer (50 mM phosphate buffer containing 0.5 mM ascorbate) at 25°C. The peroxidase reaction was then initiated by the addition of 50 μl of 4 mM H_2O_2, and APX activity was recorded by the decrease in absorbance at 290 nm.

Results

LMA, and leaf nitrogen and protein contents

The LMA, and nitrogen and protein contents per leaf area of *A. weyrichii* are shown in Fig. 1. The nitrogen and protein contents per leaf area were both significantly higher in the populations growing at higher altitudes (2850 m and 3130 m a.s.l., $p < 0.002$, Fisher's PLSD) in summer (30 July) and autumn (3 September), while LMA was almost the same at all altitudes. Nitrogen and protein contents were remarkably decreased in autumn at all altitudes. The statistical significance of the differences between summer and autumn was smaller at higher altitudes than lower altitudes (2250 and 2580 m a.s.l., $p < 0.003$; 2850 and 3130 m a.s.l., $p < 0.11$; *t*-test).

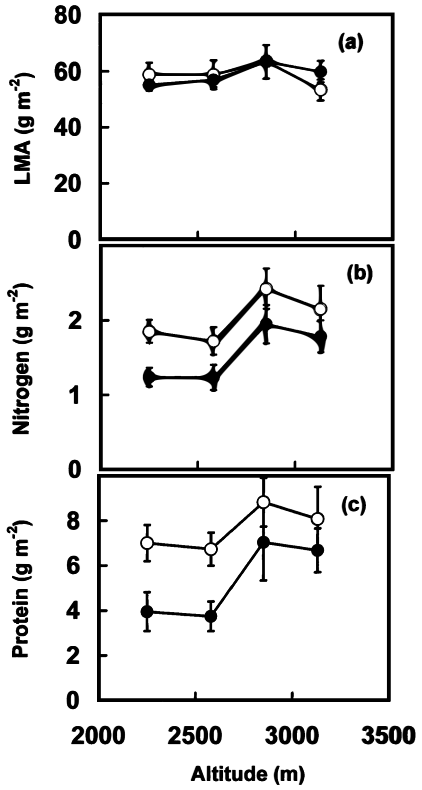

Fig. 1. Altitudinal changes in the LMA (a), and nitrogen (b) and protein (c) contents per leaf area of *Aconogonum weyrichii* in summer (○; 30 July, 2002) and autumn (●; 3 September, 2002). Points and bars indicate means±SD.

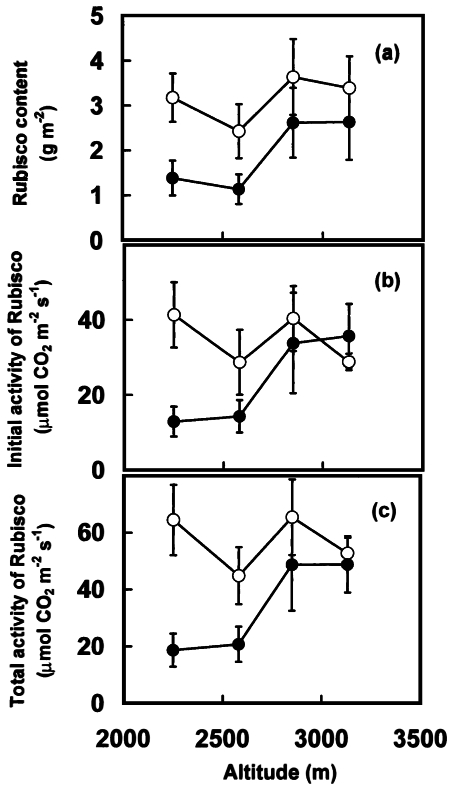

Fig. 2. Altitudinal changes in the amount of Rubisco per leaf area (a), and initial (b), and total activity of Rubisco (c). Symbols are as in Fig. 1.

Rubisco content and activity

The change in Rubisco content with altitude was almost identical to the changes in nitrogen and protein contents (Fig. 2a). Rubisco content was significantly higher in the populations growing at higher altitudes in summer and autumn ($p<0.05$; Fisher's PLSD), except at 2250 m in summer. Rubisco content also decreased in autumn at all altitudes, but the reduction was smaller at higher altitudes (2250 and 2580 m a.s.l., $p<0.004$; 2850 and 3130 m a.s.l., $p<0.19$; *t*-test). On the other hand, there were no obvious changes in the initial and total activities of Rubisco with altitude in summer (Figs. 2b and 2c). In autumn, when defoliation began at all altitudes, the initial and total activities of Rubisco did not decline in the higher populations (2850 and 3130 m a.s.l., $p>0.1$; *t*-test), but there were significant decreases in the lower populations (2250 and 2580 m a.s.l., $p<0.02$; *t*-test).

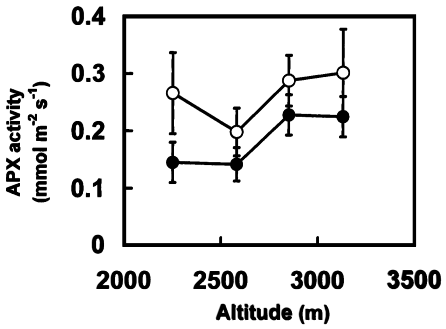

Fig. 3. Altitudinal changes in APX activity per leaf area. Symbols are as in Fig. 1.

APX activity

Figure 3 shows the altitudinal changes in APX activity. APX activity was significantly higher in the populations growing at higher altitudes in summer and autumn ($p < 0.04$; Fisher's PLSD), except 2250 m in summer.

Discussion

Altitudinal changes of leaf nitrogen and Rubisco

A larger amount of leaf nitrogen was found in populations of *A. weyrichii* growing at higher altitudes than lower altitudes, as in previous reports on other alpine plants (Körner and Diemer, 1987; Friend *et al*., 1989; Friend and Woodward, 1990; Vitousek *et al*., 1990; Westbeek *et al*., 1999). It has been suggested that the large amount of leaf nitrogen contributes to the maintenance of photosynthetic rates despite suboptimal conditions for CO_2 assimilation in alpine regions (Körner and Diemer, 1987; Cordell *et al*., 1999). However, a smaller allocation of nitrogen to the photosynthetic apparatus results in lower photosynthetic nitrogen use efficiency and suppression of photosynthesis (Field and Mooney, 1986) despite a large leaf content of nitrogen. Westbeek *et al.* (1999) found a smaller allocation of leaf nitrogen to photosynthetic apparatus in an alpine species compared with lowland species. It is suggested that the small nitrogen allocation to photosynthetic apparatus in alpine species might be caused by the large amount of nitrogenous compounds associated with physical toughness since high-speed winds are common at high altitudes (Hikosaka *et al*., 2002).

To elucidate the nitrogen allocation to protein and Rubisco in *A. weyrichii* throughout the altitude distribution, we determined the relationships between leaf nitrogen and protein (Fig. 4a) and Rubisco (Fig. 4b). The strong linear correlation between nitrogen and protein ($r^2 = 0.93$) indicates that nitrogen allocation to leaf protein did not change throughout the altitude distribution in summer and autumn. A strong linear correlation was also found between nitrogen and Rubisco ($r^2 = 0.83$). Therefore the relationship between leaf nitrogen and Rubisco did not change throughout the altitude distribution, the populations growing at higher altitudes have a larger amount of Rubisco in proportion to leaf nitrogen, which increased in higher populations.

In summer, however, the initial activity of Rubisco did not increase in the pop-

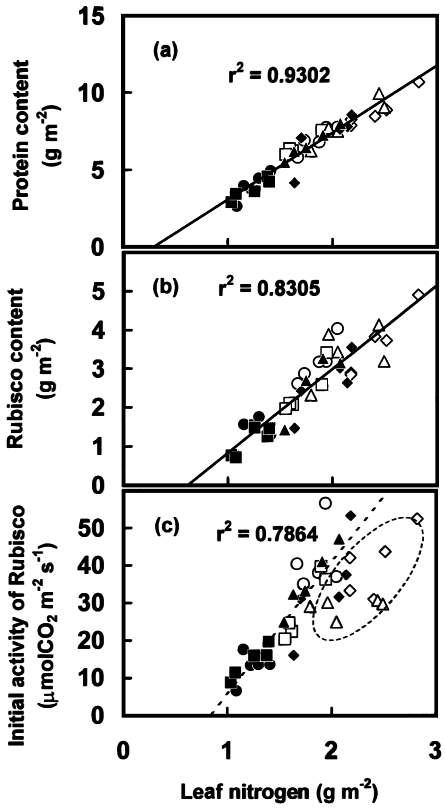

Fig. 4. Relationships between leaf characteristics and leaf nitrogen content on a leaf area basis: (a) the content of leaf protein, (b) content of Rubisco and (c) initial activity of Rubisco in summer (○, at 2250 m; □, 2580 m; ◇, 2850 m; △, 3130 m) and in autumn (●, at 2250 m; ■, 2580 m; ◆, 2850 m; ▲, 3130 m). Solid lines indicate regression lines calculated with all data from (a) and (b), and the dotted line in (c) indicates the regression line calculated without the data of the higher populations in summer (indicated by a dotted circle).

ulations growing at higher altitudes (Fig. 2b), suggesting that a large part of the Rubisco is inactive in the leaves of higher populations. The activation of Rubisco involves two processes: the carbamylation of Rubisco and the removal of inhibitors blocking carbamylation or RuBP binding to the carbamylated sites before reaction with CO_2 or O_2 (reviewed by Jensen, 2004). In summer, the altitudinal change in total activity, which was measured following carbamylation treatment, was similar to the initial activity (Fig. 2c). The inactivation of Rubisco in the higher populations is thought to be associated with inhibitors binding to Rubisco, not with differences in the carbamylation state, because the higher populations showed similar levels of total activity to the lower populations in summer.

In autumn, when the leaves of *A. weyrichii* began to turn yellow, plants from all populations seemed to begin withdrawing nitrogen from their leaves, which had remarkably smaller contents of nitrogen, protein, and Rubisco than in summer (Figs. 1b, 1c, and 2a). However, neither the initial nor total activity of Rubisco declined in the higher populations, in contrast to in lower populations (Figs. 2b and 2c). The higher altitude populations therefore maintained their Rubisco activity until immediately before defoliation as a result of recovery from Rubisco inactivation in summer.

Figure 4c indicates the strong linear correlation between nitrogen and the initial activity of Rubisco, except for the higher populations in summer. The correlation

coefficient ($r^2=0.79$), without the data of the higher populations in summer, did not differ much from the contents of Rubisco ($r^2=0.83$). When including the data of the higher populations in summer, the correlation coefficient decreased to 0.60. Since the nitrogen allocation to Rubisco was not small in the higher populations in summer (Fig. 4b), the photosynthetic nitrogen use efficiency would be decreased in the higher population in summer by inactivation of Rubisco.

The initial activity of Rubisco in higher populations of *A. weyrichii* did not increase in summer despite the larger amount of leaf nitrogen compared to lower populations as a result of inactivation of Rubisco. Thus, the PNUE of the higher populations might decrease in the summer. After that, the higher populations of *A. weyrichii* maintain substantial Rubisco activity until the end of the growing period by recovery from inactivation of Rubisco. This recovery would contribute to the survival of higher populations in alpine regions by allowing substantial photosynthetic production until the end of the growing period.

Tolerance to oxidative stress in the leaf

In the lowest population of *A. weyrichii*, the growing period inherently terminated in mid-September, and most of the leaves turned yellow by 3 September, though a co-occurring allied species, *Reynoutria japonica*, did not show significant hypoactivity in its leaves at this same time (our unpublished data). The remarkable reduction in APX activity in the lowest population in autumn (Fig. 3) suggests the lowered necessity for tolerance of oxidative stress at the end of the growing period, since this population is able to avoid the chilling season by inherent early defoliation.

In contrast, higher APX activity was observed in the higher populations, especially in autumn (Fig. 3). Maruta (1994) reported that most of *A. weyrichii* seedlings artificially sown beyond the upper altitudinal limit of distribution experienced reductions in growth rate and failed to survive in winter due to the forcedly shortened growing period. Thus, in order to maintain photosynthetic activity until the end of the growth period, the necessity of tolerance to oxidative stress would be increased with altitude in *A. weyrichii* near the upper limit of distribution. In the higher populations, the higher activity of APX would contribute to maintaining Rubisco activity and photosynthetic production until the end of the growing period, both of which are necessary for survival at higher altitudes.

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