

Chlorophyll Fluorescence in Response to Salt Stress in the Coastal Plant *Calystegia soldanella*: A Comparison between Coastal and Freshwater Populations

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Abstract Lake Biwa, Japan, is a freshwater lake that was formed about 4 MYA and harbors many coastal plants that commonly inhabit the seashore. *Calystegia soldanella* is one of the coastal plants that commonly grows on sandy seashores, but also occurs at Lake Biwa as a result of long-term isolation from coastal populations. To assess physiological differentiation between coastal and inland individuals with regard to the response to salinity stress, we measured chlorophyll fluorescence relative to photosynthetic activity after treatment with saltwater irrigation and spray. In addition, we observed the leaf morphology of inland and coastal individuals. Inland individuals at Lake Biwa showed significantly increased photochemical fluorescence as a result of saltwater sprayed onto leaf blades, corresponding to stomatal closure, whereas coastal individuals showed an insignificant decrease in the same parameter. Additionally, coastal individuals tended to have thicker leaf blades with thicker cuticles than those of inland individuals. The differences in photochemical fluorescence quenching and leaf morphology between the inland and coastal individuals imply differing physiological adaptations to each habitat.

Key words: *Calystegia soldanella*, Lake Biwa, photosynthesis, salinity stress.

Introduction

Lake Biwa, a freshwater lake located in central Japan, is one of the world's few ancient lakes and was formed about four million years ago (Yokoyama, 1984; Kawabe, 1989, 1994; Meyers *et al.*, 1993). This lake harbors many coastal plants that commonly inhabit the seashore. *Calystegia soldanella* (L.) Roem. et Schult. (Convolvulaceae) is one of these coastal plants, which grows on sandy seashores in the temperate zone including Japan. However, Lake Biwa harbors this plant as landlocked populations. Chloroplast DNA haplotype analysis and simple sequence repeat (SSR) analysis have revealed genomic signatures indicating long-term isolation of inland populations at Lake Biwa from coastal populations of *C. soldanella* (Noda *et al.*, unpublished).

The landlocked plants at the freshwater lake may have become physiologically differentiated

with regard to salt-tolerance as a result of adaptive evolution to the specific habitat over the long term. Salinity damages plants via ion toxicity, osmotic stress, mineral deficiencies, physiological and biochemical perturbations, and combinations of these stresses (Munns, 1993, 2002; Neumann, 1997; Yeo, 1998; Hasegawa *et al.*, 2000). Salinity stress decreases photosynthetic activity through stomatal and nonstomatal factors (Yeo *et al.*, 1985; Sharma and Hall, 1991; Dionisio-Sesc and Tobita, 2000). Although the nonstomatal factors are not yet fully understood, the stomatal factors imply that salinity stress may alter photosynthesis mostly due to a reduction in stomatal conductance. Decreased water-potential and subsequent stomatal closure result in a decreased capacity of the mesophyll to fix CO₂ (Seemann and Critchley, 1985; Bethke and Drew, 1992; Delfine *et al.*, 1998). Stomatal closure carries a risk of photoinhibition, and consequently, decreased photosyn-

thetic activity.

The efficiency of photosynthesis can be measured as chlorophyll fluorescence. In the photosynthetic apparatus, light is absorbed by the antenna pigments, and the excitation energy is transferred to the reaction centers of the two photosystems. There the energy drives the primary photochemical reactions that initiate photosynthetic energy conversion. In low light under optimal conditions, primary photochemistry occurs with high efficiency. A minor competing process involving the deactivation of excited pigments is the emission of chlorophyll *a* fluorescence (Krause, 1991) as a result of photosystem II (PSII) photochemistry. In general, photochemical fluorescence quenching is caused by a decrease in the photochemical efficiency of PSII attributed to damage of the PSII reaction centers caused by salinity (Masojidek and Hall, 1992; Hasson and Poljakoff-Mayber, 1981; Kura-Hotta *et al.*, 1987). Chlorophyll fluorescence can provide detailed information on PSII photochemistry, which is sensitive to environmental stresses such as strong light, low or high temperature, drought, and salinity (e.g., Larcher *et al.*, 1990). Thus, chlorophyll fluorescence is an appropriate indicator of salt stress (Godfrey and Kerr, 2000).

In this study, we used chlorophyll fluorescence analysis to detect physiological differentiation between inland and coastal individuals of *Calystegia soldanella* in terms of their response to salinity stress. In addition, we observed leaf morphology of the inland and coastal individuals, as leaf morphology is an important feature of plant salt tolerance.

Materials and Methods

Plant growth and salt treatment

Rhizomes of *Calystegia soldanella* were collected from coastal and lakeshore populations: Shimohama Beach on the coast of the Sea of Japan (39°37'N, 140°04'E) and Omimaiko at Lake Biwa (35°08'N, 135°34'E), respectively. Individuals were sampled at intervals of more than 5 m to collect materials originating from dif-

ferent genets. They were planted in 0.8-L pots filled with compost (mixture of vermiculite and gravels) and grown in a greenhouse at Kyoto University (35°13'N, 135°47'E), Japan. Voucher specimens were deposited in TNS and KYO. The plants were kept in a greenhouse during experiment. The plants were cultivated for more than one month in preparation for the experiments and were given sufficient water while nutrients were supplied by application of 1/4000 diluted liquid fertilizer (Hyponex solution) each week.

Eight individuals each from coastal and inland populations were subjected to the following conditions: 1. Saltwater (200 mM) irrigation every 3 days for 2 weeks; 2. Freshwater irrigation every 3 days for 2 weeks; 3. Saltwater (300 mM) spray every day, with saltwater (200 mM) irrigation every 3 days for 1 week; 4. Saltwater (300 mM) spray every day, with saltwater (200 mM) irrigation every 3 days for 2 weeks. Control plants were assigned to the following conditions: freshwater irrigation to inland individuals (control for inland individuals) and saltwater irrigation to coastal individuals (control for coastal individuals). Ten individuals were assigned to each control condition. Saltwater spray was conducted to provide fine mist without forming drops on the leaf surface. For the saltwater irrigation (condition 3 above), the 200 mM NaCl concentration was reached incrementally in 50-mM steps every 2 days prior to the start of the experiment.

Fluorescence measurements

Chlorophyll fluorescence was measured with a pulse amplitude modulation (PAM) unit (LI6400-40, Li-Cor Environmental Inc.). We measured basic fluorescence upon exposure to weak light after dark adaptation (F_0), maximum fluorescence (F_m), and F_v/F_m . F_v/F_m is the 'intrinsic efficiency' (Maxwell and Johnson, 2000) or the 'current photochemical capacity' of PSII (Bolh ar-Nordenkampf and  quist, 1993), where F_v is the 'variable fluorescence,' 'minimum fluorescence' is F_0 , and the 'maximum fluorescence' is F_m . Healthy leaves usually show F_v/F_m values between 0.66 and 0.8; lower values indicate

Table 1. Baseline F_v/F_m (mean \pm s.e.) of lakeshore and coastal individuals of *Calystegia soldanella*

Treatment	Lakeshore individuals		Coastal individuals	
	predawn	light	predawn	light
Freshwater irrigation	0.767 \pm 0.004	0.740 \pm 0.016	0.775 \pm 0.007	0.762 \pm 0.008
Saltwater irrigation	0.794 \pm 0.016	0.772 \pm 0.030	0.808 \pm 0.030	0.735 \pm 0.062
Saltwater spray (1 week)	0.701 \pm 0.040	0.672 \pm 0.071	0.768 \pm 0.046	0.764 \pm 0.062
Saltwater spray (2 weeks)	0.663 \pm 0.175	0.630 \pm 0.138	0.700 \pm 0.129	0.716 \pm 0.097

structural damage to PSII [calculated according to Bolh ar-Nordenkampf and  quist (1993) for a common floodplain species as described by Waldhoff *et al.* (2000)]. Measurements were made on fully expanded and mature leaves. Each sample had been pre-darkened for at least 30 min prior to the measurements to avoid nonphotochemical quenching. Other samples were measured under predawn conditions to investigate chronic photoinhibition of samples affected by their environment (Krause *et al.*, 1995, Kitao *et al.*, 2003). We collected data under two conditions, predawn and light. A red irradiance of 7000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was used for the measurements.

Light microscope analysis

To analyze morphological structure, fully expanded and mature leaves were collected, and cross sections of these leaves were investigated with a light microscope (BX-51 OLYMPUS). Photographs of the observed cross sections were taken with a digital camera (C-4040ZOOM OLYMPUS) attached to a light microscope. Fresh and mature leaves were sectioned with a razor blade. Cross sections of 50–100 μm in thickness were used for the observations.

Statistical analysis

Differences between F_v/F_m values among treatments were tested by one-way analysis of variance (ANOVA). All statistical analyses were performed with Excel 2003 for WINDOWS XP.

Results

The relative stress demonstrated by the ratio of

observed F_v/F_m is presented in Figs. 1 and 2. The data from the Lake Biwa lakeshore individuals (Fig. 1) suggested that treatment with 200 mM saltwater irrigation caused no reduction in the F_v/F_m ratio, whereas individuals subjected to saltwater spray (for 1 or 2 weeks) showed a significant decrease in this value under both sets of light conditions ($P < 0.05$, ANOVA). The two periods (1 or 2 weeks) of saltwater spray treatments were not significantly different. Individuals exposed to saltwater spray for 2 weeks had the lowest values (approximately 0.63) when measured without predawn treatment. Overall, the chlorophyll fluorescence of individuals measured under predawn conditions tended to show higher mean values than that of individuals measured without predawn treatment, although ANOVA did not demonstrate significant differentiation between the treatments (see Fig. 1).

Coastal individuals from the Sea of Japan consistently represented the highest values ($F_v/F_m > 0.7$) among all treatments, including exposure to the harshest saltwater spray for 2 weeks (Fig. 2). The only significant differentiation (as assessed by ANOVA) was between freshwater irrigation and exposure to saltwater spray for 2 weeks.

Transverse sections of leaves examined after each treatment are shown in Fig. 3. The thickness of the leaves was consistently greater in coastal individuals compared to inland individuals; however, the thickness varied greatly within and among individuals in the same treatment group.

Discussion

Our results suggest that inland individuals at

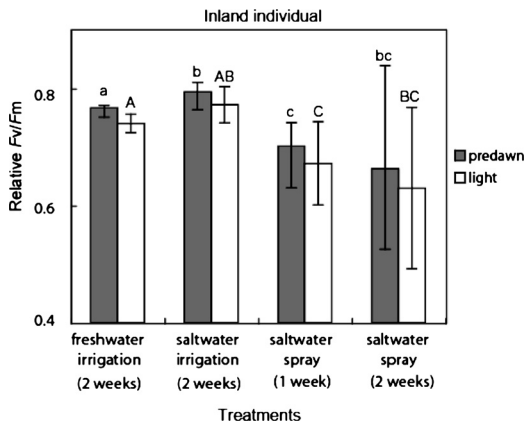


Fig. 1. Relative stress of *Calystegia soldanella* lakeshore individuals at Omimaiko, Lake Biwa. Vertical lines on the bars show the standard error. Letters (a–c, A–C) above each bar indicate significant differences as assessed by ANOVA. Scores are the ratio of the observed F_v/F_m over the baseline measurements (Table 1).

Lake Biwa have significantly inhibited F_v/F_m values after exposure to saltwater spray on leaf blades, whereas coastal individuals showed no significant decrease in the F_v/F_m value. The difference in photochemical fluorescence quenching between the inland and coastal individuals implies that their physiological characteristics related to salt tolerance in combination with the anatomy of their leaf structure may be related to the historical background of long-term isolation of coastal plants at freshwater Lake Biwa.

Effect of salinity on chlorophyll fluorescence

Inland vs. coastal differentiation of the response to salinity was identified in experiments treating leaves with saltwater spray. The photochemical efficiency of *C. soldanella* plants from coastal populations was unaffected by exposure to saltwater spray for 1 week. Conversely, the inland plants had decreased photochemical efficiency as a result of exposure to saltwater spray. Tolerance to airborne saltwater is critical to the survival of the coastal populations; in coastal plant communities, the distribution of species can sometimes be determined according to their

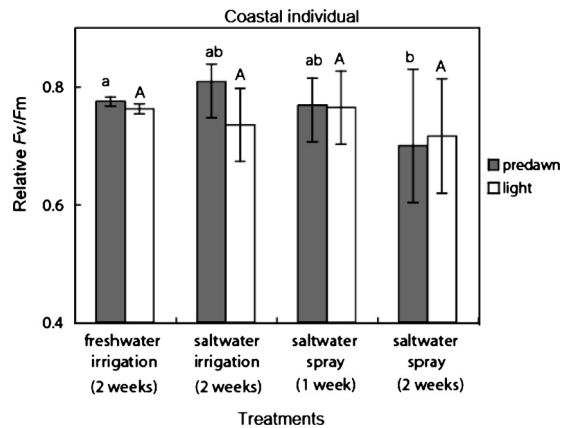


Fig. 2. Relative stress of *Calystegia soldanella* coastal individuals at Shimohama, on the coast of the Sea of Japan. Vertical lines on the bars show the standard error. Letters (a, b and A) above each bar indicate significant differences detected by ANOVA. Scores are the ratio of the observed F_v/F_m values over the baseline measurements (Table 1).

tolerance to airborne saltwater (Oosting and Billings, 1942; Oosting, 1945; Boyce, 1954; Barbour, 1978; Barbour *et al.*, 1985; Rozema *et al.*, 1985; Sykes and Wilson, 1988; Hesp, 1991; Maun, 1994; Greipsson and Davy, 1996; Wilson and Sykes, 1999). Sandblasting accompanied by saltwater spray is one of the main factors inhibiting the survival and growth of inland plants on coastal sand dunes (Ogura and Hiroshi, 2008). Salinity stress caused by saltwater spray affected only the inland individuals at Lake Biwa, and caused stomatal closure, reduction of CO_2 assimilation, and subsequent photoinhibition. These results are correlated with their adaptation to a specific habitat, i.e., coastal individuals have physiological adaptations to salinity stress, whereas inland individuals have lost those adaptations as a result of their long-term isolation at a freshwater lake.

The leaf anatomy of individuals from both habitats also suggested that anatomical structure may be related to physiological differentiation. Inland individuals had thinner leaf blades, whereas coastal ones had thicker leaf blades (see Fig. 3), possibly due to differences in the size of the

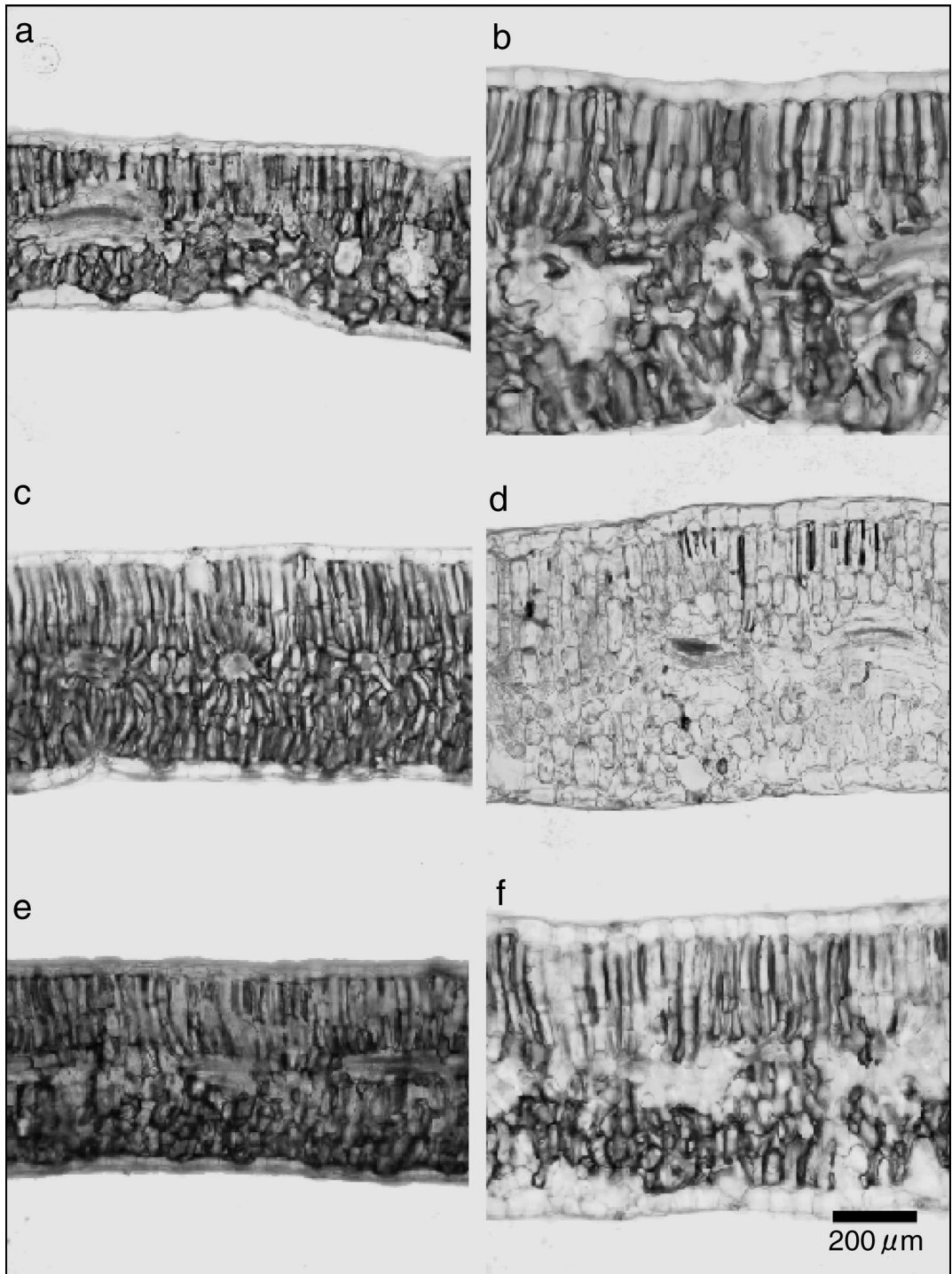


Fig. 3. Transverse leaf sections illustrating the contrast between inland and coastal populations. a, c and e: inland individuals; b, d and f: coastal individuals. a and b: freshwater irrigation for two weeks; c and d: saltwater irrigation for two weeks; e and f: saltwater spray for two weeks.

cells in the palisade and spongy tissue (i.e., cell size is larger in leaves of coastal individuals). In addition, our preliminary study on cuticle thickness of adaxial surfaces of leaf blades suggested that the cuticle layer tends to be thicker in coastal individuals (mean $0.747\ \mu\text{m}$ [$n=5$]) compared to that of inland individuals (mean $0.368\ \mu\text{m}$ [$n=5$]) based on wet-SEM observations using fresh leaves; however, the cuticle thickness was too small to measure by SEM microscopy in the current study. The cuticle on the outermost layer of the leaf blade plays an important role in determining tolerance to certain environments (Martin and Juniper, 1970). For example, *Sonchus oleraceus*, *Euphorbia peplis*, and *Eryngium maritimum* are species that repel salt droplets by their highly hydrophobic cuticle, and thus protect the leaf tissue from salt damage (Waisel, 1972). Increased leaf succulence in plants has been found to be the result of increasing salt exposure (Boyce, 1951; Hesp, 1991; Maun, 1994); e.g., Boyce (1951) found that leaf succulence in dune plants (*Cuscuta veatchii*: Cuscutaceae) was primarily due to airborne salt loading on the leaves and branches, resulting in salt-induced hypertrophy and the doubling or tripling of leaf thickness.

Molecular phylogeography of *C. soldanella* using cpDNA and nSSR makers revealed the heterogeneous genetic structure between the inland and coastal populations, corroborating the long-term isolation of coastal plants at Lake Biwa (Noda *et al.*, unpublished). Long-term isolation of coastal plants at Lake Biwa may have caused a loss of adaptations related to salinity tolerance. However, in evaluating the present findings (photochemical fluorescence quenching in milder salinity, thinner leaf blade, and thinner cuticle layer in inland individuals), we can not dismiss the possibility that these differences may be independent adaptations or correlated factors. Further study is needed to elucidate the mechanisms of the intraspecific differentiation of adaptation to inland-coastal habitats.

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