Solute patterns and diurnal variation of photosynthesis and chlorophyll fluorescence in Korean coastal sand dune plants

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Abstract

Four plant species, *Elymus mollis* Trin., *Carex kobomugi* Ohwi, *Glehnia littoralis* F. Schmidt ex Miq., and *Vitex rotundifolia* L.f., are dominant perennial species in coastal sand dunes of Korea. We examined a physiological adaptation of these species by measurements of diurnal variation in photosynthesis and chlorophyll (Chl) fluorescence and solute patterns in leaves during one season (June), which is favorable for plant growth of all four species. All four species adopted different strategies in order to utilize radiation and to maintain water status under a fluctuating microclimate. Although the lowest water contents among four plant species was found, *E. mollis* with a high Chl and K^+ content showed better photosynthetic performance, with high stomatal conductance (g_s), net photosynthetic rate (P_N), instantaneous carboxylation efficiency (CE), and water-use efficiency. Midday depression of P_N in *E. mollis* and *G. littoralis*, without a reduction of g_s, was associated with a reduction in CE and maximum photochemical efficiency of PSII, indicating nonstomatal limitation. Photosynthesis depression in both *C. kobomugi* and *V. rotundifolia*, with relatively low g_s values, could be attributed to both stomatal and nonstomatal limitations. The high storage capacity for inorganic ions in *E. molli*, *C. kobomugi*, and *G. littoralis* may play an efficient role in regulating photosynthesis and maintaining leaf water status through stomatal control, and can also play an important role in osmotic adjustment.

Additional key words: nonstomatal limitation; osmotic adjustment; physiological adaptation; stomatal limitation, gas exchange, chlorophyll fluorescence.

Introduction

Understanding the response of coastal sand dune plants to environmental stresses including drought and changes in salinity, temperature, and light intensity, is important for explaining and predicting the distribution of plant communities in coastal dune systems, and can also play a major role in the protection of natural vegetation through adequate management. Plants continuously exposed to environmental stimuli have developed different adaptive strategies for establishment and perpetuation within their habitats (Flowers and Clomer 2008). Strategies to utilize radiation and to maintain water status under field conditions vary considerably among different plant species. In general, strategies of stress-avoidance and stress-tolerance can be recognized, both involving various plant mechanisms that provide to the plants the viability under environmental-stress conditions (Levitt 1980).

Efficient use of light and water by photosynthesis and osmotic adjustment should be an important feature for plant survival under the natural environmental conditions of coastal dune regions, where plants are exposed to high direct irradiation, heat, salt spray, and a shifting sandy substrate with low water-holding capacity and changing nutrient status over the course of the day (Maun 1994, Gilbert *et al.* 2008). Photosynthesis related to plant growth and development is the most fundamental physiological process and its mechanism involves various components, including photosynthetic pigments and photosystems, the electron transport system, and the CO_2 reduction pathway (Ashraf and Harris 2013). Diurnal patterns in leaf gas

exchange are recognized as one of the best indicators of the plant ability to maintain their photosynthetic apparatus so that it can readily respond to environmental conditions (Geiger and Servaites 1994). Chl fluorescence subtly reflects the primary reactions of photosynthesis and is closely associated with PSII, which can reflect photosynthesis efficiency and provide useful information in assessing plant responses to environmental stress in the field (Krause and Weis 1991).

The ability of plants to tolerate environmental stresses is determined by multiple physiological mechanisms that facilitate retention and/or uptake of water, protect chloroplast function, and maintain ion homeostasis, making difficult to define a pattern of physiological responses to environmental stress. Coastal sand dune plants are known to be tolerant of high temperature (Ishikawa *et al.* 1990) and drought (Mooney *et al.* 1983) which can occur for a variety of reasons, such as soil dryness, high evaporation, and osmotic binding of water in saline soils (Larcher 2003). However, little is known about the contribution and relative importance of the water status and the accumulation of solutes such as soluble sugars and ions in comparative studies on photosynthetic capacity of coastal sand dune plants under field environmental conditions.

Plant water balance depends not only on stomatal regulation in order to minimize water loss (Farquhar and Richards 1984), but also on the ability of the plant to take up water for osmotic adjustment (Lilley and Ludlow 1996) and membrane stability (Tripathy *et al.* 2000). Under extreme environmental conditions, such as high light, temperature, and soil water limitation and/or high atmospheric evaporative demand, stomatal regulation is important mechanisms allowing plants to regulate and optimize CO_2 assimilation *vs.* evaporative water loss (Boyer 1982, Franks 2013). The accumulation of solutes, such as ions, soluble sugars, glycine betaine, soluble amino acids, and soluble proteins, in response to stress is important for maintaining cell turgor by reducing water potential (Silva *et al.* 2007, Farooq *et al.* 2009).

The perennial dune grasses *Elymus mollis* (Gagne and Houle 2002), *Carex kobomugi* (Ohsako 2010), and *Glehnia littoralis* (Voronkova 2008) and a perennial woody shrub *Vitex rotundifolia* (Cousins *et al.* 2010), which are tolerant to environmental stresses, such as salt and summer drought, are the dominant plant species distributed in coastal sand dune of Korea. The objectives of this study were: (*1*) to compare diurnal changes of photosynthesis characteristics, Chl fluorescence and solute patterns in leaves of four dominant plant species under field conditions; (*2*) to elucidate how these plants regulate photosynthesis and maintain leaf water status under coastal sand dune environmental conditions.

Materials and methods

Study site and plant material: Field studies were conducted in a section of the sand dunes extending from Goraebul beach to Daejin beach (*ca.* 42 km of coastline) on the eastern coast of Gyeongsangbuk-do Province, South Korea (36°34′27.9″N, 129°25′04.6″E), at established Long Term Ecological Research (LTER) site for the study of coastal ecosystems. The microtopography included a beach face, berm, beach flat, foredune, and three hummock dunes lying parallel to the shoreline. Sandy beach extended about 20 m in width and was rarely vegetated. The four species studied were: *Elymus mollis* (Poaceae) and *Carex kobomugi* (Cyperaceae), both perennial grasses with deep downward rooting and horizontal rhizomes that usually occur along foredunes and embryo dunes between 20 m and 35 m from the shoreline; *Glehnia littoralis* (Apiaceae), a perennial grass with a long taproot forming a basal patch of leaves in the interdune between the foredune ridge and hummock dunes in the semistable zone; and *Vitex rotundifolia* (Verbenaceae), a perennial woody shrub that usually dominates between the semistable zones at 45 m from the shoreline and in the further landward zone at 70 m.

The study site exhibited a total rainfall of 1.7 mm and a daily average temperature of 23.6°C from 1 June until 21 June 2010. A maximum temperature above 30°C was recorded from 2 June 2010. The measurements were conducted on 22 June 2010 which was clear day with no cloud, with a daily average temperature of 29.6°C and maximum temperature of 43.7°C. At the study site, the air temperature (T_{air}) and relative humidity (RH) during the entire day were recorded at 1-h intervals using a datalogger (*SATO*, Japan). Air vapor pressure deficit (VPD) was calculated from T_{air}, saturated vapor pressure and RH (Fig. 1*A*). The value of PAR given is hourly averages of measurements that were taken at the time when photosynthetic parameters were measured (Fig. 1*B*).

Soil properties (from 0 to 10 cm in depth) of each community were as follows; soil texture: sand (size of 100–250 μm was 1.6–5.2%, size of 250–500 μm was 66.3–77.8%, and size of 500–1,000 μm was 17.3–32.1%), pH (1:5 H_2O) 7.3–7.6, and organic matter of 0.5–0.6%. Total ionic content (TIC) calculated as NaCl equivalents varied from 0.8 ueq g^{-1}(soil) (*G. littoralis* community) to 3.0 ueq g^{-1}(soil) (*E. mollis* community and *C. kobomugi* community). Soil gravimetric water contents were less than 2% in all four communities.
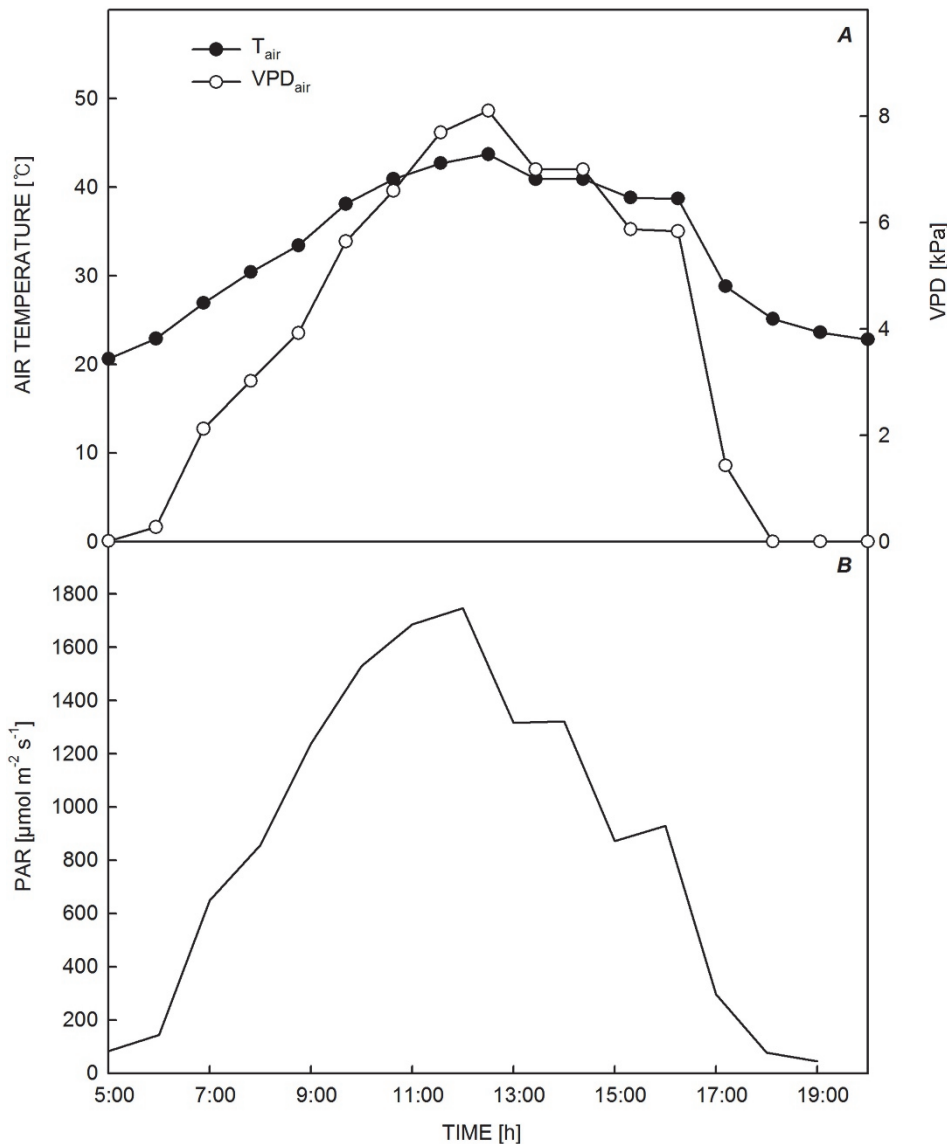
Fig. 1. Diurnal fluctuation of (*A*) air temperature (T$_{air}$) and air vapor pressure deficit (VPD$_{air}$), and (*B*) photosynthetically active radiation (PAR).

Measurement of photosynthesis and Chl fluorescence:
To study the simultaneous temperature and irradiance response, measurements of photosynthesis and Chl fluorescence were carried out with three and five mature leaves, respectively, of two individuals per species at 2-h intervals (eight times within a day) from 5:00 h to 19:00 h. Leaf gas exchange was measured under natural light with a portable CO_2 gas analyzer (*LCi, ADC BioScientific Ltd.*, Hoddesdon, UK) on fully-expanded leaves after two min of equipment acclimation on the leaf. The elapsed measurement time for collecting data on gas exchange per one species was about 15 min. Air was continuously passed through the leaf chamber to maintain CO_2 in the leaf chamber at a steady concentration. The selected leaf was placed in the leaf chamber with a known area of the leaf (6.25 cm^2) enclosed in an open-system configuration in which fresh gas was continually passed through the plant leaf chamber. The main console supplied the chamber with air at a known rate with a known concentration of CO_2 and H_2O. The CO_2 concentration atmosphere in the chamber was 376 ± 7 μmol mol^{-1}. The water vapor pressure entering the leaf chamber was 2.14 ± 0.23 kPa. PAR, net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), C_i, leaf to air vapor pressure deficit (VPD$_{leaf-air}$), and calculated leaf temperature were obtained, and using these values the instantaneous carboxylation efficiency (P_N/C_i), instantaneous water-use efficiency (P_N/E), and instantaneous intercellular to atmospheric CO_2 concentration ratio (C_i/C_a) were calculated.

109

The vapor pressure deficit between the leaf and the air, $VPD_{leaf-air}$, throughout the day was obtained by calculating the difference between the saturation (e_s) and actual (e_a) air pressure, using measurements of the leaf temperature and RH in the chamber as follows: $VPD = (e_s - e_a)$ in kPa. Saturated vapor pressure at the leaf and air was calculated by the following equation (Buck, 1981):

$$e_s = 0.61375 \exp\left[\frac{T(18.564 - T/255.4)}{T + 255.57}\right]$$

where e_s is the saturation vapor pressure [kPa] at the leaf temperature and T is the air temperature [°C] in and out of the leaf.

Chl fluorescence was measured with a portable *Handy PEA* (*Hansatech Instruments Ltd.*, Norfolk, UK). After 30 min of stabilization in the dark, minimal fluorescence (F_0) was measured. Maximal fluorescence (F_v) was obtained by a saturating pulse of light [1,500 µmol(photon) m^{-2} s^{-1}, 1 s duration]. Maximal quantum yield of PSII photochemistry (F_v/F_m) was calculated according to the equation $F_v/F_m = (F_m - F_0)/F_m$.

Measurement of pigment contents and solutes in leaves: Leaves collected for measurement of photosynthesis and fluorescence were enclosed in plastic bags and immediately stored on ice for 2 h until it was possible to transport the samples to a laboratory. Leaves sampled for Chl and Car content were extracted with dimethyl sulfoxide at 60°C for 24 h. The contents of Chl *a*, Chl *b*, and Car were estimated from absorbance at 663, 645, and 480 nm, respectively, with a spectrophotometer (*Optizen 2120, Mecasys Co. Ltd.*, Korea). Quantitative estimates of Chl and Car content were obtained using the equations of Holden (1965) and Kirk and Allen (1965), respectively. After determining the fresh mass (FM) of the four species samples, they were dried at 80°C for 72 h, and the dry mass (DM) was determined. Leaf water content as a percentage of FM was calculated according to the following equation: leaf water content [%] = 100 × (FM – DM)/FM, where DM and FM denote respectively dry matter and fresh matter of the leaves. To measure the total ionic content, osmolality, carbohydrate content, and the content of inorganic cations (K^+, Mg^{2+}, Ca^{2+}, and Na^+) and Cl^-, the dried plant material was ground into a homogenous powder and extracted at 95°C in distilled water for 1 h, and then the sample was filtered through a 0.45 µm pore size *GF/C* filter (*Whatman*, UK). TIC calculated as NaCl equivalents was determined using a conductivity instrument (*Mettler Check Mate 90, Mettler Toledo*, Billerica, USA). Osmolality was measured by cryoscopy using an osmometer (*Micro-Osmometer, Precision System Inc.*, USA). Total water-soluble carbohydrates were assayed using the phenol-sulfuric acid method (Chaplin 1994). K^+, Mg^{2+}, Ca^{2+}, and Na^+ were determined by inductively coupled plasma method (*Optima 7300DV, Perkin Elmer*, USA). The Cl^- content was measured using a chloride titrator (*Titrators DL 50*; *Mettler Toledo Inc.*, Switzerland).

Statistical analysis: All the statistical analyses were performed using *SPSS 18.0* (*SPSS*, Chicago, USA). $P<0.05$ was considered statistically significant. One-way analysis of variance (*ANOVA*) with the *Duncan's* test as a *post hoc* analysis was employed to determine the significance of difference between species for the pigment contents and solute in leaf of four species. *SPSS* was also used to calculate the *Pearson's* correlation coefficients for correlation analyses between photosynthetic variables. The data were tested for normality with the *Kolmogorov-Smirnov's* test and homogeneity of variances with *Levene's* test.

Results

Chl and Car contents in leaves: Chl and Car contents showed significant differences between the species (Table 1). The highest value for Chl *a* was found in *V. rotundifolia*, and no significant differences in Chl *a* were observed between the other three species. *E. mollis* had the highest values of Chl ($a+b$), Chl *b*, and Car, whereas *C. kobomugi* showed the lowest values. *G. littoralis* had higher amounts of Chl ($a+b$) and Chl *b* than that of *C. kobomugi*, and lower values than that of *E. mollis*. The Chl *a/b* ratio was significantly higher in *C. kobomugi* and *V. rotundifolia* than those in *E. mollis* and *G. littoralis*. The values for Car and Car/Chl in *E. mollis*, *G. littoralis*, and *V. rotundifolia* were higher than those in *C. kobomugi*.

Table 1. Comparison of chlorophyll (Chl) and carotenoids (Car) content of the four coastal sand dune plants. Values are means ± SD ($n = 6$). *Different letters* indicate significant differences between the plant species ($P<0.05$).

	E. mollis	*C. kobomugi*	*G. littoralis*	*V. rotundifolia*
Chl *a* [mg g^{-1}(FM)]	0.38 ± 0.056[B]	0.45 ± 0.11[B]	0.40 ± 0.05[B]	0.55 ± 0.07[A]
Chl *b* [mg g^{-1}(FM)]	0.51 ± 0.05[A]	0.22 ± 0.06[C]	0.33 ± 0.07[B]	0.26 ± 0.05[BC]
Chl ($a + b$) [mg g^{-1}(FM)]	0.89 ± 0.10[A]	0.67 ± 0.09[C]	0.73 ± 0.11[BC]	0.81 ± 0.10[AB]
Chl *a/b*	0.73 ± 0.08[B]	2.18 ± 0.94[A]	1.26 ± 0.21[B]	2.17 ± 0.50[A]
Car [mg g^{-1}(FM)]	47.2 ± 4.47[A]	30.2 ± 2.04[B]	47.8 ± 9.16[A]	44.9 ± 3.53[A]

Table 2. Comparison of leaf water content, osmolality, total ion content (TIC), carbohydrate content, and inorganic ion content in leaves of the four coastal sand dune plants. Values are means ± SD ($n = 6$). *Different letters* indicate significant differences between the plant species ($P<0.05$).; n.s. – values not significantly different.

Parameter	E. mollis	C. kobomugi	G. littoralis	V. rotundifolia
Leaf water content [%]	63.1 ± 1.49[D]	75.2 ± 2.13[B]	82.6 ± 1.59[A]	68.86 ± 0.57[C]
Osmolality [μOsm g^{-1}(H$_2$O)]	699. ± 41.1[n.s.]	730 ± 139.4[n.s.]	655. ± 33.7[n.s.]	655.7 ± 26.6[n.s.]
TIC [μeq g^{-1}(H$_2$O)]	679 ± 18.6[A]	600 ± 64.5[B]	522 ± 42.2[C]	307.4 ± 12.4[D]
Carbohydrate [μmol g^{-1}(H$_2$O)]	195 ± 89.1[B]	231 ± 57.8[B]	157 ± 42.5[B]	398.9 ± 138[A]
K$^+$ [μmol g^{-1}(H$_2$O)]	257 ± 19.4[A]	170 ± 38.7[B]	132 ± 21.4[B]	136.4 ± 12.76[B]
Ca^{2+} [μmol g^{-1}(H$_2$O)]	16.1 ± 4.11[B]	17.2 ± 3.99[B]	53.1 ± 11.9[A]	8.12 ± 2.38[B]
Mg^{2+} [μmol g^{-1}(H$_2$O)]	18.2 ± 1.88[B]	36.7 ± 5.42[A]	41.0 ± 9.34[A]	26.75 ± 4.18[B]
Na$^+$ [μmol g^{-1}(H$_2$O)]	58.8 ± 20.4[C]	137 ± 32.5[A]	98.1 ± 12.4[B]	27.76 ± 4.89[C]
Cl$^-$ [μmol g^{-1}(H$_2$O)]	0.14 ± 0.08[n.s.]	0.16 ± 0.08[n.s.]	0.14 ± 0.09[n.s.]	0.06 ± 0.09[n.s.]

Leaf water content, osmolality, TIC, and mineral nutrients in leaves: Leaf water content, osmolality, TIC, inorganic ions (Ca^{2+}, Na$^+$, K$^+$, Mg^{2+}, and Cl$^-$), and organic solutes (carbohydrate) in leaves of *E. mollis*, *C. kobomugi*, *V. rotundifolia*, and *G. littoralis* were measured (Table 2). *G. littoralis* showed the highest leaf water content followed by *C. kobomugi*, *V. rotundifolia*, and *E. mollis*. No significant differences in osmolality were observed between the four species. *E. mollis* had the highest TIC followed by *C. kobomugi*, *G. littoralis*, and *V. rotundifolia*. This was the same pattern as we noted for species distribution from shoreline. *E. mollis* showed a high K$^+$ content as well as high TIC, but the low Na$^+$ content compared to the other three species. *V. rotundifolia* had the highest carbohydrate contents. Similarly as *E. mollis* with its high K$^+$/Na$^+$ ratio (4.38), *V. rotundifolia* also maintained a high ratio (4.92), but K$^+$ ion content was low; in contrast, soluble carbohydrate content in *V. rotundifolia* was higher than that of K$^+$.

The highest value for Ca^{2+} was found in *G. littoralis*, and no significant differences in Ca^{2+} were observed between the other three species. Mg^{2+} was significantly higher in *C. kobomugi* and *G. littoralis* than those in *E. mollis* and *V. rotundifolia*. No significant differences in Cl$^-$ were observed between the four species.

Diurnal patterns of photosynthesis: The highest values of T_{air} (43.7°C), air VPD (8 kPa), and PAR [1747 μmol(photon) m^{-2} s^{-1}] were reached around midday (Fig. 1). Interspecific comparisons of the photosynthetic parameters (T_{leaf}, VPD$_{leaf-air}$, g_s, C_i, P_N, E, instantaneous CE, and WUE) of all four species under field conditions are shown Fig. 2. The T_{leaf} presented the lowest values in *E. mollis* with high g_s and the highest values in *V. rotundifolia* with low g_s (Fig. 2, Table 3). The VPD$_{leaf-air}$ had a negative influence on the gas exchange of the *C. kobomugi*. P_N and g_s were negatively correlated with VPD$_{leaf-air}$, though no influence on E was observed. In contrast, P_N, g_s, and E in *E. mollis* presented a positive correlation with VPD$_{leaf-air}$ (Fig. 2, Table 3). P_N and g_s in both *G. littoralis* and *V. rotundifolia* were not associated with VPD$_{leaf-air}$

(Fig. 2, Table 3). VPD$_{leaf-air}$ showed a positive correlation with transpiration of both *E. mollis* and *G. littoralis*, causing a greater cooling of the leaf, which is suggested by the negative correlation between the VPD$_{leaf-air}$ and the $T_{leaf} - T_{ch}$ (Table 3).

Between early morning and afternoon (9:00 h to around 16:00 h), g_s of both *E. mollis* and *G. littoralis* were maintained at relatively high values than those of both *C. kobomugi* and *V. rotundifolia* (Fig. 2C). As the change of the g_s after dawn in all four species presented a positive correlation with P_N and E, it was likely that g_s in these species influenced on P_N and E and it indicated that the change in P_N was related to stomatal closure in all four species (Fig. 2C,E,G).

In the absence of any change in g_s between morning and afternoon, the E increased directly with increasing VPD, as observed in *G. littoralis* (Fig. 2B,C,G). The decline in E at high VPD was observed in *C. kobomugi*, due to the closing of the stomata at high VPD, which can be efficient in restricting water losses by transpiration at high VPD (Fig. 2B,C,G), though stomatal closure can lead to the increase of T_{leaf} (Fig. 2B).

Midday depression in P_N was observed in *E. mollis*, *C. kobomugi*, and *G. littoralis*, and a recovery of P_N occurred in these three species between 13:00 and 15:00 h. However, *V. rotundifolia* showed an increase of P_N at midday and then a decrease at 13:00 h. During midday, the P_N value of *C. kobomugi* decreased to a greater extent than that of the P_N value in *E. mollis* and *G. littoralis* (Fig. 2E).

As the stomata closed, C_i in *C. kobomugi* and *V. rotundifolia* initially declined with increasing light, temperature, and drought, and then increased to a g_s of 20 and 15 mmol m^{-2} s^{-1}, respectively (Fig. 2C,D). Given that the external concentration of CO$_2$ was constant (C_a), the increase in C_i/C_a was solely due to changes in the internal concentration. P_N and instantaneous CE showed similar diurnal trends (Fig. 2, Table 3). Midday depression of P_N in *C. kobomugi* was predominantly caused by stomatal closure and subsequent lower instantaneous CE, as indicated by the marked increase in C_i despite the stomatal closure. The recovery of P_N and instantaneous CE during

111
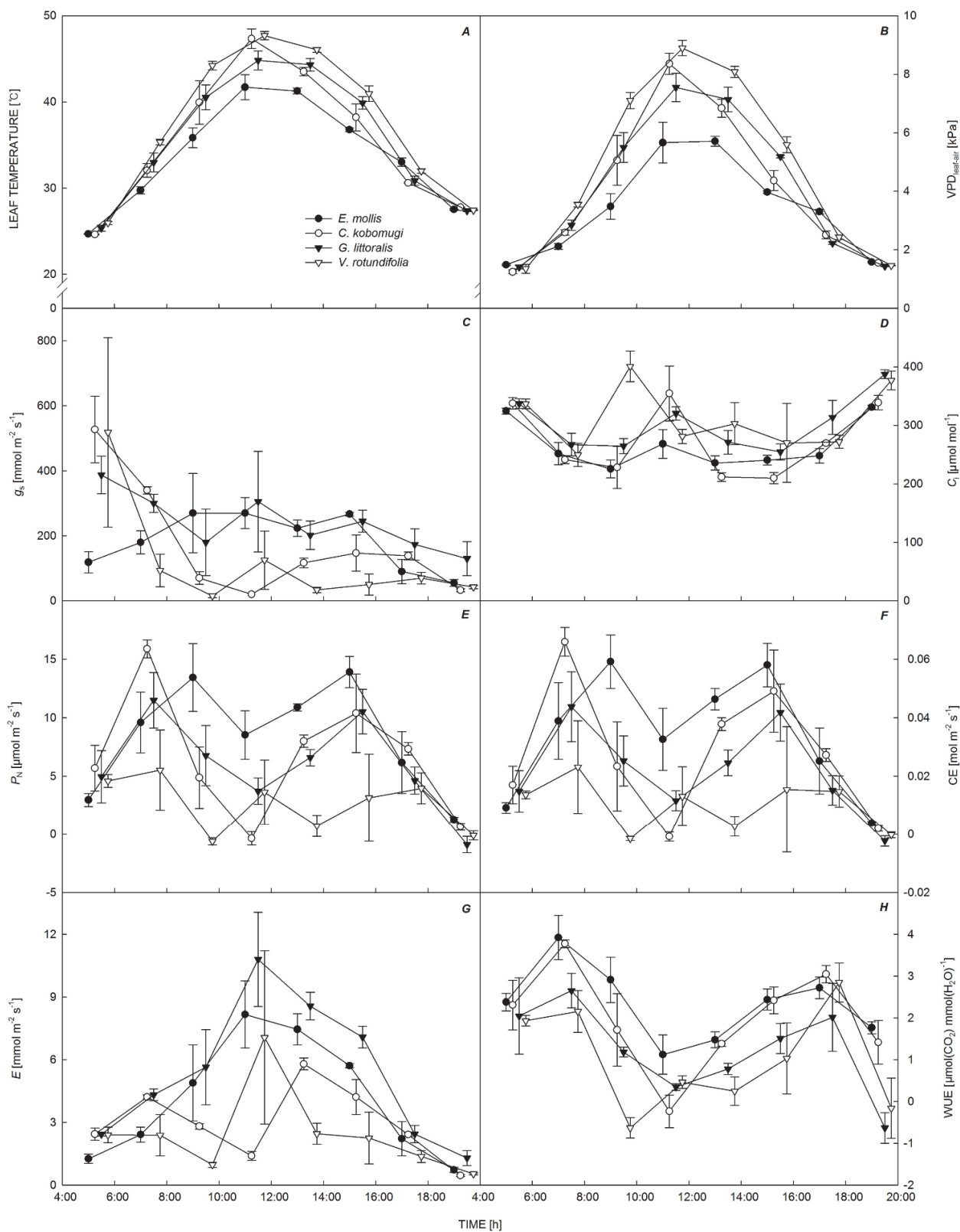
Fig. 2. Interspecies comparison of (A) leaf temperature, T_{leaf} (B) leaf to air vapor pressure deficit, $VPD_{leaf-air}$ (C) stomatal conductance, g_s, (D) intercellular CO_2 concentration, C_i, (E) net photosynthetic rate, P_N, (F) instantaneous carboxylation efficiency, CE, (G) transpiration rate, E, and (H) instantaneous water-use efficiency, WUE, in E. mollis (●), C. kobomugi (○), G. littoralis (▼), and V. rotundifolia (▽). Vertical bar indicate SD of each point which is the mean of measurements.

Table 3. *Pearson*'s correlation coefficients between leaf to air vapor pressure deficit ($VPD_{leaf–air}$), leaf temperature – leaf chamber temperature ($T_{leaf} - T_{ch}$), stomatal conductance (g_s), net photosynthetic rate (P_N), transpiration rate (E), internal concentration of CO_2 (C_i), instantaneous water-use efficiency (P_N/E), and instantaneous carboxylation efficiency (P_N/C_i) based on data after dawn (5:00 h) in *E. mollis* ($n = 37$), *C. kobomugi* ($n = 40$), *G. littoralis* ($n = 40$), and *V. rotundifolia* ($n = 40$) under field conditions of coastal sand dune.

	Variable	$T_{leaf} - T_{ch}$	g_s	P_N	C_i	E	P_N/E	P_N/C_i
E. mollis	$VPD_{leaf–air}$	– 0.457**	0.639**	0.410*	– 0.353*	0.932**	– 0.578**	0.369*
C. kobomugi		0.664**	– 0.479**	– 0.436**	0.141	0.138	– 0.778**	– 0.367*
G. littoralis		– 0.320*	0.161	0.083	– 0.383*	0.861**	– 0.269	0.071
V. rotundifolia		0.438**	0.009	– 0.197	– 0.380*	0.473**	– 0.522**	– 0.189
E. mollis	$T_{leaf} - T_{ch}$		– 0.497**	– 0.211	– 0.116	– 0.548**	0.349*	– 0.144
C. kobomugi			– 0.585**	– 0.687**	0.545**	– 0.529**	– 0.753**	– 0.666**
G. littoralis			– 0.746**	– 0.098	– 0.180	– 0.702**	0.223	– 0.050
V. rotundifolia			– 0.781**	– 0.666**	– 0.398**	– 0.534**	– 0.539**	– 0.586**
E. mollis	g_s			0.802**	– 0.405*	0.853**	– 0.147	0.748**
C. kobomugi				0.943**	– 0.445**	0.555**	0.825**	0.896**
G. littoralis				0.402*	– 0.040	0.609**	0.136	0.334*
V. rotundifolia				0.818**	0.069	0.853**	0.419**	0.741**
E. mollis	P_N				– 0.796**	0.570**	0.312	0.993**
C. kobomugi					– 0.681**	0.720**	0.855**	0.989**
G. littoralis					– 0.828**	0.199	0.776**	0.991**
V. rotundifolia					– 0.170	0.534**	0.758**	0.978**
E. mollis	C_i					– 0.316	– 0.488**	– 0.829**
C. kobomugi						– 0.769**	– 0.578**	– 0.735**
G. littoralis						– 0.239	– 0.668**	– 0.835**
V. rotundifolia						– 0.006	– 0.059	– 0.222
E. mollis	E						– 0.504**	0.516**
C. kobomugi							0.367*	0.768**
G. littoralis							– 0.220	0.157
V. rotundifolia							0.053	0.464**
E. mollis	P_N/E							0.355*
C. kobomugi								0.808**
G. littoralis								0.750**
V. rotundifolia								0.699**

the afternoon correlated with stomatal reopening. In *V. rotundifolia*, a pronounced decrease of P_N and instantaneous CE, together with a corresponding increase in g_s, also occurred during morning and afternoon, and the C_i increase indicated that the nonstomatal effects caused the decrease in P_N. As a result of the increase in g_s, both P_N and instantaneous CE were partially increased at midday. In *E. mollis* and *G. littoralis* with high and constant g_s between morning and afternoon, the P_N depression at midday was caused by a decrease of instantaneous CE as a result of nonstomatal effects.

The daily pattern of E in *G. littoralis*, *E. mollis*, and *V. rotundifolia* was bell-shaped with a peak at midday, whereas *C. kobomugi* had two peaks, one in the morning and the other in the afternoon, separated by a depression at midday (Fig. 2*G*). An increased E in both *E. mollis* and *G. littoralis* with high g_s compared to both *C. kobomugi* and *V. rotundifolia*, resulted in a decrease in instantaneous WUE. At midday, *E. mollis* exhibited higher P_N and lower E than *G. littoralis*, although both had similar g_s, which

resulted in a higher instantaneous WUE than that in *G. littoralis*. *C. kobomugi*, except at midday when it exhibited low values of P_N and E from stomatal closure, maintained a higher instantaneous WUE than that of *G. littoralis*. *V. rotundifolia* maintained comparatively lower instantaneous WUE values than the other three species due to the low values of P_N and E, except at midday (Fig. 2*H*).

Diurnal patterns of leaf Chl fluorescence: Species differences in F_0, F_m, and F_v/F_m were significantly influenced by diurnal variation in light and temperature (Fig. 3). The dawn F_v/F_m remained near 0.8 in all four species, but a midday depression was observed. The ratio decreased significantly by 63.0, 44.1, 41.1, and 16.9% in *V. rotundifolia*, *E. mollis*, *G. littoralis*, and *C. kobomugi*, respectively, during the midday compared with the dawn period. However, these decreases were recovered at the end of the afternoon up to about the dawn level (0.8). In all four species, F_m also decreased at midday and tended to recover at the end of the afternoon, but still did not recover to the

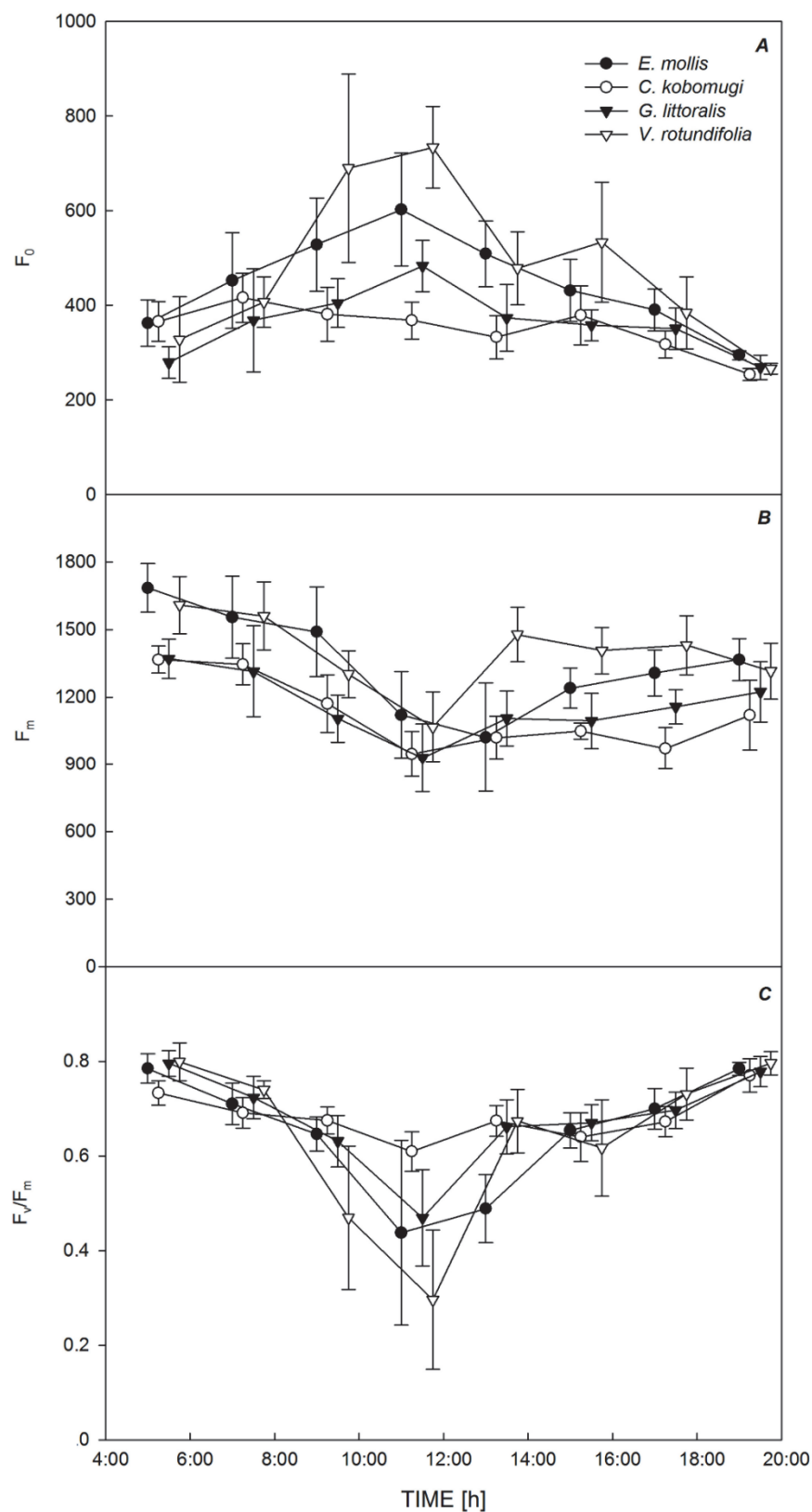Fig. 3. Diurnal changes in (*A*) minimal fluorescence yield of the dark adapted state, F_0, (*B*) maximal fluorescence of the dark-adapted state, F_m, and (*C*) maximal quantum yield of PSII photochemistry, F_v/F_m, in *E. mollis* (—●—), *C. kobomugi* (—○—), *G. littoralis* (—▼—), and *V. rotundifolia* (—▽—). Vertical bar indicate standard deviation of each point which is the mean of measurements.

dawn F_m level. Although midday depression of F_v/F_m was observed in *C. kobomugi*, this species maintained comparatively higher F_v/F_m values than the other three species. In *V. rotundifolia*, *G. littoralis*, and *E. mollis*, the decreased midday F_v/F_m was the consequence of a lower F_v with a marked increase in F_0, rather than a decrease in F_m, whereas in *C. kobomugi* it was related to a decrease in F_m without any change in F_0. In *V. rotundifolia*, *G. littoralis*, and *E. mollis*, F_0 values increased significantly at midday by 55.5, 42.2, and 39.9%, respectively, compared to their dawn values.

Discussion

Stomatal and nonstomatal limitations on photosynthesis: Decreased photosynthesis has been also reported to be dependent on stomatal or nonstomatal limitations (Hassan 2006), influenced by environmental stresses, such as high light intensity, plant water status, temperature, and VPD (Oliver *et al.* 2009, Yu *et al.* 2009). It is important to close the stomata when the benefit of water retention outweighs the negative effects on CO_2 uptake, photosynthesis, and water and nutrient uptake, as well as cooling (Arve *et al.* 2011).

Under field conditions, P_N and E showed a strong correlation with g_s in *E. mollis*, *C. kobomugi*, and *V. rotundifolia* which indicated g_s might be one of the major factors regulating photosynthesis in these three species. Diurnal trends in g_s have been associated with morning stimulation of stomatal opening by increasing PAR and temperature (Hennessey and Field 1991). However, stomatal closure at midday and the decrease of g_s during the afternoon have been associated with a response to elevated VPD (Aphalo and Jarvis 1991). *C. kobomugi* nearly closed its stomata at midday, associated with a decrease in P_N and E, preventing extreme water loss from the plant, which is an important trait of drought tolerance under high VPD (Lawlor and Tezara 2009).

The main cause of the reduced P_N may be the changes in g_s and C_i (Farquhar and Sharkey 1982, Flexas *et al.* 2004, Yan *et al.* 2011). Stomatal closure leads to decreases in C_i and restricts water loss through transpiration (Cornic 2000). If both g_s and C_i decrease simultaneously, stomatal limitations dominate in conditions of moderate drought, irrespective of any metabolic impairment (Flexas and Medrano 2002). In contrast, when g_s decreased or did not change and C_i increased, the decrease of P_N might be caused by a decrease in photosynthetic activity in mesophyll cells, indicating the predominance of nonstomatal limitations to photosynthesis (Farquhar and Sharkey 1982). Flexas and Medrano (2002) reported that the inflection point, at which C_i starts to increase, is observed predominantly at g_s around 50 mmol m^{-2} s^{-1}. With a further C_i increase, there is an inflection at a lower CE that approaches zero, where ribulose-1,5-bisphosphate (RuBP) regeneration is limiting (Galmes *et al.* 2011). Therefore, the depression in P_N in both *C. kobomugi* and *V. rotundifolia* during the morning could be explained by stomatal limitation, since the reduction in P_N was followed by the reduction in g_s and C_i. Moreover, observed increase of P_N between morning and afternoon could result from an increase in g_s, with consequent increase in E. The increase of C_i in both species by closing their stomata, when g_s fell below 50 mmol m^{-2} s^{-1}, means nonstomatal limitations on photosynthetic CO_2 assimilation. The inhibition of photosynthesis in both *E. mollis* and *G. littoralis* could not be attributed to stomatal effects, because the inhibition observed at high VPD and high E in both *E. mollis* and *G. littoralis* occurred without change in g_s. The high E in *G. littoralis* and *E. mollis* could be probably attributed to the higher g_s, and can be important to cool leaves exposed to high T_{air}, low air VPD or the heating effect of sunlight, since the VPD$_{leaf-air}$ showed a positive correlation with E, and the $T_{leaf} - T_{ch}$ showed an inverse correlation with VPD$_{leaf-air}$ (Day 2000). The P_N/C_i ratio can be considered an estimate of Rubisco activity, illustrating its limitations under stress conditions (Niinemets 2009). In contrast to *C. kobomugi* and *V. rotundifolia*, the midday depression of P_N in both *E. mollis* and *G. littoralis* with high g_s during the day might be caused by a reduction in instantaneous CE, indicating nonstomatal limitation because the reduction in P_N was not followed by changes in g_s and C_i.

A greater WUE can be attained by a lower value of C_i/C_a either by decreasing g_s, or increasing P_N, or close coordination of both (Polley 2002). Ripley and Pammenter (2004) reported that coastal dune species have high WUE because of high assimilation rates rather than low transpiration rates. A high g_s means a high E, and if there is no parallel increase in P_N, it tends to diminish the WUE (Pimentel *et al.* 2004). Although water loss occurs by stomatal opening to gain CO_2, high assimilation rates in *E. mollis* was closely related to high WUE, implying a positive balance between carbon and water exchange. In *C. kobomugi*, the stomata closure at midday resulted in the reduction in P_N and E, which indicated that the photosynthesis depended more on the availability of CO_2 than on the leaf water content until a threshold of water deficit was reached.

Photoinhibition eventually occurs under conditions of very severe drought and excessive light and almost complete stomatal closure. An increase in photorespiration by closing stomata during the midday depression in gas exchange, causing C_i reduction, may also play role in protection against photodamage (Cornic *et al.* 1989). Further reduction in g_s leads to a reduction in the biochemical pathways of photosynthesis, which under high PAR (Aro *et al.* 1993) and severe drought (Flexas and Medrano 2002) may lead to damage (*i.e.,* permanent) or adjustment (*i.e.,* reversible downregulation) to the photo-

synthetic apparatus when energy dissipation mechanisms are exceeded. In strong light, photons are abundant; this is consistent with a substantial capacity for energy processing by leaves, and hence a higher Chl a/b ratio. Car is accessory light-harvesting pigments, effectively extending the range of light absorbed by the photosynthetic apparatus, and play an essential photoprotective role *via* dissipation of potentially harmful energy (Young 1991).

The typical range for the ratio F_v/F_m is known to be 0.75–0.85 for nonstressed plants (Peterson *et al.* 1988). In our study, the midday depression in the F_v/F_m ratio of all four species indicated a reduction in the photochemical efficiency of the PSII complex, which could be due to inefficient energy transfer from the light-harvesting Chl a/b complex to the reaction center. The F_v/F_m ratio for all four species reached the lowest values at midday, and then a recovery of those values up to the initial level, around 8.0, was observed under weak light conditions after photoinhibition induced by strong light. These results indicate that photoprotection mechanism rather than photodamage occurred, and thus these species can protect PSII from excess energy. The decrease in F_v/F_m is likely to be the result of reversible inactivation or downregulation of PSII rather than photodamage to PSII (Demmig-Adams *et al.* 1996).

E. mollis, G. littoralis, and *V. rotundifolia* had higher Chl content, Car/Chl ratios, and F_0 than *C. kobomugi*, suggesting that the leaves of these three species have a more efficient light-harvesting complex and are better at dissipating thermal energy (Jeon *et al.* 2006). Although there is a difference between plant species, the decrease in F_v/F_m ratios coupled with an F_0 increase suggests the occurrence of photoinhibition in response to high temperature (Gamon and Pearcy 1989), excess photon flux density (Maxwell and Johnson 2000), and water stress (Epron *et al.* 1992). In our study, the marked reduction in F_v/F_m ratios in *E. mollis, G. littoralis*, and *V. rotundifolia* at midday was mainly due to a marked increase in F_0 rather than a decrease in F_m. The high Car content in these three species was attributed to F_0 increase at midday, and this can cause less damage to the photosynthetic apparatus because of thermal dissipation of excess excitation energy under high light conditions. *V. rotundifolia* had higher total Chl, higher Chl a content, and a higher Chl a/b ratio, as well as higher Car, but showed the lowest F_v/F_m and the highest F_0 value at midday, indicating that this species has a mechanism to protect the photosynthetic apparatus *via* dissipation of potentially harmful energy in response to high PAR, high temperature, and low RH.

A lower Chl and Car contents can reduce the absorption of light (Cao *et al.* 2006), and a higher Chl a/b ratio implies a lower amount of light-harvesting proteins and a higher amount associated with the reaction center complex (Leong and Anderson 1984). *C. kobomugi* had lower Chl and Car contents than the other three species, this species had the high Chl a/b ratio. Moreover, an obvious increase in F_0 did not appear under high light and temperature, unlike in the other three species, and F_v/F_m was higher than that in the other three species although the reduction in F_v/F_m ratio occurred due to a decrease in F_m rather than an increase in F_0. Yamane *et al.* (2008) found that a decrease in F_v/F_m due to an increase in F_0 correlates with a swelling of the thylakoids, and a decrease in F_v/F_m without an increase in F_0 is likely the result of photoprotection. Adaption mechanisms to cope with changes in light conditions can be divided into adaptions to control light-absorption capacity and adaption to deal with the light energy that has already been captured (Ruban 2009). Therefore, *C. kobomugi* that have low Chl and Car contents, high Chl a/b ratio and high F_v/F_m was more efficient at energy processing rather than light harvesting against photoinhibition of photosynthesis. Also, the rapid recovery of P_N as stomata opened under moderate light, temperature, and VPD conditions might be related to a high F_v/F_m as well as the rapid recovery of instantaneous CE.

Contribution of osmolality, soluble carbohydrate and inorganic matter to g_s and WUE: For leaf water content, the highest and lowest values were observed in *G. littoralis* and *E. mollis*, respectively. Unlike the leaf water status parameters, these two species maintained higher g_s than that of *C. kobomugi* and *V. rotundifolia* during the day. Effective control of water loss through stomatal closure, or through water uptake by osmoregulation and enhanced root growth, can improve plant water status. A decrease in osmotic potential through the accumulation of osmolytes, such as glycine-betaine, proline, and carbohydrates, in response to stress conditions improves the ability of the plant cells to maintain turgor pressure at low water potentials (Morgan 1984, Patakas and Noitsakis 1999, Silva *et al.* 2007). In the present study, although there were no significant differences in osmolality between all four species, TIC in the perennial grasses *E. mollis, C. kobomugi*, and *G. littoralis* was relatively higher than that in the perennial woody shrub *V. rotundifolia* which contained highly soluble carbohydrates. These results indicate that inorganic ions in the three grass species and organic solutes in *V. rotundifolia* could play an important role in osmotic adjustment.

Osmotic adjustment using inorganic ions is much more efficient because transport and compartmentalization of ions in the cells is less costly than the synthesis of organic molecules (Hu and Schmidhalter 1998). Li *et al.* (1992) have shown that the relative contributions of different osmotica to osmotic adjustment and their stabilizing ability is in the following order of magnitude: K^+ > soluble carbohydrates > other free amino acids > Ca^{2+} > Mg^{2+} > proline. In the present study, all four species contained higher K^+ than other cations. K^+ especially is an essential macronutrient for plant growth and development as well as playing a role as an osmotic substance (Maser *et al.* 2002). An increase in the K^+ content may also increase g_s, and water-stressed plants show greater adaptation to water deficits at higher K^+ concentrations (Premachandra *et al.*

1991). Therefore, in *E. mollis*, with the lowest water content, a high selectivity of K^+ absorption might be important to retain water in the leaves and might be responsible for the maintenance of high g_s.

In *V. rotundifolia*, a temporary stomatal opening at midday could cause a transient water deficit because of the higher E, but high soluble carbohydrate accumulation of this species could play an important role in maintaining water absorption from soil in order to reduce leaf water potential (Farooq *et al.* 2009) and could be more effective than K^+ as the main source of osmotic adjustment (Fig. 2*C,G*). Nonetheless, *V. rotundifolia*, with higher soluble carbohydrate content than the other three species, maintained low g_s except during midday hours. These results indicated that stomatal closure could more effectively maintain the water status than soluble carbohydrate accumulation.

As in previous studies of the genus *Carex*, *C. kobomugi* and *C. pumila* maintained a high selectivity of K^+ uptake relative to Na^+ uptake (Choi *et al.* 2004), but *C. scabrifolia* accumulated similar amounts of both Na^+ and K^+ ions in salty marsh habitats (Choi *et al.* 2014). In contrast to previous studies, *C. kobomugi* showed similar amounts of both Na^+ and K^+ ions in the leaves (Table 2). These differences may be due to different saline field conditions. Therefore, *C. kobomugi* may have a facultative ability to use either K^+ or Na^+ in order to maintain osmotic potential, depending upon the salinity of the rhizosphere in which the plant is situated. The genus *Carex* could regulate their mineral metabolism especially well, functioning as excluders in ion-rich and accumulators in ion-poor habitats. A balanced uptake of ions was accomplished by an efficient regulation metabolism that also prevented the uptake of potentially toxic amounts of ions (Choo and Albert 1997). Under K^+-deficient soils, Na^+ can play the role of K^+ in maintaining ionic balance (Subbarao *et al.* 2003), regulating osmotic pressure (Marschner 1995), contributing to vacuolar functions (Maser *et al.* 2002), and improving water balance *via* regulation of stomatal conductance (Marschner 1995, Gattward *et al.* 2012). Stomata of sugar beets closed more rapidly in response to drought stress when the plants were supplied with both Na^+ and K^+ compared to K^+ only (Hampe and Marschner 1982). The regulation of Na^+ import by regulation of transpiration could be a mechanism to minimize Na^+ influx into roots and subsequently into the shoots by reduction in E (Very *et al.* 1998, Chen *et al.* 2003).

In the present study, although *C. kobomugi* accumulated the highest Na^+ amount compared with the other three species, high P_N and E were observed when the stomata opened. Therefore, a decrease in E did not seem to be essential for the purpose of avoiding excessive Na^+ influx, but rather functions as an avoidance mechanism to prevent water loss under salt and drought stress, *i.e.*, an improvement in the WUE. *C. kobomugi* and *V. rotundifolia* maintained efficient stomatal control with steep decreases in g_s before midday. The stomatal response

observed in both species could be related to a conservative use of water resources, and mainly in *C. kobomugi*, which showed stomata closure at midday.

G. littoralis had higher Ca^{2+}, Mg^{2+}, and Na^+ content than those of *E. mollis* and *V. rotundifolia*. Under limited K^+ supply, Na^+, Mg^{2+}, and Ca^{2+} can replace K^+ in the vacuole as alternative inorganic osmotica (Flowers and Lauchli 1983). Osmolality and soluble carbohydrates in *G. littoralis* were similar to *E. mollis* and *C. kobomugi*, but TIC was lower in *G. littoralis*. These differences suggest that other organic solutes, such as glycine-betaine, proline, and polyols, may function as osmotica in *G. littoralis*. Accumulation of these compatible solutes in drought-tolerant species allows them to function as cytoplasmic osmotica for osmotic adjustment (Storey and Wyn Jones 1977, Smirnoff and Stewart 1985). Under field conditions, both *E. mollis* and *G. littoralis* can be described as water-spender plants that sustain high g_s and show a high E at midday, but K-efficient species, *E. mollis* with the much greater K^+ than the other cations and other compatible solutes compared to *G. littoralis* presented the greater capacity of CO_2 fixation and low E, which allowed *E. mollis* to have high WUE and maintain leaf water status effectively.

In the present study, *E. mollis*, *C. kobomugi*, and *G. littoralis* might be interpreted in terms of a very effective system of inorganic ion absorption, especially K^+ absorption in *E. mollis*, Na^+ and K^+ as well as Mg^{2+} absorption in *C. kobomugi*, and Ca^{2+}, Mg^{2+}, and Na^+ absorption in *G. littoralis*. The high storage capacity for inorganic ions in these species may play an efficient role in regulating photosynthesis and maintaining leaf water status through stomatal control, and can also play the important role in osmotic adjustment.

In conclusion, the plant species *E. mollis*, *C. kobomugi*, *G. littoralis*, and *V. rotundifolia*, growing in coastal sand dunes, adopted different strategies to utilize radiation and to maintain water status in a fluctuating microclimate. These strategies were associated with differences in photosynthesis regulation, photochemical reactions, Chl content, water status, and solute patterns in the leaves. Although the four species did not show significant differences in osmolality, their patterns of solute accumulation were different. *E. mollis* with the high K^+ content and *C. kobomugi* with the higher Na^+ and Mg^{2+} content than that of *G. littoralis* and *V. rotundifolia* maintained high instantaneous CE and WUE, except at midday, which allowed both species to gain advantage in water-holding ability and to be better adapted for survival when water is scarce. Midday depression in photosynthesis and photochemical activity of PSII under a fluctuating microclimate is the result of reversible downregulation that brings the electron transport capacity into balance with carbon metabolism (Epron *et al.* 1992). All four species were able to preserve the functional capacity of the photosynthetic apparatus against high light, temperature, and drought, thus ensuring rapid recovery of P_N and PSII upon

117

alleviation of stress under field conditions.

Photosynthesis depression in both *C. kobomugi* and *V. rotundifolia*, with relatively low g_s values could be attributed to both stomatal and nonstomatal limitations. *C. kobomugi* received less light to prevent photoinhibition, maintaining lower Chl and Car contents. Moreover, leaves of *C. kobomugi* had higher Chl *a/b* ratio and F_v/F_m values than the other three species.

F_0 increase in *E. mollis*, *G. littoralis*, and *V. rotundifolia*, with their higher Chl *b* and Car content, resulted in a reduction in photochemical efficiency but can in turn help in protecting the photosynthetic apparatus from damage due to excess energy. Apparently, the different solute accumulation, photochemical responses, and stomatal regulation found in all four species can contribute to their resistance under high light, temperature, and drought caused by low precipitation and high evaporation during the summer in coastal sand dunes.

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