

# Cold-Tolerant Crop Species Have Greater Temperature Homeostasis of Leaf Respiration and Photosynthesis Than Cold-Sensitive Species

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Some plant species show constant rates of respiration and photosynthesis measured at their respective growth temperatures (temperature homeostasis), whereas others do not. However, it is unclear what species show such temperature homeostasis and what factors affect the temperature homeostasis. To analyze the inherent ability of plants to acclimate respiration and photosynthesis to different growth temperatures, we examined 11 herbaceous crops with different cold tolerance. Leaf respiration ( $R_{\text{area}}$ ) and photosynthetic rate ( $P_{\text{area}}$ ) under high light at  $360\ \mu\text{l l}^{-1}\ \text{CO}_2$  concentrations were measured in plants grown at 15 and 30°C. Cold-tolerant species showed a greater extent of temperature homeostasis of both  $R_{\text{area}}$  and  $P_{\text{area}}$  than cold-sensitive species. The underlying mechanisms which caused differences in the extent of temperature homeostasis were examined. The extent of temperature homeostasis of  $P_{\text{area}}$  was not determined by differences in leaf mass and nitrogen content per leaf area, but by differences in photosynthetic nitrogen use efficiency (PNUE). Moreover, differences in PNUE were due to differences in the maximum catalytic rate of Rubisco, Rubisco contents and amounts of nitrogen invested in Rubisco. These findings indicated that the temperature homeostasis of photosynthesis was regulated by various parameters. On the other hand, the extent of temperature homeostasis of  $R_{\text{area}}$  was unrelated to the maximum activity of the respiratory enzyme (NAD-malic enzyme). The  $R_{\text{area}}/P_{\text{area}}$  ratio was maintained irrespective of the growth temperatures in all the species, suggesting that the extent of temperature homeostasis of  $R_{\text{area}}$  interacted with

the photosynthetic rate and/or the homeostasis of photosynthesis.

**Keywords:** Cold tolerance • Phenotypic plasticity • Photosynthesis • Respiration • Temperature acclimation • Temperature homeostasis.

**Abbreviations:** DTT, Dithiothreitol; HT, high temperature; LMA, leaf mass per area; LT, low temperature; NAD-ME, NAD-malic enzyme;  $N_{\text{area}}$ , nitrogen content per leaf area;  $P_{\text{area}}$ , net photosynthetic rate;  $P_{\text{mass}}$ , photosynthetic rate per leaf mass; PNUE, photosynthetic nitrogen use efficiency;  $R_{\text{area}}$ , dark respiration rate;  $R_{\text{mass}}$ , respiration rate per leaf mass; RM-ANOVA, repeated measures analysis of variance; RuBP, ribulose biphosphate; RNUE, respiratory nitrogen use efficiency.

## Introduction

Photosynthetic rates vary with leaf temperature (Berry and Björkman 1980, Yamori et al. 2005, Hikosaka et al. 2006). However, when plants are grown at various temperatures, the photosynthetic rate measured at their growth temperature is often maintained (Slatyer 1977, Mooney et al. 1978, Berry and Björkman 1980, Yamori et al. 2005). The case is often the same for respiration. Although respiration rates increase with leaf temperature, the respiration rate at their growth temperature is similar (Tjoelker et al. 1999a, Loveys et al. 2003, Atkin et al. 2006). These are considered as homeostatic responses to maintain a certain rate of photosynthesis

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and respiration irrespective of the growth conditions (Atkin and Tjoelker 2003, Hikosaka et al. 2006).

Temperature acclimation for photosynthesis has been related to leaf nitrogen economy, since more than half of the leaf nitrogen is in the photosynthetic apparatus and thus the photosynthetic capacity is strongly related to the leaf nitrogen content (Evans 1989, Poorter and Evans 1998, Makino et al. 2003, Hikosaka 2004). Leaf nitrogen content on a leaf area basis is greater in leaves grown at lower temperatures (either in the laboratory or in the field; Weih and Karlsson 2001, Muller et al. 2005, Yamori et al. 2005). This is considered as a compensatory response to low temperature, which decreases enzyme activity (Badger et al. 1982, Holaday et al. 1992, Strand et al. 1999). As respiration rates are also related to the leaf nitrogen content (Makino and Osmond 1991, Ryan 1995, Reich et al. 1998a, Reich et al. 1998b, Noguchi and Terashima 2006), a similar response has also been observed for the temperature acclimation of respiration (Duke et al. 1977, Atkin and Tjoelker 2003, Kurimoto et al. 2004b).

For photosynthesis, not only leaf nitrogen content but also nitrogen use within a leaf is suggested to be related to the temperature acclimation. Changes in nitrogen partitioning among photosynthetic components can be a factor responsible for changes in the temperature dependence of the photosynthetic rate, and thus may affect the photosynthetic rate at the growth temperature (Hikosaka 1997, Hikosaka et al. 2006). Some studies showed that nitrogen partitioning among photosynthetic components changes with the growth temperature (Makino et al. 1994, Steffen et al. 1995, Hikosaka 1997, Hikosaka 2005, Yamori et al. 2005). It has also been reported that the temperature dependence of Rubisco kinetics changes with growth temperatures (Huner and Macdowall 1979, Yamori et al. 2006a). Therefore, it is probable that differences in both leaf nitrogen content and nitrogen use efficiency could affect temperature homeostasis.

Temperature dependence of the respiration rate is considered to be determined by the maximum activity of respiratory enzymes, availability of substrates and/or demand for respiratory energy (Azcón-Bieto et al. 1983, Noguchi and Terashima 1997, Atkin et al. 2000, Atkin and Tjoelker 2003). It has been reported that temperature acclimation of respiration involves increases in the respiratory capacity by increasing the capacity per mitochondrion (Klikoff 1966, Klikoff 1968), or increasing the number of mitochondria (Miroslavov and Kravkina 1991), the mitochondrial density and the density of cristae within mitochondria (Armstrong et al. 2006). Thus, temperature acclimation of respiration could be linked to changes in the enzyme capacity (Atkin and Tjoelker 2003).

The extent of temperature acclimation of photosynthesis and respiration differs among species. Some species are able to acclimate, whereas others are not (Berry and Björkman

1980, Larigauderie and Körner 1995, Xiong et al. 2000, Loveys et al. 2002, Loveys et al. 2003, Atkin et al. 2006). There are reports that broad-leaved tree species exhibited a lower extent of temperature homeostasis of leaf respiration than needle-leaved tree species (Tjoelker et al. 1999b), and that a greater extent of temperature homeostasis was exhibited by herbaceous species in several studies (Yamasaki et al. 2002, Talts et al. 2004, Yamori et al. 2005, Yamori et al. 2006a, Yamori et al. 2006b, Yamori et al. 2008). Recently, Atkin et al. (2006) indicated that the extent of temperature homeostasis of leaf respiration and photosynthesis differed greatly between lowland (fast-growing) and alpine (slow-growing) *Plantago* species. Lowland species showed a greater extent of temperature homeostasis than alpine species. However, it is unclear what physiological characteristics are related to the interspecific variation of temperature homeostasis of respiration and photosynthesis. Moreover, mechanisms underlying the differences in temperature homeostasis depending on the species have still not been clarified. Recent studies have shown that interspecific variation of many leaf traits is related to the plant functional type (Wright et al. 2005). However, Loveys et al. (2003) showed that the temperature response for the respiration/photosynthesis ratio was generally similar among typical functional groups (forbs, eight species; grasses, two species; shrubs and trees, four species). Also, Campbell et al. (2007) indicated the striking similarities in the response of acclimation for respiration and photosynthesis among several functional groups (forbs, seven species; grasses, four species; shrubs and trees, eight species).

Since temperature tolerance differs depending on the plant species even in the same functional group (Long and Woodward 1989), it is possible that the extent of temperature homeostasis of respiration and photosynthesis would also differ depending on the species. Therefore, comparisons of several species with different cold tolerance would provide us with new insight into the temperature homeostasis and effects of growth temperature on the respiration/photosynthesis ratio. In the present study, we selected 11 herbaceous crops which have different cold tolerance (e.g. Larcher 1995, Huner et al. 1998, Huang et al. 2005). We grew those plants at 15°C (LT) and 30°C (HT), and measured the temperature dependences of leaf dark respiration rate and photosynthetic rate under high light of 1,500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at 360  $\mu\text{l l}^{-1} \text{CO}_2$  concentrations. Together with structural parameters such as leaf mass per leaf area (LMA), we determined leaf nitrogen content and nitrogen use efficiency for photosynthesis (PNUE) and respiration (RNUE). Moreover, we analyzed the maximal activities of Rubisco and NAD-malic enzyme (NAD-ME) as representative enzymes for photosynthesis and respiration, respectively. We addressed the following key questions. (i) Does the extent of temperature homeostasis of respiration and photosynthesis relate to cold tolerance? (ii) What factors influence the extent of temperature

homeostasis of respiration and photosynthesis? In particular, we focused on the mechanisms of temperature homeostasis from a viewpoint of nitrogen economy, and analyzed whether the interspecific difference in homeostasis is related to the plasticity in leaf nitrogen content or nitrogen partitioning within the leaf.

## Results

### Temperature homeostasis of leaf respiration and photosynthesis

Dark respiration rate ( $R_{\text{area}}$ ) and net photosynthetic rate ( $P_{\text{area}}$ ) were measured at the growth temperature in HT and LT leaves, respectively (Table 1, Supplementary Table S1). In *Cucumis sativus*, *Nicotiana tabacum* and *Oryza sativa*,  $R_{\text{area}}$  measured at 15°C in LT leaves was lower than  $R_{\text{area}}$  measured at 30°C in HT leaves, whereas other plant species exhibited similar rates at their respective growth temperatures. As an index for the extent of the respiratory homeostasis, we determined the ratio of  $R_{\text{area}}$  measured at 15°C in LT leaves to that measured at 30°C in HT leaves (LT-15°C/HT-30°C) (Table 1). When the ratio is close to 1.0, it indicates that the temperature homeostasis is high. In *C. sativus*, *N. tabacum* and *O. sativa*, the ratio of  $R_{\text{area}}$  was much lower than 1.0 and showed a value between 0.51 and 0.65. For all other species, the ratio was between 0.81 and 0.94. The average ratio of  $R_{\text{area}}$  (LT-15°C/HT-30°C) for the cold-sensitive species ( $0.67 \pm 0.05$ ) was significantly lower than that for the cold-tolerant species ( $0.88 \pm 0.05$ ).

According to Atkin et al. (2004) and Kurimoto et al. (2004a, 2004b), the extent of respiratory homeostasis was calculated as

$$H = 1 - \frac{R_{30}(30^\circ\text{C})/R_{15}(15^\circ\text{C}) - 1}{R_{30}(30^\circ\text{C})/R_{30}(15^\circ\text{C}) - 1}$$

where  $R_n(m^\circ\text{C})$  denotes a respiratory rate in  $n^\circ\text{C}$ -grown plants, which were measured at  $m^\circ\text{C}$ . The average  $H$  of  $R_{\text{area}}$

for the cold-sensitive species ( $0.69 \pm 0.18$ ) was significantly lower than that for the cold-tolerant species ( $0.90 \pm 0.06$ ;  $P = 0.016$ ). When we compared these two methods, there were significant relationships for  $R_{\text{area}}$  ( $R^2 = 0.92$ ). Therefore, it is fair to say that the trends between the two methods were the same.

$P_{\text{area}}$  measured at the respective growth temperatures also decreased with decreasing growth temperature in *C. sativus*, *N. tabacum* and *O. sativa* (Table 1, Supplementary Table S1). On the other hand, the other species exhibited similar rates irrespective of the growth temperatures. This indicates that the species with high homeostasis of  $R_{\text{area}}$  also showed high homeostasis of  $P_{\text{area}}$ , and vice versa. The average ratio of  $P_{\text{area}}$  (LT-15°C/HT-30°C) for the cold-sensitive species ( $0.67 \pm 0.06$ ) was significantly lower than that in the cold-tolerant species ( $0.92 \pm 0.06$ , Table 1).

In order to investigate the relationships between the extent of temperature homeostasis of respiration and photosynthesis, we analyzed the relationships between  $R_{\text{area}}$  and  $P_{\text{area}}$  at their respective growth temperatures (Fig. 1). The ratio of  $R_{\text{area}}$  (LT-15°C/HT-30°C) was strongly related to the ratio of  $P_{\text{area}}$  ( $r = 0.95$ ,  $P < 0.0001$ ).

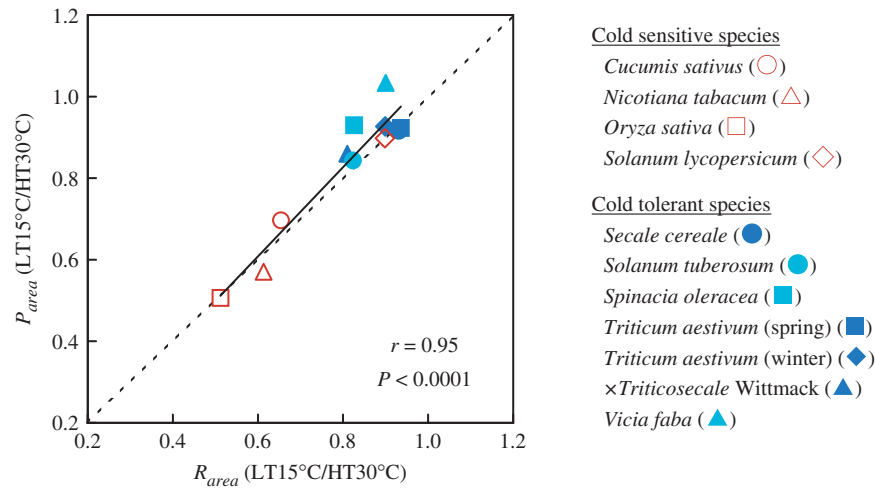
### Mechanisms of temperature homeostasis of photosynthesis and respiration

The extent of temperature homeostasis of  $R_{\text{area}}$  and  $P_{\text{area}}$  was different depending on the cold tolerance. Temperature homeostasis is basically considered as a compensation for the changes in specific activity by altering amounts of enzymes. Here, we analyzed factors affecting the temperature homeostasis of respiration and photosynthesis with a decomposition analysis (Table 2, see also Supplementary Tables S2, S3, S4 for each species). First, the effects of growth temperature, plant type and their interaction were tested with repeated measures analysis of variance (RM-ANOVA). LMA and nitrogen content per leaf area ( $N_{\text{area}}$ ) were larger in LT leaves than in HT leaves (Table 2), although there were

**Table 1** Temperature homeostasis of dark respiration and photosynthesis, and balance between the respiration and photosynthetic rates in cold-sensitive and cold-tolerant species

	$R_{\text{area}}$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )			$P_{\text{area}}$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )			$R_{\text{area}}/P_{\text{area}}$ ratio	
	LT (15°C)	HT (30°C)	Ratio (homeostasis)	LT (15°C)	HT (30°C)	Ratio (homeostasis)	LT (15°C)	HT (30°C)
Cold-sensitive species	$0.71 \pm 0.10$	$1.08 \pm 0.08$	$0.67 \pm 0.05$	$10.4 \pm 2.0$	$16.2 \pm 3.0$	$0.67 \pm 0.06$	$0.068 \pm 0.009$	$0.068 \pm 0.012$
Cold-tolerant species	$0.99 \pm 0.10$	$1.13 \pm 0.08$	$0.88 \pm 0.05$	$15.0 \pm 2.0$	$16.4 \pm 3.0$	$0.92 \pm 0.06$	$0.067 \pm 0.011$	$0.071 \pm 0.014$
Student's <i>t</i> -test	**	NS	*	**	NS	**	NS	NS

Respiratory rate ( $R_{\text{area}}$ ) and net photosynthetic rate ( $P_{\text{area}}$ ) measured at their growth temperatures in LT and HT leaves are shown for LT (15°C) and HT (30°C). The ratios of rates measured at 15°C in LT leaves to that measured at 30°C in HT leaves are also shown for 'Ratio (homeostasis)' as an index of the extent of temperature homeostasis. On the right, the balance between the respiration and photosynthetic rates ( $R_{\text{area}}/P_{\text{area}}$ ) at their respective growth temperatures in LT and HT leaves are shown. Values represent the mean  $\pm$  SD for cold-sensitive species and cold-tolerant species, respectively;  $n = 3-5$ .



**Fig. 1** Relationships of temperature homeostasis between respiration and photosynthetic rates. The extent of temperature homeostasis was determined by the ratio of  $R_{area}$  or  $P_{area}$  measured at 15°C in LT leaves to that measured at 30°C in HT leaves (LT-15°C/HT-30°C). When the ratio is close to 1.0, it indicates that the temperature homeostasis is high. The solid line represents the correlation line. The coefficient of correlation ( $r$ ) and significance of correlation ( $P$ ) are also shown. The dashed line indicates  $y = x$ . Values are the mean;  $n = 3-5$ .

variations depending on the plant species (Supplementary Table S2).  $N_{area}$  values were greater in cold-tolerant species than in cold-sensitive species at both growth temperatures. No significant differences were observed for LMA between plant types. In relation to  $R_{area}$ , the respiration rate per leaf mass ( $R_{mass}$ ), RNUE ( $R_{area}/N_{area}$ ), NAD-ME activity and NAD-ME activity/ $N_{area}$  were lower in LT leaves than in HT leaves, whereas  $R_{area}/NAD-ME$  activity was greater. However, there was no significant difference in these parameters between cold-tolerant and cold-sensitive species. There were no significant interactive effects of growth temperature and plant type in all the factors for  $R_{area}$ .

In relation to  $P_{area}$ , the photosynthetic rate per leaf mass ( $P_{mass}$ ), PNUE ( $P_{area}/N_{area}$ ), Rubisco activity and the maximum catalytic turnover rate of Rubisco ( $k_{cat}$ ) were lower in LT leaves than in HT leaves, whereas  $P_{area}/Rubisco$  activity and Rubisco contents were greater. There were significant differences in Rubisco activity,  $k_{cat}$ , Rubisco contents and  $P_{max}/Rubisco$  activity between cold-sensitive and cold-tolerant species, whereas there were no significant differences in  $P_{mass}$ ,  $P_{area}/N_{area}$  and Rubisco contents/ $N_{area}$ . We found marginally significant interactive effects ( $P < 0.1$ ) of growth temperature and plant type in  $P_{area}/N_{area}$  ( $P = 0.053$ ) and Rubisco contents/ $N_{area}$  ( $P = 0.060$ ), suggesting that the temperature response in nitrogen use was different between cold-sensitive and cold-tolerant species.

Next, we conducted a regression analysis to test what factors contributed to the temperature homeostasis in species with a great extent of the homeostasis (Fig. 2, Table 3). We treated the ratio of  $R_{area}$  or  $P_{area}$  at two growth

temperatures (i.e. the extent of temperature homeostasis) as a dependent and the ratio of the above factors as an independent. There was significant correlation between the  $R_{area}$  ratio and the  $R_{area}/N_{area}$  ratio, but there was no significant correlation between the  $R_{area}$  ratio and the other related factors (Table 3). The ratio of  $P_{area}$  was not correlated with that of  $P_{mass}$ , LMA,  $N_{area}$  and  $P_{area}/Rubisco$  activity, but was significantly correlated with that of  $P_{area}/N_{area}$ , Rubisco activity, Rubisco  $k_{cat}$ , Rubisco contents and Rubisco contents/ $N_{area}$  respectively (Fig. 2, Table 3).

### Balance between respiration and photosynthetic rates

In order to analyze the balance between respiration and photosynthetic rates, we estimated the ratio of  $R_{area}$  to  $P_{area}$  ( $R_{area}/P_{area}$ ) at their respective growth temperatures (Table 1, Supplementary Table S1). There were no differences in the average  $R_{area}/P_{area}$  ratio at their respective growth temperatures between cold-sensitive and cold-tolerant species. The  $R_{area}/P_{area}$  ratio was maintained irrespective of the growth temperatures in all the species. However, the  $R_{area}/P_{area}$  ratio differed greatly depending on the species (e.g. *O. sativa*, 0.061; *Solanum lycopersicum*, 0.078). We plotted the relationship of the  $R_{area}/P_{area}$  ratio between HT and LT leaves (Fig. 3a). The  $R_{area}/P_{area}$  ratio in HT leaves was strongly correlated with the ratio in LT leaves, irrespective of the extent of temperature homeostasis of  $R_{area}$  and  $P_{area}$  ( $r = 0.92$ ,  $P < 0.0001$ ).

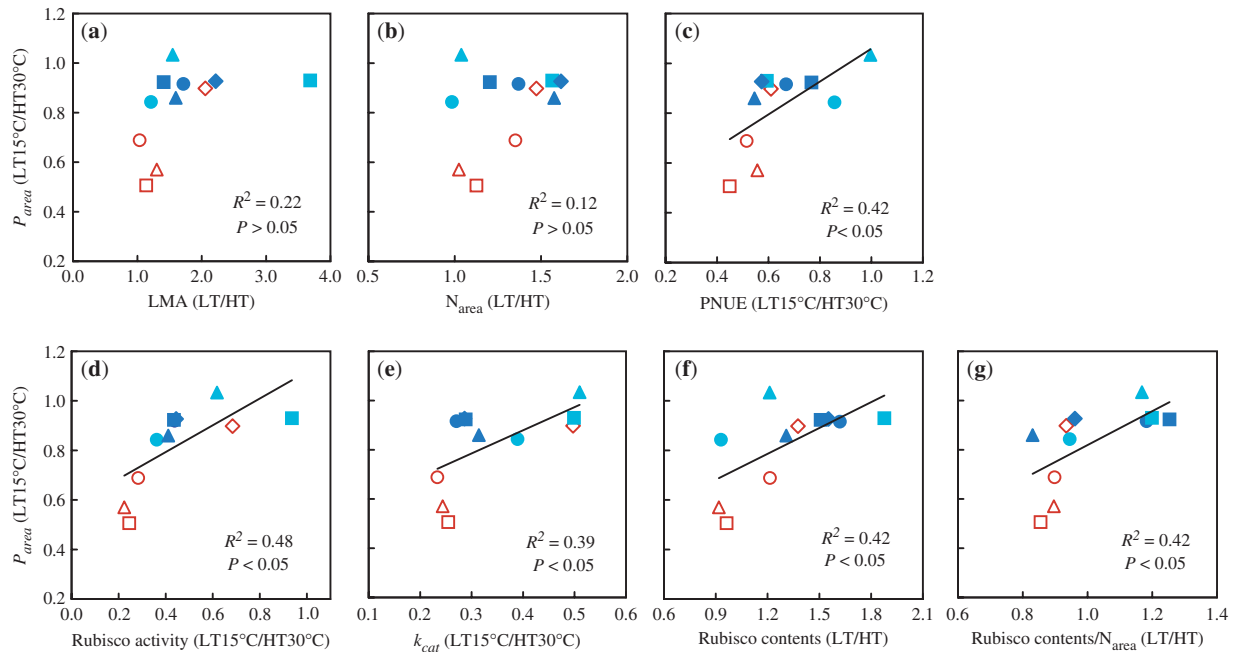
**Table 2** Effects of growth temperature, plant type and their interaction on several factors concerned with mechanisms of temperature homeostasis of respiration and photosynthesis

		Cold-sensitive	Cold-tolerant	P-value			Cold-sensitive	Cold-tolerant	P-value	
LMA (g m <sup>-2</sup> )	LT	29.7 ± 9.2	41.5 ± 17.4	GT: 0.003	<i>P</i> <sub>area</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	LT (15°C)	10.4 ± 2.0	15.0 ± 2.0	GT: <0.001	
	HT	21.8 ± 4.9	22.0 ± 2.7	PT: 0.186		HT (30°C)	16.2 ± 3.0	16.4 ± 3.0	PT: 0.086	
				GT×PT: 0.208						
<i>N</i> <sub>area</sub> (g m <sup>-2</sup> )	LT	1.28 ± 0.11	1.69 ± 0.16	GT: 0.003	<i>P</i> <sub>mass</sub> (μmol g <sup>-1</sup> s <sup>-1</sup> )	LT (15°C)	0.37 ± 0.10	0.40 ± 0.12	GT: <0.001	
	HT	1.06 ± 0.25	1.30 ± 0.23	PT: 0.008		HT (30°C)	0.76 ± 0.19	0.75 ± 0.10	PT: 0.884	
				GT×PT: 0.611						
<i>R</i> <sub>area</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	LT (15°C)	0.71 ± 0.10	0.99 ± 0.10	GT: <0.001	<i>P</i> <sub>area</sub> / <i>N</i> <sub>area</sub> (μmol g <sup>-1</sup> s <sup>-1</sup> )	LT (15°C)	8.20 ± 1.53	8.91 ± 1.19	GT: <0.001	
	HT (30°C)	1.08 ± 0.08	1.13 ± 0.08	PT: 0.008		HT (30°C)	15.6 ± 3.4	12.9 ± 2.4	PT: 0.610	
				GT×PT: 0.011						
<i>R</i> <sub>mass</sub> (μmol g <sup>-1</sup> s <sup>-1</sup> )	LT (15°C)	0.025 ± 0.007	0.026 ± 0.007	GT: <0.001	Rubisco activity (μmol m <sup>-2</sup> s <sup>-1</sup> )	LT (15°C)	10.1 ± 2.2	26.5 ± 7.7	GT: <0.001	
	HT (30°C)	0.051 ± 0.012	0.052 ± 0.007	PT: 0.878		HT (30°C)	32.4 ± 10.5	53.5 ± 13.6	PT: <0.001	
				GT×PT: 0.968						
<i>R</i> <sub>area</sub> / <i>N</i> <sub>area</sub> (μmol g <sup>-1</sup> s <sup>-1</sup> )	LT (15°C)	0.56 ± 0.15	0.59 ± 0.10	GT: <0.001	<i>k</i> <sub>cat</sub> [mol CO <sub>2</sub> (mol sites) <sup>-1</sup> s <sup>-1</sup> ]	LT (15°C)	0.45 ± 0.16	0.75 ± 0.13	GT: <0.001	
	HT (30°C)	1.06 ± 0.25	0.89 ± 0.16	PT: 0.682		HT (30°C)	1.52 ± 0.36	2.15 ± 0.58	PT: 0.006	
				GT×PT: 0.111						
NAD-ME activity (μmol m <sup>-2</sup> s <sup>-1</sup> )	LT (15°C)	17.3 ± 2.1	20.0 ± 4.2	GT: <0.001	Rubisco contents (μmol m <sup>-2</sup> )	LT	2.91 ± 0.53	4.43 ± 0.88	GT: 0.011	
	HT (30°C)	43.8 ± 16.9	38.0 ± 8.5	PT: 0.900		HT	2.69 ± 0.73	3.18 ± 0.77	PT: 0.029	
				GT×PT: 0.279						
<i>R</i> <sub>area</sub> /NAD-ME activity	LT (15°C)	0.041 ± 0.008	0.051 ± 0.010	GT: <0.001	<i>P</i> <sub>area</sub> /Rubisco activity	LT (15°C)	1.04 ± 0.12	0.59 ± 0.11	GT: <0.001	
	HT (30°C)	0.027 ± 0.011	0.031 ± 0.008	PT: 0.220		HT (30°C)	0.52 ± 0.13	0.31 ± 0.04	PT: <0.001	
				GT×PT: 0.758						
NAD-ME activity/ <i>N</i> <sub>area</sub> (μmol g <sup>-1</sup> s <sup>-1</sup> )	LT (15°C)	13.6 ± 2.7	11.8 ± 2.1	GT: <0.001	Rubisco contents/ <i>N</i> <sub>area</sub> (μmol g <sup>-1</sup> )	LT	2.29 ± 0.51	2.63 ± 0.50	GT: 0.582	
	HT (30°C)	41.1 ± 13.4	29.6 ± 5.8	PT: 0.056		HT	2.57 ± 0.65	2.45 ± 0.30	PT: 0.632	
				GT×PT: 0.370						

Gas exchange rate and enzyme activity measured at their growth temperatures in LT and HT leaves are shown for LT (15°C) and HT (30°C). *k*<sub>cat</sub> was calculated from Rubisco activity and Rubisco contents. Values represent the mean ± SD for cold-sensitive species and cold-tolerant species, respectively; *n* = 3–5. An RM-ANOVA was used after data were transformed logarithmically. *P*-values of RM-ANOVA are shown for difference between growth temperatures (GT) and between plant types (PT), and for interactions (GT×PT).

Next, in order to investigate the interaction between photosynthesis and respiration at an enzyme level, we analyzed temperature effects on activities of Rubisco and NAD-ME as representative enzymes for photosynthesis and respiration, respectively. The average ratios of NAD-ME activity to Rubisco activity (NAD-ME/Rubisco) at the growth temperatures were significantly greater in

cold-sensitive species (LT, 1.75 ± 0.36; HT, 1.34 ± 0.25) than in cold-tolerant species (LT, 0.77 ± 0.09; HT, 0.72 ± 0.07) in both HT and LT leaves (**Supplementary Tables S2, S3**). Moreover, in both cold-sensitive and cold-tolerant species, the average NAD-ME/Rubisco ratio was greater in LT leaves than in HT leaves. The NAD-ME/Rubisco ratio was strongly correlated between HT and LT leaves (*r* = 0.97, *P* < 0.0001, **Fig. 3b**).



**Fig. 2** Relationships between the extent of temperature homeostasis of photosynthesis (LT-15°C/HT-30°C) and that for leaf mass per area (LMA, a), nitrogen per leaf area ( $N_{area}$ , b), photosynthetic nitrogen use efficiency (PNUE;  $P_{area}/N_{area}$ , c), Rubisco activity (d), the maximum catalytic turnover rate of Rubisco ( $k_{cat}$ , e), Rubisco contents (f) and Rubisco contents/ $N_{area}$  (g). Abbreviations and symbols are the same as those in Fig. 1. The coefficient of determination ( $R^2$ ) and significance of regression ( $P$ ) are also shown. The solid line represents the regression line ( $P < 0.05$ ). Values are the mean;  $n = 3-5$ .

**Table 3** Regression analyses of several factors concerned with mechanisms of temperature homeostasis of respiration and photosynthesis

		$R^2$	$P$			$R^2$	$P$
$R_{area}$	$R_{mass}$	0.02	0.677	$P_{area}$	$P_{mass}$	0.02	0.718
	LMA	0.11	0.329		LMA	0.22	0.142
	$N_{area}$	0.09	0.383		$N_{area}$	0.12	0.297
	$R_{area}/N_{area}$	0.39	0.039		$P_{area}/N_{area}$	0.42	0.031
	NAD-ME activity	0.26	0.113		Rubisco activity	0.48	0.019
	$R_{area}/NAD-ME$ activity	0.00	0.967		$k_{cat}$	0.39	0.041
	NAD-ME activity/ $N_{area}$	0.22	0.144		Rubisco contents	0.42	0.031
					Rubisco contents/ $N_{area}$	0.42	0.031
			$P_{area}/Rubisco$ activity	0.27	0.102		

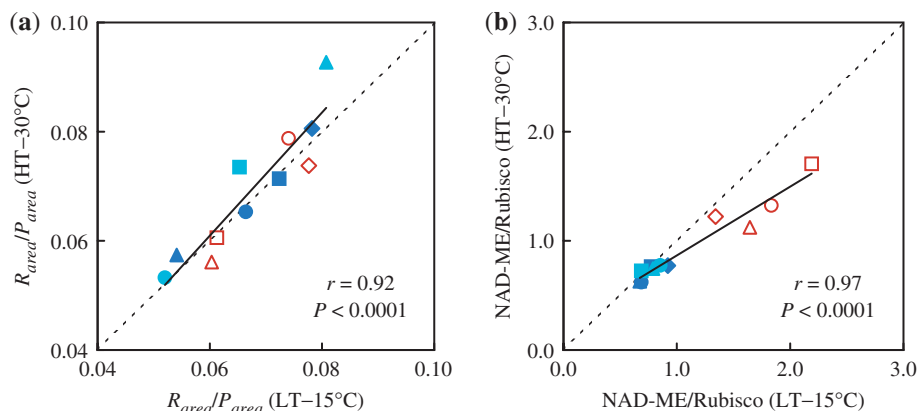
The ratio of  $R_{area}$  or  $P_{area}$  measured at their growth temperatures in LT and HT leaves (LT-15°C/HT-30°C) was treated as a dependent, and the ratio of LT leaves to HT leaves for several factors was treated as an independent.  $R^2$ , coefficient of determination;  $P$ , significance of regression.

## Discussion

### Temperature homeostasis of respiration and photosynthesis

All of the cold-tolerant species showed a great extent of temperature homeostasis of respiration and photosynthesis (Table 1, Fig. 1, Supplementary Table S1). Conversely,

most of the cold-sensitive species (*C. sativus*, *N. tabacum* and *O. sativa*) had a low extent of temperature homeostasis, with only *S. lycopersicum* having a great extent. Our studies clearly showed that cold-tolerant species exhibited high homeostasis of both respiration and photosynthesis. Cold-tolerant species in natural habitats survive at low temperature and would be subject to larger daily and seasonal fluctuations in temperature, compared with cold-sensitive species. Therefore,



**Fig. 3** Relationships between respiration and photosynthesis rates ( $R_{\text{area}}/P_{\text{area}}$ ; a) and between NAD-ME and Rubisco activities (NAD-ME/Rubisco; b) measured at the respective growth temperatures in HT and LT leaves. Abbreviations and symbols are the same as those in Fig. 1. The solid line represents the correlation line. The coefficient of correlation ( $r$ ) and significance of correlation ( $P$ ) are also shown. The dashed line indicates  $y=x$ . Values are the mean;  $n=3-5$ .

cold-tolerant species would acquire a greater extent of temperature homeostasis for both  $R_{\text{area}}$  and  $P_{\text{area}}$  than cold-sensitive species. Plants with high homeostasis would be able to maintain their relative growth rate irrespective of growth temperature (Gunn and Farrar 1999, Kurimoto et al. 2004a).

Many studies have reported a different inherent ability to tolerate cold, even in the same species, e.g. *C. sativus* (Yu et al. 2002), *O. sativa* (Saruyama and Tanida 1995) and *S. lycopersicum* (Brüggemann et al. 1994). Moreover, selective breeding of crops has been improved. Therefore, it is possible that *S. lycopersicum* examined in this study is relatively tolerant to the low temperature compared with other cold-sensitive species.

### Mechanisms of temperature homeostasis of photosynthesis

It has been argued that the temperature homeostasis of photosynthesis is related to  $N_{\text{area}}$  and LMA (e.g. Weih and Karlsson 2001, Muller et al. 2005, Yamori et al. 2005, Campbell et al. 2007). Although  $N_{\text{area}}$  and LMA increased at low growth temperature in most species (Table 2, Supplementary Table S2), this increase was unrelated to the temperature homeostasis (Fig. 2). Rather, temperature homeostasis of photosynthesis was related to  $P_{\text{area}}/N_{\text{area}}$  (PNUE, Fig. 2), suggesting that nitrogen use is more important for the temperature homeostasis than nitrogen content. Recent model analysis suggests that nitrogen partitioning has great potential for maximizing the photosynthetic rate (Zhu et al. 2007). In the present study, we clearly showed that plants which can alter nitrogen partitioning depending on the growth temperature had a great extent of temperature homeostasis for photosynthesis. Among mechanisms which contributed to the increase in PNUE, Rubisco content/ $N_{\text{area}}$ , which is the partitioning of nitrogen to Rubisco, was important

(Fig. 2). Moreover, greater increases in Rubisco contents and  $k_{\text{cat}}$  by decreasing the growth temperature were important factors (Fig. 2). Thus, the interspecific variation in the extent of temperature homeostasis of photosynthesis was related to the differences in both the quality and quantity of Rubisco. This study showed that the temperature homeostasis of photosynthesis would be regulated by various parameters.

Many studies have shown that plants grown at low temperatures have more  $N_{\text{area}}$  and Rubisco per unit leaf area to compensate for decreased activity at low temperatures (e.g. Badger et al. 1982, Holaday et al. 1992, Strand et al. 1999). However, some species (e.g. *N. tabacum*, *O. sativus* and *Solanum tuberosum*) in the present study did not increase  $N_{\text{area}}$  and Rubisco content at low growth temperature (Supplementary Table S2, S4). Therefore, an increase in  $N_{\text{area}}$  and Rubisco contents in leaves grown at low temperature is not always seen among  $C_3$  species, when plants are grown at moderate temperature (e.g. 15–30°C).

Rubisco  $k_{\text{cat}}$  was significantly different between cold-sensitive and cold-tolerant species (Table 2, Supplementary Table S4). In particular, Rubisco  $k_{\text{cat}}$  at 15°C was statistically greater in cold-tolerant species than in cold-sensitive species, when plants were grown at LT (Student's  $t$ -test,  $P<0.01$ ). Temperature acclimation would alter the concentrations of inhibitors for Rubisco and secondary metabolites in plant tissues (Kaplan et al. 2004, Zobayed et al. 2005, Parry et al. 2008), which might cause the differences in Rubisco  $k_{\text{cat}}$  between cold-sensitive and cold-tolerant species (Parry et al. 1997). However, this study strongly suggests that cold-tolerant species have Rubisco which performed efficiently at low temperature, and that Rubisco has evolved to improve its performance at the plant growth temperature. This is supported by the finding that Rubisco from plants in cool habitats had a higher  $k_{\text{cat}}$  than Rubisco

from plants in warm habitats (Sage 2002). Although Rubisco  $k_{\text{cat}}$  is known to differ between  $C_3$  and  $C_4$  species (Seemann et al. 1984, von Caemmerer and Quick 2000, Sage 2002), the variation of Rubisco  $k_{\text{cat}}$  in  $C_3$  species has been hardly highlighted (Sage 2002). Since sufficient  $\text{CO}_2$  concentration tended to be present in plants to allow Rubisco to catalyze near the  $\text{CO}_2$  saturation at low temperature, Rubisco  $k_{\text{cat}}$  would be important for increasing the photosynthetic rate and nitrogen use efficiency (Yamori et al. 2006a). In cold-tolerant species, Rubisco contents in LT leaves were greater than those in HT leaves (Table 2, Supplementary Table S4). Therefore, in cold-tolerant species, increases in Rubisco  $k_{\text{cat}}$  and content would both contribute to the increases in photosynthetic rate at low temperature, and thus to photosynthetic homeostasis. On the other hand, it is known that there is a trade-off relationship between  $k_{\text{cat}}$  and affinity for  $\text{CO}_2$  ( $K_c$ ) (von Caemmerer and Quick 2000). If this is also the case across the species in the present study, low  $k_{\text{cat}}$  in the cold-sensitive species may be a result of increasing the affinity for  $\text{CO}_2$  (decreasing  $K_c$ ). Since  $\text{CO}_2$  concentration in the carboxylation site is much lower than  $K_c$  at higher temperature, the value of  $K_c$  may be more important for efficient use of Rubisco in the cold-sensitive species.

Although the extent of temperature homeostasis of photosynthesis was not related to that of  $P_{\text{area}}/\text{Rubisco}$  activity (Table 3), there were differences in the average  $P_{\text{area}}/\text{Rubisco}$  activity depending on the species and growth temperature (Table 2, Supplementary Table S4). The average  $P_{\text{area}}/\text{Rubisco}$  activity tended to be greater in cold-sensitive species than in cold-tolerant species, and tended to be greater in HT leaves than in LT leaves.  $P_{\text{area}}/\text{Rubisco}$  activity reflects the differences in intercellular  $\text{CO}_2$  concentration, chloroplast  $\text{CO}_2$  concentration, Rubisco activation state and the limiting step of photosynthesis. Since there were no significant differences in intercellular  $\text{CO}_2$  concentration between cold-sensitive and cold-tolerant species (data not shown), other factors must affect the differences in  $P_{\text{area}}/\text{Rubisco}$  activity. In particular, in the present study, we analyzed the maximal activities of Rubisco as a representative enzyme for photosynthesis. However, it could be the case that the photosynthetic rate would be limited by the ribulose biphosphate (RuBP) regeneration rate and/or triose phosphate utilization (Sharkey 1985, Sage and Kubien 2007). Further studies are required to clarify the differences in internal conductance, Rubisco activation state and limiting steps of the photosynthetic rate between cold-sensitive and cold-tolerant species.

### Mechanisms of temperature homeostasis of respiration

The extent of temperature homeostasis of  $R_{\text{area}}$  (LT-15°C/HT-30°C) was not related to that of any respiratory parameters

that were measured, except for  $R_{\text{area}}/N_{\text{area}}$  (RNUE, Table 3). However,  $R_{\text{mass}}$ ,  $R_{\text{area}}/N_{\text{area}}$ , NAD-ME activity,  $R_{\text{area}}/\text{NAD-ME}$  activity and  $\text{NAD-ME activity}/N_{\text{area}}$  were different depending on the growth temperature (Table 2, Supplementary Table S3). Therefore, it is fair to say that the respiratory characteristics changed depending on the growth temperature, but that such responses to the growth temperature were not related to the changes in the absolute values of the respiratory rate and/or the respiratory homeostasis.

The respiration rate and the extent of temperature homeostasis of respiration were highly related to the photosynthetic rate and the extent of temperature homeostasis of photosynthesis, respectively (Table 1, Figs. 1, 3). This reflects the interdependence of photosynthesis and respiration. Maintaining the balance between photosynthesis and respiration would be important for optimal plant growth, because leaf respiration largely depends on photosynthates and supplies ATP for maintenance of the photosynthetic apparatus, whereas photosynthesis also depends on respiratory ATP supply for sucrose synthesis and energy dissipation by the ATP-uncoupling pathways (Krömer 1995, Raghavendra and Padmasree 2003). The respiration rate was stimulated by the addition of substrates such as glucose and sucrose (Azcon-Bieto et al. 1983, Atkin and Day 1990, Noguchi and Terashima 1997), and the leaf respiration rate was positively correlated with variations in leaf soluble sugar concentration (Tjoelker et al. 1999a, Griffin et al. 2002). Therefore, the temperature homeostasis of respiration would interact with the photosynthetic rate and/or the temperature homeostasis of photosynthesis. As a result, relationships between the extent of the temperature homeostasis of  $R_{\text{area}}$  (LT-15°C/HT-30°C) and RNUE could be indirectly caused by the results from the extent of temperature homeostasis of photosynthesis (Table 3). The  $R_{\text{area}}/P_{\text{area}}$  ratio was maintained irrespective of growth temperatures in all species, but differed among individual plant species (Table 1, Supplementary Table S1). This would indicate that the optimal balance of photosynthesis and respiration is different depending on the growth temperature.

In this study, we analyzed the NAD-ME activity as a representative enzyme for respiration, because NAD-ME is only located in mitochondria. When we plotted  $R_{\text{area}}$  vs. NAD-ME activity at the growth temperatures, there was no statistical correlation (data not shown). It is possible that another respiratory enzyme that is potentially rate limiting (e.g. the pyruvate dehydrogenase complex or one of the tricarboxylic acid cycle enzymes) might have provided a greater correlation with overall respiratory rates (Hill and Bryce 1992). This study indicates that the respiration rate at the growth temperature would not be limited, at least, by the capacity of NAD-ME activity. Nevertheless, the NAD-ME/Rubisco activity ratio, which indicates the balance of activities between the respiratory and photosynthetic enzymes,



was maintained irrespective of the growth temperature in both cold-sensitive and cold-tolerant species. This suggests that even respiratory enzymes which do not limit respiration would respond to changes in growth temperature to the same extent as Rubisco which would be a representative of photosynthesis. This also indicates the tight coupling of mitochondrial and chloroplastic metabolism (Krömer 1995, Raghavendra and Padmasree 2003).

### Balance between respiration and photosynthesis

The extents to which photosynthesis and respiration acclimate are clearly important determinants of plant responses to environmental change, but they are poorly understood. Our results showed that the extent of respiratory and photosynthetic acclimation differed between the cold-sensitive and cold-tolerant species (Table 1, Fig. 1, Supplementary Table S1). Nevertheless, the balance between leaf respiration and photosynthetic rate ( $R_{\text{area}}/P_{\text{area}}$ ) was constant irrespective of growth temperatures (i.e. 15 or 30°C), in both the cold-sensitive and cold-tolerant species (Table 1, Fig. 3, Supplementary Table S1). Campbell et al. (2007) showed that the  $R_{\text{area}}/P_{\text{area}}$  ratio did not remain constant when plants were exposed to chilling temperatures (e.g. 7°C). It has been suggested that the  $R_{\text{area}}/P_{\text{area}}$  ratio would change when plants were grown at extremely low and high temperatures (Loveys et al. 2003, Atkin et al. 2006, Campbell et al. 2007). Taken together, it is fair to say that the  $R_{\text{area}}/P_{\text{area}}$  ratio is homeostatic at moderate growth temperatures in many plant species, and that a large-scale carbon model is able to make broad generalizations about similar extents of temperature homeostasis for respiration and photosynthesis among the plant species (Gifford 2003).

### Conclusion

The cold-tolerant species were generally more capable of maintaining homeostasis of photosynthesis and respiration than the cold-sensitive species, indicating a clear difference in phenotypic plasticity for temperature homeostasis depending on cold tolerance. Temperature acclimation of photosynthesis has been considered to be related to changes in  $N_{\text{area}}$  and LMA. However, the extent of temperature homeostasis of photosynthesis was determined by differences in PNUE. The maximum catalytic turnover rate of Rubisco, Rubisco contents and the amount of nitrogen allocated to Rubisco were important factors that contributed to the variation in PNUE. Thus, the temperature homeostasis of photosynthesis would be regulated by various parameters. On the other hand, the extent of temperature homeostasis of respiration was considered to interact with photosynthetic rate and/or the extent of temperature homeostasis of photosynthesis.

## Materials and Methods

### Plant materials and growth conditions

Studies were conducted on 11 species, *C. sativus* L. cv. Nanshin (cucumber), *N. tabacum* L. cv. Samsun NN (tobacco), *O. sativa* L. cv. Nipponbare (rice), *Secale cereale* L. cv. Warko (winter rye), *S. lycopersicum* L. cv. House-momotarou (tomato), *S. tuberosum* L. cv. Danshaku (potato), *Triticum aestivum* L. cv. Haruyutaka (spring wheat), *T. aestivum* L. cv. Hokushin (winter wheat), *×Triticosecale* Wittmack cv. Presto (triticale) and *Vicia faba* L. cv. Nintokuissun (broad bean). In addition, data on *Spinacia oleracea* L. cv. Torai (spinach) were taken from our previous study (Yamori et al. 2005). *C. sativus*, *N. tabacum*, *O. sativa* and *S. lycopersicum* are considered to be cold-sensitive species, while *S. cereale*, *S. oleracea*, *S. tuberosum*, *T. aestivum* (spring), *T. aestivum* (winter), *Triticosecale* and *V. faba* are considered to be cold-tolerant species (e.g. Larcher 1995, Huner et al. 1998, Japan Seed Trade Association 2002, Huang et al. 2005). All plants were grown in vermiculite in 1.3 liter plastic pots. Day and night lengths were 8 and 16 h, respectively. Photosynthetically active photon flux density (PPFD) during the day time was 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Plants were grown at either 15/10°C or 30/25°C (day/night). These are referred to as low temperature (LT) and high temperature (HT) conditions, respectively. The leaves grown at LT and HT are called LT and HT leaves, respectively. All plants were able to grow at 15/10°C or 30/25°C without any injury. It is considered that a temperature of 30/25°C would be suitable to grow the cold-sensitive species, whereas a temperature of 15/10°C would be suitable to grow the cold-tolerant species. The plants, except for rice, were watered once a week and fertilized with 200 ml of a nutrient solution containing 2 mM  $\text{KNO}_3$ , 2 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.75 mM  $\text{MgSO}_4$ , 0.665 mM  $\text{NaH}_2\text{PO}_4$ , 25  $\mu\text{M}$  Fe-EDTA, 5  $\mu\text{M}$   $\text{MnSO}_4$ , 0.5  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.5  $\mu\text{M}$   $\text{CuSO}_4$ , 25  $\mu\text{M}$   $\text{H}_3\text{BO}_4$ , 0.25  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , 50  $\mu\text{M}$  NaCl and 0.1  $\mu\text{M}$   $\text{CoSO}_4$  once a week. The 1.3 liter plastic pots for rice plants were placed in a container filled with the above nutrient solution with the pH adjusted to  $5.4 \pm 0.3$ . The nutrient solution level was kept at about 10 cm from the bottom of the container. The nutrient solution was aerated continuously with an air pump, and renewed every week.

### Gas exchange measurements

Rates of dark respiration ( $R_{\text{area}}$ ) and photosynthesis ( $P_{\text{area}}$ ) of the most recently fully expanded leaves were measured using a portable gas exchange system (LI-6400; Li-Cor Inc., Lincoln, NE, USA) as described previously (Yamori et al. 2005).  $R_{\text{area}}$  and  $P_{\text{area}}$  were measured every 5°C from 10 to 35°C, and at 38°C at an ambient  $\text{CO}_2$  concentration of 360  $\mu\text{l l}^{-1}$ .  $R_{\text{area}}$  was measured after a sufficiently long dark period

(approximately 10 h).  $P_{\text{area}}$  was measured at a high light intensity of  $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

### Determinations of Rubisco and nitrogen

Immediately after gas exchange measurements, leaf discs were frozen and stored at  $-80^{\circ}\text{C}$  until biochemical measurements. The frozen leaf sample (approximately  $1.0 \text{ cm}^2$ ) was ground in liquid nitrogen and homogenized in an extraction buffer containing 100 mM sodium phosphate buffer (pH 7.0), 1.0% (w/v) polyvinylpyrrolidone, 0.1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 1.0%  $\beta$ -mercaptoethanol. The content of Rubisco was determined by the method of Yamori et al. (2005).

Some leaf discs, taken from leaves after the measurements of the gas exchange, were used for determination of leaf dry mass and leaf nitrogen contents. After the leaf discs were dried at  $70^{\circ}\text{C}$  for at least 7 d, leaf nitrogen contents were measured with an NC analyzer (CHNOS Elemental analyzer, Vario EL III, Elementar, Hanau, Germany).

### Enzyme assays

The frozen leaf sample (approximately  $1.0 \text{ cm}^2$ ) was rapidly homogenized using a chilled mortar and pestle with 0.892 ml of the extraction medium. The medium contained 100 mM HEPES-KOH (pH 7.8), 10 mM  $\text{MgCl}_2$ , 5 mM dithiothreitol (DTT) and 1 mM EDTA. The homogenate was centrifuged at  $16,000 \times g$  for 30 s at  $4^{\circ}\text{C}$ , and the supernatant was used for the Rubisco and NAD-ME assay. The enzyme activities were measured at  $15^{\circ}\text{C}$  in LT leaves and  $30^{\circ}\text{C}$  in HT leaves. The maximal Rubisco activity was assayed by monitoring NADH oxidation at 340 nm, according to the method of Yamori et al. (2006a). After Rubisco was activated for 20 min at  $4^{\circ}\text{C}$  in an activation medium that contained 375 mM HEPES-KOH (pH 7.8), 50 mM  $\text{MgCl}_2$ , 50 mM  $\text{NaHCO}_3$ , the total activity was assayed in the assay medium containing 100 mM Bicine-KOH (pH 8.2), 20 mM  $\text{MgCl}_2$ , 20 mM  $\text{NaHCO}_3$ , 5 mM ATP, 5 mM creatine phosphate,  $63 \mu\text{M}$  NADH, 0.6 mM RuBP,  $10 \text{ U ml}^{-1}$  of creatine kinase,  $10 \text{ U ml}^{-1}$  of 3-phosphoglyceric phosphokinase (PGK) and  $25 \text{ U ml}^{-1}$  of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The maximal NAD-ME activity was assayed according to Millar et al. (1998), in a reaction medium consisting of 50 mM MOPS-KOH (pH 6.5), 2 mM  $\text{NAD}^+$ , 0.025% (v/v) Triton X-100, 2 mM  $\text{MnCl}_2$ , 4 mM DTT and 10 mM malate.

### Decomposition analyses for photosynthesis and respiration

Decomposition analyses were conducted to investigate mechanisms of differences in temperature homeostasis depending on the plant species.  $P_{\text{area}}$  is divided into the  $P_{\text{mass}}$

LMA, PNUE ( $P_{\text{area}}/N_{\text{area}}$ ),  $N_{\text{area}}$ , Rubisco activity, the Rubisco  $k_{\text{cat}}$  and Rubisco contents:

$$P_{\text{area}} = P_{\text{mass}} \times \text{LMA} = \text{PNUE} \times N_{\text{area}} \quad (1)$$

and

$$\text{PNUE} = (P_{\text{area}} / \text{Rubisco activity}) \times k_{\text{cat}} \times (\text{Rubisco contents} / N_{\text{area}}) \quad (2)$$

In this study, Rubisco was analyzed as a representative enzyme for photosynthesis. The  $P_{\text{area}}/\text{Rubisco activity}$  is a parameter that is affected by the  $\text{CO}_2$  conductance from air to the carboxylation site, Rubisco activation state and the limiting step of photosynthesis. The Rubisco content/ $N_{\text{area}}$  reflects the proportion of nitrogen invested in Rubisco.

In the same manner,  $R_{\text{area}}$  is divided into the respiration rate per leaf dry mass ( $R_{\text{mass}}$ ), LMA, RNUE ( $R_{\text{area}}/N_{\text{area}}$ ),  $N_{\text{area}}$  and NAD-ME activity:

$$R_{\text{area}} = R_{\text{mass}} \times \text{LMA} = \text{RNUE} \times N_{\text{area}} \quad (3)$$

and

$$\text{RNUE} = (R_{\text{area}} / \text{NAD-ME activity}) \times (\text{NAD-ME activity} / N_{\text{area}}) \quad (4)$$

In this study, NAD-ME was analyzed as a representative enzyme for respiration, because it is located only in mitochondria.

### Statistical analyses

All data were analyzed with STATVIEW (ver. 4.58, SAS Institute Inc., Cary, NC, USA). To evaluate whether homeostasis is different between cold-sensitive and cold-tolerant species, we used a RM-ANOVA after values were transformed logarithmically. Growth temperature (within-subject factor) and cold tolerance (between-subjects factor) were treated as fixed factors, and individual species were treated as a random effect. An average value was used for each species, and the variation within a species was ignored.

### Supplementary data

Supplementary data are available at PCP online.

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