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Cold acclimation of SnRK2.2 kinases mutant *Chlamydomonas reinhardtii*

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SUMMARY

In *Chlamydomonas reinhardtii*, plant-specific serine/threonine kinases (SnRK2 kinases) play a central role in sulfur metabolism. However, their role in environmental stress has not been clearly understood. Cold stress is one of the most important factors that limit the growth, productivity, and development of photosynthetic organisms. In this report, the effect of cold stress on some physiological parameters were investigated in SnRK2.2 mutant and wild type *C. reinhardtii* culture. Our results showed that cold stress had significantly enhanced lipid peroxidation rate in wild type, while no significant change was observed in SnRK2.2 mutant culture. Our data also indicated a decline in Rubisco protein amount of wild type culture under low temperature exposure. Low temperature reduced glutathione reductase (GR) activity in the wild type, while GR activity was enhanced in SnRK2.2 mutant. This result indicated the potential of SnRK2 kinase in cold acclimation process.

Key words: cold stress, plant-specific serine/threonine kinases, Rubisco.

INTRODUCTION

Low temperature can cause cell membrane injury through changes in the cell membrane structure and lipid composition. Decreased membrane fluidity in cytoplasmic membranes and/or disruption of the ion balance are potential results of cell membrane injury (Theocharis *et al*. 2012; Los *et al*. 2013). Low temperature also results in a series of physiological, biochemical, and molecular modification, such as photoinhibition of photosystem I (PS I) (Kudoh & Sonoike 2002), and reduction in CO_2 assimilation rate (Zhou *et al*. 2012). Moreover, under low temperature, a decrease in activity of ribulose-1,5-bisphosphate carboxylase (Rubisco EC 4.1.1.39) was reported in higher plants (Zhou *et al*. 2006).

Green algae are among the group of photosynthetic organisms that are closely related to land plants (Wu *et al*. 2015). In algae, low temperature has a direct effect by inhibiting metabolic reactions. Cold-induced oxidative stress could also be observed as an indirect effect. These effects especially lead to a decreased growth rate and development process (Herma *et al*. 2007; Janská *et al*. 2010). Protein kinases and phosphatases are key regulator components in many signal transduction pathways. In the green unicellular alga *Chlamydomonas reinhardtii*, the plant-specific serine/threonine kinases, SnRK 2.1 and SnRK 2.2, have a central role in regulation of sulfur metabolism (Davies *et al*. 1999; Kimura *et al*. 2006; González-Ballester *et al*. 2008). Previous studies with mutants showed that SnRK 2.1 kinase is required for cell viability and regulation of S-responsive processes under S deprivation (González-Ballester *et al*. 2008, 2010). In addition, it is known that SnRK 2.2 kinases have an epistatic relationship with SnRK 2.1 kinases, and they have a negative modulation on gene expression of SnRK 2.1 kinases (Moseley *et al*. 2009).

In higher plants, SnRK2s family members are involved in regulation of plant tolerance to abiotic stresses (Kobayashi *et al*. 2004; Fujii *et al*. 2011; Kulik *et al*. 2011). Previous studies demonstrated that SnRK2 family include ten members in *Arabidopsis* and rice (Boudsocq *et al*. 2004). All of them, except SnRK2.9 in *Arabidopsis*, play a role under different stress conditions such as cadmium, drought, and salinity (Fujita *et al*. 2009; Kulik *et al*. 2011). Ding *et al*. (2015) demonstrated that cold stress enhanced activities of SnRK2.6 which triggered CBF-COR gene-expression cascade and freezing tolerance. However, in green algae, role of SnRK2s kinases in response to environmental stress has not clearly been demonstrated yet.

In the present study, our aim was to identify the response of SnRK2 kinases under cold stress in *C. reinhardtii.* To identify the role of SnRK2s, we analyzed rubisco protein content, MDA content and antioxidant enzyme analysis under cold stress in wild type and SnRK2.2 mutant cell culture of *C. reinhardtii.*

MATERIAL AND METHODS

Culture conditions

The *C. rehindratii* strains used in this study was wild type (WT, CC1010) and SnRK2.2 (CC-4270) (Davies *et al*. 1994). They were obtained from *Chlamydomonas* Research Center culture collection. SnRK2.2 mutant were obtained by insertional mutagenesis from an allele at SAC3 locus, which encoded a Snf1-like serine/threonine kinase. The cultures were grown in a TAP medium under 120 μmol m^{-2} s^{-1} light intensity and in the presence of a continuous light source at

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25°C and kept in an exponential phase of growth by sub-culturing with freshly prepared medium at about 5-day intervals. All experiments were carried out using cells in exponential phase, and five 250 mL Erlenmeyer flasks of *C. rehindratii* were used for the experiments. WT and SnRK2.2 mutant were grown in a TAP medium under 120 μmol $m^{-2}s^{-1}$ light intensity and in the presence of a continuous light source at 24°C for 24, 48 and 72 h (as control). WT and SnRK2.2 mutant were then incubated in a growth chamber under continuous illumination at 120 μmol $m^{-2}s^{-1}$ light intensity and at 10°C for 24, 48 and 72 h (low temperature treatment). Optical density of cell growth was measured by UV-spectrophotometer (Optizen UV/VIS, Optizen POP, Daejeon, South Korea) at 750 nm, and cell number was counted by using Neubauer hemocytometer. Each experiment was repeated for three times.

Lipid peroxidation analysis

Determination of malondialdehyde (MDA) by thiobarbituric acid reactive substances (TBARS) method was performed according to Heath and Packer (1968). Absorbance differences ranging from 532 to 600 nm were used to calculate MDA formation as a product of lipid peroxidation. Each analysis was comprised of three replicates.

Rubisco protein amount analysis

The optical density of each cell culture was arranged to an OD value of 0.2, and cells were collected by centrifugation. Rubisco protein amount was measured using the plant ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) ELISA Kit (Cat.No:CK-E91378). The Rubisco protein amount was measured at 450 nm. Each analysis was comprised of three replicates.

Antioxidant enzyme analysis

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to Beyer and Fridowich (1987). One unit of SOD activity was defined as the quantity of SOD that caused a 50% inhibition of NBT, and the specific enzyme activity was expressed as units mg $protein^{-1}$. Catalase (CAT; EC 1.11.1.6) activity was measured according to Bergmeyer (1970). The disappearance of H_2O_2 was determined at 240 nm. One unit of CAT activity was defined as 1 μmol H_2O_2 destroyed per minute. APX activity (APX; EC 1.11.1.11) was measured by the decrease in absorbance of the substrate at 290 nm (Nakano & Asada 1981). The concentration of oxidized ascorbate was determined by using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX activity was defined as μmol mL^{-1} oxidized ascorbate per min. Glutathione reductase (GR, EC 1.6.4.2) activity was assayed according to Carlberg and Mannervik (1985) by the oxidation of $NADPH_2$ at 340 nm. The antioxidant enzyme activities were expressed as the specific activity by measuring the protein content (Bradford 1976).

Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) and Pearson's correlation test. Comparisons with P values <0.05 were validated as significantly different.

RESULTS

The effect of low temperature on growth is shown in Figure 1. The growth rate of wild type and SnRK2.2 mutant was increased during the 3 days at normal conditions (Fig. 1). In addition, low temperature stress increased the cell number slowly in both cultures. After 72 h under low temperature, the cell number of WT at 10°C showed a significant decrease ($P < 0.001$) when compared to that of WT control. Moreover, SnRK2.2 mutant cultures grew faster than WT cultures under cold stress (Fig. 1).

Figure 2 shows MDA content of *C. reinhardtii* under low temperature for 24, 48 and 72 h. The lipid peroxidation rate of WT at 10°C increased 1.6-, 2- and 2.21-fold within 24, 48, and 72 h, respectively as compared to WT control ($P < 0.05$). MDA content of SnRK2.2 at 10°C was not significantly different as compared to SnRK2.2 and WT controls.

As shown in Figure 3, the Rubisco protein amount decreased gradually in the samples under low temperature for 24, 48 and 72 h (Fig. 3). In *C. reinhardtii*, the Rubisco protein amount decreased approximately 1.73-fold under low temperature at 72 h. However; the Rubisco protein amount was not significantly altered in the SnRK2.2 mutant under low temperature.

The effects of low temperature on antioxidant enzyme activities of WT and SnRK2.2 mutant was illustrated in Figure 4a–d. As shown in Figure 4a, the APX activity of WT was increased due to low temperature exposure; however, a decrease was observed in SnRK2.2 mutant when compared to their control groups. The APX activity of WT was remarkably increased at 24 h (1.40-fold higher than that of the control group, $P < 0.05$). SOD activity did not show a significant difference in SnRK2.2 mutant culture under low temperature; however, a significant decrease in SOD activity of WT was determined at the end of 48 h (1.16-fold) and 72 h (1.14-fold) when compared to the control group ($P < 0.05$) (Fig. 4b). Unlike the APX activity, the GR activity of WT was reduced; however, the activity of SnRK2.2 mutant increased

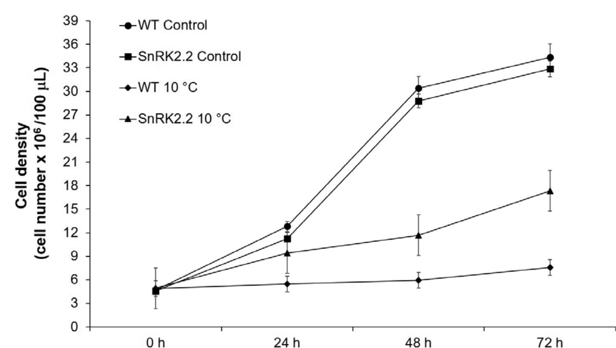

Fig. 1. Cell density of SnRK2.2 mutant and wt (+) *C. reinhardtii* in culture.

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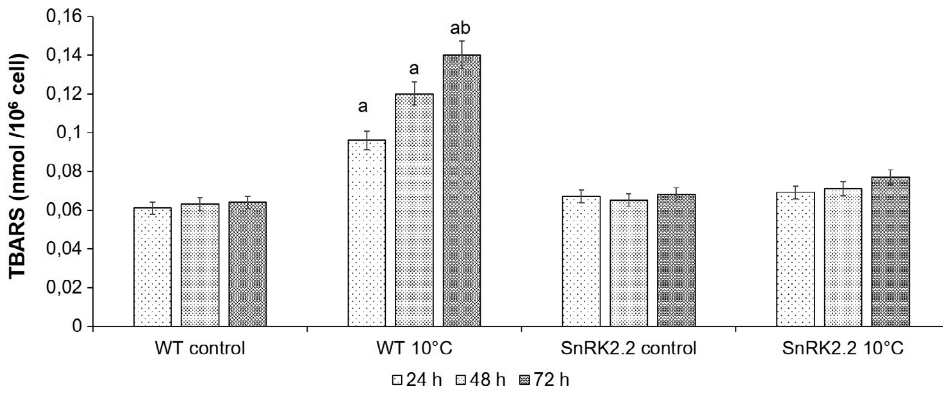

Fig. 2. Lipid peroxidation of SnRK2.2 mutant and wt (+) *C. reinhardtii* culture under cold stress. Represents a statistically significant difference of $P < 0.05$ when compared with the control.

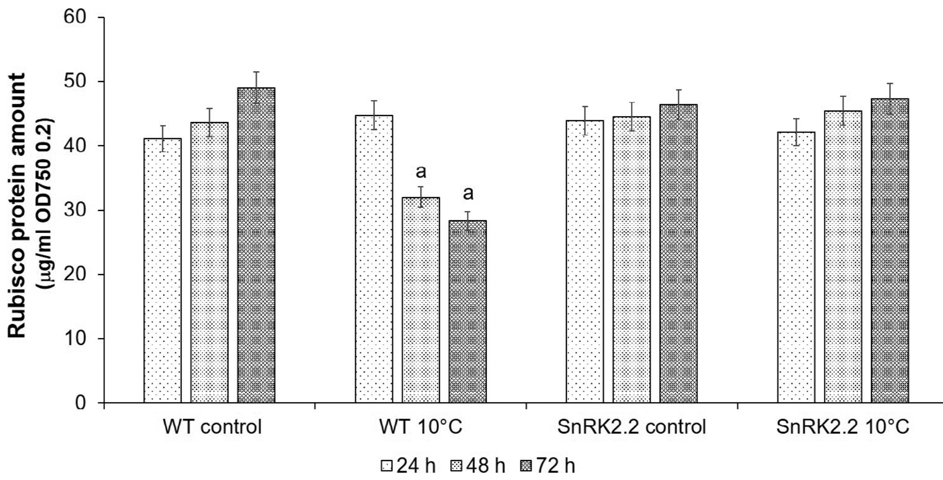

Fig. 3. Rubisco protein amount of SnRK2.2 mutant and wt (+) *C. reinhardtii* culture under cold stress. Represents a statistically significant difference of $P < 0.05$ when compared with the control.

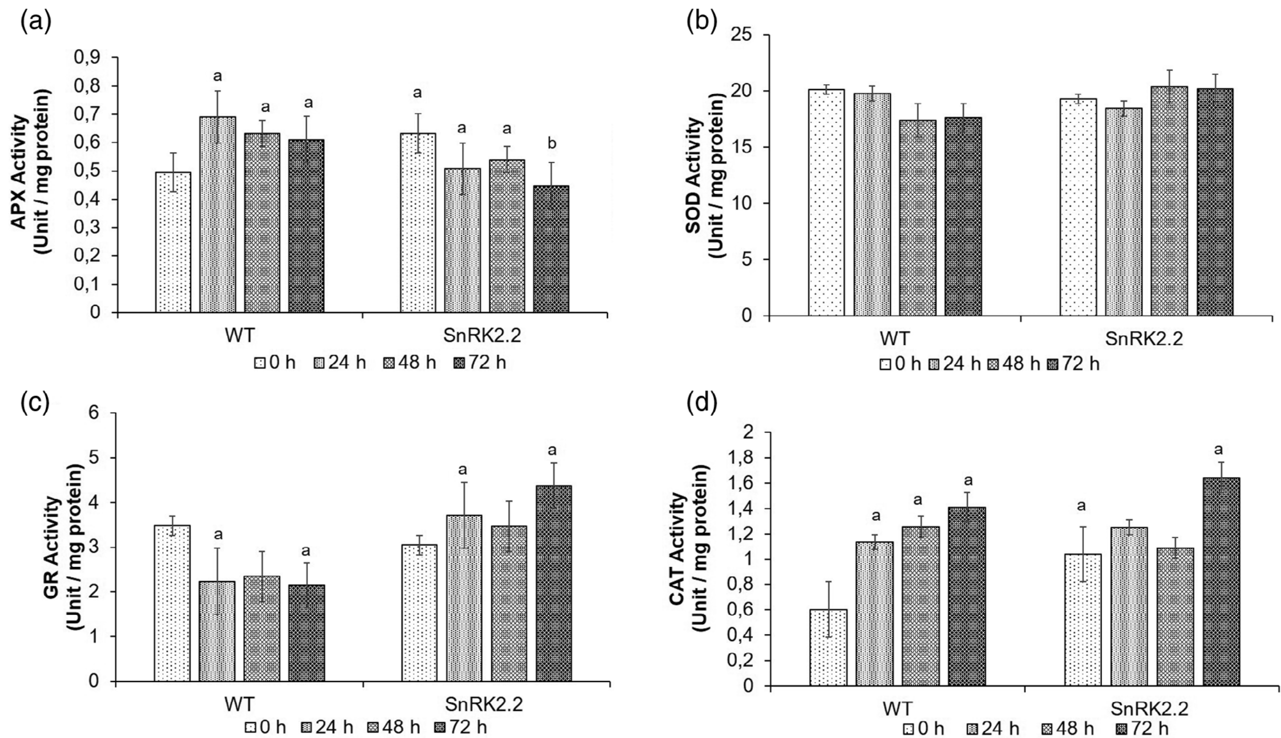

Fig. 4. Antioxidant enzyme activity of SnRK2.2 mutant and wt (+) *C. reinhardtii* culture under cold stress, (a) APX activity, (b) SOD activity, (c) GR activity and (d) CAT activity. Represents a statistically significant difference of $P < 0.05$ when compared with the control.

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due to low temperature treatments (Fig. 4c). We also found a significant increase in GR activity of SnRK2.2 mutant at 72 h (1.44-fold higher than the control group, $P < 0.05$). The CAT activity was enhanced in WT and SnRK2.2 mutant under low temperature when compared to their control groups ($P < 0.05$) (Fig. 4d).

DISCUSSION

Previous studies demonstrated that cold stress lead to a slow growth rate in *C. reinhardtii* culture (Valledor *et al*. 2013). Renaud *et al*. (1995) showed that *Nitzschia paleacea* had a low temperature tolerance while the cells could grow slowly at 10°C. Nedwell and Rutter (1994) also reported that under low temperature a decreased rate of nutrient uptake from the environment could be the rate-limiting step for the growth of microorganisms. Therefore, nitrogen limitation could cause a slower growth rate in algal culture under low temperature conditions (Sakamoto & Bryant 1998). Similarly, our data demonstrate that the growth rate at 10°C was significantly slower in the WT when compared to those cultivated at 25°C (Fig. 1). However, SnRK2.2 mutant showed a higher growth rate than the WT cultures under cold stress. The data indicated that cold acclimation of SnRK2.2 mutant was better than that of WT culture under cold stress.

Cold stress causes compositional, structural, and functional changes in plasma membranes of cells via increased lipid peroxidation in photosynthetic organisms (Hara *et al*. 2003; Gülen *et al*. 2008). Protection of the membrane stability is very important for cold acclimation and abiotic stress tolerance in photosynthetic organisms (Uemura *et al*. 2006). Previous studies showed that low temperature could induce oxidative stress and lead to a loss of membrane stability (Gülen *et al*. 2008). Similarly, in the present study, the rate of lipid peroxidation increased depending on the exposure time in the WT culture. SnRK2.2 mutant had a lower lipid peroxidation rate than WT, which was grown at 10°C (Fig. 2). Our data indicated that the SnRK2.2 mutant could have a better survival ability than the WT under low temperature conditions.

The rate of CO_2 assimilation decreases under low temperature conditions in higher plants (Savitch *et al*. 1997; Zhou *et al*. 2006), and cold stress also has an impact on the photosynthetic process, including carbon reduction cycle (Byrd *et al*. 1995). Previous studies reported that low temperature caused a reduction in levels of *rbcL* and *rbcS* transcripts, Rubisco content and initial Rubisco activity (Zhou *et al*. 2006, 2007). In this study, Rubisco protein amount was decreased gradually in the WT culture at 10°C. In contrast, no significant changes were observed in the Rubisco protein amount of SnRK2.2 mutant when compared to the control and WT culture at 10°C (Fig. 3). According to our results, we can suggest that Calvin cycle was not negatively influenced by cold treatment in SnRK2.2 mutant.

Enzymatic and non-enzymatic cellular antioxidant mechanisms play essential roles in cold acclimation (Brüggemann *et al*. 1999; Pastori *et al*. 2000). Antioxidant enzymes are known to protect against ROS which is induced by cold stress. SOD enzymes catalyse the first step by converting superoxide radicals to H_2O_2 and O_2 (Baek & Skinner 2003). In this study, we did not find a significant difference between SOD activities of the WT and SnRK2.2 mutant (Fig. 4b) under low temperature. CAT plays a significant role in the chilling tolerance in maize plants (Prasad 1997), and it is especially important for the removal of hydrogen peroxide in C3 plants (Willekens *et al*. 1997). In a previous study, it was reported that, in *C. reinhardtii*, mercury exposure triggered the activities of SOD, CAT and APX indicating the response of *C. reinhardtii* against ROS accumulation (Elbaz *et al*. 2010). Aksmann *et al*. (2014) also indicated that an increase in CAT and SOD activities in *C. reinhardtii* could be involved in recovery of photosynthesis within 12–24 h under heavy metal stress. Moreover, an increase in hydrogen peroxide accumulation could also be responsible for elevated SOD, CAT and APX activities of *C. reinhardtii* (Vavilala *et al*. 2015).

Table 1. Pearson's correlation test between physiological parameter

	r value			
	GR	*APX*	*SOD*	*CAT*
Rubisco amount	0.7021**	–0.4292	0.9166**	–0.2331
MDA content	–0.753**	0.5215*	–0.753**	0.445

Single asterisks denote statistically significant correlation coefficients ($P < 0.05$) whilst double asterisks denote statistically significant correlation coefficients ($P < 0.01$).
If the r value is near the ±1; a perfect correlation (increase (if positive) or decrease (if negative)).
If the r value is between ±0.50 and ±1; a strong correlation.
If the r value is between ±0.30 and ±0.49; a medium correlation.
If the r value is ±0.29; a small correlation.
No correlation: When the value is zero.

In tobacco plants, Gechev *et al*. (2003) demonstrated that a chilling treatment decreased CAT activity. Pastori *et al*. (2000) also reported a decrease in CAT activity, while APX activity increased in the leaves of maize grown at 15°C. In our study, the activities of CAT and APX increased under cold stress (Fig. 4a–d). Pearson's correlation results indicated no correlation of CAT activity regardless of all physiological parameters (Table 1). However, APX activity showed positive correlation with lipid peroxidation level, while SOD activity showed strongly negative correlation.

GR is also crucial for adaptation during chilling and cold acclimation in higher plants (Kocsy *et al*. 2001). GR activity plays an important role in Halliwell-Asada pathway (Noctor & Foyer 1998), and it is involved in maintaining high ratios of GSH/GSSG associated with increased oxidative stress tolerance. Moreover, previous studies demonstrated that high GSH content and GR activity were related to low temperature tolerance and cold acclimation in several plants (Leipner *et al*. 1999; Kocsy *et al*. 2001). Leipner *et al*. (1999) reported that higher GR activity could occur in chilling-tolerant tomato plants than in sensitive ones. Similarly, in SnRK2.2 mutant, GR activity was much higher in WT culture grown at 10°C (Fig. 4c). In addition, Pearson's correlation test results demonstrated that GR activity showed strong positive correlation with Rubisco protein amount and strong negative correlation with lipid peroxidation level (Table 1). Our results indicated that GR activity could play a role in response to cold acclimation in SnRK2.2 mutant.

In conclusion, plant-specific kinases are involved in the regulation of sulfur metabolism (González-Ballester *et al*.

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2010), and they are considered to be important in response to environmental stress in higher plants (Kulik *et al.* 2011). In this study, we showed that SnRK2s took part in the response to abiotic stress in *C. reinhardtii* as it does in higher plants. Therefore, we can suggest the potential of SnRK2 kinase in cold acclimation process. However, further studies should be done to determine the relationship between environmental stress and sulfur responsive gene in SnRK2.1 and SnRK2.2 single and double mutants in *C. reinhardtii* under various stress conditions.

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