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Chilling tolerance in *Zizania latifolia*

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ABSTRACT

The tumescent stems of *Zizania latifolia* are consumed as vegetable in southern and eastern Asia. This study aimed to compare photosynthesis and chilling tolerance parameters between two well-known *Zizania latifolia* cultivars: Longjiao 2 and Zhejiao 911, which are chilling tolerant and sensitive, respectively. We found that severe cold stress induced photosynthesis inhibition (5°C) resulted from non-stomatal factors. However, net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (Tr) of the cultivar Longjiao 2 were higher than that of Zhejiao 911 with more gradual variations. Six parameters of chlorophyll fluorescence including latent activity of PSII (Fv/F0), efficiency of primary photochemistry (Fv/Fm), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (qN), quantum yield of electric transport (Yield) and the ratio of electric transport at PSII (ETR) were analyzed in the two cultivars. We found that Longjiao 2 had significantly higher Fv/Fm and qN than Zhejiao 911 while qP values were only slightly different for the two lines at severe cold stress. In our experiments, Yield and ETR changed in a similar way in both Longjiao 2 and Zhejiao 911. In addition, the ability of heat dissipation of Longjiao 2 was statistically higher than that found in Zhejiao 911 when treated at 5°C for one day. These data suggest that cultivar Longjiao 2 induces chilling tolerance by modulating critical pathways including photosynthesis and energy dissipation.

Keywords: Longjiao 2, Zhejiao 911, cold stress, photosystem, photoinhibition.

RESUMO

Tolerancia ao frio em *Zizania latifolia*

Os caules intumescidos de *Zizania latifolia* são consumidos como vegetais no sul e leste da Ásia. Este estudo teve como objetivo avaliar os parâmetros de fotossíntese e de tolerância ao frio entre duas cultivares conhecidas de *Zizania latifolia*: Longjiao 2 e Zhejiao 911, que são tolerante e sensível ao resfriamento, respectivamente. Descobrimos que indução severa de estresse ao frio induziu à inibição da fotossíntese (5°C), como resultado de fatores não-estomáticos. No entanto, a taxa líquida fotossintética (Pn), a condutância estomática (Gs) e a taxa de transpiração (Tr) da cultivar Longjiao 2 foram maiores do que de Zhejiao 911 com mais variações graduais. Seis parâmetros de fluorescência da clorofila, incluindo a atividade latente de PSII (Fv/F0), a eficiência da fotoquímica primária (Fv/Fm), o coeficiente fotoquímico de têmpera (Qp), o coeficiente de extinção não-fotoquímica (qN), rendimento quântico de transporte elétrico (Yield) e a razão de transporte elétrico em PSII (ETR) foram analisados nas duas cultivares. Longjiao 2 teve significativamente maior Fv/Fm e qN que Zhejiao 911 enquanto que os valores qP foram apenas ligeiramente diferentes para as duas cvs. sob estresse severo causado pelo frio. Nas nossas experiências, Yield e ETR alteraram-se de forma semelhante em ambas as cultivares. Além disso, a capacidade de dissipação de calor de Longjiao 2 foi estatisticamente superior àquela encontrada em Zhejiao 911 quando tratada a 5°C durante um dia. Estes dados sugerem que cv. Longjiao 2 induz tolerância ao frio através da modulação de rotas críticas, incluindo a fotossíntese e a dissipação de energia.

Palavras-chave: Longjiao 2, Zhejiao 911, estresse ao frio, fotossistema, fotoinibição.

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Environmental stresses are caused by conditions that are unfavorable for the optimal growth and development of plants (Guy, 1999). Low temperature, or chilling stress is one of the most detrimental environmental stresses affecting plant growth and its unique environmental impact on crop plant physiology has been recognized for over 70 years (Kratsch & Wise, 2000). The effects of chilling stress vary with plant species (Valluru *et al.*, 2012) and can cause injury and even cell death in cold-sensitive plants (Wang *et al.*, 2001). However, plants exposed to low but non-freezing temperatures enhance cold tolerance via many complex physiological and biochemical changes (Chen *et al.*, 2015). A clear understanding of the molecular mechanisms through which plants respond to low temperatures is of fundamental importance to the transgenic strategies to improve stress tolerance in crops (Xu, 2012). Temperature affects a broad spectrum of cellular components and metabolism, and extreme temperatures impose stresses of variable severity depending on the rate of temperature change, intensity and duration (Iba, 2002).

The chilling tolerance of plants is known to be closely related to photosynthesis (Sun *et al.*, 2006). Low temperature stress plays an important role in a series of physiological and biochemical functions (Sun *et al.*, 2006). Photosynthesis is an important assimilation pathway in plants and has been widely used as a physiology index. It is known that low temperatures could impact photosynthesis as well as the accumulation of matter (Crafts-Brander *et al.*, 1990). Indeed, the effects of low temperatures on plant physiology and especially the photosynthetic capacity were reported a few decades ago (Wataru *et al.*, 2014). The effect of low temperatures on photosynthetic capacity was shown by decreasing the activity of various enzymes involved in the photosynthesis and inhibiting the regeneration of PSII (Liu *et al.*, 2012). Sometimes this can cause irreversible damages in the photosynthetic apparatus and reduce the efficiency of energy utilization (Xie *et al.*, 2008). Chlorophyll can be regarded as an intrinsic fluorescent probe of the photosynthetic system and has been used as an important tool in plant physiology (Mohammed *et al.*, 1995). Indeed, chlorophyll fluorescence measurements have been widely utilized to study the effects of various parameters on photosynthesis, including light quality (Shibuya *et al.*, 2012) and low temperature stress (Hu *et al.*, 2008).

Most studies dealing with the molecular responses of plants to cold stress were carried out in the cold-tolerant plant *Arabidopsis thaliana* (Seki *et al.*, 2001). These studies are of great interest for other cold-tolerant species such as cereals. However, little is known about the induction mechanisms of cold tolerance in cold-sensitive plants, which can help to understand the cold-acclimation processes in such species. *Zizania latifolia*. (Poaceae) is a perennial and hydrophytic plant which has been cultivated for approximately one thousand years. *Z. latifolia* is rich in proteins, carbohydrates, coarse-fibers and vitamins, and is highly appreciated for its high nutritious value. It grows in South China with the suitable growth temperature of 15-30°C. However, there are some differences among the *Zizania latifolia* cultivars. For example, cultivar Longjiao 2, a mutant with high chilling-tolerance and other valuable features (He *et al.*, 2009), is harvested in December, later in the year than cultivar Zhejiao 911, a normal and cold-sensitive cv. Therefore, it will be interestingly to understand the differentiated chilling tolerance of cultivars Longjiao 2 and Zhejiao 911.

In our study, we analyzed varied photosynthesis changing patterns between cultivars Longjiao 2 and Zhejiao 911 at low temperatures. The results confirmed that Longjiao 2 has a higher chilling tolerance than Zhejiao 911. Using multiple parameters of photosynthesis, we attempted in this work to dissect possible explanation for the chilling tolerance induction in cultivar Longjiao 2.

MATERIAL AND METHODS

Plant materials and chilling treatments - Two *Zizania latifolia* cultivars were used in these experiments, including the chilling tolerant cv. named Longjiao 2 and Zhejiao 911, which is more sensitive to cold stress. Suckering plants of the two cvs. (obtained from a planting base in Longxiang Town, Tongxiang City, Zhejiang Province, China) were transplanted to flowerpots (80×60×50cm) at 5-leaf stage and grown at normal temperatures for a period of 15 days. After that, plants were transferred to automatic climate chambers at 5 or 10°C for 5 days with a 16 h photoperiod (80 μmol/m^2/s), at a relative humidity of 70-80% , and further grown at 25°C for 5 days for recovery. Plants grown at 25°C for 10 days in automatic climate chambers were selected as control.

At 1, 3, and 5 days after plants treated with temperatures (5, 10 and 25°C) or resumed growth at 25°C, the middle parts of the second leaves from the lasts were chosen for measurement. The whole experiment was repeated for a total of three times.

Measurements of photosynthesis - Net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and transpiration rate (Tr) of leaf segments were measured with a portable LI-6400 apparatus (LI-COR Biosciences, USA), following the manufacturer's instructions.

Chlorophyll fluorescence measurements - Chlorophyll fluorescence was recorded with a pulse amplitude modulation fluorometer (PAM-2000, Heinz-Walz-GmbH, Effeltrich, Germany) as previously described (Flexas *et al.*, 2002).

Assessment of energy dissipation - Energy dissipation was evaluated according to well established protocols (Schreiber *et al.*, 1986; Adams III *et al.*, 1999) .

Statistical analyses - The Microsoft Excel software was used to calculate the mean values and standard errors. Multiple comparisons were performed by the Duncan's method ($p<0.05$).

RESULTS AND DISCUSSION

Effects of low temperature on photosynthesis parameters - When grown at low temperatures, the same trends of variation were observed for photosynthesis parameters in the two cultivars with a decrease of Pn, Gs and Tr while Ci values were increased (Figure 1). These findings indicated that photosynthesis inhibition by severe low temperature stress resulted from non-stomatal limitations.

However, there were clear differences between the absolute values obtained for the various parameters in the two cultivars. When treated at 5°C for one day, Pn, Gs and Tr values were sharply reduced and close to zero in Zhejiao 911 while in Longjiao 2 these three parameters were significantly higher and showed only slight changes (Figure 1). In parallel, the Ci values were significantly elevated in 'Zhejiao 911' in comparison with 'Longjiao 2' at this time point. These data indicate a higher photosynthesis rate in 'Longjiao 2' compared with 'Zhejiao 911' at low temperatures, in good accordance with the chilling tolerance capacity of the two cultivars. It was implied that cultivar Longjiao 2 exhibited higher chilling tolerance (photochemical efficiency and the ability of heat dissipation) than Zhejiao 911, and was better protected at low temperatures.

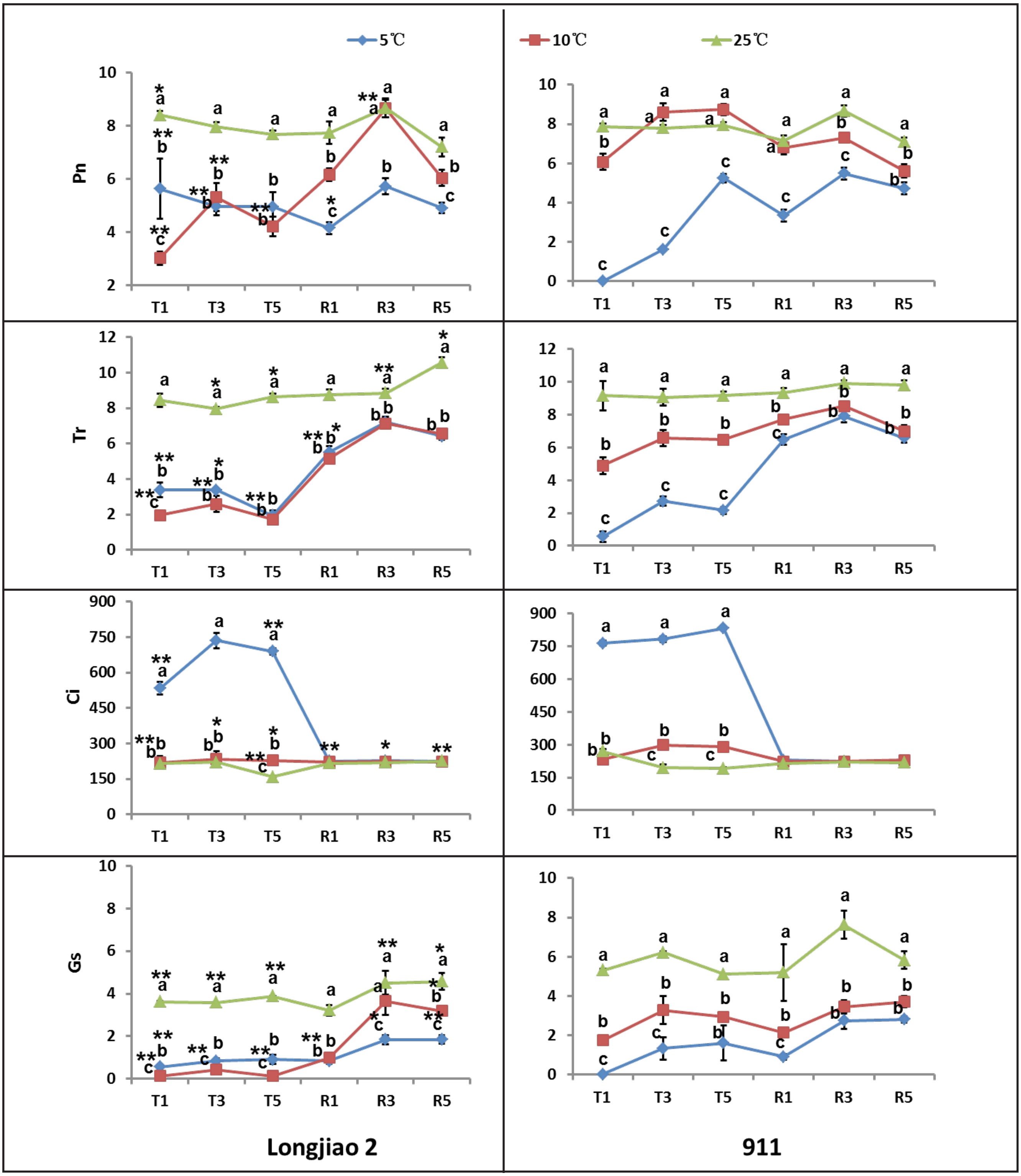

Figure 1. Changes in Pn, Gs, Ci and Tr after treatment of 'Longjiao 2' and 'Zhejiao 911' with different temperatures (variações no Pn, Gs, Ci e Tr após o tratamento de 'Longjiao 2' e 'Zhejiao 911' com diferentes temperaturas). T1, T3, T5: 1d, 3d and 5d after treatment (T1, T3, T5: 1d, 3d e 5d após o tratamento); R1, R3, R5: 1d, 3d and 5d of recovery at 25°C (recuperação de R1, R3, R5: 1d, 3d e 5d a 25°C); Pn: net photosynthetic rate (taxa fotossintética líquida); Gs: stomatal conductance (condutância estomática); Tr: transpiration rate (taxa de transpiração); Ci: intercellular CO_2 concentration (concentração intercelular de CO_2); The error bars represent the standard error (±SE) for three replications {as barras de erro representam o erro padrão (±SE) para três repetições}. * and ** indicate significant differences at levels 0.05 and 0.01 with respect to 'Zhejiao 911' (* e ** indicam diferenças significativas no nível de 0,05 e 0,01 para 'Zhejiao 911'). Different characters indicate significant differences at level 0.05 with statistical comparison among 5, 10, and 25°C (letras diferentes indicam diferenças significativas ao nível de 0,05 na comparação estatística entre 5, 10, e 25°C). No characters indicate no statistically differences at 0.05 among treatments (nenhum caractere indica não haver diferenças estatistica ao nível de 0,05 entre os tratamentos). Hangzhou, Zhejiang, China Jiliang University, 2014.

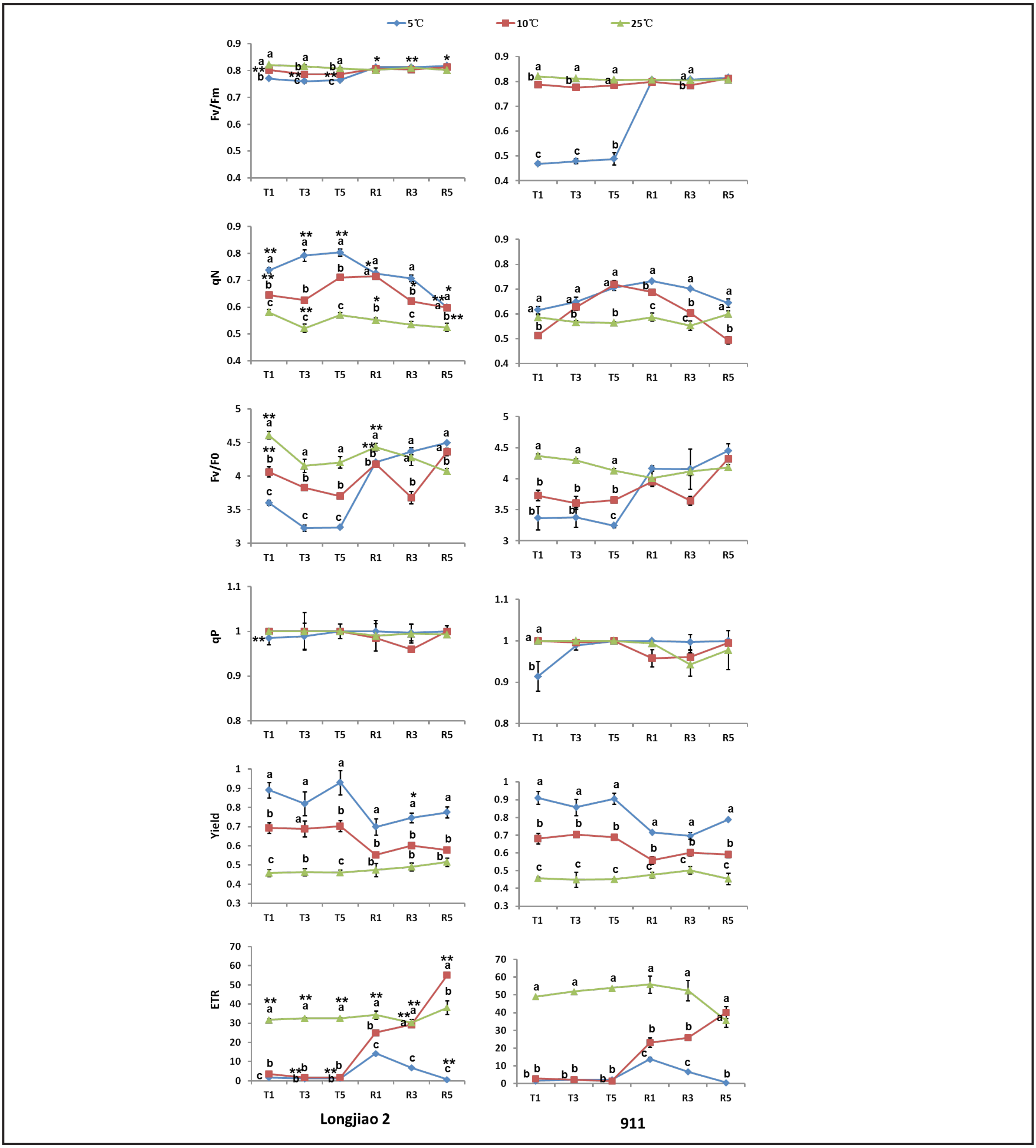

Figure 2. Changes in the parameters of chlorophyll fluorescence during growth of *Zizania latifolia* of 'Longjiao 2' and 'Zhejiao 911' in different temperature conditions (variações nos parâmetros da fluorescência da clorofila durante o crescimento de *Zizania latifolia*, cvs. Longjiao 2 e Zhejiao 911 em diferentes condições de temperatura). T1, T3, T5: 1d, 3d and 5d after treatment (T1, T3, T5: 1d, 3d e 5d após o tratamento); R1, R3, R5: 1d, 3d and 5d of recovery at 25°C (recuperação de R1, R3, R5: 1d, 3d e 5d a 25°C); Fv/F0: latent activity of PSII (atividade latente de PSII); Fv/Fm: efficiency of primary photochemistry (eficiência da fotoquímica primária); qP: photochemical quenching (extinção fotoquímica); qN: non-photochemical quenching (extinção não-fotoquímica); Yield: quantum yield of electric transport (rendimento quântico de transporte elétrico); ETR: ratio of electric transport at PSII (razão de transporte elétrico em PSII); The error bars represent the standard error (±SE) for three replications {as barras de erro representam o erro padrão (±SE) para três repetições}. * and ** indicate significant differences at level 0.05 and 0.01 with respect to 'Zhejiao 911' (* e ** indicam diferenças significativas no nível de 0,05 e 0,01 com relação ao 'Zhejiao 911'). Different characters indicate significant differences at 0.05 in statistical comparison among 5, 10, and 25°C (caracteres diferentes indicam diferenças significativas no nível de 0,05 na comparação estatística entre 5, 10, e 25°C). No characters indicate no statistically differences at level 0.05 among treatments (nenhum caractere indica não haver diferenças estatisticamente a 0,05 entre os tratamentos). Hangzhou, Zhejiang, China Jiliang University, 2014.

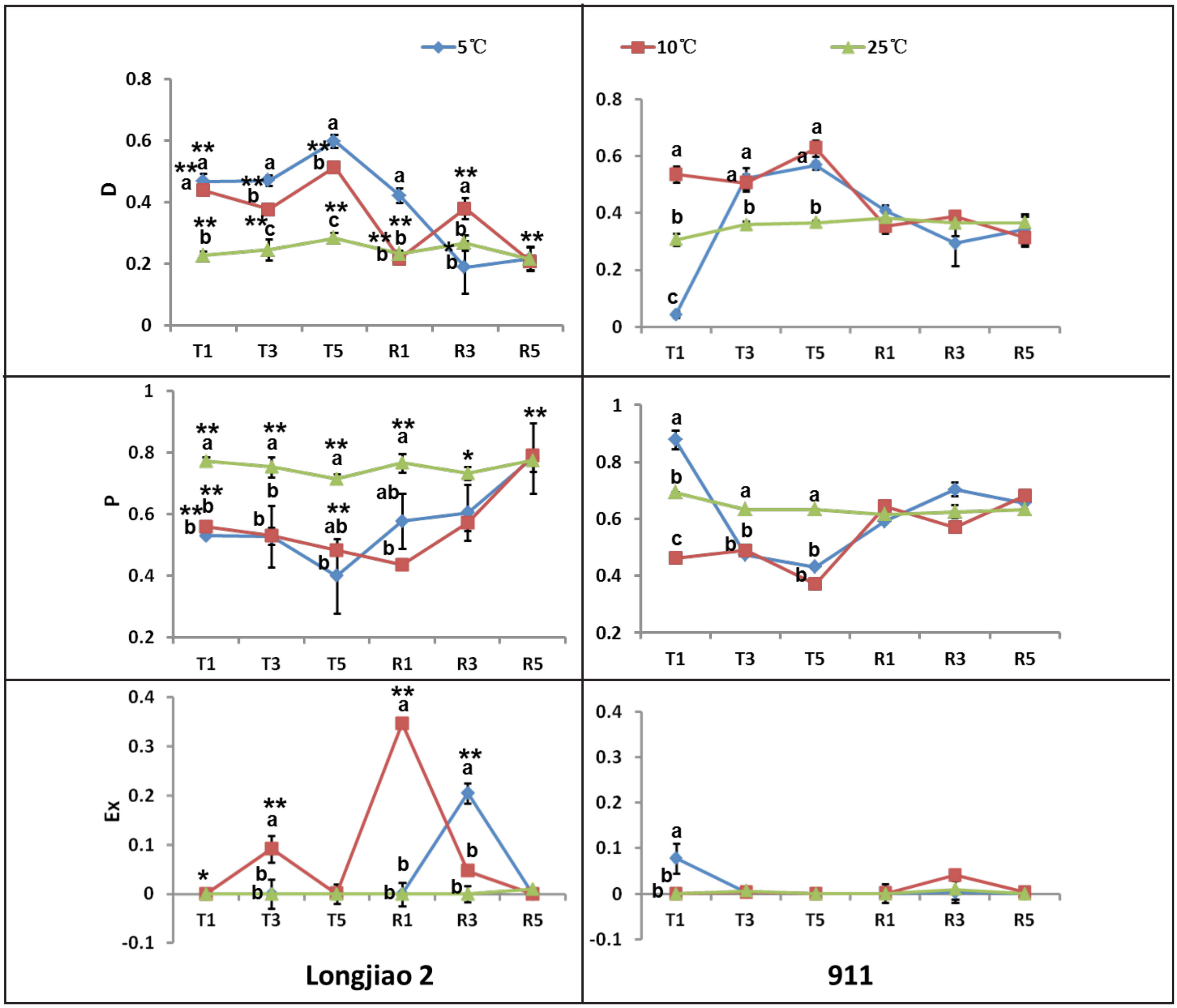

Figure 3. Energy dissipation changes in *Zizania latifolia* cultivars Longjiao 2 and Zhejiao 911 at different temperatures (alterações da dissipação de energia em *Zizania latifolia* cvs. Longjiao 2 e Zhejiao 911 em diferentes temperaturas). T1, T3, T5: 1d, 3d and 5d after treatment (T1, T3, T5: 1d, 3d e 5d após o tratamento); R1, R3, R5: 1d, 3d and 5d of recovery at 25°C (R1, R3, R5: recuperação de 1d, 3d e 5d a 25°C); Ex: portion of excess energy (porção de excesso de energia); D: portion of antenna thermal dissipation energy (porção de dissipação de energia térmica antenna); P: portion of absorbed light energy utilized in the PSII photochemistry (parte da energia luminosa absorvida utilizada na fotoquímica PSII); The error bars represent the standard error (±SE) for three replications (barras de erro representam o erro padrão (±SE) para três repetições). * and ** indicate significant differences at 0.05 and 0.01 with respect to 'Zhejiao 911' (* e ** indicam diferenças significativas a 0,05 e 0,01 com relação à cultivar Zhejiao 911). Different characters indicate significant differences at 0.05 in statistical comparison among 5, 10, and 25°C (caracteres diferentes indicam diferenças significativas a 0,05 na comparação estatística entre 5, 10, e 25°C). No characters indicate no statistical differences at 0.05 among treatments (nenhum caractere indica não haver diferença estatistica a 0,05 entre os tratamentos). Hangzhou, Zhejiang, China Jiliang University, 2014.

Nevertheless plants have mechanisms for "self-protection" to compensate the effects of low temperature including heat dissipation, irreversible photo-damage of photosystem II (PSII) reaction centers, and Mehler reaction (Demmig-Adams *et al.*, 1990). In order to detect the self-protection of two lines, we prolonged growth at the low temperature. The reduced photosynthesis gradually recovered as reflected by the measured parameters. However the recovery was not total since the values of photosynthetic parameters (except Ci) were significantly lower than levels obtained at 25°C for both cultivars. And there was no significant deviation detected for these parameters (except Gs) between two cultivars at 5 days of recovery after treated at 5 or 10°C. Despite the chilling tolerance of 'Longjiao 2', low temperatures caused damages in plant photosynthesis.

Effects of low temperature on chlorophyll fluorescence - Chlorophyll can be regarded as an intrinsic fluorescent probe of the photosynthetic system and its fluorescence measurements are increasingly applied to various fields of plant physiology. In the leaf, the fluorescence yield is influenced in a very complex manner by events directly or indirectly related to photosynthesis (Patrick *et al.*, 2014). In our study, various parameters of chlorophyll fluorescence were evaluated including latent activity of PSII (Fv/F0), efficiency of primary photochemistry (Fv/Fm), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (qN), quantum yield of electric transport (Yield) and ratio of electric transport at PSII (ETR) in the two cultivars (Figure 2).

We found that chilling-induced photoinhibition was remarkable in the two cvs. At low temperature (5°C), Fv/F0 and Fv/Fm were significantly decreased in both studied cvs and the change was more pronounced in 'Zhejiao 911'. After the plants were transferred to temperature of 25°C, both lines recovered to the levels of control rapidly (Figure 2). Interestingly, the values of Fv/Fm obtained for 'Longjiao 2' were significantly higher than that of 'Zhejiao 911' during cold stress (5°C), indicating that the chilling-induced photoinhibition was less pronounced in 'Longjiao 2' in comparison with 'Zhejiao 911'.

The qP values reflect the efficiency of excitation energy captured by open PSII reaction centers. No significant change was observed in qP values of cv. Longjiao 2 after treatment with low temperature. This was not the case for 'Zhejiao 911' which showed significantly decreasing in qP when treated at 5°C for one day. Indeed, in cold stress conditions (5°C) qP values of 'Zhejiao 911' at first day were statistically lower compared with 'Longjiao 2' (Figure 2). These changes observed in qP show that 'Longjiao 2' was less affected by low temperature.

Due to light loss of reaction centers and the reduction of the efficiency of excitation energy captured by open PSII reaction centers at low temperature, PSII reaction centers and the activity of electric transport antenna at PSII were injured and resulted in inhibition of photochemistry (Mauro *et al.*, 1997). As a mechanism of self-protection, the qN could prevent the organization of photosynthesis from injury. It reflected the energy of heat dissipation in the excitation energy captured by PSII antenna pigment which was not utilized in electric transport. Upon exposure to low temperature, qN values were significantly higher in both cvs. in comparison to controls except 'Zhejiao 911' at 5°C for one day. Although both of them fully recovered after transfer to natural temperature, there were obvious differences in the adjustment patterns of qN in the two cvs. qN increased gradually in 'Longjiao 2' during the treatment at 5°C and a gradual decrease was observed after transfer into natural conditions of 25°C (Figure 2). In contrast, qN continued to increase in 'Zhejiao 911' until the first day of recovery treatment before decreasing thereafter. In addition, 'Longjiao 2' showed qN values significantly higher than 'Zhejiao 911' under cold stress (5°C). These findings indicate a higher ability and sensitivity of self-protection of 'Longjiao 2' when grown at low temperatures compared with 'Zhejiao 911'. Our data show a decrease in the photochemical quenching of Zhejiao 911 and this cultivar displayed a significantly lower non-photochemical quenching than 'Longjiao 2' at low temperatures. Therefore, decrease in the photochemical quenching of 'Zhejiao 911' is likely due to the accumulation of the excess excitation energy and the inhibition of photosynthetic capacity caused by the deactivation of reaction centers and the damage of antenna system at low temperatures.

In our experiments, we found that the changes in Yield and ETR were similar in both cultivars except for the last day of treatment at 25°C (Figure 2). Interestingly, Yield and ETR were significantly higher and lower, respectively, at low temperature of 5°C in comparison with control plants grown at 25°C. The yield of photochemical quantum was inversely proportional to the decrease growth temperature and therefore decreased quickly once transferred in natural conditions. When compared between two cultivars, ETR of 'Longjiao 2' was significantly higher than that of 'Zhejiao 911' at the end of treatments, indicating better recovery in transferring exciting light energy to the PSII reaction center in 'Longjiao 2'.

Effects of low temperature on changes in energy dissipation - It is well known that effective heat dissipation prevents chilling-induced inhibition of photosynthesis (Wang & Guo, 2005). Cold stress can cause irreversible damages in the photosynthetic apparatus with reduced efficiency in the utilization of energy (Xie *et al.*, 2008). In this work, the changes in energy dissipation of leaves were evaluated in cultivar Longjiao 2 as well as cultivar Zhejiao 911 at different temperatures and the results are summarized in Figure 3. We found that the portion of energy dissipation by antenna pigments of the light energy captured by PSII reaction centers (D) was increased in both cvs. The portion of absorbed light energy utilized in the PSII photochemistry (P) decreased in the two cvs. as well although the patterns of change were different in both varieties (Figure 3). Interestingly, 87.8% of light energy was utilized in the photochemical reaction at first day of 5°C and then sharply decreased to 47.4% in 'Zhejiao 911'. These findings are consistent with many studies and indicate that an increase in the thermal dissipation in the PSII antennae competes with the excitation energy transfer from the PSII antennae to PSII reactions centers, thus resulting in a decrease in the efficiency with the excitation energy captured by "open" PSII reaction centers (P). The component of absorbed light not going into either P or D is labelled as "excess energy" (Ex). Ex includes not only "excess" energy but also reflects non-light induced quenching processes. This portion of energy has the greatest potential to cause PSII photoinactivation because it represents light energy that is trapped by closed PSII reaction centers. Indeed, there was an increase in the excess energy in the photosynthetic apparatus during the reduction of photosynthetic rate. While the excess of light energy (Ex) increased only in the 'Zhejiao 911' at first day of 5°C and 'Longjiao 2' at third day of 10°C when treated with low temperature. Different to relatively stable variation of energy dissipation of 'Zhejiao 911' at recovering period, 'Longjiao 2' showed gradually increased P, significantly varied D and Ex. At 5 days of recovery, 'Longjiao 2' reached a significantly higher P and lower D than 'Zhejiao 911'. These indicated that 'Longjiao 2' exhibited chilling tolerance by maintaining relatively higher photosynthetic energy capture and utilizing the increased antenna thermal energy dissipation at low temperature. 'Longjiao 2' demonstrated a significantly higher photosynthetic capacity than 'Zhejiao 911' by a higher photosynthetic rate and low energy dissipation in antenna system at 5 days of recovery.

In these studies, the physiological changes observed in both cvs. suggest that inhibition of photosynthesis at low temperatures of 5°C and 10°C resulted from non-stomatal limitations. Interestingly, photosynthesis inhibition in 'Longjiao 2' was not as pronounced as 'Zhejiao 911'. In addition, chlorophyll fluorescence showed that 'Longjiao 2' had a higher chilling tolerance than 'Zhejiao 911' with higher Fv/Fm and qN and less important changes in qP values under the similar variations of Yield and ETR. In summary, we confirmed that 'Longjiao 2' had higher chilling tolerance than 'Zhejiao 911' under severe low temperature stress (5°C) and suggested that cultivar 'Longjiao 2' induced chilling tolerance by modulating pathways including photosynthesis and energy dissipation.

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