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Photoinhibition of oxygen-evolving complex and photosystem II at chilling stress in the tropical tree species *Dalbergia odorifera*

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Abstract

Photosystem II is sensitive to chilling stress in tropical tree species. However, the underlying mechanism is not clear. In this study, we examined the effects of chilling and light stress on activities of PSII and oxygen-evolving complex (OEC), in order to resolve the controversy of whether chilling-induced PSII photoinhibition is attributed to the excess-energy model or the two-step scheme. We determined changes in chlorophyll *a* fluorescence transient and energy distribution in PSII during treatment at chilling-light stress for the tropical tree species *Dalbergia odorifera*. At chilling temperature, the light-use efficiency was largely inhibited. During chilling treatment, the maximum quantum yield of PSII gradually decreased. Meanwhile, the relative fluorescence intensity at K-step (300 μs) significantly increased, indicating the photodamage to OEC. A tightly positive correlation was found between photodamage to OEC and PSII photoinhibition, suggesting that photodamage to OEC was a rate-limiting step for PSII photoinhibition. Furthermore, additional generation of reactive oxygen species did not aggravate PSII photoinhibition at chilling temperature. These results suggest that two-step model is responsible for chilling-induced PSII photoinhibition in the tropical tree species *D. odorifera*.

Additional key words: chlorophyll fluorescence; JIP-test; low temperature; photosynthesis.

Introduction

Chilling temperatures above zero are the major limitation to the distribution of tropical plants at higher latitudes and altitudes. Due to the exacerbation of tropical forest fragmentation and increasing demand on tropical hardwood timber, forestation using tropical high-quality timber species in marginal tropical areas is presently under practice. Occasionally, cold snaps invade these areas for several days and cause severe damage to the introduced tropical crops and plants. Our previous studies indicated that chilling temperature associated with moderate light intensity caused strong photodamage to PSII but little photodamage to PSI in tropical tree species (Huang *et al*. 2010a,b), similar to pumpkin, spinach (Barth and Krause 1999), and *Paphiopedilum* species (Yang *et al*. 2017). However, the underlying mechanism of chilling-induced photoinhibition of PSII in tropical tree species is not well known.

Light is the driving power of photosynthesis. However, excess light energy absorbed by leaves, being neither utilized by photochemistry nor dissipated harmlessly as heat by nonphotochemical quenching, induces the generation of reactive oxygen species (ROS), which could induce photoinhibition of PSII. At present, there are two proposed schemes to explain the molecular mechanism of photoinhibition of PSII. In the excess-energy scheme, photodamage of PSII is a one-step process: ROS cause direct oxidative damage to PSII complexes (Vass *et al*. 1992, Telfer *et al*. 1994, Okada *et al*. 1996, Chow and Aro 2005, Krieger-Liszkay *et al*. 2008, 2011; Vass 2011). However, the more recent two-step scheme demonstrates

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Abbreviations: F_t – fluorescence level at time t; F_v/F_m – maximum quantum yield of PSII after dark adaptation; F_v'/F_m' – maximum quantum yield of PSII after light adaptation; MV - methyl viologen; q_P – photochemical quenching coefficient; ROS – reactive oxygen species; V_K - relative variable fluorescence at 300 μs; W_K – the ratio of variable fluorescence F_K to the amplitude $F_J – F_0$; V_t - relative variable fluorescence at time t; $Y_{(II)}$ – effective quantum yield of PSII photochemistry; $Y_{(NO)}$ – quantum yield of nonregulated energy dissipation in PSII; $Y_{(NPQ)}$ – quantum yield of regulated energy dissipation in PSII *via* nonphotochemical quenching.
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that the primary photodamage to PSII occurs at the oxygen-evolving complex (OEC) with release of manganese ions (Mn^{2+}) (Hakala *et al*. 2005, Zsiros *et al*. 2006, Antal *et al*. 2009, Murata *et al*. 2012). The disruption of the manganese cluster upon absorption of light is a primary event in photodamage (Tyystjärvi 2008, Zavafer *et al*. 2015, 2017). Following photodamage to OEC, the supply of electrons from water to the primary electron donor of PSII ($P_{680}^{+\bullet}$) is blocked, and, as a result, the level of $P_{680}^{+\bullet}$ remains high (Murata *et al*. 2007, Takahashi and Murata 2008, Takahashi and Badger 2011). P_{680}^{+} is a strong oxidant and at high levels might damage the PSII reaction centers (Takahashi and Murata 2008). Furthermore, in the two-step scheme, ROS inhibit the repair of photodamaged PSII but do not aggravate the rate of photodamage (Nishiyama *et al*. 2001, 2004, 2005, 2006, 2011; Allakhverdiev and Murata 2004, Takahashi *et al*. 2009). Interestingly, Oguchi *et al*. (2011) suggest that both mechanisms lead to photodamage of PSII under high light, the underlying mechanism of PSII photodamage in higher plants is largely controversial (Murata *et al*. 2012, Vass 2012).

Net PSII photoinhibition occurs only when the rate of PSII photodamage exceeds the rate of recovery. At chilling temperature, the linear electron flow was largely inhibited (Huang *et al*. 2011, 2016a; Yang *et al*. 2018), resulting in inhibition of ATP synthesis (Huang *et al*. 2017). Consequently, the ATP-dependent fast *de novo* synthesis of D1 protein was largely inhibited at chilling temperature (Allakhverdiev and Murata 2004). Therefore, the extent of PSII photoinhibition at chilling stress is mainly determined by the rate of PSII photodamage. According to the excess-energy scheme, singlet oxygen generated in PSII damages the D1 protein of PSII (Vass 2011). In the addition of methyl viologen, which accepts electrons from PSI to O_2, superoxide anion radicals damage PSII in isolated thylakoids of tobacco (Krieger-Liszkay *et al*. 2011). By comparison, at chilling temperature, visible light damages OEC prior to photodamage to the PSII reaction center in isolated PSII membrane fragments of spinach (Zavafer *et al*. 2015, 2017). As a result, the mechanisms of PSII photoinhibition induced by chilling-light stress have not yet been clarified.

To test the scheme, which is responsible for the chilling-induced photoinhibition of PSII in the tropical tree species *Dalbergia odorifera*, we determined the effects of chilling-light stress on photodamage of PSII and OEC by measurements of chlorophyll (Chl) *a* fluorescence transient and light-adapted Chl fluorescence. The following questions were addressed: (*1*) Is photodamage to OEC a rate-limiting step for PSII photoinhibition? (*2*) Do additional ROS accelerate PSII photoinhibition at chilling temperature?

Materials and methods

Plant materials and growth conditions: The chilling-sensitive tropical tree *Dalbergia odorifera* T. Chen (Fabaceae) is a light-demanding tree species occurring in secondary forests and is native to the Hainan Island, China. It produces high-quality timber. Sun leaves represent the leaves of 3-year-old seedlings grown in an open site with full sunlight. Shade leaves represent the leaves of 2-year-old seedlings grown under a shaded environment with 10% sunlight controlled by a shade net. These seedlings were cultivated under good water and nutrition conditions. They exhibit good growth in Xishuangbanna Tropical Botanical Garden (21°54'N, 101°46'E, and altitude 570 m) located in the northern boundary of tropical zone. The highest PPFD at midday is up to 1,850 μmol(photon) m^{-2} s^{-1} in summer and 1,350 μmol(photon) m^{-2} s^{-1} in winter, respectively. Mean air temperatures are approximately 34/24°C (day/night) in summer and 25/14°C (day/night) in winter.

Photoinhibitory treatment: Sun leaves on branches, which were immersed in water, were placed in a thermostatic 4°C chilling storage room and were illuminated under light (LED optical source, *Chengdu Maiyue Technology Ltd.*, China). To examine the effect of chilling stress on OEC, Chl *a* fluorescence transient OKJIP was determined in sun leaves during chilling treatment at 4°C under a PPFD of 430 μmol(photon) m^{-2} s^{-1}. To examine the effect of ROS on PSII photodamage in *D. odorifera* illuminated at chilling temperature, detached shade leaves were vacuum infiltrated with either H_2O or 300 μM methyl viologen (MV) to promote electrons from PSI to O_2 at 25°C for 1 h in darkness and then illuminated at 4°C for 2 h under a light of 430 μmol(photon) m^{-2} s^{-1}. MV can abolish cyclic electron flow by promoting electrons from PSI to O_2. The effect of MV on PSII photodamage in sun leaves of *D. odorifera* includes at least two mechanisms: abolishing cyclic electron flow, and increasing production of ROS. Because cyclic electron flow activity was much lower in the shade leaves than that of the sun leaves (data not shown), the effect of MV on PSII photodamage in the shade leaves can be mainly interpreted by increasing generation of ROS. Therefore, we chose the shade leaves in order to examine the effect of ROS on PSII photodamage in *D. odorifera* illuminated at chilling temperature.

Chl fluorescence measurements: Chl *a* fluorescence transient is a useful noninvasive technique to monitor and predict photosynthetic responses to various abiotic stresses (Li *et al*. 2009, Swoczyna *et al*. 2010, Dąbrowski *et al*. 2015, 2016, 2017; Mathur *et al*. 2016). In this study, Chl *a* fluorescence transient was determined by a *Dual-PAM100* (*Heinz Walz*, Effeltrich, Germany) after dark adaptation at 25°C for 30 min. Each transient obtained from the dark-adapted samples was analyzed according to the JIP-test (Strasser *et al*. 2000, 2004; Kalaji *et al*. 2018): (*1*) the fluorescence intensity at 30 μs (F_0, when all reaction centers of PSII are open); (*2*) the maximum fluorescence intensity (F_m, when all reaction centers of PSII are closed), and (*3*) the fluorescence intensities at 300 μs (K-step), 2 ms (J-step), and 30 ms (I-step).

The maximum quantum yield of PSII after dark adaptation was calculated as $F_v/F_m = (F_m - F_0)/F_m$. The relative variable fluorescence intensity was calculated as $V_t = (F_t - F_0)/(F_m - F_0)$, where F_t is the fluorescence intensity at any time upon illumination with a pulse [10,000 μmol(photon) m^{-2} s^{-1} and 300 ms]. The change in V_t during

chilling treatment compared with the control was calculated as $\Delta V_t = [(F_t - F_0)/(F_m - F_0)]_{treatment} - [(F_t - F_0)/(F_m - F_0)]_{control}$. In this study, $[(F_t - F_0)/(F_m - F_0)]_{control}$ represents the value of $(F_t - F_0)/(F_m - F_0)$ measured before chilling treatment. The ratio of variable fluorescence F_K to the amplitude $F_J - F_0$ (W_K) was calculated as $W_K = (F_K - F_0)/(F_J - F_0)$.

The energy distribution in PSII was also determined by the *Dual-PAM100*. F_0 and F_m was determined after dark adaptation at 25°C for 30 min. Leaves on detached branches were light adapted at 25°C and 430 μmol(photon) m^{-2} s^{-1} for at least 20 min. Afterwards, PSII parameters were measured at 25 and 4°C. The following chlorophyll fluorescence parameters were calculated: $F_v/F_m = (F_m - F_0)/F_m$, $F_v'/F_m' = (F_m' - F_0')/F_m'$, $F_0' = F_0/(F_v/F_m + F_0/F_m')$ (Oxborough and Baker 1997), $q_P = (F_m' - F_s)/(F_m' - F_0')$, $Y_{(II)} = (F_m' - F_s)/F_m'$ (Genty *et al*. 1989), $Y_{(NPQ)} = F_s/F_m' - F_s/F_m$, $Y_{(NO)} = F_s/F_m$ (Hendrickson *et al*. 2004, Kramer *et al*. 2004). F_0 and F_0' are the dark-adapted and light-adapted minimum fluorescence. F_m and F_m' are the dark-adapted and light-adapted maximum fluorescence upon illumination with a pulse (300 ms) of saturating light [10,000 μmol(photon) m^{-2} s^{-1}], respectively. F_s is the light-adapted steady-state fluorescence. F_v'/F_m' is the maximum quantum yield of PSII after light adaptation. The parameter $1 - q_P$ can be used to indicate the excitation pressure in PSII or the proportion of closed PSII reaction centers. $Y_{(II)}$ is the effective quantum yield of PSII. $Y_{(NPQ)}$ corresponds to the fraction of energy dissipated in form of heat *via* the regulated photoprotective NPQ mechanism. $Y_{(NO)}$ is the fraction of energy that is passively dissipated in form of heat and fluorescence. A high value of $Y_{(NO)}$ indicates that the excess light energy in PSII cannot be quenched by photochemistry and NPQ. All measured and calculated fluorescence parameters were listed in the text table.

Statistical analysis: The results were displayed as mean values of four independent experiments. One-way analysis of variance (*ANOVA*) test was used at α=0.05 significance level to determine whether significant differences existed between different treatments.

Results

Light-adapted PSII parameters: We first determined the light-adapted PSII parameters at 25 and 4°C. When shifted from 25 to 4°C, the light-adapted maximum quantum yield of PSII (F_v'/F_m') significantly decreased at the chilling temperature of 4°C (Fig. 1*A*). The value of $1 - q_P$ increased largely from 0.13 (at 25°C) to 0.84 (at 4°C) (Fig. 1*B*), indicating that the most of PSII reaction centers were closed at the chilling temperature of 4°C. Because of the large decreases in F_v'/F_m' and q_P, $Y_{(II)}$ largely decreased from 0.63 (at 25°C) to 0.10 (at 4°C) (Fig. 1*C*). These results suggest that the photosynthetic CO_2 assimilation was strongly inhibited at the chilling temperature of 4°C, which caused depression of $Y_{(II)}$. The value of $Y_{(NPQ)}$ (fraction of energy dissipated in form of heat *via* the regulated photoprotective NPQ mechanism) largely increased when transferred abruptly from 25 to 4°C (Fig. 1*D*), indicating that the activation of NPQ was a rapid photoprotective response at chilling temperature. The value of $Y_{(NO)}$ slightly increased at 4°C when compared to 25°C (Fig. 1*E*). This increase in $Y_{(NO)}$ indicates excess excitation energy in PSII that cannot be quenched by photochemistry and NPQ (Busch *et al*. 2009), thus increasing the risk of PSII photoinhibition during the chilling treatment.

Chl *a* JIP-test: Next, we measured the Chl *a* fluorescence transient in order to examine the effects of chilling

Parameter	Name and basic physiological interpretation
Basic values and JIP-test parameters from fast chlorophyll fluorescence induction	
F_t	Fluorescence level at time t
$F_0 = F_{30\mu s}$	Minimum fluorescence at O-step
$F_m = F_P$	Maximum fluorescence at P-step
$V_t = (F_t - F_0)/(F_m - F_0)$	Relative variable fluorescence at time t (V_K at 300μs)
$W_K = (F_K - F_0)/(F_J - F_0)$	The ratio of variable fluorescence F_K to the amplitude $F_J - F_0$
Chlorophyll fluorescence parameters derived from the saturation pulse analysis	
F_0	Minimum fluorescence after dark adaptation
F_m, F_m'	Maximum fluorescence after dark or light adaptation respectively
F_s	Steady-state fluorescence after light adaption
$F_v/F_m = (F_m - F_0)/F_m$	Maximum quantum yield of PSII photochemistry after dark adaptation
$F_0' = F_0/(F_v/F_m + F_0/F_m')$	Minimum fluorescence after light adaptation
$F_v'/F_m' = (F_m' - F_0')/F_m'$,	Maximum quantum yield of PSII after light adaptation
$q_P = (F_m' - F_s)/(F_m' - F_0')$	Proportion of open PSII reaction centers
$Y_{(II)} = (F_m' - F_s)/F_m'$	Effective quantum yield of PSII photochemistry
$Y_{(NPQ)} = F_s/F_m' - F_s/F_m$	Quantum yield of regulated energy dissipation in PSII *via* NPQ
$Y_{(NO)} = F_s/F_m$	Quantum yield of nonregulated energy dissipation in PSII

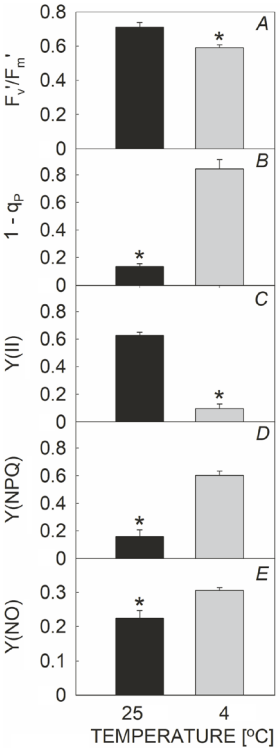

Fig. 1. Effects of chilling temperature on F_v'/F_m' (*A*), $1 - q_P$ (*B*), $Y_{(II)}$ (*C*), $Y_{(NPQ)}$ (*D*), and $Y_{(NO)}$ (*E*) in leaves of *Dalbergia odorifera*. All parameters were measured under a PPFD of 430 μmol(photon) m^{-2} s^{-1}. Values are means ± SD (n = 4). Asterisk indicates significant difference between 25 and 4°C. F_v'/F_m' – maximum quantum yield of PSII after light adaptation; $1 - q_P$ – proportion of closed PSII reaction centers; $Y_{(II)}$ – effective quantum yield of PSII photochemistry; $Y_{(NPQ)}$ – quantum yield of regulated energy dissipation in PSII *via* NPQ; $Y_{(NO)}$ – quantum yield of nonregulated energy dissipation in PSII.

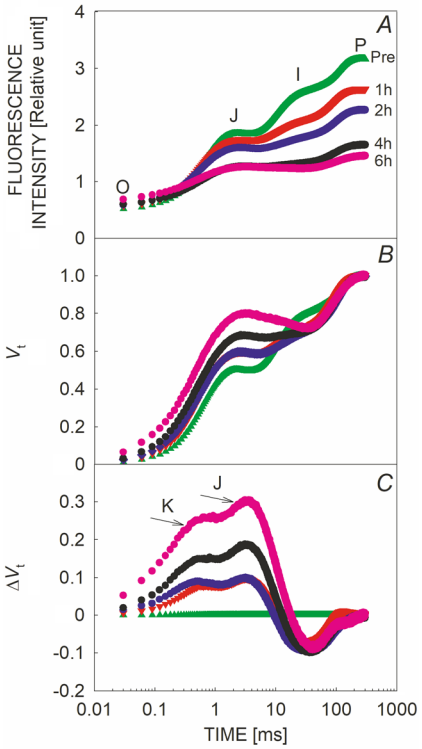

Fig. 2. Changes in chlorophyll *a* fluorescence OJIP (*A*), relative variable fluorescence at time t (V_t) (*B*), and the change in V_t (ΔV_t) (*C*) in *Dalbergia odorifera* during the chilling treatment at 4°C under a light of 430 μmol(photon) m^{-2} s^{-1}. $V_t = (F_t - F_0)/(F_m - F_0)$, $\Delta V_t = [(F_t - F_0)/(F_m - F_0)]_{treatment} - [(F_t - F_0)/(F_m - F_0)]_{control}$. In this study, $[(F_t - F_0)/(F_m - F_0)]_{control}$ represents the value of $(F_t - F_0)/(F_m - F_0)$ measured before chilling treatment. The average was calculated from four independent leaves.

treatment on PSII and OEC. During the chilling treatment, the Chl fluorescence emission at the J, I, and P levels (F_J, F_I, and F_P) all decreased (Fig. 2*A*). The relative fluorescence intensity at O–J level gradually increased during the chilling treatment (Fig. 2*B*), indicating the photodamage to donor side of PSII. Furthermore, the difference in relative Chl fluorescence intensity (ΔV_t) at 300 μs (K-step) gradually increased during the chilling treatment (Fig. 2*C*), suggesting that this chilling-light stress caused significant photodamage to the OEC (Srivastava *et al*. 1997, Strasser 1997, Strasser *et al*. 2000, 2004; De Ronde *et al*. 2004, Li *et al*. 2009).

During the chilling-light treatment, F_0 slightly increased with the increase in treatment time (Fig. 3*A*). Meanwhile, F_m largely decreased. After chilling treatment for 6 h, F_m decreased from 3.2 to 1.5 (Fig. 3*B*). Consequently, the maximum quantum yield of PSII after dark adaptation (F_v/F_m) gradually decreased with the increase in chilling treatment time (Fig. 3*C*). As showed in Fig. 3*C*, F_v/F_m decreased from 0.83 to 0.50 after this chilling treatment for 6 h. The decline in both F_m and F_v/F_m strongly indicated the severe PSII photoinhibition induced by this chilling-light stress. Furthermore, the relative Chl fluorescence intensity at 300 μs (V_K) gradually increased during the chilling treatment (Fig. 4*A*), indicating that the extent of photodamage to OEC was accelerated with the increase in treatment time. Pooling the data obtained during chilling treatment, F_v/F_m was strongly and negatively correlated with V_K and W_K (Fig. 4*B*,*C*), suggesting that photodamage to the OEC was significantly correlated with the total PSII photoinhibition.

Effect of methyl viologen on PSII photoinhibition: In order to examine whether additional ROS can accelerate PSII photoinhibition at chilling temperature, we determined the effect of MV on PSII photoinhibition during chilling treatment. MV promotes the production of superoxide anion radicals in the chloroplast stroma by accepting electrons from PSI. After treatment at 4°C for 2 h under a moderate light of 430 μmol(photon) m^{-2} s^{-1}, there was no significant difference in F_v/F_m between MV-treated and H_2O-treated samples in shade leaves (Fig. 5). Therefore, the supplemental generation of ROS in the chloroplast stroma did not accelerate PSII photodamage in the shade leaves illuminated at chilling temperature.

Discussion

Chilling-light stress induces photodamage to OEC and PSII

In the present study, we examined the effect of chilling-light stress on the extent of photoinhibition of PSII and OEC. Our results indicated that chilling-light stress induced significant increase in the relative fluorescence intensity at 300 μs (V_K) in the tropical tree species *D. odorifera* (Fig. 2). Because the K-step correlates with damage to the donor side of PSII (Strasser 1997, De Ronde *et al*. 2004, Strasser *et al*. 2004), and OEC is responsible for the splitting of water molecules and provides electrons

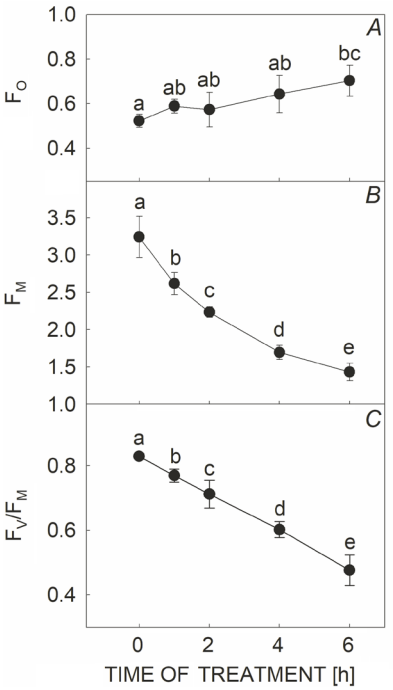

Fig. 3. Time course of minimum fluorescence (F_0) (*A*), maximum fluorescence (F_m) (*B*), and maximum quantum yield of PSII (F_v/F_m) (*C*) in *Dalbergia odorifera* during treatment at 4°C and 430 μmol(photon) m^{-2} s^{-1}. Values are means ± SD (n = 4). *Different letters* indicate significant differences between different treatments.

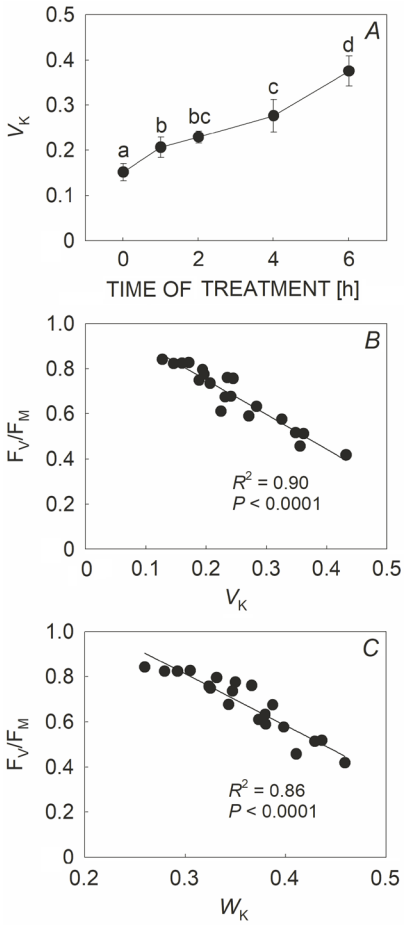

Fig. 4. Time course of the relative fluorescence intensity at 300 μs (V_K) in *Dalbergia odorifera* during the chilling treatment at 4°C and 430 μmol(photon) m^{-2} s^{-1} (*A*). Changes in maximum quantum yield of PSII (F_v/F_m) as functions of V_K (*B*) and the ratio of variable fluorescence F_K to the amplitude $F_J - F_0$ (W_K) (*C*) in *Dalbergia odorifera* during the chilling treatment. Values are means ± SD (n = 4). *Different letters* indicate significant differences between different treatments.

to PSII reaction centers, the increase in V_K indicated the photodamage to OEC (Huang *et al*. 2016b, Kalaji *et al*. 2018). Furthermore, with the increase in chilling treatment time, V_K gradually increased, suggesting that the extent of photodamage to OEC was dependent on the total light energy absorbed by photosynthetic pigments. Although manganese compounds show very low light absorbance at visible light spectra (Hakala *et al*. 2005), this result indicated that the slight visual light energy absorbed by the manganese compounds was still sufficient to damage OEC.

During the chilling treatment, the decrease in F_v/F_m was linearly correlated with treatment time course, indicating that the repair process was inhibited entirely at the chilling temperature of 4°C, consistent with the result of Allakhverdiev and Murata (2004). As reported in previous study, the rate of PSII repair is largely dependent on the ATP synthesis of ATP *via* PSI and PSII (Allakhverdiev *et al*. 2005, Huang *et al*. 2018, Murata and Nishiyama 2018). At chilling temperature, the photosynthetic electron flow through PSII was largely reduced in the studied species *D. odorifera* (Fig. 1). Furthermore, the total electron transport rate through PSI was also reduced by the chilling temperature (Huang *et al*. 2011). Therefore, the chilling-light stress resulted in the inhibition of ATP synthesis, which in turn restricted the recovery of photodamaged PSII. Consequently, the rate of total PSII photoinhibition at chilling-light stress was mainly determined by the rate of photodamage.

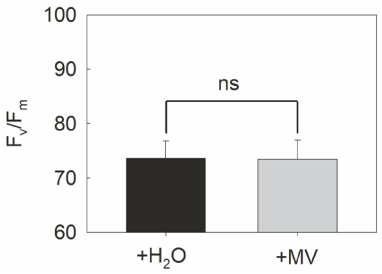

Fig. 5. Effect of methyl viologen (MV) on the decrease in maximum quantum yield of PSII (F_v/F_m) induced by chilling and light treatment in the shade (10% sunlight) leaves of *Dalbergia odorifera*. After treated with 300 μM MV or water, leaf samples were illuminated at 4°C for 2 h under 430 μmol(photon) m^{-2} s^{-1}. All values were expressed relative to the controls measured before the chilling treatment. Values are means ± SE (n = 4). ns – no significant difference.

If the rate of photodamage to PSII was controlled by ROS, the rate of PSII photoinhibition would not be linearly correlated with the rate of photodamage of OEC, because ROS hardly cause oxidative photodamage to OEC (Hakala *et al*. 2005, Ohnishi *et al*. 2005). However, if the rate of photodamage of PSII is limited by the rate of photodamage of OEC, the decrease in F_v/F_m would be linearly correlated with the increase in V_K. Interestingly, our present study indicated that F_v/F_m was strongly negatively correlated with V_K (Fig. 4*B*). This result suggested that the chilling-induced PSII photodamage in the studied species was mainly limited by the photodamage to OEC, but not by the ROS. Inactivation of OEC induces suppression of electron supply from water to P_{680}, and then leads to high levels of $P_{680}^{+\bullet}$. Since $P_{680}^{+\bullet}$ is a strong oxidant, high levels of $P_{680}^{+\bullet}$ might damage the PSII reaction center (Takahashi and Murata 2008, Takahashi and Badger 2011). Therefore, our results indicated that photodamage to OEC is a rate-limiting step for chilling-induced photodamage of PSII for the studied tropical species, which provides new evidence to support the two-step scheme of photodamage of PSII over the excess-energy scheme.

ROS did not aggravate PSII photodamage at chilling temperature

During the chilling treatment, $Y_{(II)}$ was largely inhibited and $Y_{(NPQ)}$ gradually decreased (Fig. 1*C*,*D*), leading to the increase in $Y_{(NO)}$ (Fig. 1*E*). $Y_{(NO)}$ consists of nonphotochemical quenching due to photoinactivation of PSII and constitutive thermal dissipation that is very stable despite environmental stresses (Busch *et al*. 2009). Thus, the increase in $Y_{(NO)}$ indicates an increase in production of ROS. With the increase in treatment time, ROS would be gradually accumulated. If ROS caused direct oxidative damage to PSII, the rate of photodamage would increase gradually with the increase in treatment time. In contrast, the decrease in F_v/F_m was linearly correlated with the time course of chilling treatment (Fig. 3*C*). Therefore, these results suggest that ROS hardly caused direct oxidative photodamage to PSII at chilling-light stress for the studied tropical species.

We further examine the effect of ROS on PSII photoinhibition in shade leaves by adding MV. Since MV promotes electrons from PSI to O_2, the production of ROS should be much greater in the MV-treated samples than that of the H_2O-treated samples. If the chilling-induced photodamage to PSII in the studied tropical tree species can be affected by ROS, the supplemental addition of ROS should aggravate PSII photodamage. In fact, the MV-treated samples did not show greater PSII photoinhibition than the H_2O-treated samples (Fig. 5). It should be noted that MV also abolish cyclic electron flow around PSI. In order to exclude the effect of CEF in the shade leaves of *D. odorifera*, we examined the CEF activity and found that the shade leaves showed much lower CEF activity than that of sun leaves (data not shown). As a result, the effect of MV on PSII photodamage in the shade leaves is mainly attributed to the additional generation of ROS (Takahashi *et al*. 2009). Our results indicated that ROS did not aggravate PSII photodamage in shade leaves of the studied species, which again supports the two-step scheme of PSII photodamage (Murata *et al*. 2012).

In summary, we found that chilling temperature associated with moderate light intensity significantly induced photodamage to the OEC in a tropical tree species *D. odorifera*. The photodamage to OEC was a rate-limiting step for chilling-induced PSII photoinhibition. Furthermore, supplemental production of reactive oxygen species by infiltration with methyl viologen did not aggravate PSII photoinhibition. These results strongly suggested that the chilling-induced PSII photoinhibition in the studied tropical species was attributed to the two-step photodamage model.

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