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Moderately elevated temperature eliminates resistance of rice plants with enhanced expression of glutathione reductase to intensive photooxidative stress

R. KOUŘIL[*], D. LAZÁR[*,+], H. LEE[**], J. JO[**,++], and J. NAUŠ[*]

Laboratory of Biophysics, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic[*]
Department of Animal Science, Kyungpook National University, Daegu 702701, South Korea[**]

Abstract

We developed transgenic rice plants (*Oryza sativa* L. cv. Daeribbyeo) overproducing cytosolic glutathione reductase (GR) using a GR gene from *Brassica campestris* and studied their response to photo-oxidative stress in the presence of methyl viologen (MV, 10 and 50 μM concentrations) under room (25 °C) and moderately elevated (35 °C) temperature by analysis of chlorophyll (Chl) *a* fluorescence parameters (F_V/F_M, q_N, and q_P) and of Chl content. Elevated temperature enhanced and accelerated the photo-oxidative damage to photosynthetic apparatus expressed mainly by a fast decrease of q_N. Higher temperature supported the protective reaction in transformed rice plants for lower MV concentration (10 μM) and eliminated the enhanced tolerance of photosystem 2 photochemistry to photooxidative stress for higher (50 μM) MV concentration. Different mechanisms and temperature dependence of oxidative and protective reactions explain the results.

Additional key words: active oxygen species; chlorophyll fluorescence; *Oryza sativa*; photochemical and non-photochemical fluorescence quenching; transgenic plants.

Introduction

In general, exposure of plants to biotic or abiotic stress leads to the increased production of active oxygen species (AOS) which can induce a structural and functional damage of photosynthetic enzymes and components of photosynthetic apparatus (for reviews see Foyer and Harbinson 1994, Foyer *et al.* 1994a, Foyer and Noctor 2000). In chloroplasts, several effective mechanisms that eliminate formation of AOS and protect photosynthetic machinery from oxidative damage were found. Involvement of β-carotene or zeaxanthin in quenching of excited state of singlet oxygen or triplet chlorophylls plays an important role in protection of photosystem 2 (PS2) against oxidative stress under high irradiance (for reviews see Foyer *et al.* 1994a,b, Pospíšil 1997). On the reducing side of photosystem 1 (PS1), superoxide generated in Mehler reaction (Mehler 1951) is effectively scavenged by superoxide dismutase (SOD), producing H_2O_2, which is consequently reduced to water through the action of ascorbate peroxidase (APX). Regeneration of ascorbate can proceed *via* the reduction by ferredoxin, by the action of monodehydroascorbate reductase, or *via* the ascorbate-glutathione cycle, which includes the regeneration of glutathione catalysed by glutathione reductase (GR), at the expense of NADPH (for reviews see Asada 1999, 2000, Foyer and Noctor 2000).

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[+]To whom correspondence for photo-oxidative stress should be addressed; fax: +420 585225737, e-mail: lazard@seznam.cz
[++]To whom correspondence for plant genetic engineering should be addressed; fax: 82-53-950-6750, e-mail: jkjo@knu.ac.kr
Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; Chl, chlorophyll; DHA, dehydroascorbate; F_0, minimal Chl fluorescence intensity of the dark-adapted leaf segment; F_M, maximal Chl fluorescence intensity of the dark-adapted leaf segment; F_0', minimal Chl fluorescence intensity of the light-adapted leaf segment; F_M', maximal Chl fluorescence intensity of the light-adapted leaf segment; $F_V/F_M = (F_M - F_0)/F_M$, maximal quantum yield of photosystem 2 photochemistry; GR, glutathione reductase; MV, methyl viologen; PAR, photosynthetically active radiation; PS, photosystem; Q_A, primary stable quinone acceptor of PS2; q_P and q_N, photochemical and non-photochemical Chl fluorescence quenching coefficients; SOD, superoxide dismutase; WT, wild type.
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In the past decade, genetically modified plants with altered contents of specific enzymes of antioxidant system were under extensive study with respect to their response to photo-oxidative stress. It was found that over-expression of chloroplastic SOD results in enhanced tolerance of transgenic potato and tobacco to photo-oxidative stress in the presence of methyl viologen (MV) in comparison with control, non-transformed plants (Perl *et al.* 1993, Slooten *et al.* 1995, Van Camp *et al.* 1996, Arisi *et al.* 1998). A protective role of elevated content of chloroplast SOD in transgenic tobacco plants against inhibition of photosynthetic rate was observed under high irradiance and chilling temperature (Sen Gupta *et al.* 1993a,b). On the other hand, no protection of over-expressed SOD on PS2 activity was found after photoinhibition carried out at room temperature in transgenic poplars (Tyystjärvi *et al.* 1999). Enhanced content of cytosolic or chloroplastic APX in transgenic tobacco plants suppressed membrane damage induced by MV treatment in comparison with non-transformed control plants (Allen *et al.* 1997). In addition, a greater restoration of oxygen evolution after photoinhibitory treatment at low temperature was also observed in the above transgenic tobacco plants (Allen *et al.* 1997). Overproduction of cytosolic and chloroplastic GR in transgenic tobacco plants exhibited lower susceptibility to treatment of MV with respect to control plants (Aono *et al.* 1991, 1993, Foyer *et al.* 1995). Protective role of over-expressed GR against photoinhibition was observed at both low and room temperature in transgenic poplar and tobacco plants (Foyer *et al.* 1995, Tyystjärvi *et al.* 1999).

In the present study, we have developed transgenic rice plants (*Oryza sativa* L. cv. Daeribbyeo) overproducing cytosolic GR using a GR gene from *Brassica campestris* and studied their response to photo-oxidative stress in the presence of MV under room and moderately elevated temperature.

Materials and methods

Plants: Wild type (WT) and transformed rice (*Oryza sativa* L. cv. Daeribbyeo) overproducing GR in the cytosol were cultivated in a greenhouse under natural irradiation and at temperature around 20–29 °C during day and 12–16 °C during night. 14~16 weeks old rice plants were used for experiments.

Bacterial strain and vector construction: A 1.8 kb XbaI-SacI fragment containing the cytosolic GR cDNA (BcGR1; Lee *et al.* 1998) from *Brassica campestris* was ligated into the XbaI-SacI digested binary vector pIG121-Hm under the control of the CaMV 35S promoter. The vector contains *nptII* gene and *hpt* gene conferring resistance to kanamycin and hygromycin, respectively. The resulting vector pIG-BcGR1 was introduced into the *Agrobacterium tumefaciens* strain EHA101 by freeze-thaw method (Horsch *et al.* 1978) and was used for rice transformation.

Rice transformation: At 21 d after anthesis, seeds of the used rice cultivar were de-hulled, sterilised with 70 % ethanol for 30 s, 1 % NaOCl for 30 min, and washed three times with sterile distilled water. Calli were induced from the scutellum of immature seeds on the N6 medium (Chu *et al.* 1975) containing 2 g m^{-3} 2,4-D, 30 kg m^{-3} sucrose, and 5 kg m^{-3} gelrite for 4 weeks in the darkness at 25 °C. An *Agrobacterium* strain EHA101 harbouring the pIG-BcGR1 was grown for 3 d in an AB liquid medium (Chilton *et al.* 1974) supplemented with 30 g m^{-3} hygromycin. The bacteria were collected by centrifugation and suspended in AA medium (Toriyama and Hirata 1985) containing 100 μM acetosyringone at a density of $3–5\times10^9$ cells per cm^3. Four-week-old calli were co-cultivated with *Agrobacterium* on N6 medium containing 2 g m^{-3} 2,4-D, 30 kg m^{-3} sucrose, 10 kg m^{-3} glucose, 1 kg m^{-3} casamino acids, 100 μM acetosyringone, and 5 kg m^{-3} gelrite for 3 d in the darkness at 25 °C. The co-cultivated calli were washed with sterile water containing 100 g m^{-3} cefotaxime, and incubated on N6 medium containing 40 g m^{-3} hygromycin and 100 g m^{-3} cefotaxime for 3 weeks. The hygromycin-resistant calli were transferred onto the regeneration medium, N6 supplemented with 1 g m^{-3} NAA, 5 g m^{-3} kinetin, 30 kg m^{-3} sucrose, 40 mg m^{-3} hygromycin, and 100 g m^{-3} cefotaxime. After 4-6 weeks under continuous irradiation, regenerated plantlets (T_0) were potted and grown in a growth chamber with 10 h irradiation per day. The T_0 transformants were selfed to produce T_1 seeds. For segregation analysis, T_1 seeds were surface sterilised and sown in Petri dishes containing N6 medium supplemented with hygromycin (40 g m^{-3}). The numbers of hygromycin-resistant green seedlings and hygromycin-sensitive bleached plants were scored 3 weeks after sowing. The hygromycin-resistant plants of T_1 generation were used for further analysis.

DNA isolation and PCR analysis: Rice genomic DNA was extracted from leaves according to the CTAB methods (Murray and Thompson 1980) and 100 ng of each sample was subjected to PCR analysis. PCR was performed with specific primers either for 35S promoter and BcGR1 cDNA (Fig. 1). The four primers used for PCR analysis were 35S sense-1 (5'-TTCAACAAAG GGTAATATCCGG-3'), 35S sense-2 (5'-CCCACCCAC GAGGAGCATC), 35S antisense-1 (5'-CGAAGGATAG TGGGATTGTGC-3'), and GR antisense-1 (5'-CTAGCATCCTCAAGTTCACC-3'). The cycling parameters were an initial de-naturation at 95 °C for 5 min, 35 cycles of de-naturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min.

Amplified DNA was separated by electrophoresis in a 1.2 % agarose gel.

Northern blot analyses: Total RNA was extracted from leaves according to the guanidine thiocyanate method (McGookin 1984). Total RNAs (15 μg) were fractionated on a 1.2 % formaldehyde agarose gel. Gels were blotted onto *Hybond-XL* nylon membranes (*Amersham*, UK) with 10× SSC. The membrane was hybridised with a ^{32}P-labeled full-length BcGR1 cDNA probe, washed with 0.2× SSC and 0.1 % SDS at 55 °C for 1 h, and then auto-radiographed.

Photo-oxidative stress and determination of Chl content: Small leaf segments (approx. 0.4×3 cm) were cut out of the central part of rice leaves. Infiltration of the segments with MV at given concentrations (10 and 50 μM) were carried out in Petri dishes by floating of segments on the surface of solution. Leaf segments in distilled water were used as a reference. At first, samples were incubated at given solution 1 h in the dark and then were exposed to "white light" [120 μmol(photons of PAR) m^{-2} s^{-1}] for 20 h. All treatments were carried out at 25 or 35 °C.

Determination of Chl *a*+*b* content was performed by spectrophotometric method in 80 % acetone according to Lichtenthaler (1987).

Chl *a* fluorescence measurements were performed by kinetic imaging fluorometer *FluorCam 700 MF* (*Photon Systems Instruments*, Brno, Czech Republic) described in detail in Nedbal *et al.* (2000). Briefly, fluorescence was measured during 10 μs long measuring flashes (λ = 650 nm) generated by two panels of light-emitting diodes (LED; 345 LEDs in each panel). The same LED panels also provided continuous actinic irradiance of 130 μmol(photons of PAR) m^{-2} s^{-1}. Saturating "white" pulse of 800 μmol(photons of PAR) m^{-2} s^{-1} was provided by a 250 W halogen lamp. Minimal fluorescence F_0 was taken as fluorescence signal initiated by measuring flashes placed 1 s apart in dark with dark-adapted (10 min) plant material. Maximal fluorescence F_M (dark-adapted state) was measured in the middle of 1.6 s-long saturating pulse. One minute after the end of saturating pulse, continuous actinic radiation was switched on to initiate fluorescence induction. After 2 min of continuous actinic irradiation when fluorescence signal reached its steady-state level, the saturating pulse was applied to measure maximal fluorescence F_M' for light-adapted state. Fluorescence signal detected during continuous irradiation just before application of the saturating pulse to determine F_M' was taken as fluorescence signal in steady state, F_S. As the instrument does not possess far-red radiation, the minimal fluorescence F_0' for light-adapted state was calculated according to Oxborough and Baker (1997) as $F_0' = F_0/[(F_V/F_M) + (F_0/F_M')]$. From the above basic fluorescence values the following fluorescence parameters were evaluated: Maximal quantum yield of PS2 photochemistry expressed as F_V/F_M (Kitajima and Butler 1975), photochemical fluorescence quenching expressed as $q_P = (F_M' - F_S)/F_V'$ (Bilger and Schreiber 1986), and non-photochemical fluorescence quenching expressed as $q_N = (F_V - F_V')/F_V$ (Bilger and Schreiber 1986), where variable fluorescence is expressed as $F_V = F_M - F_0$ and $F_V' = F_M' - F_0'$.

To decrease an effect of handling with leaf segments on the determination of fluorescence parameters, and to obtain more accurate values of the parameters, we utilised advantage of imaging fluorometers to capture fluorescence signal from large area during one measurement. Hence, after incubation of samples in particular solution for particular time, three leaf segments of each leaf type (WT and over-expressed) were adapted to dark for 10 min at 25 ºC and then used for measurements of fluorescence parameters at 25 ºC. In this case, a value of fluorescence was obtained and averaged for one leaf type from about 3 500 pixels.

Statistical testing: The statistical significance of the difference in the fluorescence parameters between the WT and T_1 was tested by *t*-test calculated in *MS Excel*. The *p*-values of the test indicated by the asterisks are presented in Figs. 2–4.

Results

Development of transgenic rice plant expressing BcGR1 gene: The full-length cytosolic GR gene (BcGR1) was cloned into the pIG121-Hm vector under the control of a CaMV 35S promoter in sense orientation and was introduced into rice using *Agrobacterium*-mediated transformation. The transgenic rice plants were selected with hygromycin and 17 independent rice plants were regenerated. The transgenic rice plants were identified for the presence of 35S promoter and BcGR1 cDNA by PCR amplification performed with internal primers to the 35S promoter and BcGR1 cDNA (Fig. 1*A*-*C*). PCR analysis confirmed that all the 17 independent rice plants selected were transformed as expected. Moreover, Southern blot analysis of the genomic DNA probed with native BcGR1 cDNA also certified the introduction of BcGR1 gene in transgenic plants (data not shown). The transgenic plants did not show any phenotypic changes and had similar overall morphology as the wild-type plants. To evaluate the expression of BcGR1 gene in transgenic plants, total RNAs from both transgenic and wild-type plants were subjected to Northern blot analysis. As shown in Fig. 1*D*, the BcGR1 transcripts were detected in the transgenic plants with different levels among the plants.

To establish the T_1 plants, transformants were allowed to self-fertilisation and T_1 progenies were sown on hygromycin-containing medium. All transformants exhibited a 3 : 1 segregation ratio (data not shown), suggesting that the transgene was inserted at a single locus. Hygromycin-resistant individuals were transferred to the greenhouse and were used for further analysis.

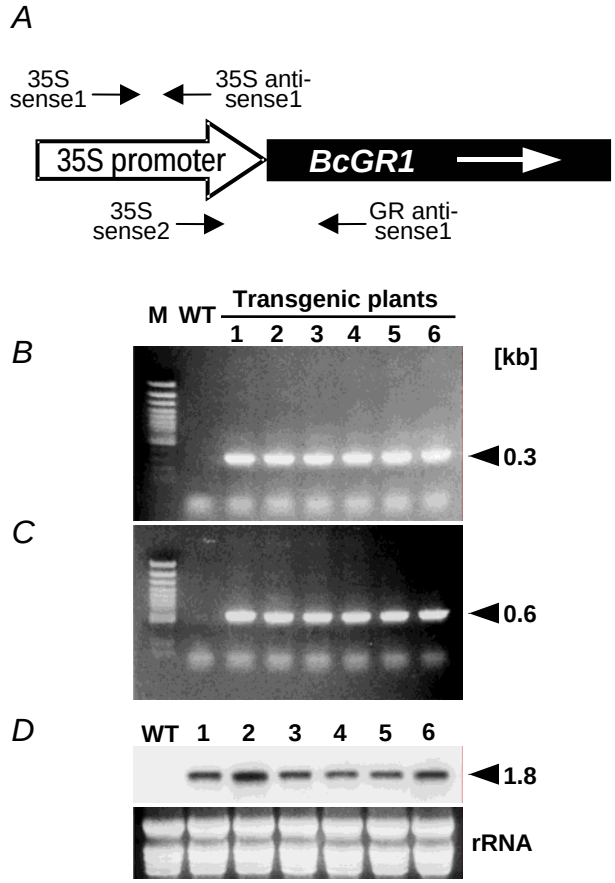

Fig. 1. Confirmation of transgenic rice plants by PCR and Northern blot analyses. *A*: Schematic diagram for PCR amplification of 35S promoter and *BcGR1* cDNA fragment for identification of transformation. *B*: PCR amplification with 35S sense1 and 35S antisense1 primers. M, DNA size marker (100 bp ladder); WT, wild-type. Numbers indicate independent transgenic lines. *C*: PCR amplification with 35S sense2 and GR antisense1 primers. *D*: Expression of the *BcGR1* in transgenic tobacco plants. Total RNA was isolated from the leaves of wild-type (WT) and transgenic rice plants. Ethidium bromide-stained ribosomal RNA (rRNA) served as a loading control.

Effect of photo-oxidative stress on maximal quantum yield of PS2 photochemistry: Irradiation of leaf segments of both the WT and T_1 rice plant samples infiltrated with MV led to a pronounced decrease in the F_V/F_M ratio (Fig. 2, *second and third rows*) compared to reference samples (Fig. 2, *first row*). When the photooxidative stress was applied at room temperature in the solution of 10 μM MV, a slower decrease in the F_V/F_M ratio was detected for T_1 rice plants, especially after 4 h of irradiation (Fig. 2, *left column*). Increment of MV concentration to 50 μM did not intensify any longer the inhibition of the PS2 photochemistry in both types of samples.

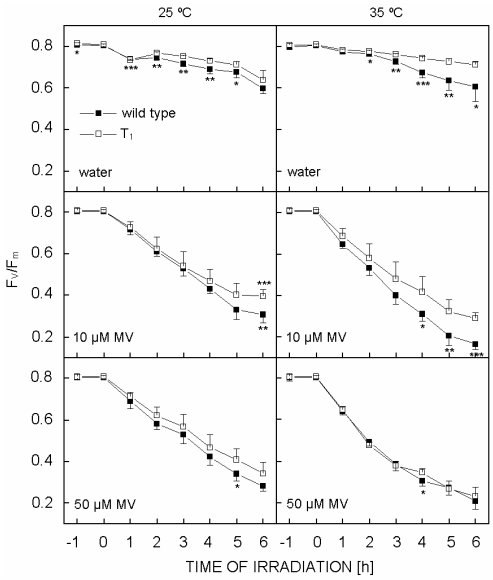

Fig. 2. Changes of the maximal quantum yield of PS2 photochemistry (F_V/F_M ratio) of dark-adapted leaf segments of WT (*solid squares*) and transformed T_1 (*open squares*) rice plants treated with distilled water (used as the reference) and MV (10 and 50 μM) under "white light" [120 μmol(photons of PAR) m^{-2} s^{-1}] at 25 °C (*left column*) or 35 °C (*right column*). During the first hour (the –1 h in the graphs), the infiltration of the leaf segments with solution of MV or distilled water was carried out in dark. Zero time indicates measurement with dark-adapted leaf segments without any infiltration (used as the control for all three rows). *Vertical bars* indicate standard deviation and the asterisks indicate statistical significance (the *p*-level of the test) of difference between the WT and T_1 leaf segments at following levels: $^{*}0.01 \le p \le 0.05$; $^{**}0.001 \le p < 0.01$; $^{***}p < 0.001$. For other conditions see Materials and methods.

Increase in temperature of treatment to 35 °C led to a faster and more pronounced decrease in the F_V/F_M ratio in both the WT and T_1 in the presence of MV (Fig. 2, *right column*) compared to treatment at 25 °C (Fig. 2, *left column*). In the presence of 10 μM MV, a greater extent of inhibition of the F_V/F_M ratio was observed in WT sample in comparison with T_1 during the whole treatment period. However, in the presence of 50 μM MV, the same course of inhibition of the F_V/F_M ratio was observed for both the WT and T_1 rice plants (Fig. 2, *right column*). Importantly, the shift of treatment temperature from 25 to 35 °C did not induce any pronounced changes in the F_V/F_M ratio in the reference samples (Fig. 2, *first row*). The inhibitory effect of MV on the F_V/F_M ratio was observed only in light and not in darkness (data not shown).

Effect of photooxidative stress on photochemical and non-photochemical Chl *a* fluorescence quenching:

Treatment with MV (10 and 50 μM) at 25 °C induced a decrease in the q_P in both the WT and T_1 rice plants, but with a greater extent in WT plants (Fig. 3, *left column*). Increase in temperature to 35 °C did not induce any additional changes in the response of q_P to photooxidative stress in the presence of 10 or 50 μM MV in both types of samples (Fig. 3, *right column*) although the difference between the WT and T_1 plants was more pronounced for 50 μM MV. Further, no marked changes and approximately the same values of the q_P parameter (0.6) were observed in the reference samples of both the WT and T_1 plants within 6 h of photo-oxidative treatment at 25 °C or 35 °C (Fig. 3, *first row*). Here, again, the values of q_P for T_1 plants had tendency to be higher.

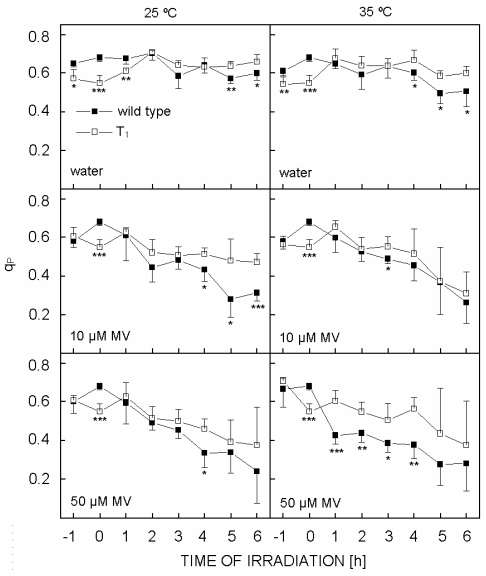

Fig. 3. Changes of steady-state photochemical fluorescence quenching (q_P) of leaf segments of WT (*solid squares*) and transformed T_1 (*open squares*) rice plants treated with distilled water (used as the reference) and MV (10 and 50 μM) under "white light" [120 μmol(photons of PAR) m^{-2} s^{-1}] at 25 °C (*left column*) or 35 °C (*right column*). During the first hour (the –1 h in the graphs), the infiltration of the leaf segments with solution of MV or distilled water was carried out in dark. Zero time indicates measurement with dark-adapted leaf segments without any infiltration (used as the control for all three rows). Other conditions and meaning as in Fig. 2.

Photo-oxidative stress in the presence of MV induced marked inhibition of q_N in WT and T_1, but the kinetics of q_N inhibition was different and it also depended on temperature of treatment (Fig. 4). In WT, q_N reached its minimal value (about 0.20) after approx. 2 h of irradiation and a decrease in the q_N value was accelerated in the presence of 50 μM MV and/or at higher temperature of treatment (Fig. 4). In the case of T_1 treated with MV (10 and 50 μM) at 25 °C, a higher value of q_N parameter was observed within the first 3 h of irradiation compared to the WT sample. After 4 h of irradiation, a decrease in q_N to a similar value (0.25) as in the WT was observed in T_1

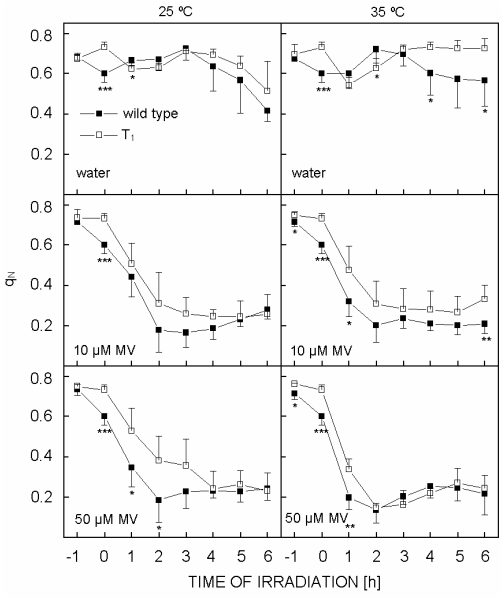

Fig. 4. Changes of steady-state non-photochemical fluorescence quenching (q_P) of leaf segments of WT (*solid squares*) and transformed T_1 (*open squares*) rice plants treated with distilled water (used as the reference) and MV (10 and 50 μM) under "white light" [120 μmol(photons of PAR) m^{-2} s^{-1}] at 25 °C (*left column*) or 35 °C (*right column*). During the first hour (the –1 h in the graphs), the infiltration of the leaf segments with solution of MV or distilled water was carried out in dark. Zero time indicates measurement with dark-adapted leaf segments without any infiltration (used as the control for all three rows). Other conditions and meaning as in Fig. 2.

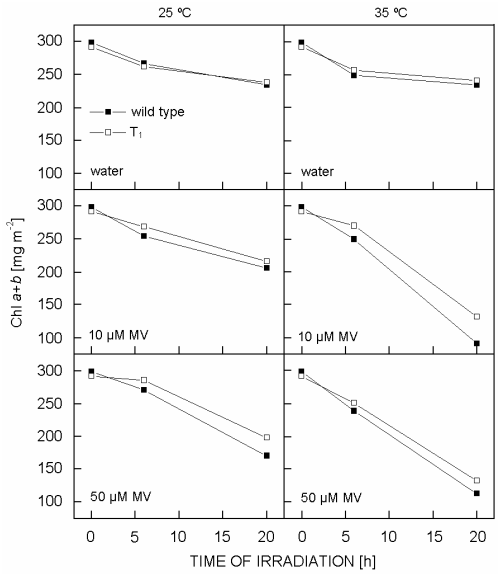

Fig. 5. Changes of chlorophyll (Chl) *a+b* content in leaf segments of WT (*solid squares*) and transformed T_1 (*open squares*) rice plants induced by the photooxidative stress in the presence of MV (10 and 50 μM) and "white light" [120 μmol(photons of PAR) m^{-2} s^{-1}] under 25 (*left column*) or 35 °C (*right column*). Leaf segments treated with distilled water were used as a reference. For other conditions see Materials and methods.

under the above experimental conditions. Treatment of T_1 with 10 μM MV at 35 °C did not change the course of q_N when compared to the treatment of T_1 with 10 μM MV at 25 °C (Fig. 4, *second row*). On the other hand, in T_1 treated with 50 μM MV at 35 °C there was a more pronounced decrease of q_N during the first three hours of the treatment when compared to T_1 treated with 50 μM MV at 25 °C (Fig. 4, *third row*). In addition, q_N of T_1 treated with 50 μM MV at 35 °C was similar as in the WT sample (Fig. 4). In reference samples, q_N did not markedly change during 6 h of irradiation and the course of q_N was independent of treatment temperature. Nevertheless, after 4 h of irradiation, a slightly higher value of q_N was observed in T_1 compared to WT sample both at 25 and 35 °C (Fig. 4, *first row*). Similarly as in the case of F_V/F_M, the presence of light was indispensable for observed inhibitory effect of MV on the q_P and q_N parameters (data not shown).

Changes of Chl content during the photo-oxidative stress: Fig. 5 shows that a prolonged time of photo-oxidative treatment led to a decrease in the amount of Chl *a*+*b* in both WT and T_1 rice plants under all experimental conditions, when a more pronounced loss of Chl content was observed in WT in comparison with T_1. In addition, higher temperature in the presence of MV intensified a photo-oxidative damage of Chls in both types of used rice plants.

Discussion

Sensitivity of fluorescence parameters to photo-oxidative stress: As shown in Figs. 2–4, the most sensitive Chl *a* fluorescence parameter reacting to photo-oxidative stress caused by MV was q_N representing non-photochemical fluorescence quenching. The q_N value had fallen within 1 h of photo-oxidative process to half or less of its initial value. This decrease was more pronounced at higher temperature and for higher MV concentration (Fig. 4).

The most important component of non-photochemical quenching in our case seems to be the energy-dependent quenching (q_E) related to the difference of pH across thylakoid membrane (Govindjee and Spilotro 2002, Kanazawa and Kramer 2002; see also Horton *et al.* 1996 and Pospíšil 1997 for reviews). The photoinhibitory quenching (q_I) can be discarded due to low irradiance used. The quenching due to the state transition (q_T) is usually generated within several minutes and in addition, it would not yield such a drastic change. A decrease of q_N can then be explained as a consequence of damage to thylakoid membrane leading to an increase in proton permeability and proton leakage. An increase in electrolyte leakage observed during photo-oxidative stress in the presence of MV (Aono *et al.* 1995, Allen *et al.* 1997) supports our interpretation. A partially protective role of over-expressed cytosolic GR against increase in electrolyte leakage (Aono *et al.* 1995) is probably responsible for the slower decrease in the q_N in our transformed plants during the initial phase of photo-oxidative treatment (Fig. 4). Both lipids and proteins are exposed to photo-oxidation. However, lipids are usually thought the most sensitive ones undergoing de-esterification reactions or peroxidation in a chain reaction or amplified chain reaction (McKersie 1996). We assume the dominant role of lipid peroxidation although our results cannot supply a more detailed information about these reactions.

On the other hand, the two parameters related to photochemical electron transfer in thylakoid membrane, F_V/F_M and q_P, reacted with much slower kinetics to photo-oxidative action than q_N. The decrease of these values was rather gradual and proceeded within the whole time interval of the experiment. Two circumstances may help to explain a higher resistance of the photochemical events to photo-oxidation than the non-photochemical processes. Protein sub-units in complexes such as PS2, PS1, or cytochrome b_6/f are less prone to be damaged by external photo-oxidation than the freely moving and abundant lipids. The other reason follows from the fact that MV ($E^{0'} = -0.446$ mV) acts as an effective electron acceptor from reduced ferredoxin or other acceptors of PS1 driving in this way the linear electron transport through the whole chain in thylakoid membrane. This transport tends to keep Q_A oxidised and slows the decrease of q_P.

Temperature dependence of the photo-oxidative damage: The increase of temperature from 25 to 35 °C caused an acceleration of q_N decrease as it is clearly seen by the values that appeared one hour after the onset of photo-oxidation (Fig. 4). The courses of F_V/F_M and q_P were less sensitive to the temperature increase.

This distinctly different behaviour indicates again differences in molecular mechanisms. As has been elucidated in our model of fluorescence temperature dependence (Pospíšil and Nauš 1998), the processes having high activation energy are more temperature-dependent than those of low activation energy. Hence, upon increasing temperature the activity of the former may strongly increase. The enzymatic processes lower the activation energy of their specific reactions and these reactions are less temperature-dependent. The reactions leading to a decrease in q_N are probably the non-enzymatic spontaneous reactions with higher activation energy. The reactions related to photochemistry and protective reactions are mostly of enzymatic nature with lower dependence on temperature. Although the rate of enzymatic protective reactions should also increase with increasing temperature, the increase should not be very large. The protective processes thus do not cope with the damaging oxidative reactions.

The effect of mutation: The over-expression of cytosolic GR should lead to a higher proportion of reduced glutathione (GSH) in cytosol. Glutathione is present in plant cells mostly (70–90 %) in the reduced form under normal conditions (Smith *et al.* 1985, Bielawski and Joy 1986). A higher amount of GR produced in transgenic plants may thus only change the situation within relatively narrow limits, increasing the amount of reduced glutathione.

The reduced glutathione molecule can act in anti-oxidative processes by several mechanisms (McKersie 1996, Noctor *et al.* 1998). It may directly scavenge the reactive oxygen species, reduce dehydroascorbate (DHA) by non-enzymatic reaction at alkaline pH, or reduce DHA in an enzymatic reaction at the assistance of the enzyme dehydroascorbate reductase. The mechanism of strengthening the antioxidative processes in chloroplasts (*e.g.* the water-water cycle also called the Halliwell-Asada antioxidative pathway) remains to be explained.

Our results show that a higher expression of cytosolic GR (Fig. 1) led to an improvement of the protection against photo-bleaching of Chls (Fig. 5) and against photo-oxidative action of MV in thylakoid membranes at 25 °C. For the latter case, the increased temperature (35 °C) had a positive effect in the case of 10 μM MV concentration (Figs. 2–4). However, the situation at 35 °C and for the higher MV concentration (50 μM) was different. Although a tendency to improve q_P was maintained, the F_V/F_M and q_N values had shown nearly no difference between the WT and transgenic plants. For the latter case, the positive influence of mutation demonstrated at 25 °C was at 35 °C overwhelmed by damaging processes accelerated more intensively by the higher temperature. Thus a certain kind of negative feedback might have also contributed to damages at higher MV concentrations. A better functioning linear electron transport increases the production of reactive oxygen species and consequently increases the potential damage to the membrane. The elimination of positive effect of mutation by higher temperature and higher MV concentration indicated also that the increment of reduced glutathione or its products in transgenic plants took part in chloroplasts mostly in the enzymatic protective reactions.

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