DOI: 10.1007/s10535-017-0732-9 BIOLOGIA PLANTARUM 61 (4): 659-666, 2017

A protoporphyrinogen oxidase gene expression influences responses of transgenic rice to oxyfluorfen

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Abstract

We compared the defense against photoinhibitions and oxidative stress in transgenic rice expressing a modified *Myxococcus xanthus* protoporphyrinogen oxidase (*MxProtox*) gene and in wild-type (WT) rice. Although the *MxProtox* transgenic lines had higher content of protoporphyrin IX (Proto IX) than the untreated controls, they did not exhibit a drastic accumulation of Proto IX as in the WT after 2 d of 50 μM oxyfluorfen (OF) treatment. In the transgenic lines S4 and S11, the transcriptions of *OsProtox* and modified *MxProtox* genes were almost sustained in response to OF, although transcription of *OsProtox* was greatly down-regulated in the WT. The excess Proto IX in the WT plants treated with OF generated a severe stress mediated by singlet oxygen ($^1O_2$), leading to a prominent increases in electrolyte leakage and malondialdehyde production. This stress in the WT necessitated not only a substantial accumulation of zeaxanthin and antheraxanthin, but also strong increases in activities of superoxide dismutase, catalase, and peroxidase as well as transcriptions of *CatalaseB*, *Ascorbate Peroxidase*, and *Heme Oxygenase2* genes. By contrast, the transgenic plants did not result in any noticeable increase in these parameters. Our results demonstrate that the transgenic rice expressing modified *MxProtox* efficiently prevented accumulation of photosensitizing Proto IX through sustaining higher transcriptions of porphyrin biosynthetic genes, thereby reducing the stress imposed by OF.

Additional key words: antioxidant enzyme, ascorbate peroxidase, catalase, photodynamic stress, superoxide dismutase, xanthophylls.

Introduction

Tetrapyrroles play an essential role in plants because they are involved in light harvesting, energy transfer, signal transduction, and detoxification (Von Wettstein *et al*. 1995, Molina *et al*. 1999, Tanaka and Tanaka 2007). The last common step in the tetrapyrrole pathway to heme and chlorophyll is the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX), a reaction catalyzed by protoporphyrinogen oxidase (Protox) (Beale and Weinstein 1991, Tanaka and Tanaka 2007). Intermediates in the porphyrin biosynthetic pathway, such as Proto IX and its various Mg^{2+} derivatives interact with oxygen producing reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), which is harmful to the cell and causes the peroxidation of membrane lipids (Reinbothe *et al*. 1996, Tripathy and Oemüller 2012). The biosynthesis of porphyrin is tightly regulated to avoid the accumulation of porphyrin intermediates (Papenbrock and Grimm 2001). However, plants suffer severe photoinhibition if these control mechanisms failed, for example, in plants treated with peroxidizing herbicides (Pham *et al*. 2015, Phung and Jung 2015a) or environmental stresses (Phung *et al*. 2011, Phung and Jung 2015b).

Peroxidizing herbicides, such as oxyfluorfen (OF) and acifluorfen, inhibit Protox and so the accumulation of Protogen IX, which diffuses out of chloroplasts to the cytoplasm (Jacobs *et al*. 1991, Nandihalli *et al*. 1992). In the cytoplasm, Protogen IX is oxidized to Proto IX *via* peroxidase-like enzymes. Cytoplasmic Proto IX interacts with oxygen under light to form $^1O_2$, which causes the photobleaching in chloroplasts (Jacobs *et al*. 1991). Genes for the Protox enzyme have played a pivotal role in plants resistant to Protox-inhibiting herbicides (Lermontova and Grimm 2000, Jung *et al*. 2004, Yang

Submitted 22 August 2016, *last revision* 22 January 2017, *accepted* 27 January 2017.

Abbreviations: APX - ascorbate peroxidase; CAT - catalase; MDA - malondialdehyde; *Mx*Protox - *Myxococcus xanthus* protoporphyrinogen oxidase; OF - oxyfluorofen; POD - peroxidase; Proto IX - protoporphyrin IX; Protox - protoporphyrinogen oxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; WT - wild-type.

Acknowledgements: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D1A3A01020365).

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et al. 2006). In our previous study, rice plants transformed with the modified, low G+C content *Myxococcus xanthus* (*Mx) Protox* gene (a decrease in G+C content from 71 to 49 %) had resistance to OF as high as rice plants expressing the native *MxProtox* gene despite a lower *Protox* expression (Yang *et al*. 2006). The G+C content was optimized to more closely resemble that of plants (49 and 58 % in *Arabidopsis* and rice, respectively) without changing the *MxProtox* amino acid sequence by synthesizing the full-length coding sequence for *Mx*Protox.

The balance between the production of ROS and their quenching by antioxidants is often upset and this results in oxidative damage (Foyer and Noctor 2009, Suzuki *et al*. 2012). To cope with oxidative stress, plants contain substantial amounts of carotenoids that serve as non-enzymatic scavengers of ROS. The protection of photosynthetic apparatus from photo-oxidative destruction may be afforded by down-regulation of the photo-chemical efficiency *via* the action of the xanthophyll cycle pigments, zeaxanthin and antheraxanthin, which mediate non-radiative energy dissipation at photosystem II (Demmig-Adams *et al*. 1996, Foyer and Noctor 2009, Jia *et al*. 2013). The metabolism of ROS is also dependent on several functionally interrelated antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). SOD is believed to play a crucial role in antioxidant defense because it catalyzes the dismutation of O_2^- into H_2O_2, whereas CAT, POD, and ascorbate peroxidase (APX) destroy H_2O_2 (Suzuki *et al*. 2012, Noctor *et al*. 2014).

In this study, we attempted to clarify differential herbicidal responses between the transgenic rice expressing a modified *MxProtox* (S line) and the wild-type (WT) rice. The aim was to find how the transcription of *Protox* gene is regulated to overcome photoinhibition caused by OF and to compare the contributions of enzymatic and non-enzymatic antioxidants in the resistance of transgenic plants against oxidative stress.

Materials and methods

Plant growth and oxyfluorfen treatment: The modified, low G+C-content *MxProtox* gene construct pGA1611:synthetic *MxProtox* was transformed into rice using *Agrobacterium tumefaciens*-mediated transformation (Yang *et al*. 2006). The wild-type and the homozygous transgenic rice lines expressing the modified *MxProtox* gene (S4, S11, and S19) were used for physiological experiments. The seeds of rice (*Oryza sativa* L.) plants were sown in pots filled with commercial greenhouse compost and grown in a greenhouse at a temperature of 28 to 30 ºC for 4 weeks. Three days before treatments, they were transferred to a growth chamber maintained at day/night temperatures of 28/25 ºC, a 14-h photoperiod, a photosynthetic photon flux density (PPFD) of 200 µmol m^{-2} s^{-1}, and an air humidity of 70 %. Technical-grade OF (*Gyungnong*, Gyeongju, Korea) was used for electrolyte leakage measurement. Commercially available OF (*Goal*®, *Kyungnong*) containing surfactants, which reduce the surface tension of the spray mixture and improve movement into the waxy cuticle, was used for the foliar application test. Four-week-old plants were sprayed with 50 µM OF, placed in darkness for 12 h, and then exposed to a normal photoperiod for 2 d. Control plants were treated with solvent only (30 %, v/v, acetone and 0.01 %, v/v, *Tween 20*). The youngest, fully developed leaves from control and OF-treated plants were sampled at 38 h after the treatment.

Electrolyte leakage: The rice leaf tissues were treated with OF as described previously (Lee *et al*. 1995) by cutting 4-mm leaf squares (0.1 g) with a razor blade and then placing them in a 6-cm diameter polystyrene Petri dish containing 5 cm^3 of 1 % (m/v) sucrose, 1 mM MES (pH 6.5) with or without OF dissolved in acetone. The controls contained the same amount of the solvent. The tissues were incubated with various concentrations of OF (0, 0.1, 1, 10, and 100 µM) in a growth chamber at 25 ºC in darkness for 12 h and then exposed at 250 µmol m^{-2} s^{-1} PPFD for 24 h. Electrolyte leakage into the bathing medium was determined periodically by the conductivity meter (*Cole-Parmer Instruments*, Vernon Hills, IL, USA).

Determination of malondialdehyde: Lipid peroxidation was estimated according to the malondialdehyde (MDA) production using a slight modification of the thiobarbituric acid (TBA) method described by Buege and Aust (1978). The leaf tissue (0.1 g) was homogenized with a mortar and pestle in 5 cm^3 of a solution of 0.5 % (m/v) TBA in 20 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 20 000 *g* for 15 min, the supernatant was heated in a boiling water bath for 25 min, and then cooled in an ice bath. Following centrifugation at 20 000 *g* for 15 min, the resulting supernatant was used for spectrophotometric (UV-2500, *Shimadzu*, Kyoto, Japan) determination of MDA.

Determination of protoporphyrin IX: To measure the Proto IX content, plant tissue (0.1 g) was ground in 2 cm^3 of methanol + acetone + 0.1 M NaOH (9:10:1, v/v/v), and the homogenate was centrifuged at 10 000 *g* for 10 min to remove cell debris and proteins (Lermontova and Grimm 2000). Porphyrins were separated by HPLC *Nova-Pak C18* column (4-µm particle size, 4.6 × 250 mm, *Waters*, Milford, MA, USA) at a flow rate of 1 cm^3 min^{-1}. Porphyrins were eluted with 0.1 M ammonium phosphate (pH 5.8) in methanol. The column eluate was monitored with a fluorescence detector (type *2475*, *Waters*) at excitation and emission wavelengths of 400 and 630 nm, respectively.

Determination of xanthophylls: For xanthophyll analysis, leaf tissues were ground in a solution of 100 % acetone containing 10 mg $CaCO_3$. The extracts were centrifuged at 16 000 *g* for 10 min and the resulting supernatants were collected. The pigments were separated by HPLC as previously described by Gilmore and Yamamoto (1991) using a *Waters 2690* System equipped with a *Waters 2487* absorbance detector. Solvent A (acetonitrile + methanol + 0.1 M Tris-HCl buffer, pH 8.0, 72:8:3, v/v/v) was run isocratically from 0 to 4 min followed by a 2.5 min linear gradient to 100 % solvent B (methanol + hexane, 4:1, v/v) at flow rate of 2 cm^3 min^{-1}. The detector was set at 440 nm for the integration of peak areas. The chlorophyll content was spectrophotometrically determined according to the method of Lichtenthaler (1987).

Assays for antioxidant enzymes: Soluble proteins were extracted by homogenizing the leaf tissues (0.25 g) in 2 cm^3 of 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 1 % (m/v) polyvinylpyrrolidone, and 1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 15 000 *g* and 4 ºC for 20 min. Equal amounts of protein were electrophoresed on 10 % non-denaturing polyacrylamide gels at 4 ºC and a constant current of 30 mA for 1.5 h. Gels were stained for SOD isoforms by soaking in 50 mM potassium phosphate (pH 7.8) containing 2.5 mM nitroblue tetrazolium (NBT) in darkness for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 28 mM NBT and 28 µM riboflavin in darkness for 30 min (Rao *et al*. 1996). The gels were then exposed to PPDF of 130 µmol m^{-2} s^{-1} for approximately 30 min. The CAT activity was detected by incubating the gels in 3.27 mM H_2O_2 for 25 min, rinsing them in water, and staining them in a solution of 1 % potassium ferricyanide and 1 % ferric chloride for 4 min (Woodbury *et al*. 1971). The staining of POD isozymes was achieved by incubating gels in sodium citrate buffer (pH 5.0) containing 9.25 mM *p*-phenylenediamine and 3.92 mM H_2O_2 for 15 min. For APX, 2 mM ascorbate was added to the electrode buffer and the electrophoresis system was pre-run for 30 min before the sample was loaded. Isoforms of APX were visualized by incubating the gels for 30 min in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate. The gels were then incubated in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min, and then soaked in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) and 2.45 mM NBT for 15 min with gentle agitation (Rao *et al*. 1996).

RNA Extraction and reverse transcriptase quantitative PCR: Total RNA was prepared from leaf tissues using *Trizol* reagent (*Invitrogen*, Waltham, MA, USA), and 5 µg of RNA from each sample was used for the reverse transcription reaction (*SuperScript III* first-strand synthesis system, *Invitrogen*). Subsequently, 50 ng of cDNA was used for qPCR analysis carried out with the *7300 Real-Time* PCR system (*Applied Biosystems*, Waltham, MA, USA) using *Power SYBR Green PCR Master Mix* (*Applied Biosystems*) and specific primers (Table 1 Suppl.). The PCR program consisted of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A melting curve analysis was performed after every PCR reaction to confirm the accuracy of each amplified product. All reactions were set up in triplicate. The sample of WT control was used as the calibrator, with the expression of the sample set to 1. *Actin* was used as the internal control.

Results and discussion

The transgenic rice expressing a modified, but not native, *MxProtox* gene was generated by transforming the WT rice with the binary vector pGA1611:modified *MxProtox* (Yang *et al*. 2006). Despite the decreased expression of modified *MxProtox* gene, the seedling growth test with OF suggested that the modified gene conferred herbicide tolerance as high as that of the native *MxProtox* gene. In the present study, we further evaluated the effect of the modified *MxProtox* gene on the resistance of transgenic rice to OF induced oxidative stress. The OF resistance, as indicated by changes in electrolyte leakage, in the modified *MxProtox* transgenic lines S4 and S11 was compared with that of the WT. When leaf squares were incubated in the OF concentrations ranging from 0 to 100 µM, the great increase in conductivity was observed only in the WT plants (Fig. 1). After 24 h at PPDF of 100 µmol m^{-2} s^{-1} and OF concentrations above 10 µM, leaf squares of the WT began to exude electrolytes and the leakage further increased at 100 µM. Membrane

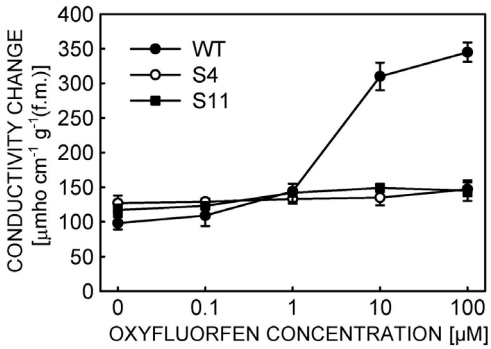

Fig. 1. Effect of oxyfluorfen (OF) on electrolyte leakage in the wild-type (WT) and the transgenic rice plants (S4 and S11) expressing a modified *MxProtox* gene. Conductivity was measured in the bathing solution after leaf segments were treated with various concentrations of technical-grade OF and exposed to PPDF of 250 µmol m^{-2} s^{-1} for 24 h following a 12-h dark. Means ± SEs, $n = 3$.

disruption by lipid peroxidation can destroy cellular compartments, causes loss of solutes and water, and finally leads to cell death (Mock *et al*. 1999, Pham *et al*. 2015). Significant electrolyte leakage was not detected in the transgenic lines S4 and S11 up to 100 μM OF (Fig. 1), indicating the increased tolerance of these transgenic rice plants to OF.

When four-week-old rice plants were exposed to foliar application of 50 μM OF, the WT plants showed typical symptoms of leaf necrosis at 2 d after the treatment (Fig. 2*A*). This was followed by a 3-fold increase in MDA content (Fig. 2*B*), which may derive from lipid peroxidation due to excess accumulation of the photosensitizer Proto IX. Obviously, the oxidative lipid damage is a marker of OF-induced stress in WT plants. By contrast, the transgenic lines S4 and S11 did not exhibit necrotic lesions on the treated leaves and significantly increased MDA content (Fig. 2). Next, we examined the effect of modified *MxProtox* gene on the content of Proto IX. The transgenic lines S4, S11, and S19 exhibited 2- to 3-fold greater content of Proto IX compared to the WT plants (Table 1). This appears to derive from the increased expression of Protox protein in the transgenic rice (Yang *et al*. 2006). Upon foliar application of 50 μM OF, content of Proto IX increased up to 66-fold in the WT plants (Table 1), which resulted from cytosolic accumulation of Proto IX by nonspecific oxidation of Protogen IX. Proto IX present in excess leads to highly toxic $^1O_2$ formation; it triggers membrane lipid peroxidation, protein denaturation, and chlorophyll bleaching (Beale and Weinstein 1991, Reinbothe *et al*. 1996, Tripathy and Oemüller 2012). All the transgenic lines treated with OF also increased Proto IX content, but the increase was much lower. Our results confirm that the expression of the modified transgene led to increased resistance to OF, although the metabolic imbalance of porphyrin biosynthesis by OF can induce photoinhibiton and oxidative stress.

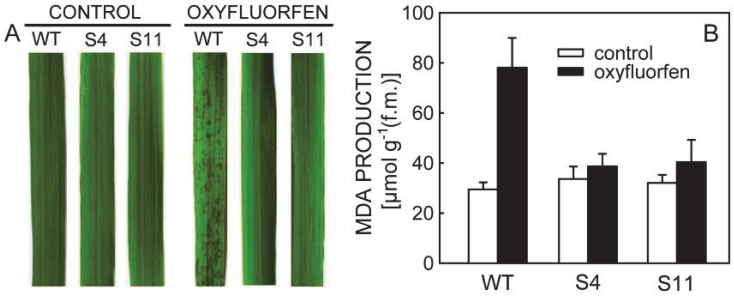

Fig. 2. Effect of oxyfluorfen (OF) on photoinhibition, as indicated by leaf phenotypes (*A*) and oxidative stress as indicated by malondialdehyde (MDA) production (*B*), in the wild-type (WT) and the transgenic rice lines (S4 and S11)expressing a modified *MxProtox* gene. Four-week-old plants were sprayed with 50 μM OF. Photographs were taken 2 d after OF treatment. Means ± SEs, $n = 3$.

Table 1. Effect of oxyfluorfen (50 μM for 2 d) on protoporphyrin IX content [nmol g^{-1} (f.m.)] in leaves of the wild-type (WT) and the transgenic rice lines (S4, S11, and S19) expressing a modified *MxProtox* gene. Means ± SEs, $n = 3$.

Plants	Control	Oxyfluorfen
WT	4.3 ± 0.3	282.5 ± 27.3
S4	7.6 ± 0.4	45.4 ± 4.8
S11	9.8 ± 2.1	25.5 ± 3.5
S19	14.4 ± 3.2	26.6 ± 2.2

To gain an insight into the molecular mechanisms underlying the *Protox* overexpression-induced changes in stress tolerance, we analyzed the expression of the enzymes with known regulatory roles in porphyrin biosynthesis. The untreated transgenic lines S4 and S11 exhibited a strong expression of modified *MxProtox*, whereas the WT did not express this transgene (Fig. 3). On the other hand, the transcription of endogenous *OsProtox* was not different in the WT and transgenic lines. During stress imposed by OF, the WT plants greatly decreased the transcription of *OsProtox*, whereas the transcription of both *OsProtox* and modified *MxProtox* in the transgenic lines remained almost constant. We also compared the expression of other key genes in the porphyrin biosynthetic pathway. The transcription of *Fe-Chelatase2* (*FC2*), which encodes the plastidic isoform of Fe-chelatase, was markedly down-regulated in the WT plants after 2 d of OF treatment, whereas the transgenic plants did not show any noticeable change (Fig. 3). The induction of Fe-chelatase plays a positive role in acifluorfen-induced oxidative stress tolerance of the transgenic rice expressing *Bradyrhizobium japonicum FC* (Kim *et al*. 2014). After OF treatment, the transcription of *Heme Oxygenase2* (*HO2*), encoding heme oxygenase (Muramoto *et al*. 2002), slightly increased in the WT plants, but not in the transgenic plants. HO is suggested as a part of the antioxidant machinery of the cell due to its production of biliverdin IXα preventing oxidative cell damage (Noriega *et al*. 2004, Kim *et al*. 2014).

We examined the photoprotective responses of the WT and the transgenic rice lines to counteract OF-induced photoinhibition. Carotenoids function mainly in excess radiation dissipation and contribute to non-enzymatic scavenging of ROS (Jahns and Holzwarth

2012, Jia *et al*. 2013). In comparison with untreated WT plants, the content of violaxanthin and lutein was slightly greater in the transgenic lines S4 and S11, whereas the content of other carotenoids did not significantly differ (Table 2). In response to foliar application of 50 μM OF, the content of neoxanthin, violaxanthin, and β-carotene greatly decreased in the WT plants, but not in the transgenic plants (Table 2). The OF treatment triggered a 3-fold increase in antheraxanthin and a new formation of zeaxanthin only in the WT plants (Table 2), indicating that the WT plants were partly protected from the photoinhibition through increasing content of antheraxanthin and zeaxanthin. Zeaxanthin is the major player in the deactivation of excited singlet chlorophyll and essentially important for plants to minimize photo-oxidative damage of membrane lipids (Gilmore and Yamamoto 1991, Demmig-Adams *et al*. 1996, Jahns and Holzwarth 2012). However, this regulatory mechanism of the xanthophyll-cycle pigments was not sufficient to overcome the photoinhibition in the WT plants.

In addition, the extent of stress-induced damage can be attenuated by the action of the antioxidant enzymes capable of scavenging ROS (Suzuki *et al*. 2012, Gallie 2013, Noctor *et al*. 2014). To test whether the OF-induced stress brings about increased antioxidants, enzyme activities and transcription of respective genes were determined. The activities of SOD, CAT, POD, and APX were similar in the untreated WT and the transgenic lines S4 and S11 (Fig. 4). After the administration of 50 μM OF for 2 d, the WT plants increased the activities of SOD isozymes 2 and 3, POD isozyme 3, and all CAT

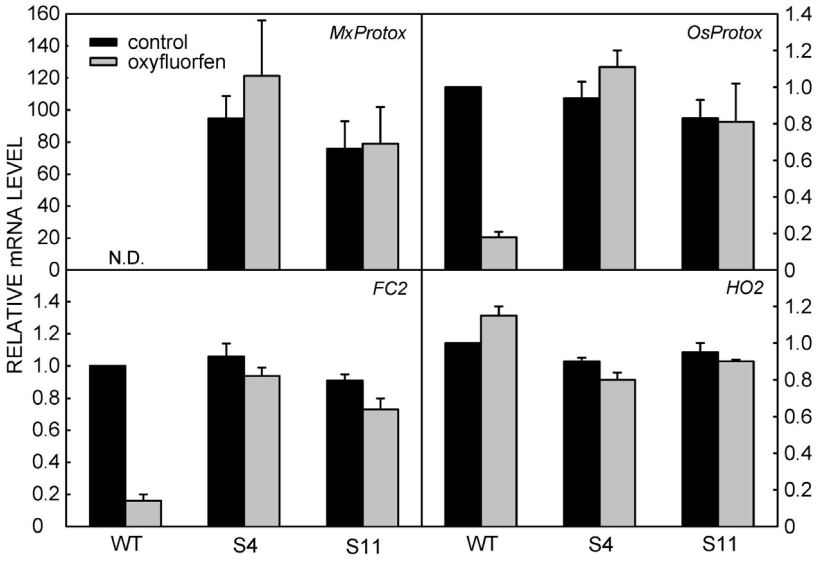

Fig. 3. Oxyfluorfen-induced changes in the expression of the modified *MxProtox* gene, constitutive *OsProtox* gene, and other key genes encoding the porphyrin pathway enzymes *Fe-Chelatase2* (*FC2*) and *Heme Oxygenase2* (*HO2*) in leaves of the wild-type (WT) and the transgenic rice lines (S4 and S11) expressing a modified *MxProtox* gene. Reverse transcriptase quantitative PCR was used and *Actin* as an internal control. The WT control was used for normalization with the expression set to 1. N.D. - not detectable. Means ± SEs, $n = 3$.

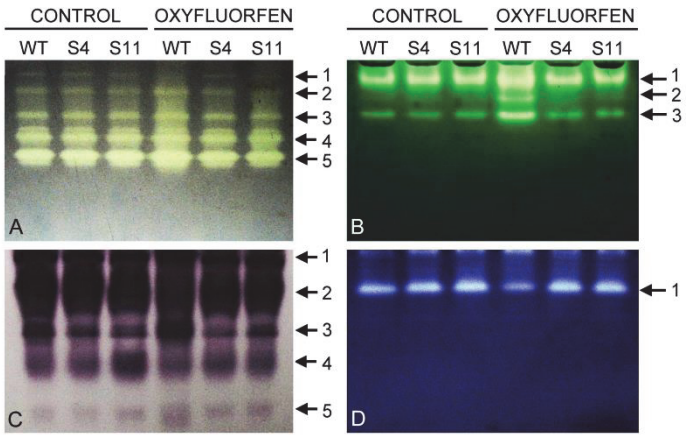

Fig. 4. Effect of oxyfluorfen (50 μM, 2 d) on the isozyme profiles of superoxide dismutase (*A*), catalase (*B*), peroxidase (*C*), and ascorbate peroxidase (*D*) in the wild-type (WT) and the transgenic rice lines (S4 and S11) expressing a modified *MxProtox* gene.

Table. 2. Effect of oxyfluorfen (50 μM for 2 d) on relative content of xanthophylls and β-carotene [mmol mol^{-1}(Chl *a*)] in leaves of the wild-type (WT) and the transgenic rice lines (S4 and S11) expressing a modified *MxProtox* gene. Means ± SEs, *n* = 3. N.D. - not detectable.

Treatment	Plants	Neoxanthin	Violaxanthin	Antheraxanthin	Lutein	Zeaxanthin	β-carotene
Control	WT	29.6 ± 0.5	41.7 ± 2.3	1.7 ± 0.1	80.0 ± 7.2	N.D.	62.9 ± 6.0
	S4	34.4 ± 1.6	58.7 ± 1.0	2.3 ± 0.1	101.8 ± 10.2	N.D.	76.2 ± 3.2
	S11	32.0 ± 1.8	52.1 ± 3.2	2.0 ± 0.5	95.4 ± 0.5	N.D.	72.8 ± 1.8
Oxyfluorfen	WT	19.1 ± 0.9	23.0 ± 1.2	5.0 ± 1.1	81.4 ± 4.1	2.4 ± 0.7	29.9 ± 1.5
	S4	29.8 ± 3.5	49.2 ± 9.3	1.4 ± 0.3	81.8 ± 6.3	N.D.	61.9 ± 5.1
	S11	29.3 ± 1.8	44.2 ± 2.9	1.3 ± 0.1	85.2 ± 1.9	N.D.	69.5 ± 4.4

isozymes (strong increases in isozymes 2 and 3), whereas the activity of APX decreased (Fig. 4). Particularly, the increases of POD and CAT activities would be predominantly responsible for controlling free radical production in the stressed WT plants, but the increased activities were not enough to overcome the damage. In contrast to the WT, the transgenic lines S4 and S11 treated with OF exhibited no change in the activities of all antioxidant enzymes examined (Fig. 4). Cells express a set of H_2O_2-decomposing enzymes, namely CAT and APX, which are heme enzymes with Proto IX moieties (Feierabend 2005, Mittler and Poulos 2005). The WT plants responded to OF treatment by up-regulating the transcription of *Catalase* (*Cat*) *B*, whereas the transcription of *CatC* decreased (Fig. 5). In the OF-treated WT plants, there was the discrepancy between the increase in *Ascorbate Peroxidase* (*APX*) *a* and *APXb* transcription and the decline in APX activity (Figs. 4 and 5), which can be ascribed to the inactivation of APX under severe stress. On the other hand, the transgenic lines S4 and S11 did not noticeably alter the transcription of genes coding antioxidant enzymes in response to OF (Fig. 5). Overall, transgenic rice expressing modified *MxProtox* had the lower content of non-enzymatic and enzymatic antioxidants, which may result from less production of ROS compared to the WT plants.

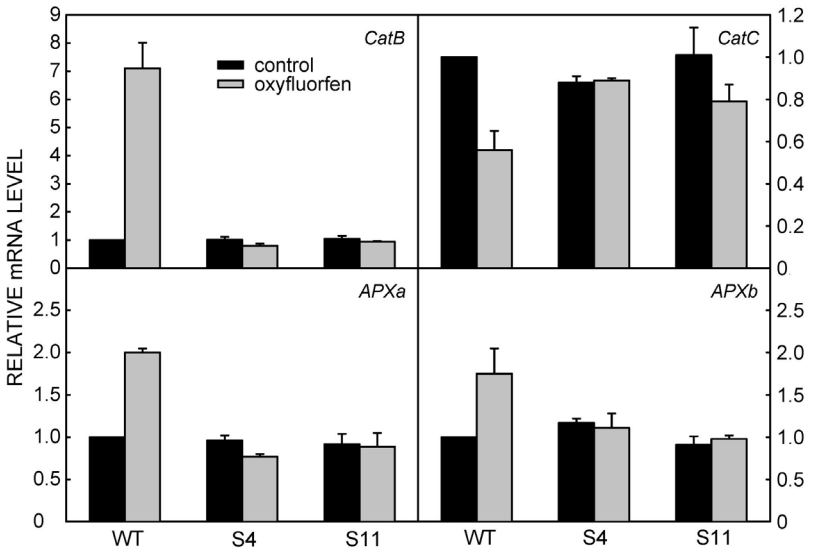

Fig. 5. Effect of oxyfluorfen (50 μM, 2 d) on expression of genes encoding antioxidant enzymes in leaves of the wild-type (WT) and the transgenic rice lines (S4 and S11) expressing a modified *MxProtox* gene. Reverse transcriptase quantitative PCR was used and *Actin* as an internal control. The WT control was used for normalization with the expression set to 1. Means ± SEs, *n* = 3.

Conclusion

During OF action, the transgenic rice sustained the transcription of both *OsProtox* and modified *MxProtox*, which appear to contribute to the reduced accumulation of Proto IX. By contrast, the transcription of *OsProtox* in the WT was greatly down-regulated in response to OF and the over-accumulated Proto IX triggered severe stress, necessitating the substantial increases not only in zeaxanthin and antheraxanthin, but also in the activities of SOD, CAT, and POD. The transcription of *HO2* as well as *CatB* and *APX* also increased only in the

OF-treated WT, which could be indicative of a build-up of protective mechanisms. However, in the transgenic plants, the antioxidants were not noticeably enhanced in response to OF, mainly due to the lower ROS formation. Our results suggest that the transgenic rice expressing a modified *MxProtox* regulated transcription of porphyrin biosynthetic genes and efficiently prevented accumulation of photosensitizing Proto IX, thereby suffering reduced photoinhibition and oxidative stress imposed by OF, compared to the WT.

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