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ORIGINAL RESEARCH

Overexpression of Rice Ferrochelatase I and II Leads to Increased Susceptibility to Oxyfluorfen Herbicide in Transgenic Rice

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Abstract Protoporphyrin IX is a photosensitizer and a causative agent of rice membrane lipid peroxidation in plant cells. Protoporphyrinogen IX oxidase (PPO) is the molecular target of PPO-inhibiting herbicides, which trigger a massive increase in protoporphyrin IX. Thus, any possible method to decrease the levels of protoporphyrin IX upon challenge with PPO-inhibiting herbicides could be employed to generate plants resistant to such herbicides. We generated transgenic rice plants overexpressing rice ferrochelatase isogenes encoding ferrochelatase enzymes, which convert protoporphyrin IX into protoheme, to see whether the transgenic plants have phenotypes resistant to PPO-inhibiting herbicides. The resulting transgenic rice plants were all susceptible to oxyfluorfen (a diphenyl-ether-type PPO-inhibiting herbicide), as judged by cellular damage with respect to cellular leakage, chlorophyll loss, and lipid peroxidation. In particular, the transgenic plants expressing rice ferrochelatase II without its plastid targeting sequence showed higher transgene expression and oxyfluorfen susceptibility than lines expressing the intact ferrochelatase II. Possible susceptibility mechanisms to oxyfluorfen herbicide in the transgenic rice plants are discussed.

Keywords Herbicide resistance · Oxyfluorfen · Photosensitizer · Rice ferrochelatase · Transgenic rice

Abbreviations
FC Ferrochelatase
PPO Protoporphyrinogen IX oxidase

Introduction

Protoporphyrin IX is a photosensitizer that produces singlet oxygen and provokes membrane lipid peroxidation leading to cellular death via a light-dependent mechanism (Duke et al. 1991). The production of protoporphyrin IX in plant cells is a causative agent that kills plants on treatment with protoporphyrinogen IX oxidase (PPO)-inhibiting herbicides. In normal healthy plants, the level of protoporphyrin IX is tightly regulated to prevent accumulation in plant cells. Because protoporphyrin IX is not only an enzymatic product of the PPO enzyme but also a substrate for the next step enzymes (e.g., ferrochelatase and magnesium chelatase) in chlorophyll and the heme biosynthetic pathway, the cellular levels of protoporphyrin IX will be affected by either PPO activity or chelatase activities. For example, changes in PPO enzyme activities cause rice plants to form necrotic leaf lesions (Molina et al. 1999; Jung et al. 2008) due to the high level of protoporphyrin IX. Also, suppression of ferrochelatase led to accumulation of photosensitizing protoporphyrin IX (Papenbrock et al. 2001). So far, many attempts have been made to find PPO-inhibiting herbicide-resistant plants by overexpressing various types of PPO genes that prevent accumulation of protoporphyrin IX upon herbicide treatments (Li et al. 2003; Jung et al. 2004; Li and Nicholl 2005).

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Ferrochelatase is the last enzyme of heme biosynthesis that catalyzes the insertion of ferrous iron (Fe^{2+}) into protoporphyrin IX to form protoheme (Moulin and Smith 2005). Thus, it is expected that ferrochelatase overexpression would chelate protoporphyrin IX into protoheme and eventually decrease the levels of protoporphyrin IX that accumulate upon treatment with PPO-inhibiting herbicides. As an initial study to see whether ferrochelatase overexpression shows resistance toward PPO-inhibiting herbicides such as oxyfluorfen, we introduced rice ferrochelatase genes into the rice genome under control of the maize ubiquitin promoter in sense orientation. The integration and expression of the transgenes was analyzed in the T_0 generation by Southern and Northern blot analyses in transgenic rice plants. The transgenic rice plants overexpressing various ferrochelatase isogenes in T_0 leaves were susceptible to oxyfluorfen treatment, as judged by various biochemical analyses such as cellular leakage, chlorophyll loss, and lipid peroxidation. This is the first report, to our knowledge, both on the generation of transgenic rice plants expressing ferrochelatase genes and on their characteristics in oxyfluorfen-susceptible phenotypes.

Materials and Methods

Vector Construction and Rice Transformation

Two full-length cDNA fragments of rice ferrochelatases were provided by the National Institute of Agrobiological Sciences, Japan (http://www.rgrc.dna.affrc.go.jp/). They were AK068174 (ferrochelatase I) and AK073873 (ferrochelatase II). The full-length cDNA of ferrochelatase I (*FC1*) was amplified by polymerase chain reaction (PCR), using the cDNA clone as a template, with 5′-d(CTGGAGCTC**ATG** GAGTGCGTCCGCTCC)-3′ as the forward primer (*Sac*I restriction site underlined and translation start codon in bold) and 5′-d(ACCGTTAAC**CTA**CAACAGGGTGTTCCG)-3′ as the reverse primer (*Hpa*I restriction site underlined and translation stop codon in bold). For ferrochelate II (*FC2*), the forward primer was 5′-d(CTGGAGCTC**ATG**TGGTCGTC GAGCCAG)-3′ (*Sac*I restriction site underlined and translation start codon in bold), and the reverse primer was 5′-d (TTGGGTACC**TCA**GTGGAACAAAGGCAG)-3′ (*Kpn*I restriction site underlined and translation stop codon in bold). ΔFerrochelatase II (*FC3*), which has its plastid transit sequence removed, was PCR amplified with the FC2 cDNA clone as a template, with 5′-d(CTGGAGCTC**ATG**TG CAAGTTGGGATGGTCT)-3′ as the forward primer (*Sac*I restriction site underlined and translation start codon in bold) and 5′-d(TTGGGTACC**TCA**GTGGAACAAAGGCAG)-3′ as the reverse primer (*Kpn*I restriction site underlined and translation stop codon in bold). Three PCR products were digested with corresponding restriction enzymes, gel purified, and ligated into pBluescript SK that was predigested with the same restriction enzymes (Stratagene, La Jolla, CA). After verification of the DNA sequences, the cDNA fragments were ligated into the pGA1611 binary vector between the maize ubiquitin promoter and the nopaline synthase terminator. The resulting pGA1611:FC1, pGA1611:FC2, and pGA1611:FC3 vectors were transformed into *Agrobacterium tumefaciens* LBA4404. Rice transformation was performed as previously described (Lee et al. 2000).

Isolation and Analysis of Nucleic Acids

Genomic DNA was isolated using DNAzol ES (Molecular Research Center, Cincinnati, OH) from 5-week-old leaves of transgenic and wild-type rice plants. Genomic DNA (5 μg) extracted from the transgenic and wild-type leaves was digested with the *Sac*I restriction enzyme. The resulting gDNA was size fractionated by electrophoresis on an 0.8% agarose gel and blotted onto a nylon membrane (Nylon 66 plus; Amersham Pharmacia Biotech, Piscataway, NJ). Total RNA (10 μg) was isolated from 3-week-old leaves of transgenic and wild-type rice and then ground in liquid nitrogen with TRI reagent (Sigma Chemical Co., St. Louis, MO). The total RNA was fractionated on a 1% agarose gel that contained formaldehyde, using 20 mM 3-(*N*-morpholino)propanesulfonic acid as the running buffer. The gel was blotted onto a nylon membrane and hybridized with the rice *FC1* and *FC2* cDNA clones that were radiolabeled using the Prime-It kit (Stratagene). Hybridization was carried out at 60°C in 0.25 M sodium phosphate buffer (pH 7.5), 7% SDS (sodium dodecyl sulfate), 1% bovine serum albumin, and 1 mM EDTA. After hybridization, the RNA blot was washed at 55°C twice with 2× SSC (150 mM sodium chloride, 15 mM sodium citrate)/0.1% SDS and then twice with 0.2× SSC/0.1% SDS (Lee et al. 2000). The RNA was stained with ethidium bromide prior to blotting. Quantification was performed with the PhosphorImager BAS2000 image analyzer (Fuji, Tokyo, Japan).

Chlorophyll Determination

The T_0 transgenic and wild-type rice leaf tissues from 5-week-old plants were treated with various oxyfluorfen concentrations by cutting 4-mm leaf squares (0.2 g fresh weight) with a razor blade and then placing them in a 6-cm diameter polystyrene Petri dish containing 7-mL 1% sucrose, 1 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5) with or without the herbicide dissolved in acetone. The control contained the same amount of the solvent without the herbicide (Lee et al. 1995). The final concentration of acetone in all dishes was 1% (v/v). The tissues were incubated in a growth chamber at 25°C in darkness for 12 h and then exposed to continuous white

light at 250 μmol $m^{-2}s^{-1}$ photosynthetically active radiation for 2 days. No toxic effects of acetone alone on the tissues were detected during the experiments (data not shown). Chlorophyll content was spectrophotometrically determined according to the method of Lichtenthaler (1987).

Cellular Leakage and Malondialdehyde Determination

The tissues were treated with oxyfluorfen and incubated in the same manner as for the measurements of chlorophyll. Cellular leakage was determined periodically by detection of electrolyte leakage into the bathing medium using a conductivity meter (Cole-Parmer Instruments Co., Vernon Hills, IL) as previously described (Lee et al. 1995). Malondialdehyde (MDA) was measured using the thiobarbituric acid test. Transgenic and wild-type leaves (0.1 g) were homogenized in a 2-mL solution of 0.5% thiobarbituric acid in 20% trichloroacetic acid. The supernatants, after 20,000×*g* centrifugation for 15 min, were subjected to spectrometric analysis as described previously (Lee et al. 2000). All analyses for each measurement were done in triplicate.

Results and Discussion

Generation of Transgenic Rice Plants and DNA Blot Analysis

Rice contains two distinct ferrochelatase (*FC*) genes, as does *Arabidopsis*. Their deduced polypeptides share an overall identity of 73%. Ferrochelatase I (*FC1*; AK068174) consists of 482 aa with no apparent transit sequence in its amino terminus. Ferrochelatase II (*FC2*; AK073873) has 526 aa and a typical transit sequence for plastid targeting of about 70 aa in length according to TargetP analysis (Emanuelsson et al. 2000). FC1 from *Arabidopsis* has a unique feature to translocate FC1 precursor protein into both chloroplasts and mitochondria, whereas FC2 is only directed into chloroplasts (Chow et al. 1997). A total of three rice FC isogenes were transformed into the rice genome. These were *FC1*, *FC2*, and *FC3* (ΔFerrochelatase II), of which *FC3* represents the ferrochelatase II gene encoding a mature form of rice ferrochelatase II. The transit sequence was deduced based on both the TargetP analysis of rice FC2 and comparisons of other FC polypeptides. Transgenic rice plants expressing the rice *FC* genes were generated by transforming wild-type rice (*Oryza sativa* cv. "Dongjin") with the binary vectors pGA1611:FC1, pGA1611:FC2, and pGA1611:FC3 (Fig. 1). Transgene *FC* was designed to be expressed constitutively under the control of the constitutive maize (*Zea mays*) ubiquitin promoter. Seven transgenic rice plants (T_0) were selected for production of T_1 seeds from among at least 20

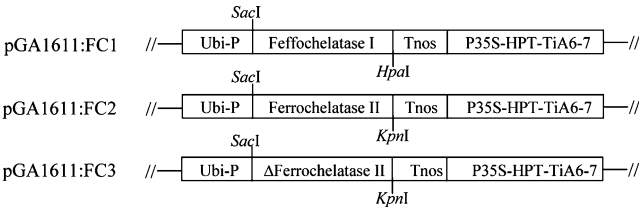

Fig. 1 Schematic diagram of the T-DNA region of the binary vector pGA1611 harboring various rice ferrochelatase cDNAs. Ubi-P, maize ubiquitin promoter; Tnos, nopaline synthase terminator; P35S, cauliflower mosaic virus 35 S promoter; HPT, hygromycin phosphotransferase; TiA6-7, TiA6-7 terminator; ΔFerrochelatase II, ferrochelatase II without the native transit sequence of 70 amino acids

independent transgenic lines per each construct regenerated in vitro under hygromycin selection. The phenotypes of the T_0 transgenic lines were almost indistinguishable during the vegetative growth stages, whereas the transgenic mature leaves (T_0) of around 13-week-old plants were slightly bleached compared to the same stage of wild-type leaves.

The site of transgene integration was analyzed in the FC transgenic rice lines using genomic DNA blots. DNA was extracted from 5-week-old T_0 transgenic and wild-type rice plants, digested with *Sac*I, and hybridized with ^{32}P-labeled rice *FC1* or *FC2* cDNA. As shown in Fig. 2, three hybridizing bands appeared in the wild type upon *FC1* probe, whereas two bands were found in the wild type with the *FC2* probe, confirming that the rice genome contains two copies of *FC*. Three hybridizing bands were generated in the *FC1* probe because *FC1* had an internal *Sac*I restriction site within cDNA. In FC1 transgenic lines, all lines showed one to three copies of *FC1* transgenes other than the three bands detected in the wild type. Lines 1 and 4 seemed to have one copy of the transgene inserted into the rice genome, but lines 1, 2, and 7 harbored two copies of the transgene. Some lines, such as lines 5 and 6, had more than three copies of the transgene. Similarly, the *FC2* transgene was found in FC2 and FC3 transgenic genotypes. Among FC2 transgenic lines, one-copy insertion was frequently observed, whereas two-copy insertion also occurred in two lines (lines 6 and 7). In contrast, FC3 transgenic lines were introduced with multiple copies of the transgene, of which line 6 seemed to have at least five copies of the transgene. All Southern blot analysis data clearly suggested that the ferrochelatase transgene was successfully inserted into the rice genome in the three different genotypes. All lines appeared to be independent because the size and intensity of bands differed on Southern blots.

Expression Levels of Ferrochelatase mRNA by Northern Blot Analysis

To examine whether the stable integrations of the three ferrochelatase transgenes were closely coupled to their

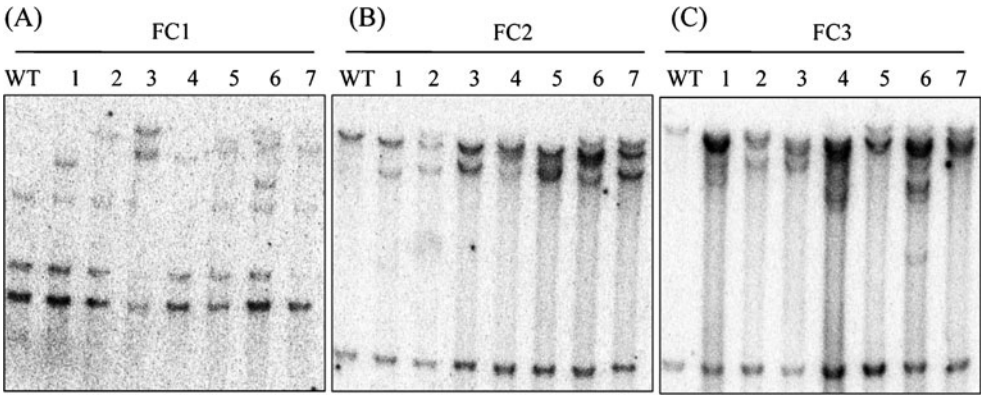

Fig. 2 Southern blot analysis of transgenic and wild-type rice plants. Five micrograms of genomic DNA extracted from various genotypes were digested with the *Sac*I restriction enzyme, size fractionated by electrophoresis, and transferred to nylon membranes. FC1 gDNA was probed with ferrochelatase I cDNA, whereas FC2 and FC3 were probed with ferrochelatase II cDNA. WT, wild type; 1–7, transgenic lines

transcriptional levels in the three transgenic genotypes, we performed Northern blot analyses using total RNA extracted from 5-week-old T_0 transgenic rice lines and the wild type. As shown in Fig. 3, transgene *FC* mRNA was detected in total RNA from the leaves of all T_0 transgenic lines, with varying levels of *FC* mRNA, as well as from the wild type. In the wild type, *FC1* and *FC2* mRNA was expressed at a low level, whereas in all three transgenic genotypes, *FC1* and *FC2* mRNA was expressed in abundance. In FC1 transgenic lines, most lines showed high levels of *FC1* mRNA, whereas line 3 had the lowest expression level. Although both FC2 lines and FC3 lines expressed the *FC2* cDNA transgene, the relative expression levels between the two transgenic genotypes were strikingly different. The overall expression levels of *FC2* mRNA were higher in the FC3 transgenic genotype than in the FC2

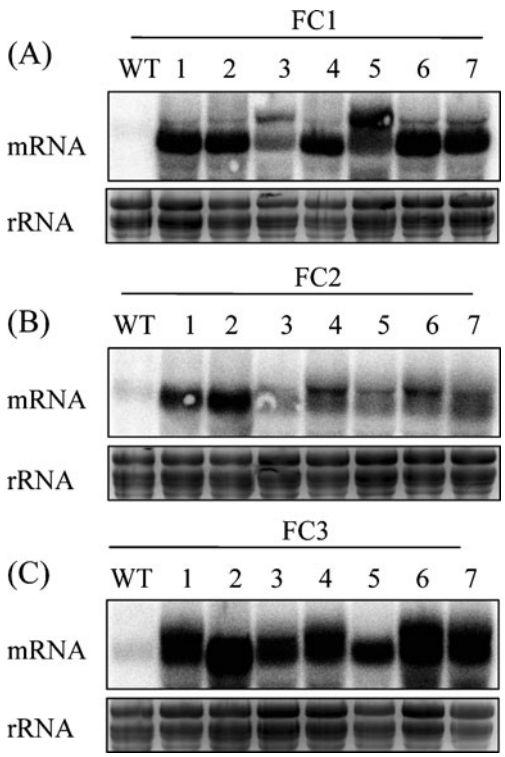

Fig. 3 Northern blot analysis of ferrochelatase mRNA in various transgenic lines and in the wild type. Total RNA (10 μg) was isolated from 5-week-old leaves of T_0 transgenic or wild-type rice and blotted onto a nylon membrane. The FC1 blot was probed with ferrochelatase I cDNA, whereas FC2 and FC3 blots were probed with ferrochelatase II cDNA. Equal loading of RNA samples was checked using ethidium bromide staining prior to blotting. WT, wild type; 1–7, transgenic lines

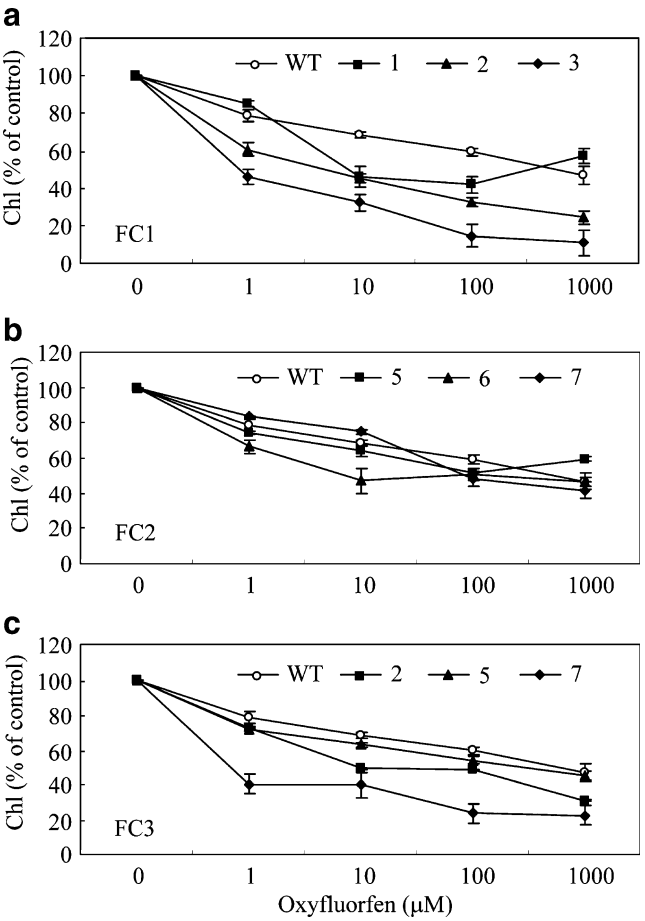

Fig. 4 Effect of oxyfluorfen on chlorophyll loss from leaf squares of the wild-type and the T_0 transgenic rice plants. The tissues were exposed to continuous light at 250 μmol $m^{-2}s^{-1}$ at 25°C for 2 days, following a 12-h dark incubation. All treatments for each measurement were in triplicate (mean ± SE, n=3). WT, wild type; 1–7, transgenic lines

transgenic genotype. This implies that the *FC2* gene without the transit sequence was more stably expressed at the mRNA level than the intact *FC2* gene. These data are in contrast with prior notions, in which the gene harboring the plastid transit sequence is more stably expressed than the gene without the transit sequence (Lee et al. 2000; Ha et al. 2004). Taken together, all three transgenic genotypes showed high expression of *FC* mRNA, suggestive of stable and abundant transcription after the introduction of *FC* transgenes into the rice genome.

Evaluation of Oxyfluorfen Resistance of the Transgenic Rice Plants

To show the role of ferrochelatase overexpression in the transgenic rice plants, we further selected representative transgenic plants to evaluate of their responses upon oxyfluorfen herbicide application. Leaf disks prepared from 5-week-old wild-type and transgenic plants were exposed to various concentrations of oxyfluorfen ranging from 1 to 1000 μM. After a 12-h dark incubation followed by a 2-day light exposure, leaf disks were subjected to chlorophyll analysis. The control leaf squares prior to oxyfluorfen treatment had similar levels of chlorophyll in wild type and all transgenic lines. As shown in Fig. 4, all transgenic genotypes had less chlorophyll than the wild type upon oxyfluorfen treatments. Although there were no dramatic differences among genotypes in chlorophyll levels when challenged by oxyfluorfen, the *FC2* genotype that overexpressed the full-length *FC2* cDNA exhibited the least oxyfluorfen susceptibility, followed by the FC3 genotype. In general, the low susceptibility to oxyfluorfen of the FC2 genotype is probably associated with the relatively low levels of transgene expression compared to the other genotypes. However, the expression levels of the transgene within a genotype were not closely matched with the symptoms of oxyfluorfen susceptibility. For example, line 3 from FC1 had the lowest transgene expression, but this line had the highest susceptibility to oxyfluorfen. Based on the chlorophyll data of oxyfluorfen treatments, all transgenic genotypes expressing various forms of ferrochelatases exhibited a single phenotype with oxyfluorfen susceptibility, suggesting that the overexpression of ferrochelatase does not play a role in enhancing resistance toward PPO-

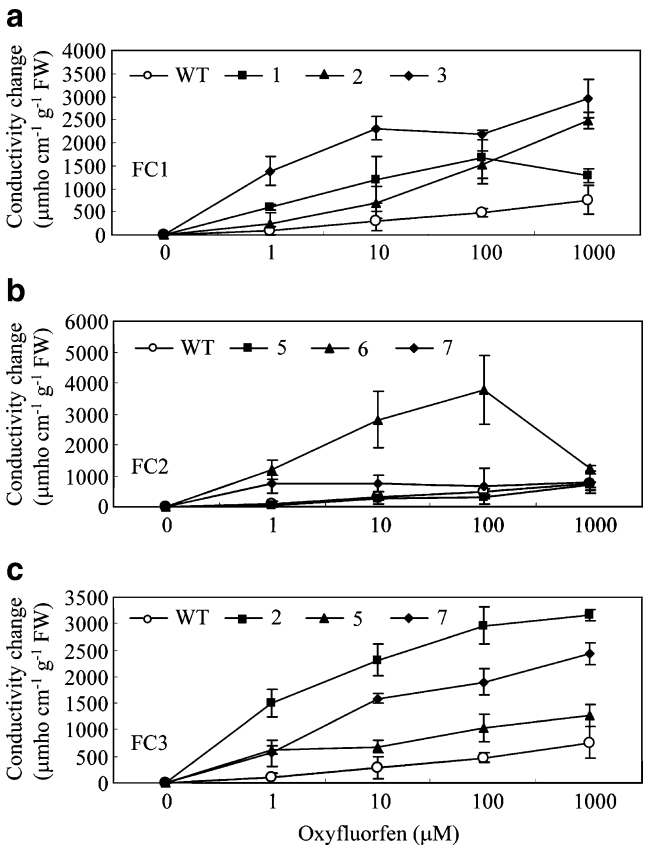

Fig. 5 Effect of oxyfluorfen on cellular leakage from leaf squares of the wild-type and the T_0 transgenic rice plants. The tissues were treated as shown in Fig. 4. All treatments for each measurement were in triplicate (mean ± SE, n=3). WT, wild type; 1–7, transgenic lines

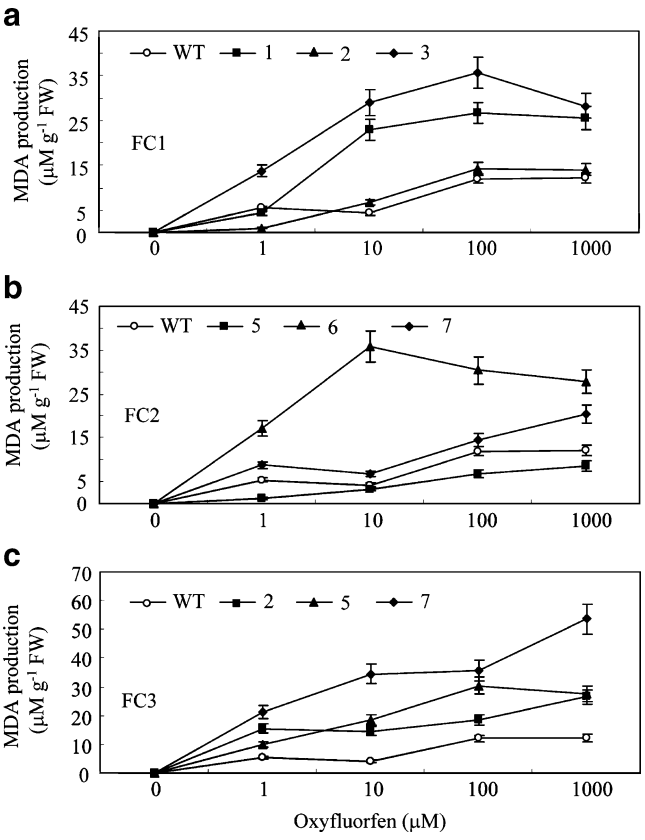

Fig. 6 Effect of oxyfluorfen on MDA production in leaf squares of the wild-type and the T_0 transgenic rice plants. The tissues were exposed to the same conditions as shown in Fig. 3. All treatments for each measurement were in triplicate (mean ± SE, n=3). WT, wild type; 1–7, transgenic lines

inhibiting herbicides by chelating protoporphyrin IX. In addition to chlorophyll loss, oxyfluorfen caused remarkable cellular leakage from the treated leaf squares of the nontransgenic rice plants (Fig. 5). The severity of cellular leakage depended on the concentrations of oxyfluorfen herbicide used. Compared to the wild type, all transgenic genotypes, FC1, FC2, and FC3, showed high cellular leakage in all concentrations of oxyfluorfen treatment. A relatively higher level of cellular leakage was observed in FC3 genotypes than in FC2 genotypes, which is consistent with the chlorophyll loss data. FC2 genotypes showed a comparable response as the wild type in terms of cellular leakage, except for line 6, which showed a high level of cellular leakage unlike two other lines (5 and 7). This also indirectly suggests that FC2 without the transit sequence functioned intensely in rice cells due to its high expression strength. These electrolyte leakage data clearly suggest that the transgenic lines were less resistant to oxyfluorfen than the wild type. Finally, we measured levels of MDA, an indicator of lipid peroxidation, following oxyfluorfen treatment (Fig. 6). Like the results for chlorophyll loss and electrolyte leakage analyses, all transgenic lines produced relatively high MDA amounts compared to the wild type, except line 5 in the FC2 genotype, which had less MDA than the wild type (Fig. 6b). Taking all these biochemical analyses together, we conclude that overexpression of ferrochelatase in rice plants leads to the generation of transgenic rice genotypes that are more susceptible to oxyfluorfen herbicide than the wild type. The exact mechanism by which ferrochelatase overexpression increases susceptibility to oxyfluorfen herbicide remains to be investigated. There are reports that overexpression of tetrapyrrole biosynthetic genes triggers adverse effects due to dysfunction in tetrapyrrole biosynthesis. For example, overexpression of Mg-chelatase resulted in a decrease in chlorophyll levels due to reduced Mg-chelatase activity (Papenbrock et al. 2000). Also, the overexpression of PPO produced a high level of protoporphyrin IX in an age-dependent manner, leading to the formation of severe necrotic spots and growth retardation (Jung et al. 2008). In addition, it was reported that suppression of ferrochelatase by antisense techniques in tobacco led to an increase in protoporphyrin IX, which resulted in a necrotic phenotype. However, the overexpression of ferrochelatase has not previously been described; the current report is the first on a transgenic plant expressing the ferrochelatase gene. Although the underlying mechanism by which the overexpression of ferrochelatase caused herbicide sensitivity is not yet known, either changes of enzyme activities in the tetrapyrrole biosynthetic pathway or altered levels of tetrapyrrole intermediates, such as protoporphyrin IX, may be involved in giving rise to such phenotypes. Further detailed studies are needed to elucidate this phenomenon by employing stable homozygous transgenic lines.

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