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Role of the Ascorbate–Glutathione Cycle in Paraquat Tolerance of Rice

Tai-You Tseng, Jen-Fu Ou, and Ching-Yuh Wang*

Out of 1,343 mutant lines of rice mutated by sodium azide from the parental Japonica-type variety 'Tainung 67' (TNG67), a paraquat-susceptible line 1192 and a paraquat-tolerant line 72 were selected using whole seedlings at the four-leaf stage and leaf segments at the tillering stage as test materials. Further selection from progenies of these two mutant lines yielded the susceptible 1192-11 (S) and tolerant 72-16 (T), which were studied herein. Chlorophyll fluorescence, electrolyte leakage, and lipid peroxidation were measured for leaf segments of rice following treatment with 0.1 mM paraquat. A comparison of these responses among the three rice lines (TNG67, 72-16, and 1192-11), revealed a higher tolerance to paraquat in the tolerant mutant line 72-16 and the parental variety TNG67 than in the susceptible mutant 1192-11. Analysis of the antioxidative system in paraquat-treated leaf segments showed that the reduced form of glutathione (GSH) and the ratio of GSH to total glutathione increased by 3.5-fold within 6 h after treatment (HAT) and up to 5-fold 9 HAT in the T line, as compared with the S line. In view of the high activities of both dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in paraquat-treated leaves of TNG67 and the T line, the antioxidative effect of the ascorbate–glutathione cycle is hereby proposed to play an essential role in paraquat tolerance of rice. Pretreatment of rice segments with spermine enhanced DHAR and GR activities as well as paraquat tolerance of the S line. These results suggest that the activity of ascorbate–glutathione cycle induced by spermine is involved in rice tolerance to this herbicide. Although kinetics studies showed no significant difference among the three rice lines in paraquat inhibition of GR, a lower affinity of enzyme to substrate (K_m) in TNG67 and the T line and a higher maximal reaction rate (V_{max}) in the T line for the oxidized glutathione substrate (GSSG) were detected. These observations further implicate the importance of glutathione reductase in paraquat tolerance of rice.

Nomenclature: Paraquat; rice, *Oryza sativa* L.

Key words: Antioxidant, glutathione cycle, tolerant mutant.

Paraquat is a widely used nonselective herbicide for controlling annual weeds in both cultivated and noncultivated land throughout Taiwan, and the recommended rate is 1.08 to 1.44 kg ai ha^{-1} (Plant Protection Manual 2010). Most weeds in paddy fields, such as barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], junglerice [*Echinochloa colona* (L.) Link], monochoria [*Monochoria vaginalis* (Burm. f.) C. Presl ex Kunth], as well as other annual weeds within the Amaranthaceae, Asteraceae, Cyperaceae, Fabaceae, and Gramineae families, can be controlled.

The main mechanism of paraquat action is its competition for electrons with nicotinamide adenine dinucleotide phosphate (NADPH)-dependent ferredoxin reductase on the thylakoid membrane of chloroplasts. When paraquat captures an electron from photosystem I to become a paraquat radical, a cycle is initiated where the paraquat radical reacts with molecular oxygen to produce an oxygen radical and concomitantly regenerates the parent paraquat molecule (Iannelli et al. 1999). These oxygen radicals can cause peroxidative damage to cell membranes (Ekmekci and Terzioglu 2005; Hung et al. 2002), DNA denaturation, and protein reduction (Chagas et al. 2008; Ekmekci and Terzioglu 2005; Guo et al. 2007; Kuk et al. 2006; Norman and Fuerst 1997). Due to the high penetrating ability of paraquat into the cuticular layer of the leaf, the photosynthetic rate decreases eventually and the membrane system breaks down within a few hours after its application, subsequently leading to plant death (Chagas et al. 2008; Iturbe-Ormaetxe et al. 1998). In general, the efficiency of photosystem II (PSII) photochemistry has been used as an indicator of the damage on photosystem I caused by paraquat. The maximal chlorophyll fluorescence (F_m), maximal efficiency of PSII photochemistry (variable fluorescence/maximal chlorophyll fluorescence [F_v/F_m]), and electron transfer rate (ETR) of sugarcane (*Saccharum officinarum* L.) were significantly inhibited 18 h after 2 mM paraquat treatment (Chagas et al. 2008). Similar results showing photosystem destruction were also observed in wheat (Ekmekci and Terzioglu 2005).

Since the first introduction of chemical herbicides in the 1940s, the number of herbicide-tolerant weeds has increased year by year, and more than 200 weed species tolerant to various herbicides have been reported. About 22 species have been proven tolerant to paraquat, and their tolerance mechanisms include reduced uptake in hairy fleabane [*Conyza bonariensis* (L.) Cronq.], perennial ryegrass (*Lolium perenne* L.), and smooth barley (*Hordeum glaucum* Steud.); sequestration and limited translocation in horseweed [*Conyza canadensis* (L.) Cronq.], tall fleabane [*Conyza floribunda* Kunth], and hare barley (*Hordeum murinum* L. ssp. Leporinum (Link) Arcang.) (Yu et al. 2004); enhanced paraquat metabolism in rehmannia [*Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. & C. A. Mey] (Chun et al. 1997); and increased antioxidative ability in tall fleabane (Chiang et al. 2008). It appears that more than one mechanism of paraquat tolerance can exist simultaneously in the same weed species (Shaaltiel and Gressel 1986; Shaaltiel et al. 1988). In view of the fast action of this nonselective herbicide on target plants, the antioxidative mechanism, which effectively scavenges the free radicals generated, has been proposed to play an important role in paraquat resistance (Chiang et al. 2008).

In response to peroxidative stress, antioxidative systems, including enzymatic proteins and nonenzymatic compounds, are either activated or induced in plants. It is well known that activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), dehydroascorbate reductase (DHAR, EC 1.8.5.1), peroxidase (POD, EC 1.11.1.7), and catalase (CAT, EC 1.11.1.6) increase under oxidative stress (Foyer et al. 1997; Ye and Gressel 2000). Additionally,

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antioxidants, such as α-tocopherol and carotenoids as well as reduced glutathione and ascorbate, are able to protect cell membranes from the attack of toxic superoxide anions (Bowler et al. 1992; Noctor and Foyer 1998).

Chiang et al. (2008) proposed that the ascorbate–glutathione cycle in paraquat-tolerant tall fleabane coped with the peroxidative stress generated by this herbicide possibly via an indirect scavenging of the active oxygen species such as OH^-, H_2O_2 and O_2^-, as found in hairy fleabane (Ye and Gressel, 2000). Following paraquat application, glutathione reductase activity in a paraquat-tolerant biotype of this weed increased significantly, as compared with activities of other antioxidative enzymes, such as SOD and APX (Chiang et al. 2008). Ding et al. (2009) reported that tobacco, transformed using RNA interference technique to reduce GR activity, showed much more severe paraquat damage when compared with nontransformed plants. Apparently, this ascorbate–glutathione cycle plays an essential role in paraquat tolerance of plants.

In the ascorbate–glutathione cycle, DHAR and GR are the main enzymes driving electron transport. The former enzyme, which is responsible for GSH oxidation and dehydroascorbate reduction, catalyzes the generation of potent antioxidant reduced ascorbate (Asada 1999). Nevertheless, the relationship of DHAR and GR with paraquat tolerance seems obscure. In chloroplasts of paraquat-treated radish (*Raphanus sativus* L.), both malondialdehyde (MDA) accumulation and increase of DHAR activity suggest that DHAR activity was induced during the development of peroxidation damage (Ahn and Jin 2004). Similar results have also been found in tobacco (*Nicotiana tabacum* L.) seedlings after paraquat treatment (Ding et al. 2009). However, taking into consideration the observations of radish plants under paraquat stress (DHAR activity was maintained at certain level with polyamines [PAs] pretreatment, whereas DHAR activity was decreased without PAs pretreatment, and paraquat tolerance was improved by PAs), it is reasonable to propose the involvement of this antioxidative enzyme in paraquat tolerance (Kim and Jin 2006). Also, the GR enzyme using NADPH as an electron donor to reduce GSSG in the cytosol and chloroplast is able to enhance antioxidative ability (Ekmekci and Terzioglu 2005; Jiménez et al. 1997). This close relationship between GR activity and paraquat resistance has also been found in tall fleabane (Chiang et al. 2008).

The primary lethal action of paraquat described above also can be caused by environmental stress. It has been reported that the exogenous application of antioxidants, such as ascorbic acid, polyphenols, flavonoids, tannins, and saponins, on plants has successfully overcome some environmental stresses (Chiang et al. 2008; Shalata and Neumann 2001; Vinson et al. 1998). Shalata and Neumann (2001) found that the survival rate of tomato (*Solanum lycopersicum* L.) seedlings under sodium chloride stress was significantly increased and its lipid peroxidation prevented by ascorbate treatment. Also, it has been reported that polyamines are able to act as radical scavengers (Benavides et al. 2000), and compounds with antioxidative and metal ion chelating functions can scavenge free radicals directly or indirectly (Kuthanová et al. 2004). Under salt stress, application of spermine effectively improves salt tolerance of rice seedlings (Chattopadhayay et al. 2002). Subsequently, Duan et al. (2008) found that activities of antioxidative enzymes SOD, POD, and CAT in cucumber (*Cucumis sativus* L.) under salt (NaCl) treatment were all induced by spermidine (Spd). With pretreatments of polyamines, including putrescine, Spd, and Spm, leaf protein content of rice plants treated with paraquat remained relatively unchanged (Chang and Kao 1997). A Spm pretreatment also lowered the contents of thiobarbituric acid-reactive substances (TBARS), which were generated by paraquat in sunflower leaf discs (Benavides et al. 2000). Obviously, polyamines act through triggering antioxidation to improve stress tolerance of plants.

In general, an alteration of target protein conformation may result in changes of enzyme activity as reflected in various kinetic parameters, such as the velocity of the maximal reaction rate (V_{max}), and affinity of enzyme to substrate (K_m) (Dowd and Riggs 1965; Lee and Wilson 1971). In this study, we wanted to find out if the enzymatic kinetics of key enzymes such as GR in paraquat-tolerant and -susceptible rice mutants is different, thus influencing antioxidative ability.

In 2008, we found that, in a paraquat-tolerant (T) biotype of tall fleabane, an active antioxidative system, especially the GSH/GSSG cycle and GR activity, plays an important role in its resistance to paraquat (Chiang et al. 2008). In addition, a simultaneous application of ascorbate enhanced the paraquat tolerance of tall fleabane via an activation of this antioxidative system (Chiang et al. 2008). However, whether this mechanism also exists in other paraquat-tolerant species needs to be clarified. In Taiwan, a major source of the rice mutant pool is obtained through sodium azide treatment, and several of the resultant mutants exhibited characteristics of tolerance to pathogens, insects, herbicides, and environmental stress (Wang et al. 2002). Paraquat-tolerant and -susceptible mutant lines selected from 1,343 inbred lines of mutant (M7 generation, obtained after seven selfing processes) in a preliminary study were used to explore the tolerance mechanism of paraquat in rice plants. In view of the previous findings that a ^{14}C-paraquat tracing experiment showed no difference in uptake, translocation, and metabolism of paraquat between T and S lines (C. C. Hsu and C. Y. Wang, unpublished data), the role of ascorbate–glutathione cycle in paraquat tolerance for rice plants was studied.

Materials and Methods

Screening of Paraquat-Susceptible and -Tolerant Rice Mutants. A total of 1,343 rice mutant lines mutated by sodium azide (NaN_3) from the parental variety Tainung 67 (TNG67), a popular variety with the largest cultivation area in Taiwan, were screened for paraquat- susceptible and -tolerant mutants. A preliminary experiment of dose response of parental TNG67 seedlings at the four-leaf (V4) stage showed that the median effective dose (ED_{50}) values of paraquat causing injury and death within 14 d ranged from 0.11 mM to 0.25 mM.

Taking into consideration the high susceptibility of rice seedlings to this herbicide, a specific screening procedure (Figure 1) was designed to screen the l,343 mutant lines from the M7 generation (obtained after seven selfing processes) for paraquat-susceptible and -tolerant lines. The rice was cultivated in pots filled with sandy-clay loam soil (pH 7.3 and 1.09% organic matter, passed through 2-mm sieve) in the greenhouse until the V4 stage was reached for paraquat spraying. In order to acquire a homogenized spraying effect on these young seedlings with erect leaves, an autosprayer system consisting of automobile orbital sprayer with a running speed

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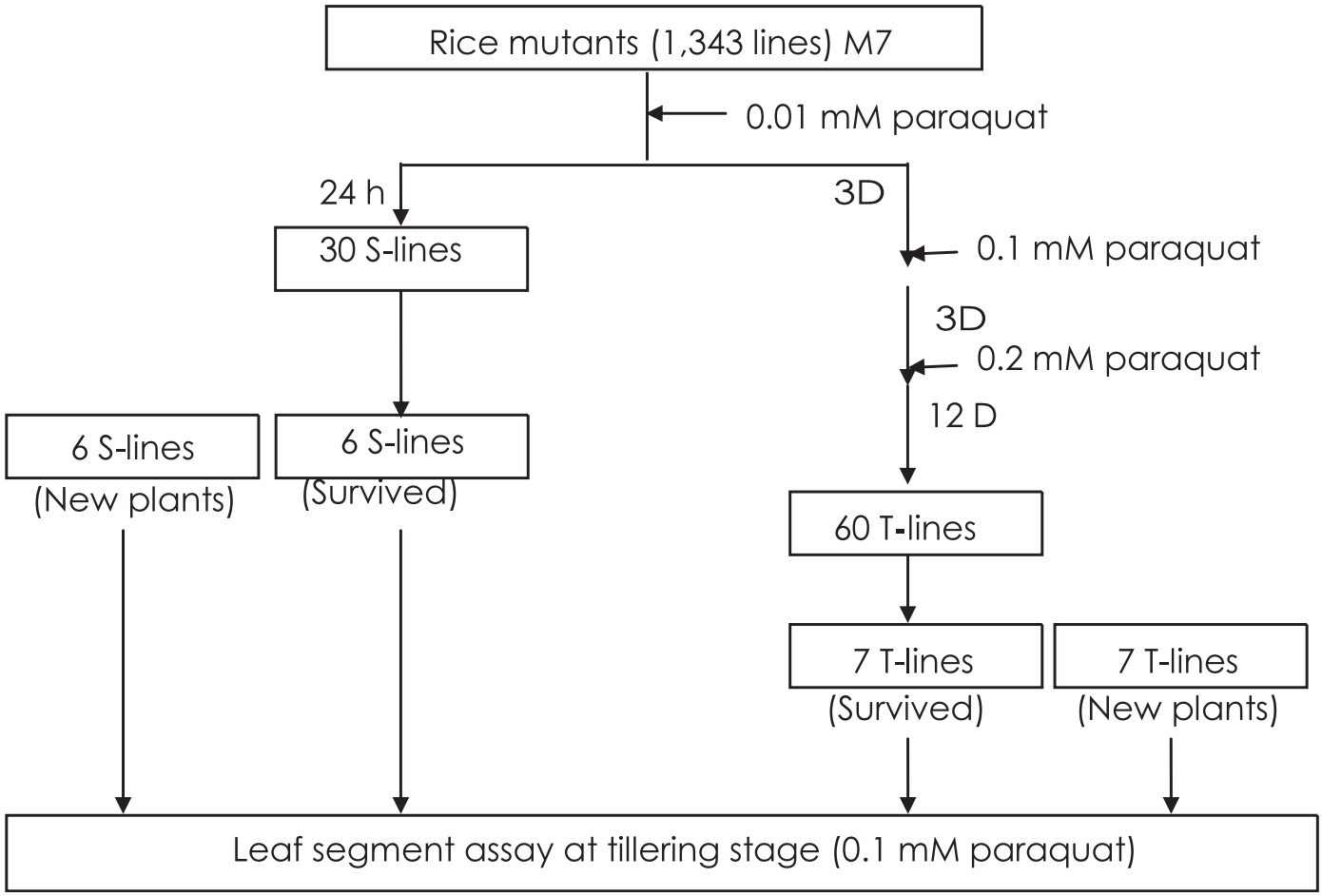

Figure 1. Screening process for the paraquat-susceptible and -tolerant rice mutants from 1,343 rice mutant lines at the four-leaf (V4) and tillering stage.

of 187 cm min^{-1}, a high-pressure compressor providing 0.20 MPa pressure with a flow rate of 1 m sec^{-1}, and three nozzles at 45 cm height above plants for generating a droplet particle with a 30 µm diameter, was used. The herbicide delivered from the nozzle was 4.0 L h^{-1}. Paraquat (Gramoxone, Syngenta Co., Greensboro, NC) at 0.01, 0.1, and 0.2 mM, was applied at different times (Figure 1).

After foliar spraying of paraquat on rice seedlings at the M7 generation, six putative S lines and seven putative T lines were selected based on the injury index of the whole plant, ranging from 0 to 5, where 0 indicates a healthy plant (no damage) and 5 indicates death caused by paraquat. All selected M7 lines were transplanted to new pots and cultivated to the tillering stage for leaf segment assay. Meanwhile, M7 seeds corresponding to the S line and T line were planted and grown to the tillering stage for a leaf segment assay. Due to a limited supply of rice seeds from the mutant pools, leaf segments were taken at the tillering stage instead of using the whole plant at the seedling stage. The large number of leaf segments thus satisfied the statistical requirement of the experimental design. The parental Japonica-type variety TNG67 was also included as a test material, in view of the need of further work on gene expression of paraquat tolerance (N. H. Shih, C. Y. Wang, and C. W. Wang, unpublished data).

For the leaf segment assay, 0.1 mM paraquat was applied on filter paper (5C, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) lining a 9-cm-diam Petri dish; 1.5 cm leaf segments, excised from the center of completely expanded leaves of rice plants at the tillering stage, were then placed into the Petri dish. These leaf segments were left for 6, 9, and 12 h under a light intensity of 300 µmol m^{-2} s^{-1}. The injury index, ranging from 0 to 5, where 0 indicates a normal leaf segment with deep green color and 5 indicates a leaf segment with a severe etiolation, was recorded at the end of the testing period. For the chlorophyll fluorescence measurement, leaf segments treated with paraquat were blotted dry with tissue paper and fixed to the sensor clip of a chlorophyll fluorescence analyzer (OS1-FL, Opti-Science, Inc., Hudson, NH) for 10 min in the dark before assaying the maximal efficiency of PSII photochemistry (F_v/F_m) (Guo et al. 2007).

Paraquat Susceptibility of Susceptible and Tolerant Rice Mutants. Rice plants, including TNG67, the S-line 1192-11, and the T-line 72-16 were grown in pots filled with sandy-clay loam soil in the greenhouse until the tillering stage for the leaf segment assay. Paraquat at 0.1 mM was applied to filter paper lining a 9-cm-diam Petri dish; 1.5-cm leaf segments were then excised from the center of completely expanded leaves of rice plants at tillering stage and placed into the Petri dish. These leaf segments were left for 0, 3, 6, and 9 h under a light intensity of 300 µmol m^{-2} s^{-1}, incubated indoors with an ambient temperature that ranged from 15 C to 20 C (January to April). Because in a previous experiment responses to paraquat of rice leaf segments between the S and T lines could be clearly differentiated within 9 h, and thereafter the detached leaf segments of the controls gradually became aged, the period for measuring paraquat susceptibility using rice leaf seg-

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ments was terminated at 9 h. All leaf segments were harvested as samples for subsequent determination of indicators of herbicidal toxicity, i.e., chlorophyll fluorescence, electrolyte leakage, and malondialdehyde (MDA).

Chlorophyll Fluorescence. Leaf segments treated with paraquat were blotted dry with tissue paper and fixed to the sensor clip of chlorophyll fluorescence analyzer for 10 min in the dark before assaying the maximal efficiency of PSII photochemistry (F_v/F_m) (Guo et al. 2007).

Electrolyte Leakage. Eight fresh leaf segments (each 1.0 cm by 1.0 cm, for a total of ca. 0.1 g fresh weight [FW]) were placed into a test tube containing 8 ml of double-distilled water; the tube then was shaken gently for 24 h before the conductivity of the water solution was determined with a conductivity meter (SC-170, SunTex Instruments Co., Taipei, Taiwan). Subsequently, the test tube was heated in a boiling water bath for 15 min and cooled for the second assay of conductivity. The electrolyte leakage was calculated as the ratio of conductivity prior to and after boiling (Guo et al. 2007).

Malondialdehyde. About 0.1 g fresh leaf tissue was ground in liquid nitrogen and extracted with 1.0 ml of 5% trichloroacetic acid (TCA); the homogenate then was centrifuged (Himac SCR 20, HITACHI, Tokyo, Japan) at 12,000 × *g* for 15 min at 4 C. An aliquot of 0.5 ml supernatant was mixed with 0.5 ml 0.5% thiobarbituric acid (dissolved in 20% TCA), and incubated at 95 C for 30 min. The reaction was terminated by placing the mixture in an ice bath. After centrifugation at 12,000 × *g* for 10 min at 4 C and standing at room temperature for 15 min, absorbances of the solution were read at 532 and 600 nm (Spectrophotometer, U-2001, Hitachi., Tokyo, Japan). MDA content was calculated based on an extinction coefficient 155 mM^{-1} cm^{-1} (Ekmekci and Terzioglu 2005).

Antioxidative Ability of Rice Mutants. Two rice mutants, 1192-11 (S) and 72-16 (T), and TNG 67 were grown until the tillering stage in a greenhouse on the campus of National Chung-Hsing University. After a 0.1 mM paraquat treatment for 0, 3, 6, and 9 h (described previously), leaf segments were taken for determination of glutathione content and antioxidative enzyme activity.

Total and Reduced Glutathione. Fresh leaf tissue (0.1g) was ground in liquid nitrogen and homogenized with 1 ml 5% (v/v) TCA, and then centrifuged at 10,000 × *g* for 15 min at 4 C. A 20 µl aliquot of the supernatant was mixed with 260 µl 150 mM NaH_2PO_4 (pH 7.4) for neutralization, and used for glutathione determination (Guo et al. 2007; Smith 1985). For total glutathione quantification, 20 µl of neutralized extract was placed into a 96-well plate and mixed with an equal volume of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) solution (2 mg DTNB dissolved in 3 ml 100 mM potassium phosphate buffer [pH 7.5] containing 5 mM ethylenediamine tetraacetic acid [EDTA]), 120 µl 2 unit ml^{-1} of glutathione reductase (dissolved in 100 mM sodium phosphate buffer, pH 7.5), and 60 µl β-NADPH (2 mg β-NADPH dissolved in 3 ml 100 mM potassium phosphate buffer [pH 7.5] containing 5 mM EDTA). After 10 min, the absorbance at 415 nm was recorded.

For measurement of the reduced form of glutathione, 20 µl neutralized supernatant was mixed with an equal volume of DTNB solution (2 mg DTNB dissolved in 3 ml 100 mM potassium phosphate buffer [pH 7.5] containing 5 mM EDTA), 120 µl potassium phosphate buffer containing EDTA (KPE) solution (100 mM potassium phosphate buffer [pH 7.5] containing 5 mM EDTA), and 60 µl β-NADPH (2 mg β-NADPH dissolved in 3 ml 100 mM potassium phosphate buffer [pH 7.5] containing 5 mM EDTA). After 10 min, absorbance of reduced glutathione was recorded at 412 nm.

Activity of Dehydroascorbate Reductase (DHAR; EC 1.8.5.1). Fresh leaf (0.1 g) was ground in liquid nitrogen and homogenized with 0.5 ml 50 mM potassium phosphate buffer (pH 7.8) containing 8% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA. After centrifugation at 12,000 × *g* for 20 min at 4 C, a 100 µl enzyme extract was mixed with 800 µl 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM Na_2EDTA, and reacted with 80 µl 2.5 mM reduced glutathione (GSH) and 80 µl 0.2 mM dehydroascorbate (DHA); the absorbance at 265 nm was measured continuously for 2 min (Ananieva et al. 2004). Protein was quantitated according to the method of Bradford (1976). The activity was expressed as mmole ascorbate min^{-1} mg^{-1} protein.

Activity of Glutathione Reductase (GR; EC 1.6.4.2). Fresh leaf (0.1g) was ground in liquid nitrogen and homogenized with 1 ml 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM Na_2EDTA, and mixed with 2% polyvinylpyrrolidone (PVP). After centrifugation at 12,000 × *g* for 20 min at 4 C, the supernatant was used as a crude enzyme extract. GR activity measurement was modified from Ekmekci and Terzioglu (2005). The crude enzyme extract (100 ml) was mixed with 1 ml of 200 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.5 mM oxidized glutathione (GSSG), and 50 µM NADPH. Within 5 min, absorbance at 340 nm was measured.

Effect of Polyamine Application on Paraquat Tolerance and Antioxidative Ability. Leaf segments excised from the susceptible rice mutant line 1192-11 were treated with 5 mM spermine in a Petri dish in the dark for 6 hr, and was then followed by a 0.1 mM paraquat treatment in the light, coupled with incubation indoors with the ambient temperature ranging from 25 C to 30 C (May to August). Nine h later, the paraquat tolerance of the leaf segments was determined on the basis of leaf chlorophyll fluorescence and MDA, and the antioxidative ability, i.e., GR and DHAR activities, as well as total glutathione and reduced glutathione content, were determined as described above.

Enzymatic Kinetics of Glutathione Reductase in Rice. Glutathione reductase activity in leaves of the two rice mutants, 1192-11 (S) and 72-16 (T) and the parental TNG 67, at the tillering stage was determined. About 5 g of fresh leaves were ground in liquid nitrogen and homogenized in 10 ml 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM Na_2EDTA and 2% PVP. After filtration through four layers of gauze, the homogenate was centrifuged at 12,000 × *g* for 20 min at 4 C. Ammonium sulfate was added to the supernatant to 40% saturation for 5 min. After

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Table 1. Injury indices of the sodium azide (NaN_3)-induced rice mutants (1,343 lines; M7 generation) caused by the foliar spraying of 0.01 mM paraquat at the four-leaf stage. Injury indices of the top 30 lines susceptible to paraquat were recorded 24 h after spraying.

No.	Mutant line	Injury index[a]	No.	Mutant line	Injury index
1	19	2.5	16	948	2.5
2	81	2.5	17	1081	2.5
3	191	2.5	18	1192	2.5
4	226	2.5	19	1200	2.5
5	227	2.5	20	1247	2.5
6	238	2.5	21	1335	2.5
7	356	2.5	22	1378	2.5
8	365	2.5	23	231	2.0
9	407	2.5	24	366	2.0
10	802	2.5	25	685	2.0
11	803	2.5	26	1035	2.0
12	804	2.5	27	1078	2.0
13	865	2.5	28	270	1.5
14	873	2.5	29	379	1.5
15	875	2.5	30	585	1.5

[a] Injury index ranging from 0 to 5, where 0 indicates a healthy plant and 5 indicates plant death.

centrifugation at 10,000 × *g* for 20 min, the pellet was resuspended in 0.5 ml 100 mM potassium phosphate buffer (pH 7.0) before desalting through a Sephadex G-25 column (Ekmekci and Terzioglu 2005). The eluate was used for the enzymatic kinetics study described below.

Kinetics Analysis of Glutathione Reductase. The experiments were carried out using 50 μM NADPH and five concentrations of GSSG between 0.10 to 1.25 μM. After the addition of NADPH, absorbance at 340 nm was recorded within 2 min. Enzyme activity was expressed as μmol NADPH min^{-1} mg^{-1} protein. Values for V_{max} and K_m were calculated using the Michaelis–Menten equation and the Lineweaver–Burk equation, respectively (Whiteley 1997). In addition, the inhibition constant of paraquat against glutathione reductase (K_i) was calculated with the addition of four concentrations of this herbicide (up to 1.0 μM).

Data Analysis. Data were subjected to ANOVA, and Fisher's Protected LSD at 5% probability was used to compare treatment means. All experiments, including kinetics coefficient measurements, were conducted three times with at least three replicates for each treatment.

Results and Discussion

Screening of Paraquat-Susceptible and -Tolerant Rice Mutants. *Seedling Screening at the V4 Stage*. Considering the ED_{50} value of paraquat causing plant death for V4 rice seedlings of TNG67 observed in the preliminary experiment, a lower dosage (0.01 mM) was used to distinguish paraquat-susceptible from -tolerant mutant lines (Figure 1). Twenty-four h after 0.01 mM paraquat application, 30 lines more susceptible to paraquat were collected (Table 1) from 1,343 mutant lines in the M7 generation. Six of these selected mutants with an injury index of 2.5 that survived 3 d after treatment (lines 81, 802, 875, 948, 1081, and 1192) were selected as candidates of S-line for a subsequent tolerance assay at the tillering stage. In addition, the remaining 1,313 lines more tolerant to 0.01 mM paraquat were treated with 0.1 mM paraquat 3 d after the first application, and with an additional 0.2 mM paraquat application 3 d after the second application. Finally, 60 lines more tolerant to the two consecutive paraquat applications were collected (Table 2), and seven of these mutants with an injury index 2.5 or less (lines 72, 713, 881, 882, 883, 986, and 1067) were selected as candidates of the T line for subsequent tolerance assay at the tillering stage. Due to the "one plant, one mutant line" situation, there was no statistical analysis of variation in Tables 1 and 2; thus, we conducted a more complete and replicable assay on the paraquat tolerance of rice plants at the tillering stage with more leaf segments available to satisfy the requirement of statistical analysis.

Table 2. Injury indices of the sodium azide (NaN_3)-induced rice mutants (1,313 lines; M7 generation) caused by the foliar spraying of 0.01 mM paraquat at the four-leaf stage, followed by a second application of 0.1 mM paraquat 3 d later, and then followed by a 0.2 mM paraquat treatment 3 d after the second application. Injury indices of the top 60 (5%) lines tolerant to paraquat were recorded 12 d after the last spraying.

No.	Mutant line	Injury index[a]	No.	Mutant line	Injury index
1	1067	0.0	31	908	3.0
2	72	2.0	32	946	3.0
3	712	2.0	33	961	3.0
4	713	2.0	34	1089	3.0
5	881	2.0	35	1097	3.0
6	882	2.0	36	1112	3.0
7	101	2.5	37	1170	3.0
8	279	2.5	38	1330	3.0
9	355	2.5	39	1336	3.0
10	659	2.5	40	80	3.5
11	720	2.5	41	82	3.5
12	746	2.5	42	140	3.5
13	883	2.5	43	145	3.5
14	897	2.5	44	175	3.5
15	986	2.5	45	268	3.5
16	1027	2.5	46	287	3.5
17	1082	2.5	47	292	3.5
18	1138	2.5	48	326	3.5
19	1386	2.5	49	327	3.5
20	139	3.0	50	376	3.5
21	221	3.0	51	408	3.5
22	457	3.0	52	456	3.5
23	459	3.0	53	466	3.5
24	467	3.0	54	468	3.5
25	500	3.0	55	501	3.5
26	502	3.0	56	503	3.5
27	770	3.0	57	643	3.5
28	876	3.0	58	649	3.5
29	888	3.0	59	650	3.5
30	900	3.0	60	657	3.5

[a] Injury index ranging from 0 to 5, where 0 indicates a healthy plant and 5 indicates plant death.

Leaf Segment Screening at the Tillering Stage. All surviving seedlings from the previous stage, including six putative S lines and seven putative T lines, were cultivated to the tillering stage for leaf segment assay. After a 0.1 mM paraquat treatment, injury indices of both the putative S lines and T lines all increased with time within 12 HAT (Figure 2). Lines 1081 and 1192 from putative S lines were more susceptible to paraquat, whereas lines 72, 713, and 1067 from putative T lines were more tolerant to paraquat. Because the subsequent growth of surviving rice mutants were more or less inhibited by paraquat treatment during the screening process, lines 1192 and 72 were finally selected based on their growth vigor and yield capacity, which are very important for seed production of the next generation (M8).

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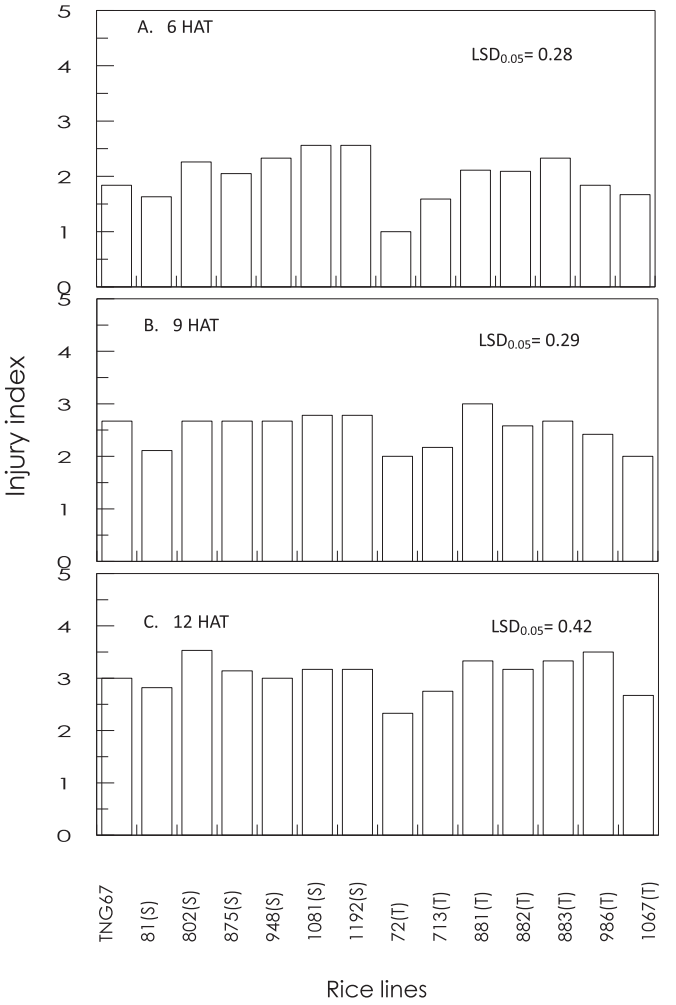

Figure 2. Changes in injury index of leaf segments excised from rice plants at the tillering stage after treatment of paraquat at 0.1 mM. Six susceptible (S: 81, 802, 875, 948, 1081, 1192) and seven tolerant (T: 72, 713, 881, 882, 883, 986, 1067) lines of rice mutants survived after pretreatment with paraquat, at 0.01 mM and 0.01 + 0.1 + 0.2 mM, respectively, for S and T lines, on four-leaf seedlings.

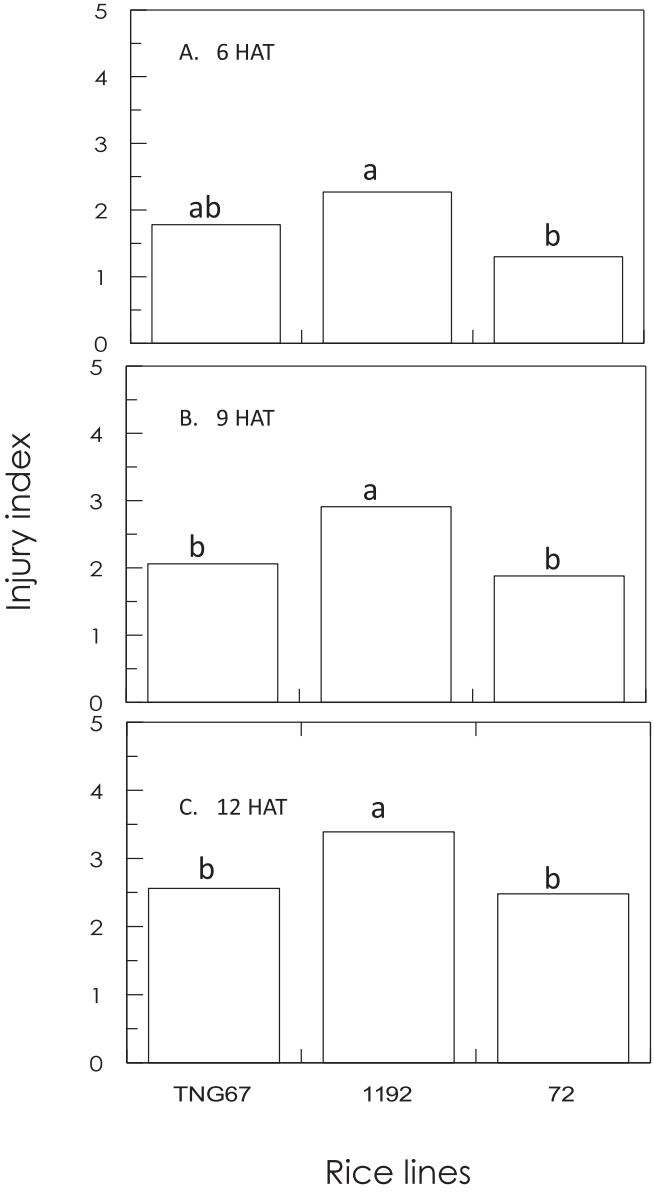

Figure 3. Changes in injury indices of leaf segments excised from rice plants at the tillering stage, after treatment with paraquat at 0.1 mM. The plants were not treated with paraquat before the leaf segment assay.

In order to define the tolerance difference between lines 1192 and 72, leaf segments collected at the tillering stage from rice plants without paraquat treatment were used. After treatment with 0.1 mM paraquat, injury indices of three rice lines (including the parental line) all increased with time, and in line 1192, was considerably higher than in the other two lines 9 h after treatment (Figure 3), revealing the significant difference of paraquat tolerance between the S-line 1192 and T-line 72 mutants.

To clarify the heritable stability of paraquat tolerance in these two mutant lines, both M7 and M8 generations were tested, based on the responses of the maximal efficiency of PSII photochemistry (F_v/F_m) to paraquat. Due to the requirement of objective and accurate measurement for determining paraquat tolerance, an instrumental measurement of chlorophyll fluorescence is better than the injury index measurement, which is judged by visual perception.

A measurement of PSII inhibition caused by 0.1 mM paraquat showed that F_v/F_m values of leaf segments excised from the M7 and M8 generations of S-line 1192 ranged from 40% to 45% of control, and that from the M7 and M8 generations of T-line 72 was 80% of control, similar to the parental variety TNG67 (Figure 4). These results confirm a heritable stability of paraquat tolerance between the two generations.

To obtain mutant progenies either more susceptible or more tolerant to paraquat for subsequent study on the tolerance mechanism, paraquat tolerance of 22 and 20 lines (M8) from S-line 1192 and T-line 72 (M7), respectively, were further tested. Changes of F_v/F_m values of leaf segments after 0.1 mM paraquat treatment showed that the maximal efficiency of PSII photochemistry of 20 S-lines (except two lines) was decreased to less than 60% of the control except two lines, and all 20 T-lines still maintained at F_v/F_m values higher than 70% of the control (Figure 5). Therefore, lines 1192-11 and 72-16 from the M8 generation were selected as the paraquat-susceptible and -tolerant mutants. Because the herbicidal damage of rice seedlings caused by paraquat is serious and irreversible, and because limited amount of rice seeds produced by mutant lines, the leaf segment assay at the tillering stage was the only way to meet the requirement for statistical analysis.

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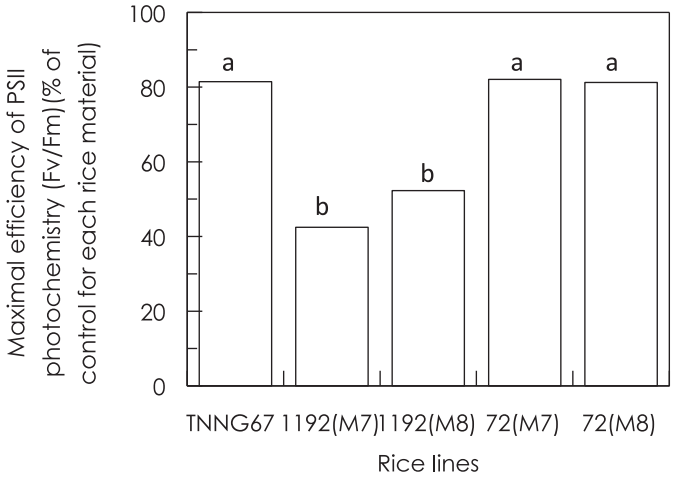

Figure 4. Photosystem II (PSII) inhibition of leaf segments excised from rice plants at the tillering stage, with 0.1 mM paraquat at 9 h after treatment. Rice cv. TNG 67, and both susceptible (S; 1192) and tolerant (T; 72) mutant lines between two generations (M7 and M8), were compared.

Paraquat Susceptibility of Susceptible and Tolerant Rice Plants. The maximal efficiency of PSII photochemistry (F_v/F_m), a sensitive physiological trait reflecting rapid damage to the photosystem of rice plants at the tillering stage, was adopted as an injury index. No significant difference in F_v/F_m of rice leaf segments treated with 0.1 mM paraquat was observed among the two rice mutant lines and the parental TNG67 within 6 h; however, F_v/F_m of the S mutant line was significantly lower than that of the T mutant line and TNG67 9 h after treatment (Figure 6A). Similar results have been found in an earlier study of tolerant rice cultivar Xiangnuo No. 1 and susceptible cv. 'IR-50', with F_v/F_m remaining at a normal value in the tolerant but not in the susceptible rice cultivar 72 h after 0.01 mM paraquat treatment (Guo et al. 2007). Changes in F_v/F_m of wheat plants under paraquat stress also reflected their respective differential levels of paraquat tolerance (Ekmekci and Terzioglu 2005).

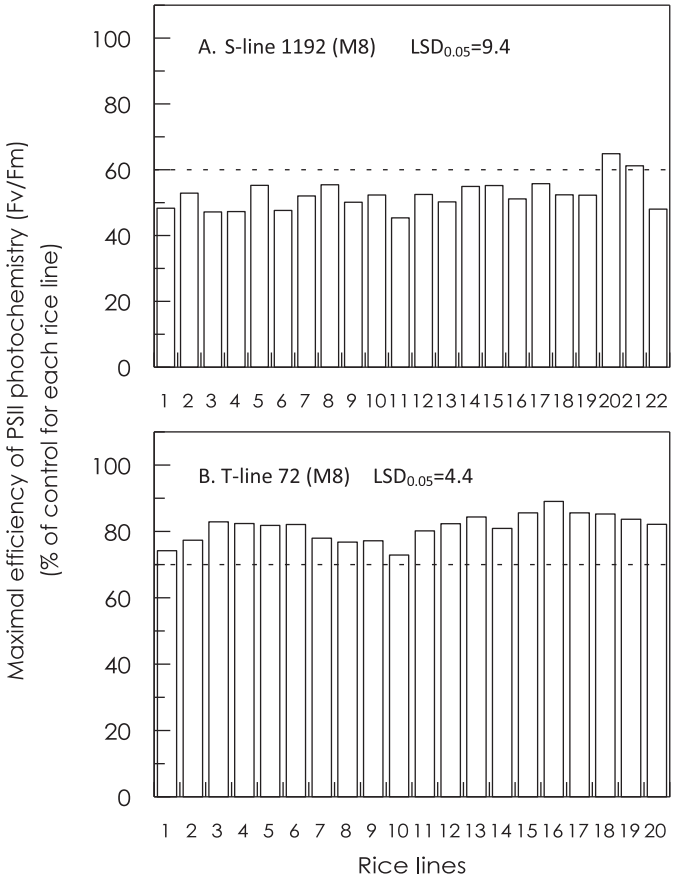

Figure 5. Maximal efficiency of photosystem II (PSII) photochemistry in leaf segments excised from rice plants at the tillering stage, with 0.1 mM paraquat at 9 h after treatment. Variations in (A) 22 lines of susceptible mutant (1192; generation M8), and (B) 20 lines of tolerant mutant (72; generation M8), were compared.

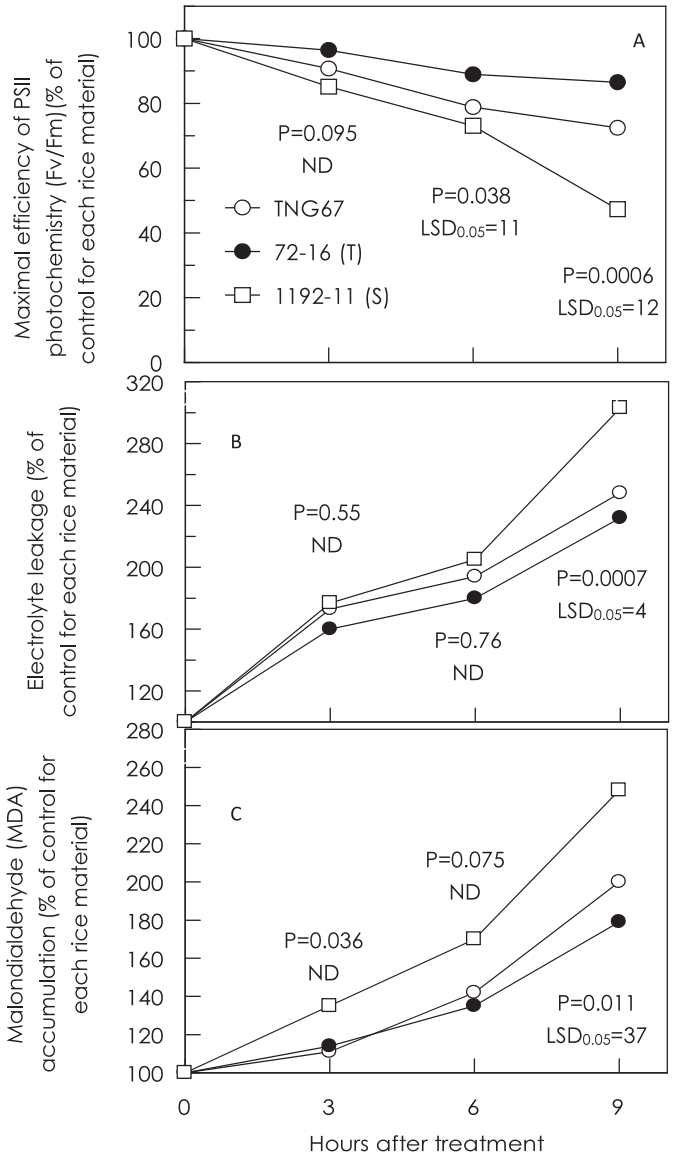

Figure 6. (A) Maximal efficiency of photosystem II (PSII) photochemistry, (B) electrolyte leakage, and (C) malondialdehyde (MDA) accumulation in leaf segments excised from rice cv. Tainung 67 (TNG67) and two rice mutants, 1192-11 (susceptible [S]) and 72-16 (tolerant [T]) at the tillering stage after treatment with 0.1 mM paraquat. Original F_v/F_m values of TNG67, and mutants 1192 and 72 were 0.88, 0.89, and 0.89, respectively. Original electrolyte leakage of TNG67, and mutants 1192-11 and 72-16 were 11.0, 12.2, and 8.5 μs cm^{-1}, respectively. Original MDA contents of TNG67 and mutants 1192-11 and 72-16 were 10.6, 10.1, and 9.7 nmol g^{-1} fresh weight [FW], respectively.

Free radicals caused by paraquat in plants cause peroxidation of lipids, including the phospholipids of the cell membrane, leading to the destruction of membrane integrity and subsequent electrolyte leakage, and this electrolyte leakage

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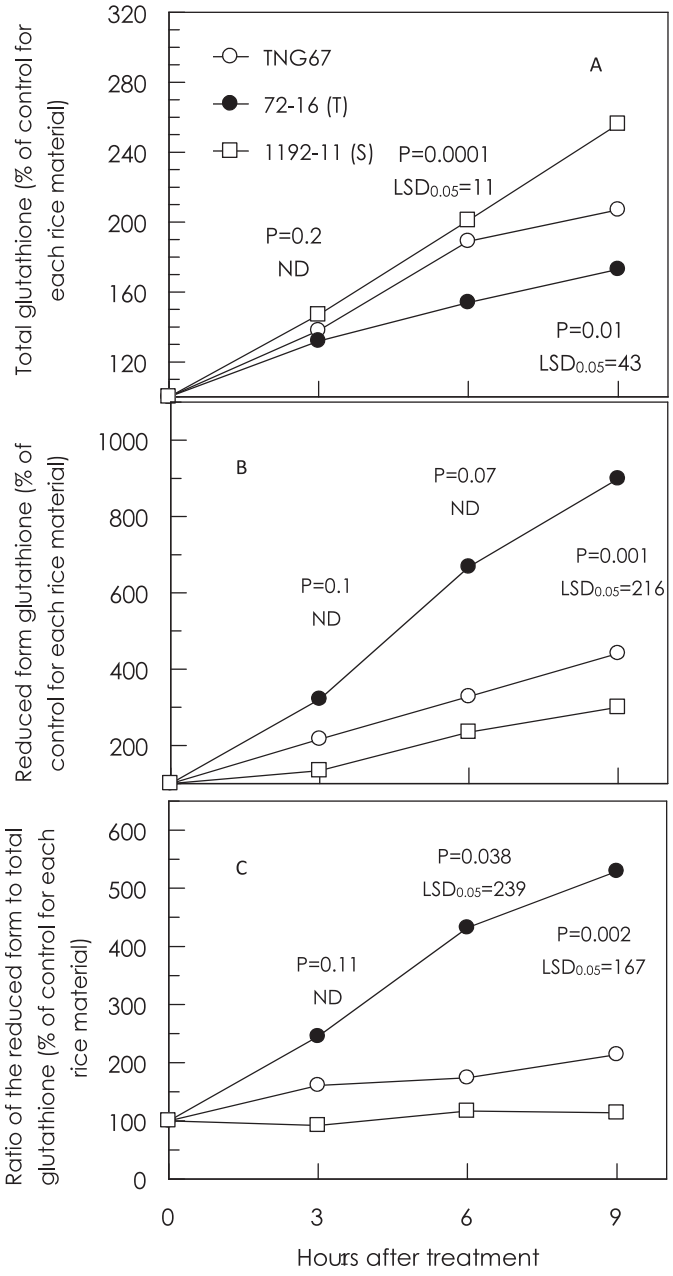

Figure 7. (A) Total glutathione, (B) reduced form glutathione, and (C) the ratio of the reduced form to total glutathione in 0.1 mM paraquat-treated leaf segments excised from rice cv. Tainung 67 (TNG67), and two rice mutants, 1192-11 (susceptible [S]) and 72-16 (tolerant [T]) at the tillering stage. The original total glutathione in control plants of TNG67, 1192-11, and 72-16 were 1.0, 1.0, and 0.7 μmol g^{-1} fresh weight (FW), respectively. The original contents of reduced glutathione of TNG67, 1192-11, and 72-16 were 0.24, 0.48, and 0.06 μmol g^{-1} (FW), respectively. The original ratio of the reduced-form to total glutathione of TNG67, and mutants 1192-11 and 72-16 were 0.23, 0.45, and 0.10, respectively.

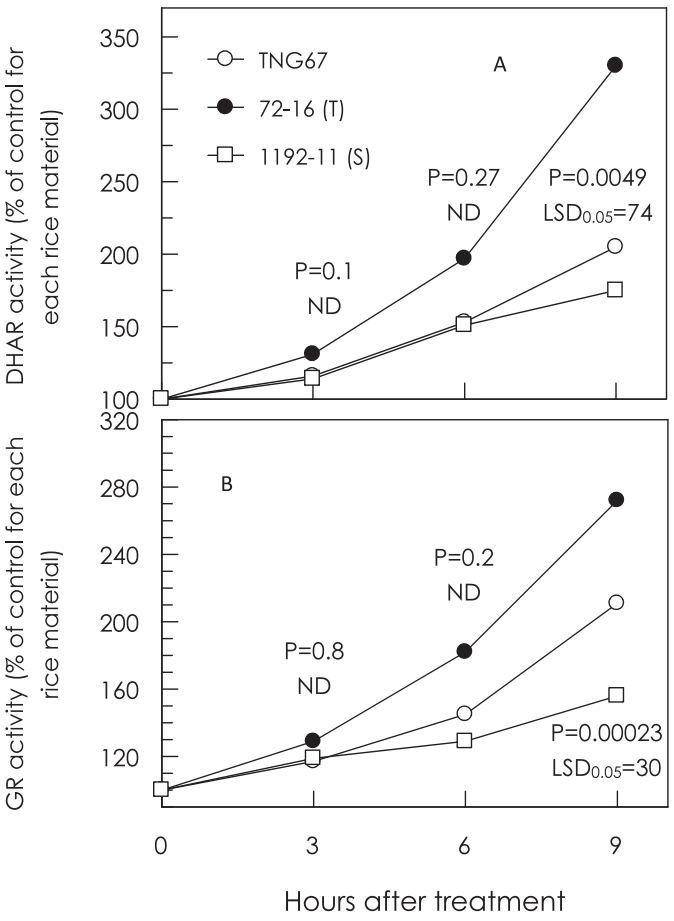

Figure 8. (A) Relative activities of dehydroascorbate reductase (DHAR) and (B) glutathione reductase (GR) in leaf segments excised from rice cv. Tainung 67 (TNG67) and two rice mutants, 1192-11 (susceptible [S]) and 72-16 (tolerant [T]) at the midtillering stage, after treatment with 0.1 mM paraquat. The original DHAR activities of TNG67 and mutants 1192-11 and 72-16 were 8.1, 6.9, and 7.7 mmol ascorbate min^{-1} mg $protein^{-1}$, respectively. The original GR activities of TNG67 and mutants 1192-11 and 72-16 were 0.22, 0.25, and 0.38 μmol NADPH min^{-1} mg $protein^{-1}$, respectively.

of leaf segments increased up to 80% within 6 h. Although no significant differences among the three rice lines was noted 6 HAT, electrolyte leakage in the S line was significantly higher 9 HAT, as compared to the other two rice lines (Figure 6B), a response similar to that of the F_v/F_m trait. In addition, there was no difference in the amount of MDA, a product of lipid peroxidation, among the three rice lines within 6 h after 0.1 mM paraquat treatment. However, by 9 HAT, 30% more MDA was observed in the S line than in TNG67 and the T lines (Figure 6C). In rehmannia, corn (*Zea mays* L. cv. 'Dekalb'), and soybean [*Glycine max* (L.) Merr. cv. 'Kwangkyo'], the increase of MDA with time after paraquat treatment also reflected herbicidal damage (Chun et al. 1997). Polos et al. (1988) reported that under 10 μM paraquat treatment, MDA increased in susceptible but not in tolerant biotypes of horseweed. The change in MDA content, which is related to the extent of lipid peroxidation in cells, apparently could discriminate the paraquat susceptibility between the two biotypes (Chun et al. 1997).

Antioxidative Abilities of Susceptible and Tolerant Rice Plants. *Glutathione.* Total glutathione content of leaf segments treated with 0.1 mM paraquat increased with time for all three rice lines. At 6 HAT, total glutathione in the S line and TNG67 increased 110% and 90%, respectively, but only increased 56% in the T line (Figure 7A). The difference between the S and T lines widened at 9 HAT, with total glutathione in the T line remaining approximately 35% lower than in the S line. It has been found that at high temperature or ionic stress, more glutathione accumulated in apple [*Malus pumila var. domestica* (Borkh.) C. K. Schneid. cv. 'Gala'] leaves (Ma et al. 2008) and Indian mustard [*Brassica juncea*

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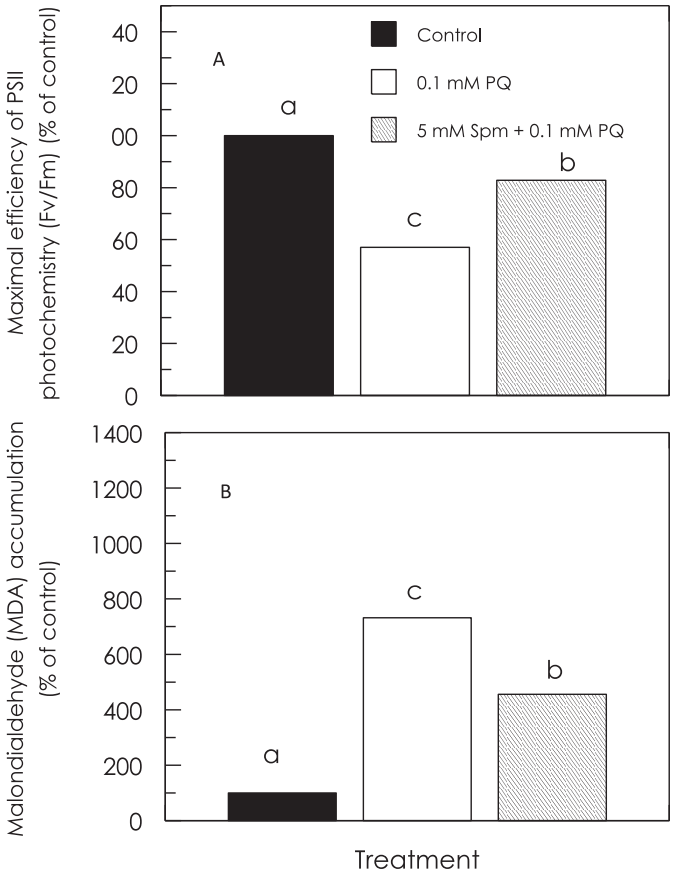

Figure 9. (A) Maximal efficiency of photosystem II (PSII) photochemistry and (B) malondialdehyde (MDA) accumulation in leaf segments excised from rice mutant 1192-11(susceptible [S]) at the tillering stage, 9 h after treatment with 0.1 mM paraquat. The effect of 5 mM spermine pretreatment for 6 h before the paraquat treatment was also included. The original F_v/F_m ratio for the control was 0.8. The P value of difference among these treatments is 9.04×10^{-7}, and LSD at 0.05 was 4.27%. The original data of MDA in the control leaf segment was 6.3 nmol g^{-1} fresh weight (FW). The P value of differences among the three treatments was 5.26×10^{-6}, and LSD at 0.05 was 84%.

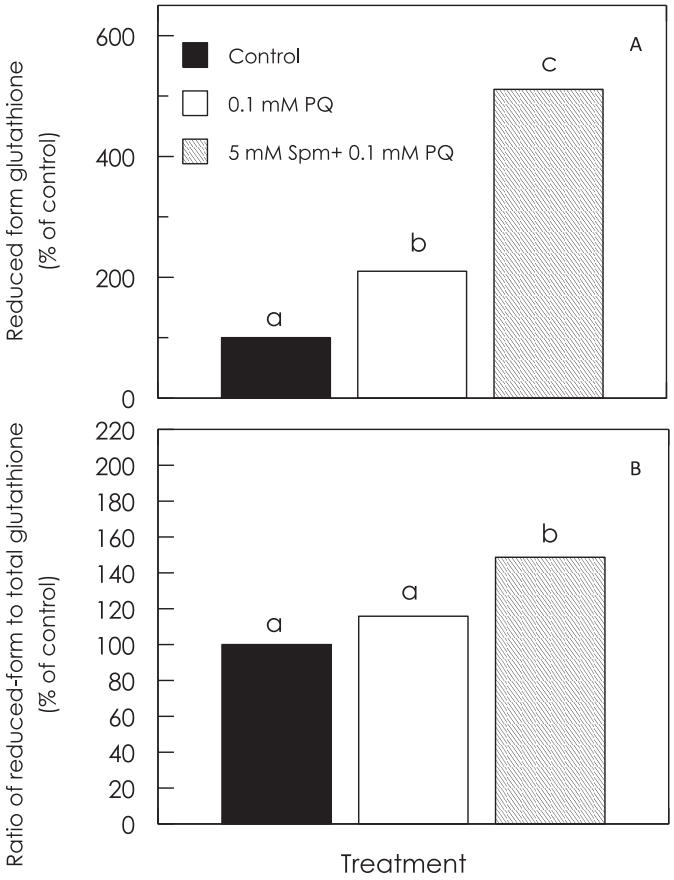

Figure 10. (A) Reduced form glutathione and (B) the ratio of reduced-form to total glutathione in leaf segments excised from rice mutant 1192-11(susceptible [S]) at the tillering stage 9 h after treatment with 0.1 mM paraquat. The effect of 5 mM spermine pretreatment for 6 h before the paraquat treatment was also included. The original glutathione content of the control plant was 3.3 μmol g^{-1} fresh weight (FW). The P value of difference among the three treatments was 7.45×10^{-5}, and LSD at 0.05 was 89%. For the ratio (B), the P value of difference among the three treatments was 0.005, and LSD at 0.05 was 23%.

(L.) Czern.] seedlings (Prasad et al. 1999). May et al. (1998) suggested that under environmental stress, the glutathione homeostasis in plants was changed, and the reactive oxygen species (ROS) generated might inhibit oxidative phosphorylation (Seyfried et al. 1999). Thus the significance of total glutathione fluctuation in paraquat tolerance of plants remains to be resolved.

The reduced form of glutathione was increased by two- to sixfold for all three rice lines within 6 h of paraquat treatment; and although the T line had the highest level of glutathione, the difference among them was not statistically significant (Figure 7B). At 9 HAT, GSH in the T line increased ninefold, suggesting the involvement of this antioxidative compound in paraquat tolerance. Due to the inconsistent total glutathione contents in leaves of the three rice lines tested, i.e., 0.24, 0.06, and 0.48 μmol g^{-1} FW, respectively, in control leaves of TNG67, 72-16, and 1192-11, the ratio of GSH : total glutathione was subsequently examined to estimate the regeneration ability of GSH. Experimental results showed that within 6 HAT, this ratio increased by 4.3-fold in the T line, whereas only 1.7- and 1.2-fold increments were observed, respectively, in TNG67 and the S line (Figure 7C). It has been reported that the GSH : total glutathione ratio in a paraquat-tolerant biotype was higher than that in a S biotype of tall fleabane after paraquat treatment (Chiang et al. 2008). This larger increment of the ratio of GSH : total glutathione in the T-line than in the S-line after paraquat treatment supports the suggestion that an efficient recycling of glutathione immediately after paraquat treatment could be a prerequisite for paraquat tolerance.

Dehydroascorbate Reductase and Glutathione Reductase. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) is responsible for the recycling of ascorbate to scavenge reactive oxygen species (Asada 1999), and glutathione reductase (GR; EC 1.6.4.2) contributes to the subsequent reduction of oxidized form glutathione using NADPH as substrate. Both enzymes have key roles in the operation of ascorbate–glutathione cycle to protect plants from oxidative stress (Foyer et al. 1994). Analysis of enzyme activity showed that DHAR activities in all three rice lines were induced within 9 h after 0.1 mM paraquat treatment (Figure 8A), with a significant 3.25-fold increment in the T line as compared with 1.7- and 2.0-fold increments for the two other rice lines. Ye and Gressel (2000) also reported that DHAR activity in tolerant hairy fleabane increased significantly 24 h after 1 μM paraquat treatment. It is reasonable to suggest that the high activity of DHAR might increase antioxidative ability of rice plants through the activation of the ascorbate–glutathione cycle.

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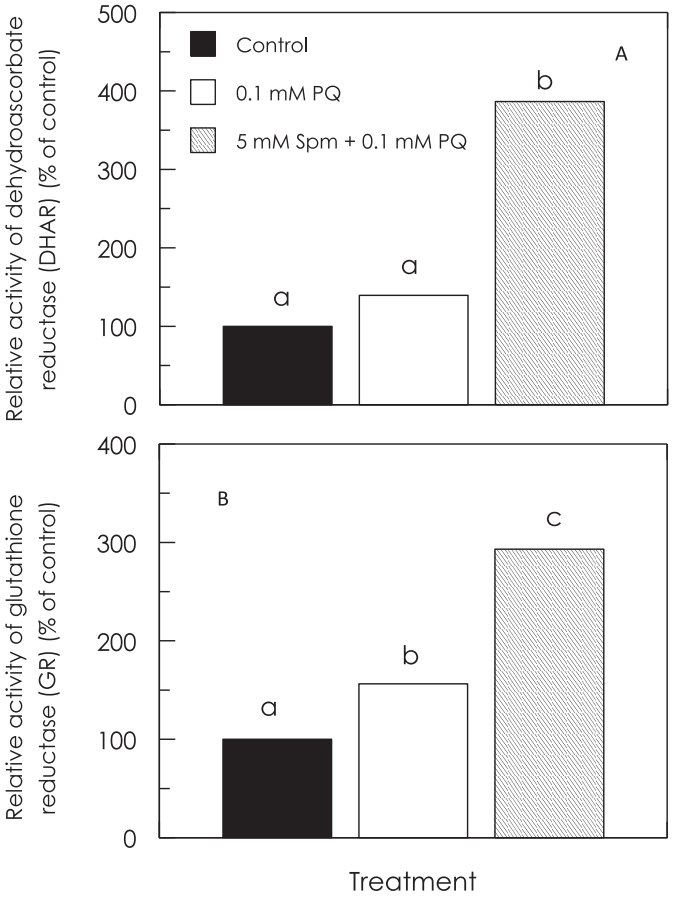

Figure 11. (A) Relative activities of dehydroascorbate reductase (DHAR) and (B) of glutathione reductase (GR) in leaf segments excised from rice mutant 1192-11(susceptible [S]) at the tillering stage, 9 h after treatment with 0.1 mM paraquat. The effect of 5 mM spermine pretreatment for 6 h before paraquat treatment was also included. The original activity of DHAR for control was 0.8 mmol ascorbate min^{-1} mg $protein^{-1}$. The P value of difference among the three treatments was 1.27×10^{-5}, and the LSD at 0.05 was 48%. The original activity of GR for control was 0.5 µmol NADPH min^{-1} mg $protein^{-1}$. The P value of difference among the three treatments was 8.58×10^{-7}, and the LSD at 0.05 was 19%.

In this study, changes of GR activities in all three rice lines paralleled those of DHAR activities after paraquat treatment (Figure 8B). This increased GR activity in paraquat-tolerant plants has also been observed in tall fleabane (Chiang et al. 2008) and hairy fleabane (Ye and Gressel 2000). Based on the high expression of both enzyme activities, an activated ascorbate–glutathione cycle is proposed to confer strong antioxidative ability to alleviate damage of rice plants under paraquat stress.

Effect of Exogenous Spermine on Paraquat Tolerance. Excised from S mutant rice, leaf segments treated with 5 mM spermine for 6 h and subsequently with 0.1 mM paraquat for 9 h showed an 18% reduction of the maximal efficiency of PSII photochemistry, whereas leaf segments without Spm pretreatment showed a 43% reduction (Figure 9A). Although the reduction value of the maximal efficiency of PSII photochemistry caused by paraquat from May to August was a little less than similar treatment from January to April (Figure 6A), it is obvious that Spm exerted a preventive effect on the oxidative damage of paraquat against rice. Benavides et al. (2000) also reported an alleviating effect on the reduction of chlorophyll in paraquat-treated sunflower by Spm pretreatment. Polyamines have been known as antioxidants to protect plants from various environmental stresses (Benavides et al. 2000). Spm, when exogenously applied to common sunflower (*Helianthus annuus* L.), could prevent oxidative damage by paraquat more effectively than some other polyamines, e.g., spermidine and putrescine.

Table 3. Kinetics for the glutathione reductase (GR) of rice cv. TNG67 and two mutants.[a]

Rice line	K_m(GSSG)	K_i(paraquat)	V_{max}
	µM		µmol NADPH (min^{-1} mg^{-1} protein)
TNG67	0.017	0.67	4.4
72-16(T)	0.043	1.04	6.0
1192-11(S)	0.250	1.43	4.7
P value	< 0.001	0.06	0.01
$LSD_{0.05}$	0.027	ND	0.88

[a] Abbreviations: K_m, affinity of enzyme to substrate; K_i, inhibition constant of paraquat against glutathione reductase; V_{max}, velocity of maximal reaction rate; T, tolerant; S, susceptible; ND, no significant difference at the 0,05 level.

Decrease of Malondialdehyde (MDA) Accumulation by Exogenous Spermine. MDA in leaf segments excised from susceptible rice mutant was increased by 7.3-fold after 0.1 mM paraquat treatment for 9 h (Figure 9B). As compared with the similar experiment conducted under the lower temperature from January to April, this large increase of lipid peroxidation might be promoted by the higher ambient temperature from May to August. Purba et al. (1995) reported that the survival rate of a paraquat-resistant biotype of hare barley was dramatically decreased if it was treated with paraquat in summer, as compared with in winter. Higher temperature enhanced the development of paraquat damage. Nevertheless, pretreatment of 5 mM Spm 6 h before paraquat treatment effectively alleviated this MDA increment to 4.6-fold, still showing a significant antioxidation ability. Groppa et al. (2001) reported that the MDA accumulations caused by cadmium chloride or copper chloride decreased 1.6- and 5.4-fold, respectively, by Spm pretreatment.

Activation of the Ascorbate–Glutathione Cycle by Exogenous Spermine. In this study, the reduced form of glutathione in leaf segments excised from the S-line mutant, was increased by twofold only after 0.1 mM paraquat treatment as compared with the control; and yet, Spm pretreatment prior to paraquat treatment resulted in a fivefold increase of GSH (Figure 10A). Under paraquat application, the reduced form of glutathione was increased 2.5-fold by Spm pretreatment, leading to a 20% increase of the GSH : total glutathione ratio (Figure 10B). Exogenous Spm has also been reported to increase GSH content in chickpea (*Cicer arietinum* L.) seedlings under environmental stress, e.g., low temperature and drought (Nayyar and Chander 2004). Greater relative increase of GSH in the T mutant (Figure 7B) might allow the rice plants to adapt to oxidative stress caused directly and indirectly by environmental factors.

In this study, DHAR activity in leaf segments was not changed by paraquat, but it was increased fivefold under paraquat treatment coupled with Spm pretreatment (Figure 11A). The induction of DHAR activity by Spm has also been reported in

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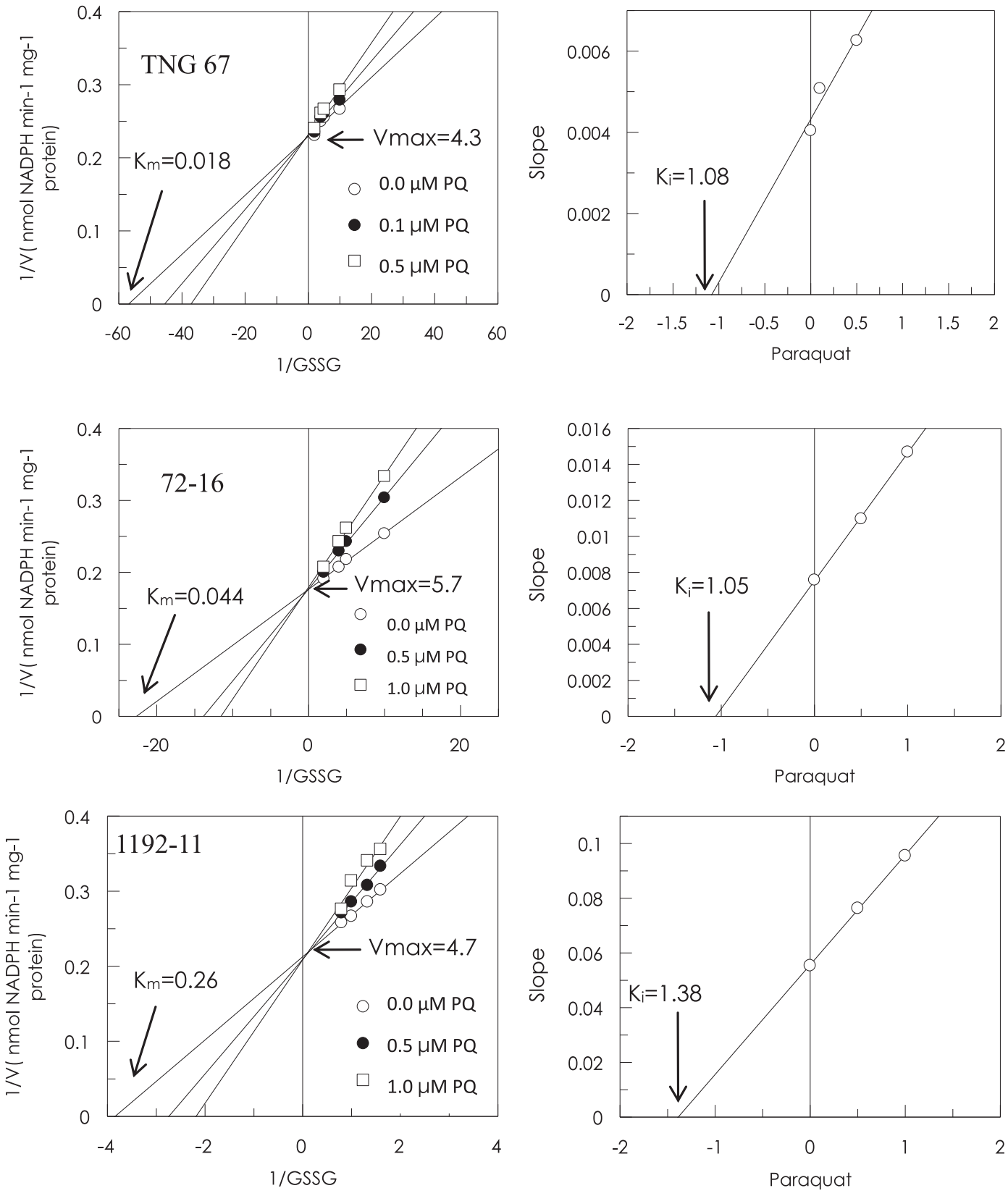

Figure 12. Enzymatic kinetics for the competitive inhibition of glutathione reductase (GR) by paraquat in leaf segments excised from rice cv. Tainung 67 (TNG 67) and two rice mutants, 1192-11 (susceptible [S]) and 72-16 (tolerant [T]) at midtillering stage.

radish (Kim and Jin 2006). GR activity in leaf segments treated with 0.1 mM paraquat was increased only 50%; however, a threefold increment of GR activity was achieved by 5 mM Spm pretreatment (Figure 11B). In common sunflower with peroxidation stress caused by exposure of excessive cadmium or copper, GR activity was also increased by the Spm pretreatment, suggesting a function of Spm protecting plants from oxidative damage (Groppa et al. 2001). The above results provide strong

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evidence showing that an active ascorbate–glutathione cycle promoted by spermine, via enhanced DHAR and GR activities, is a critical process for paraquat tolerance in rice plants.

Kinetics of Glutathione Reductase in Paraquat-Susceptible and -Tolerant Rice Plants. The high expression of GR activity in tolerant rice mutants justifies the key role of this detoxifying enzyme in the three rice lines. Kinetic studies showed that K_m of GR for the T mutant and TNG67 was significantly lower than that of the S mutant (Table 3), revealing a higher affinity of the former two for the substrate GSSG. Besides, V_{max} of the T mutant was significantly higher than that of the other two rice materials, indicating that under a saturable supply of substrate, this enzyme in the T mutant has the maximal catalytic capacity. However, there was no difference of K_i values for paraquat among the three rice lines, a phenomenon anticipated because paraquat is not an inhibitor of GR. Although GR is an enzyme responsible for scavenging free radicals induced by this herbicide, instead of a target enzyme of attack, a high affinity and catalytic rate of this enzyme for its substrate in the T mutant might very possibly assist in alleviating damages generated by paraquat. In a comparison of kinetics traits of GR in T and S mutants, using the Lineweaver–Burk double-reciprocal plot method, it appeared that paraquat and GSSG might compete for the same binding site on GR protein in all three rice lines (Figure 12).

In conclusion, an active antioxidative system, especially GR and DHAR, the two key enzymes of the ascorbate–glutathione cycle, plays an important role in endowing T-mutant rice with paraquat tolerance. A higher expression of antioxidative ability in paraquat-tolerant rice and tall fleabane (Chiang et al. 2008) indicate that this tolerance mechanism might exist in other plant species. Pretreatment of paraquat-susceptible plants with spermine, resulting in an enhancement of paraquat tolerance via activation of this antioxidative system, provided more solid evidence to confirm the significance of antioxidative system. With respect to the key enzyme GR, the high V_{max} and low K_m of this enzyme for its substrate in the T mutant suggests a higher catalytic efficiency under a saturable supply of substrate to promote recycling of glutathion.

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