

# A Paraquat Tolerance Mutant in Rice (*Oryza sativa* L.) Is Controlled by Maternal Inheritance

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## Abstract

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) tolerance is an important trait in the weed control during crop production. The paraquat tolerant (Pq72) and susceptible (Pq1192) mutants are pure lines derived from the mutation pool of rice cultivar TNG67. Two reciprocal crosses, Pq72/Pq1192 and Pq1192/Pq72, were conducted between Pq72 and Pq1192 mutant lines for studying the genetic of paraquat tolerance by investigations of physiological characteristics related to paraquat tolerance including leaf injury index, leaf chlorophyll fluorescence (Fv/Fm) and electrolyte leakage in the F<sub>2</sub> populations of two reciprocal crosses after paraquat treatment. The results suggested that a maternal inheritance of paraquat tolerance is existed in these mutants. Further analysis found that the F<sub>2</sub> population of Pq72/Pq1192 segregated 3:1 (tolerant to susceptible) in both Fv/Fm and electrolyte leakage, respectively. This result implies that the paraquat tolerance of the Pq72 mutant is controlled by a single dominant gene.

## Keywords

Paraquat, Tolerance, Leaf Chlorophyll Fluorescence, Electrolyte Leakage, Maternal Effect

## 1. Introduction

Weed control has become a problem for farmers in most rice production areas. Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridinium; methyl viologen) is a rapid acting and non-selective herbicide for controlling weeds in cultivated as well as non-cultivated land throughout Taiwan [1]. PQ can cause membrane damage,

DNA denaturation, protein reduction by transferring electron from photosystem I (PSI) to oxygen producing toxic reactive oxygen species (ROSs), efficiently induce the death of plant cells [2] [3] [4] [5]. Therefore, paraquat can kill a wide range of annual grasses, broad-leaved and perennial weeds upon contact, but it becomes biologically inactive and less toxic toward roots as well as rhizomes when entering the soil environment [3] [6]. Up to now, more than 30 paraquat-resistant weed species, *i.e.* *Conyza bonariensis* (L.) Corng. *Lolium rigidum*, *Arctotheca calendula*, *Rehmannia glutinosa* and *Mazus pumilus* have been reported in the world (<http://www.weedscience.org/>; [7]). In Arabidopsis, several paraquat resistant mutants including photoautotrophic salt tolerance 1 (*psf1*), radical-induced cell death1 (*rcd1*), paraquat resistant 1 (*par1*), paraquat resistant 2 (*par2*), pleiotropic drug resistance 11 (*atpdr11*) and resistant to methyl viologen 1 (*rvm1*) [8]-[13] have been identified and characterized.

Fuerst and Vaughn [14] proposed mechanisms of paraquat resistance as follows: reducing quantities of paraquat absorbed by leaf surface; reducing the efficiency of electron acceptance of paraquat, *i.e.* modification of photosystem I; increasing paraquat metabolic rate; increasing the enzymatic activities for free radical scavenging; and sequestration of paraquat. The first three mechanisms still need to be defined experimentally, however, the increase of enzymatic activities for free radical scavenging and sequestration of paraquat have been considered as the critical mechanisms for paraquat resistance [15].

A mutant pool of rice variety TNG67 contains more than 3000 mutants with many desirable traits were developed by sodium azide mutagenesis at the Taiwan Agricultural Research Institute [16]. In our previous study, two pure mutant lines, the paraquat tolerant 72 (T, Pq72) and susceptible 1192 (S, Pq1192), have been screened from this mutant pool [17], and the physiological mechanism study on the paraquat tolerance mutants revealed no difference in uptake, translocation, and metabolism of paraquat between T and S lines (C. S. Wang, unpublished data). However, the induction of enzymatic activities for free radical scavenging played a critical role in paraquat tolerance [15]. Therefore, it is interesting to explore the expression of genes encoding anti-oxidation related enzymes and the mutations induced by sodium azide. In this study, the inheritance of paraquat tolerance in rice mutant was studied based on the tolerance expression in F<sub>2</sub> populations from the reciprocal crosses of Pq72 (T) and Pq1192 (S) pure lines.

## 2. Materials and Methods

### 2.1. Rice Materials

Six paraquat-susceptible (S) lines (No. 81, 802, 875, 948, 1081 and 1192) and 7 tolerant (T) lines (No. 72, 713, 881, 882, 883, 986 and 1067) have been selected from 1343 pure mutants at M7 generation from the mutant pool of TNG67 rice variety [17]. After further identification of paraquat tolerance was conducted for three selfed generations, two most stable pure lines, the tolerant 72 (T, Pq72)

and susceptible 1192 (S, Pq1192), were confirmed in the M10 generation and selected as the materials for studying the inheritance of paraquat tolerance. The rice plants of parental and F<sub>1</sub> generations used for inheritance analysis were transplanted into pots at the three-leaf (V3) stage and grown to 21 days in greenhouse for tolerance test. All rice plants of F<sub>2</sub> populations used for inheritance analysis were grown in the paddy field according to traditional management.

## 2.2. Paraquat Treatment

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) treatment was performed as previous described [17]. To acquire a homogenized spraying effect on the tested pot-grown plants, an auto sprayer system consisting of automobile orbital sprayer with a speed of 187 cm·min<sup>-1</sup>, high-pressure compressor providing 0.2 MPa with a flow rate of 1 m·sec<sup>-1</sup>, and three nozzles at 45 cm above plants producing droplet of 30 µm diameter, were applied in this work in our herbicide spraying facility in the greenhouse at the NCHU main campus.

## 2.3. Genetic Crossing

Two paraquat mutants, Pq72 (T) and Pq1192 (S), were selected as parents to make reciprocal crosses to generate the offspring for the genetic analysis of paraquat tolerance. The F<sub>1</sub> plants from the reciprocal crosses between Pq72 and Pq1192 were analyzed by at least two polymorphic SSR markers (data not shown) to identify the true hybrid and then selfed to obtain the F<sub>2</sub> generation. Responses of these plants to paraquat were determined (Table S1).

*Duration and location.* All the experiments were conducted during 2010 to 2013 either in greenhouse at the main campus of NCHU or in the paddy field at the experimental farm of Agronomy Department of NCHU at Wu-Feng, Taichung, Taiwan according to the experimental requirement.

## 2.4. Evaluation of Plant Response to Paraquat

The parameters of paraquat tolerance were analyzed by investigations of injury index, chlorophyll fluorescence [18] and electrolyte leakage [19]. At tillering stage, nine completely expanded leaves for each rice plant were harvested and the 1.5 cm-leaf segments were excised 5 cm distance from leaf tip for the following measurements.

*Injury index.* The paraquat treatment in this injury analysis was performed as previous method [17]. An aliquot of 15 mL 100 µM paraquat was applied to a Petri dish (diameter 15 cm) lined with filter paper and then the leaf segments of rice plants were placed on the paper. These samples were placed under a light intensity of 200 µ·mole·m<sup>-2</sup>·s<sup>-1</sup> for 9 hrs and the injury indices were determined. The injury index could be classified from 0 to 7, where 0 indicates healthy leaf segment without any herbicidal damage and 7 indicates the completely etiolated leaf segment caused by paraquat (Figure S1).

*Chlorophyll fluorescence.* The paraquat-treated leaves were blotted with tissue paper for drying and then fixed to the sensor clip of portable Chlorophyll Fluorimeter (OS1-FL, Opti-Sciences, and Tyngsboro, USA) for 10 min in the dark before analyzing the maximal efficiency of PSII photochemistry (Fv/Fm), and a sensitive physiological trait reflecting rapid damages to photosystem.  $F_v/F_m = (F_m - F_o)/F_m$ , where  $F_o$  and  $F_m$  indicate the minimal and maximal fluorescence after dark acclimation, respectively [20].

*Electrolyte leakage.* The paraquat-treated leaves were placed in a tube containing 8 mL deionized water and shaken gently for 24 hrs. The electric conductivity of the solution was determined by a conductivity meter (SC-170, SUNTEX INSTRUMENTS CO., Taipei, Taiwan). Subsequently, samples were heated in a boiling water bath for 15 min and then cooled for the second assay of conductivity. The electrolyte leakage was calculated as the ratio of conductivity prior to and after boiling.

## 2.5. Data Analysis

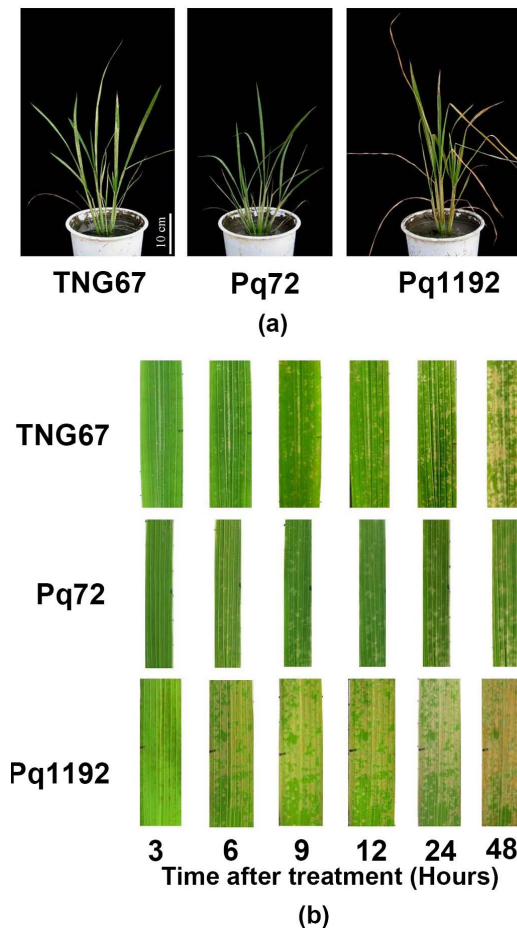
A completely randomized design (CRD) with three replications was conducted in this work. Data was subjected to ANOVA, and the difference among treatments was compared statistically based on the least significant difference at 5% level ( $LSD_{0.05}$ ). In genetic analysis,  $\chi^2$  test was applied to analyze the fitness of segregation ratio for paraquat tolerance of  $F_2$  populations from two crosses, Pq72/Pq1192 and Pq1192/Pq72.

## 3. Results and Discussion

### 3.1. Responses of the Pq1192 and Pq72 Mutants to Paraquat

Rice mutants Pq1192 (S) and Pq72 (T) have been selected from the M7 generation of TNG67 mutation pool by 100  $\mu$ M paraquat treatment [17] and continued selfing to obtain the M10 pure lines. In order to understand the response against paraquat, the rice plants, TNG67, Pq1192 and Pq72, at tillering stage were treated with 100  $\mu$ M paraquat. At 3 days after treatment, Pq72 displayed an almost unaffected appearance in contrast with Pq1192 showed a serious injurious appearance (**Figure 1(a)**). Besides, some significant water soaking injured spots were observed on the leaves of TNG67 after treatment. These results indicate that Pq72 has a better paraquat tolerance than the other two lines.

The morphology of paraquat-treated leaves was investigated at 0, 3, 6, 9, 12, 24 and 48 h after treatment to reveal the progress of paraquat damage of these lines (**Figure 1(b)**). Some water soaking injured spots were observed on the leaves of Pq1192 at 3 h after treatment. The necrosis spots appeared on all leaves of TNG67, Pq1192 and Pq72 after 6 h of treatment, and the Pq1192 leaves showed the most serious injury. At the 48 h after treatment, the severe desiccation occurred on the leaf tissues of TNG67 and Pq1192 and large necrosis lesion were observed on the leaves. The Pq72 leaves showed a less injury than TNG67 and Pq1192 after paraquat treatment. These results indicate that Pq72 effectively



**Figure 1.** Responses of three rice plants to paraquat, injury of paraquat-susceptible mutant appeared 21 days after 100  $\mu$ M paraquat treatment (a). Injury of leaf segments excised from 21-days-old rice TNG67, Pq72(T) and Pq1192(S), respectively, caused by 100  $\mu$ M paraquat (b).

delayed the formation of water soaking injured spots and show tolerance to paraquat treatment.

The maximal efficiency of photosystem II (PSII) photochemistry (Fv/Fm) can be applied as an indicator to distinguish the injury level of plants under stress [21]. For example, the Fv/Fm values of health leaves were distributed in the range 0.72 - 0.85 and the less Fv/Fm denoted that the PSII has been damaged. In general, the Fv/Fm < 0.6 denoted the plants is injured but could be recovered; otherwise, the Fv/Fm < 0.3 denoted the plants is injured severely and could not be recovered [22]. In order to understand the injury level of TNG67, Pq72 and Pq1192 under paraquat treatment, Fv/Fm values of rice plants at tillering stage were determined (Table 1). No significant difference in Fv/Fm of leaf segments excised from TNG67, Pq1192 and Pq72 before treatment, all the Fv/Fm values were about 0.8. However, the Fv/Fm of Pq1192(S) line was reduced to 0.596 (74.1%) which is lower than TNG67 (0.662; 81.3%) and Pq72 (0.687; 85.0%) at 3 h after treatment. At the same time, significant water soaking spots were also

**Table 1.** Photosystem II inhibition of leaf segments, excised from 21-day-old rice plants, after treatment with 100  $\mu$ M paraquat. Rice cv. TNG67, and both tolerant (Pq72) and susceptible (Pq1192) mutants were compared for the evaluation of paraquat tolerance.

Line	Fv/Fm						
	Time after treatment (Hours)						
	0	3	6	9	12	24	48
TNG67	0.814 $\pm$ 0.005 <sup>ax</sup>	0.662 $\pm$ 0.028 <sup>a</sup>	0.554 $\pm$ 0.034 <sup>a</sup>	0.521 $\pm$ 0.045 <sup>b</sup>	0.555 $\pm$ 0.043 <sup>b</sup>	0.500 $\pm$ 0.043 <sup>b</sup>	0.442 $\pm$ 0.076 <sup>ab</sup>
Pq72	0.808 $\pm$ 0.008 <sup>a</sup>	0.687 $\pm$ 0.019 <sup>a</sup>	0.621 $\pm$ 0.014 <sup>a</sup>	0.645 $\pm$ 0.035 <sup>a</sup>	0.677 $\pm$ 0.029 <sup>a</sup>	0.635 $\pm$ 0.041 <sup>a</sup>	0.587 $\pm$ 0.060 <sup>a</sup>
Pq1192	0.805 $\pm$ 0.007 <sup>a</sup>	0.596 $\pm$ 0.045 <sup>b</sup>	0.447 $\pm$ 0.056 <sup>b</sup>	0.463 $\pm$ 0.044 <sup>b</sup>	0.503 $\pm$ 0.044 <sup>b</sup>	0.437 $\pm$ 0.041 <sup>b</sup>	0.365 $\pm$ 0.094 <sup>b</sup>

\*Values are mean of Fv/Fm  $\pm$  SD. Values with the same letter are not significantly at  $P < 0.05$ .

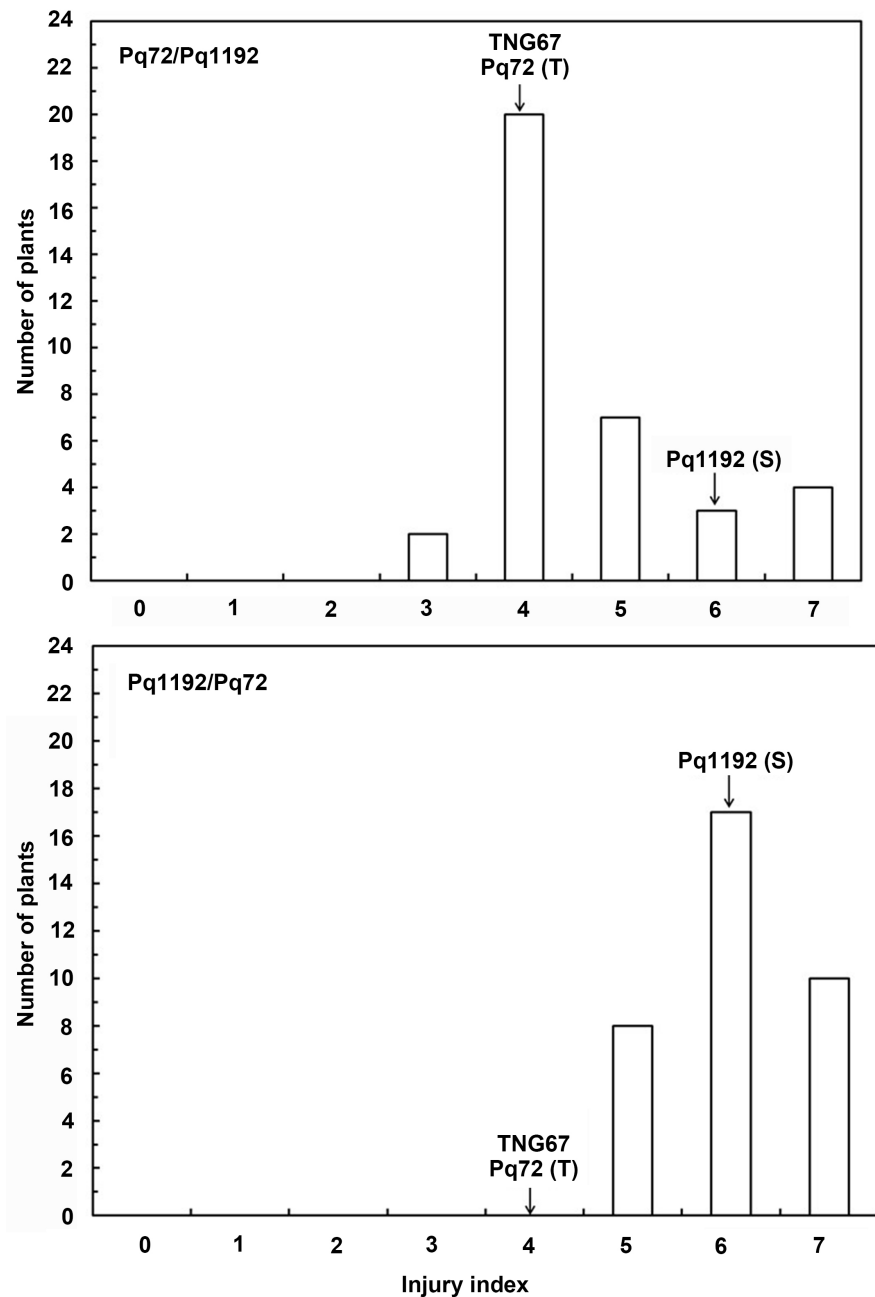
observed on the leaves of Pq1192. At 6 h after treatment, the Fv/Fm values of all lines were significantly reduced and the injury spots were observed on the leaves. However, the injury of Pq72 delayed from 6 to 24 h and Fv/Fm ratio maintained above 0.6 (**Table 1**).

At any time during treatment, the Fv/Fm values of Pq72 were almost higher than the ones of TNG67 and Pq1192. Even at 48 h, the Fv/Fm ration of Pq72 was 0.587 (72.6%) still significantly higher than those of TNG67 (0.442; 54.3%) and Pq1192 (0.365; 45.4%). These results revealed that Pq72 was also injured slightly by paraquat in the initial stage but the injury development was significantly delayed. These results are also consistent with the suggestion that the Pq72 is more tolerant than the other lines, and Pq72 efficiently delays the injury of paraquat [17].

### 3.2. Inheritance of Paraquat Tolerance

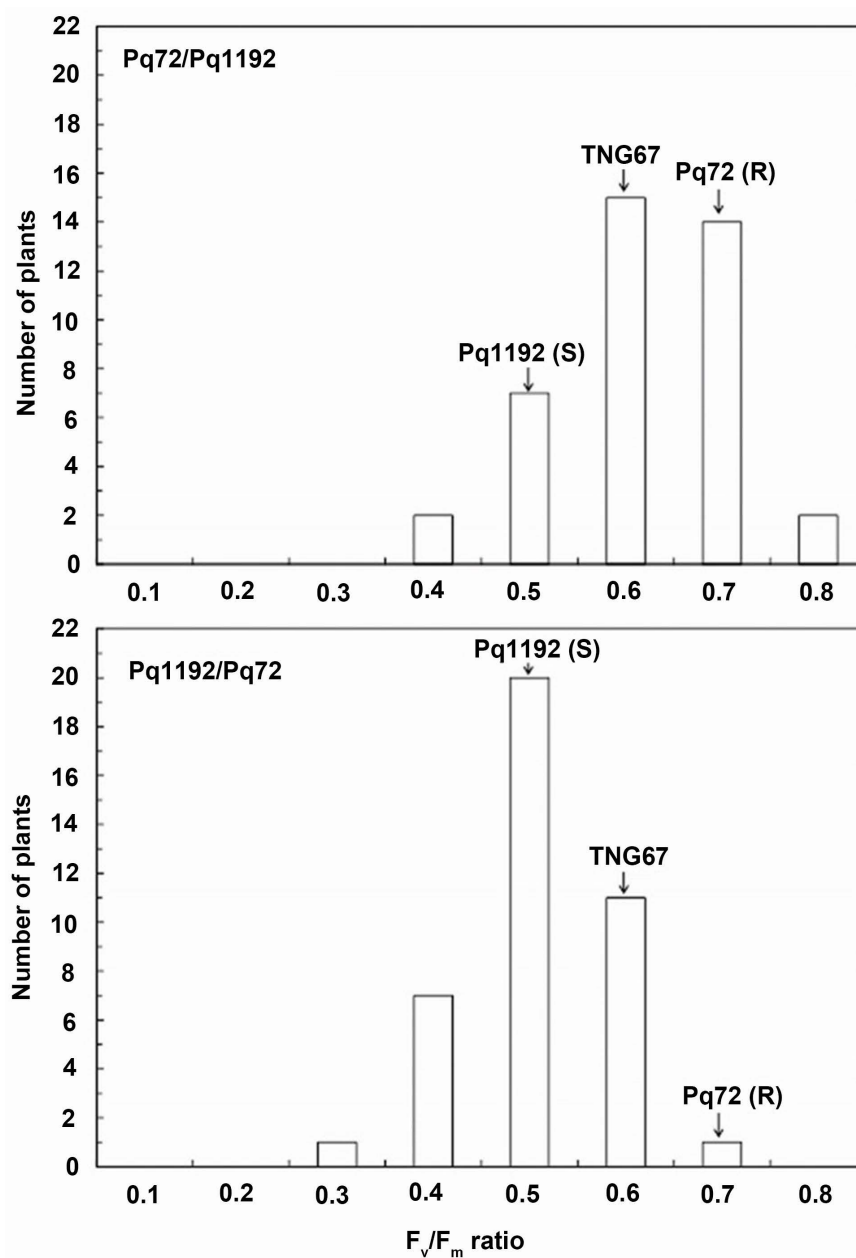
In order to determine the inheritance of paraquat tolerance, reciprocal crosses were made between Pq1192 and Pq72 and the  $F_1$  progeny was confirmed by SSR (simple sequences repeat) markers (data not shown). There were 36 and 42 of true bred  $F_1$  individuals selected from Pq72/Pq1192 and Pq1192/Pq72, respectively. After paraquat treatment, the frequency distribution of injury index of  $F_1$  populations was shown in **Figure 2**. The  $F_1$  plants of Pq72/Pq1192 show significant less injury index than that of Pq1192/Pq72 cross. The significant differences in injury index of two reciprocal crosses suggest the maternal effect on paraquat tolerance.

In the  $F_2$  population, a total of 120 individuals were planted in the field and 40 individuals were selected randomly for determination of chlorophyll fluorescence and electrolyte leakage after paraquat treatment. In chlorophyll fluorescence analysis, the Fv/Fm values of TNG67, Pq72 and Pq1192 were 0.665, 0.724 and 0.508, respectively. The Fv/Fm values of the  $F_2$  populations from Pq72/Pq1192 and Pq1192/Pq72 distributed among 0.354 - 0.732 and 0.254-0.602, respectively (**Figure 3**). The mean Fv/Fm values showed significant differences from the  $F_2$  populations of Pq72/Pq1192 and Pq1192/Pq72 were 0.574 and 0.457, respectively.



**Figure 2.** Frequency distribution of injury index of the leaf segments excised from the individuals of the reciprocal crosses, Pq72/Pq1192 and Pq1192/Pq72, in F<sub>1</sub> generation.

The responses of rice in electrolyte leakage assay, the percentages of electrolyte leakage (PELs) from three rice lines TNG67, Pq72 and Pq1192 were 30.2%, 20.4% and 44.6%, respectively. The frequency distributions of PELs of F<sub>2</sub> populations, Pq72/Pq1192 and Pq1192/Pq72, were shown in **Figure 4**. The PELs of the F<sub>2</sub> populations from Pq72/Pq1192 and Pq1192/Pq72 distributed among 16.4% - 50.5% and 27.3% - 58.7%, respectively. The mean PELs of the F<sub>2</sub> populations from Pq72/Pq1192 and Pq1192/Pq72 were 32.3% and 44.6%, respectively. These results also display a significant difference in the results of reciprocal

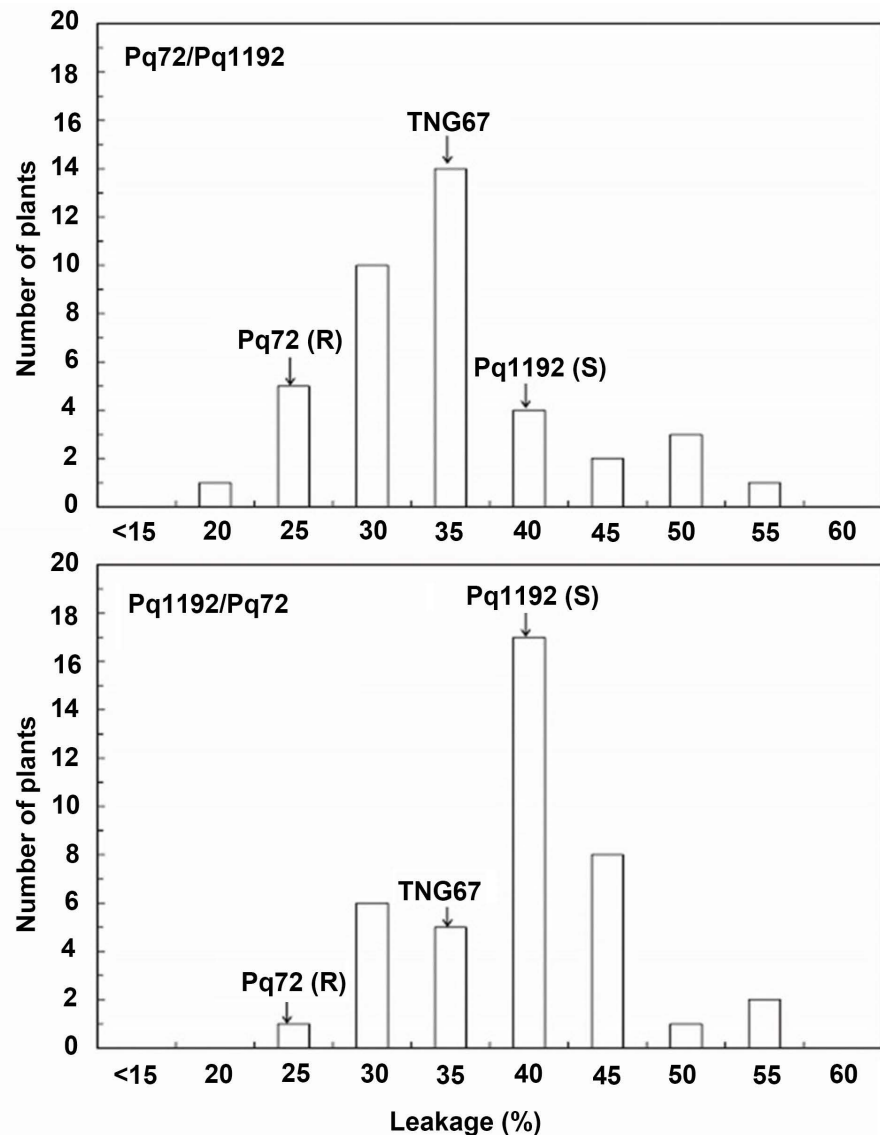


**Figure 3.** The frequency distribution of  $F_v/F_m$  of the leaf segments excised from the individuals of the two reciprocal crosses, Pq72/Pq1192 and Pq1192/Pq72, in  $F_2$  generation.

crosses and are similar to the results in the frequency distributions of injury index of  $F_1$  populations. These findings suggested that the influence of maternal effect on paraquat tolerance is present.

Furthermore, the individuals with  $F_v/F_m$  value less than 0.5 were defined as susceptibility; otherwise the ones higher than 0.5 were defined as tolerance. At the same time, the individuals with PLEs higher than 35% were defined as susceptibility; otherwise the ones lower than 35% were defined as tolerance. The Chi-square test fits the expected ratio in  $F_2$  population (Table 2). The results suggested that the  $F_2$  population of Pq72/Pq1192 segregated to 3:1 (T:S)





**Figure 4.** Frequency distribution of electrolyte leakage percentages of the leaf segments excised from the individuals of the two reciprocal crosses, Pq72/Pq1192 and Pq1192/Pq72, in  $F_2$  generation.

according to Fv/Fm and leakage investigation. However, the  $F_2$  population of reciprocal cross, Pq72/Pq1192, did not segregate to theoretical ratio, 1:3. It implies that the paraquat tolerance of Pq72 is maternally controlled by a single dominant gene.

Most studies displayed that genetic characteristic of paraquat resistance was controlled by the nuclear genes, such as *Conyza bonariensis* [23], *Erigeron philadelphicus* [24], *Erigeron Canadensis* [25], *Hordeum glaucum* [26], *Hordeum leporinum* [27], *Arctotheca calendula* [27] and *Lolium rigidum* [28]. However, the paraquat tolerance inheritance of Pq72 is influenced by the maternal effect. This result is similar to the findings that the inheritances of triazine resistance of some weeds were the examples of cytoplasmic inheritance [29] [30] [31].

**Table 2.** Segregation for paraquat tolerances of F<sub>2</sub> populations from Pq72/Pq1192 and Pq1192/Pq72.

Physiological traits	F <sub>2</sub> generation	No. of F <sub>2</sub> plants			Genetic ratio tested	$\chi^2$
		T	S	Total		
F <sub>v</sub> /F <sub>m</sub> value	Pq72/Pq1192	31	9	40	3:1	0.133 <sup>ns</sup>
	Pq1192/Pq72	12	28	40	1:3	0.533 <sup>ns</sup>
Leakage	Pq72/Pq1192	30	10	40	3:1	0 <sup>ns</sup>
	Pq1192/Pq72	12	28	40	1:3	0.533 <sup>ns</sup>

Critical value:  $\chi^2$  (1, 0.05) = 3.84; <sup>ns</sup> means that the probability is not significant.

The mechanism of triazines resistance was postulated that the electron transfer in photosystem II is hindered by this kind of herbicide, and a G264S mutation of D1 protein in photosystem II causes the triazine resistance of *Brassica napus* L. [32]. On the other hand, the action mechanism of paraquat was proposed that the electron transfer in photosystem I of plants was interfered by paraquat [2] [3] [4] [5]. The genetic analysis also displays that the paraquat tolerance of Pq72 is controlled by a single dominant gene. According to these findings, we propose that the paraquat tolerance gene of Pq72 might participate in the electron transfer in the chloroplastic thylakoid.

It has been found that the paraquat resistant mutant (*par1*) of Arabidopsis is contributed by a strong transporter protein localized to the Golgi apparatus due to an amino acid mutation caused the reduction of intracellular transportation of paraquat and accumulation into chloroplast [11]. Transgenic rice plants carry the RNA interference of the *PARI*-like gene, *OsPARI*, showed significant paraquat resistance by reduction of paraquat transportation and accumulation less into chloroplasts but no difference in paraquat uptake was observed [11]. Same results were reported in our previous report that no differences were detected in the uptake and transportation of paraquat after application in the three tested rice lines and the tolerance is due to the delay of cellular damage in the tolerance mutant [17]. These results supported that the organelles or cytoplasmic factors [7] [33] [34] of the tolerance lines may contribute to paraquat tolerance. Therefore, our results showed significant maternal effects in all the physiological traits detected. However, it is difficult to map the paraquat tolerance gene of Pq72, because of cytoplasmic inheritance of Pq72 paraquat tolerance. Our ongoing molecular genetic study on the paraquat T/S mutants will provide a clear information for the mechanism of paraquat tolerance in mutants.

In this study, the properties and inheritance of paraquat tolerance in mutant Pq72 was characterized. These results demonstrate that Pq72 is a useful germplasm for studying the mechanism of paraquat tolerance in rice improvement. The increasing activities of anti-oxidation enzymes were proposed to explain to be responsible for paraquat tolerance [17]. Therefore, the expression profiles of the genes involved in the anti-oxidation pathway of Pq72 will be de-

terminated through transcriptomics analysis and molecular genetics to clarify the mechanism of paraquat tolerance in Pq72.

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### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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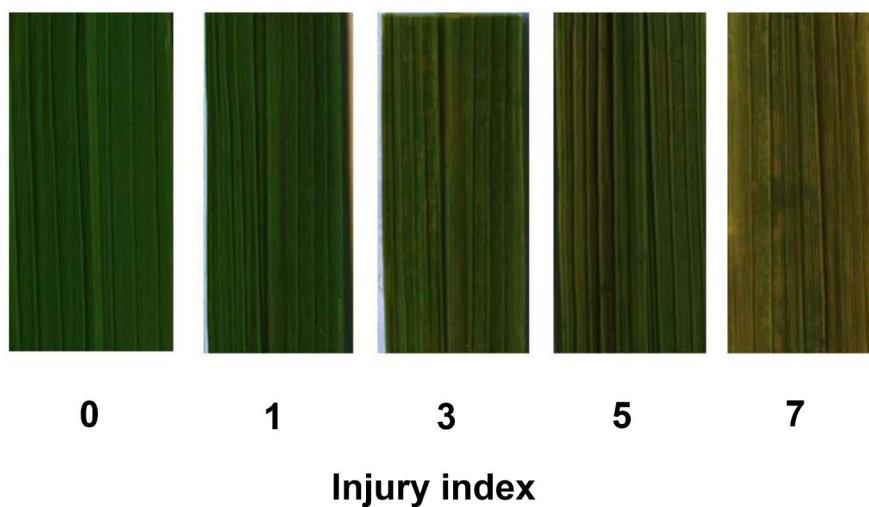
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## Supplemental

**Table S1.** The 21 polymorphic SSR markers between TNG67, Pq72 (T) and Pq1192 (S).

Marker	Chromosome	Position	PCR product (bp) in	
		(cM)	TNG67 or Pq72(T)	Pq1192(S)
RM495	1	2.8	150	136
RM1	1	29.7	82	97
RM259	1	54.2	163	146
RM307	4	0	119	113
RM507	5	0	256	247
RM413	5	26.7	63	69
RM161	5	96.9	168	152
RM178	5	118.8	105	107
RM334	5	141.8	132	173
RM133	6	0	111	109
RM125	7	24.8	117	114
RM11	7	47	116	131
RM455	7	65.7	122	119
RM118	7	96.9	148	151
RM408	8	0	121	115
RM105	9	32.1	94	101
RM215	9	99.4	147	143
RM271	10	59.4	86	99
RM287	11	68.6	212	250
RM19	12	20.9	104	115
RM277	12	57.2	115	119



**Figure S1.** The injury index used in this study. It was classified from 0 to 7, where 0 indicates healthy leaf segment without any herbicidal damage and 7 indicates the completely etiolated leaf segment caused by paraquat.