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Optimization of *Epsps* Gene and Development of Double Herbicide Tolerant Transgenic PGMS Rice

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ABSTRACT

The alternative use of non-selective herbicides in different years will reduce the possibility of weeds resistance to herbicide by avoiding the crops constant exposure to herbicide selection pressure. The photoperiod-sensitive genic male sterile (PGMS) rice with herbicide resistance will be convenient to the mechanization of hybrid seed production and weed control. In order to develop double herbicide tolerant transgenic PGMS rice, the optimized *Epsps* gene and the *Bar* gene were jointly introduced into 7001S, a *japonica* PGMS rice, by *Agrobacterium*-mediated transformation. The coding sequence of *Epsps* gene was optimized based on the codon usage of rice genes and the function of optimized *Epsps* gene was validated by real-time PCR analysis, enzyme-linked immunosorbent assay of EPSPS protein, and herbicide resistant assay in the obtained independent transformants. The highest relative expression level of EPSPS protein in leaf reached 9.02% of the total soluble proteins. The transformants of T_2 generation withstood at least 3.332 g glyphosate /m^2 at seedling stage, which was 2.7 times higher than that reported by Monsanto Company. The transformants harboring the optimized *Epsps* gene and *Bar* gene were also resistant to glyphosate and glufosinate simultaneously at seedling stage; the resistant dosages were 0.375 g glufosinate m^{-2} and 0.833 g glyphosate m^{-2}.

Keywords: Codon optimization, Glyphosate resistance, Glufosinate resistance, Photoperiod-sensitive genic male sterile (PGMS) rice.

INTRODUCTION

The weeds in the fields inflict serious damage on crop production. Chemical weeding using selective herbicides is simple, fast, and effective for weed control. However, different types of weeds in the crop fields need different selective herbicides, which results in the chemical weeding complexity and cost. Transgenic crops resistant to non-selective herbicide grow normally after spraying non-selective herbicides while weeds are all killed. Such plants have effectively resolved the problems resulting from using selective herbicides in non-genetically modified crops (Xiao *et al.*, 2007). In 2011, the planting area of transgenic non-selective herbicide resistant crops reached 93.9 million hectares, accounting for 59% of 160 million hectares of the world's total cultivated area of genetically modified (GM) crops (James, 2011). But, only two herbicide resistant lines, namely, LLRice06 (ACS-OS ØØ1-4) and LLRice62 (ACS-OS ØØ2-5) were approved to be planted in USA by 1999 (USDA-APHIS, 1999) and the planting area was very small.

Glufosinate [2-amino-4-(hydroxymethylphosphinyl) butanoic acid] and glyphosate [N-(phosphonomethyl) glycine] are highly efficient, lowly toxic, and non-selective herbicides applied mostly in crop production. Glufosinate inhibits

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glutamine synthetase (GS), which results in the rapid accumulation of intracellular ammonia, metabolic disruption, and plant death (Manderscheid and Wild, 1986). The *Bar* gene cloned from *Streptomyces hygroscopicus* encodes phosphinothricin acetyltransferase (PAT) that converts glufosinate to a detoxicated form in plant by acetylation of free amino group of glufosinate (Thompson *et al.*, 1987). The *Bar* gene has not only been used in commercial GM crops as a target gene to provide herbicide resistance (Reddy *et al.*, 2011), but also increasingly has been used as a highly efficient selection marker in development of GM crops (Miki and McHugh, 2004). Glyphosate inhibits EPSPS and blocks the biosynthesis of aromatic amino acids and aromatic compounds, which disrupts normal amino acid synthesis and nitrogen metabolism of living organisms to result in their death. Currently, the major strategies to obtain glyphosate-resistant crops through genetic engineering include reducing affinity of EPSPS to glyphosate, overexpressing the EPSPS and degrading glyphosate into non-toxic products (Thompson *et al.*, 1987; Pline-Srnic, 2006; Duke, 2011). The *Epsps* gene from *Agrobacterium* sp. CP4 encodes a class II EPSPS which shares less than 50% amino acid similarity with the class I enzymes of *Escherichia coli* and *Salmonella typhimurium* (Fitzgibbon and Braymer, 1990). The class II EPSPS-overexpressed transgenic plants were found to keep normal metabolism after absorption of glyphosate (Kishore *et al*., 1992), and were successfully applied to a variety of commercial transgenic glyphosate-resistant crops (Dill *et al*., 2008).

In addition, the problem of herbicide-resistant weeds emerged due to the use of single herbicide year-by-year and has always been a concern (Neve, 2007; Kuk *et a1*., 2008). So far, it has been discovered that 375 biotypes of 202 weed species (117 dicots and 85 monocots) have resistance to 21 types of chemical herbicide throughout the world (Heap, 2012). If the crops are provided with two or more kinds of herbicide resistance, the alternative use of herbicides will be realized, and the possibilities of weed resistance to herbicide will be reduced by avoiding the crops constant exposure to herbicide selection pressure. In the present research, the glufosinate-resistance gene *Bar* and the glyphosate-resistant gene *Epsps* optimized by rice preferred codons were transformed into the 7001S, a *japonica* PGMS line, to generate a new transgenic rice germplasm resistant to both glyphosate and glufosinate with higher commercial potential.

MATERIALS AND METHODS

Optimization of *Epsps* Gene

Modification and optimization in nucleotide sequence of *Epsps* gene (Genebank No. AB209952.1) were carried on the premise of keeping the amino acid sequence of the EPSPS protein constant. Firstly, the rare codons of rice were displaced by preferred codons of rice. Secondly, the AT-rich regions, poly(A) addition signal sequences, intron-exon boundary sequences that may influence transcription, translation and mRNA stability, and frequently-used restriction endonuclease sites were avoided. Thirdly, the sequence of optimized *Epsps* gene was determined after analyzing the mRNA secondary structure and eliminating hairpin loop. Next, the tobacco chloroplast transit peptide sequence *TSP* (Wang *et al*., 2003) and the terminator sequence *Nos* were added to 5’ end and 3’ end of the optimized *Epsps* gene, respectively. Finally, the *Sma*I site (cccggg) and *Sac*I site (gagctc) were appended to 5’ end and 3’ end of the above sequence, respectively, to form the fused sequence (*Tsp-Epsps-Nos*) finally. The fused sequence was synthesized by Takara Biotechnology (Dalian) Co. Ltd. and cloned in pMD19-T simple vector, named as pM19-Epsps.

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Construction of Vector and Transformation of Rice

The plasmids of pM19-Epsps and pC3300-ubi-Ω-OsbHLH1 (with *Bar* gene) modified from pCAMBIA3300 (CAMBIA, Canberra, Australia) by our laboratory were digested with the *Sam*I and *Sac*I restriction enzymes. Then, the *OsbHLH1* gene of pC3300-ubi-Ω-OsbHLH1 was replaced by the fused *Tsp-Epsps-Nos* sequence from pM19-Epsps. The resulting plasmid, whose T-DNA cassette is shown in Figure 1, was named as pC3300-Epsps and the *Bar* gene was used as selection maker.

The plasmid pC3300-Epsps was introduced into *Agrobacterium tumefaciens* EHA105 by the freeze-thaw protocol (Sambrook and Russell, 2001). The receptor was 7001S, a *japonica* PGMS rice, developed by Anhui Rice Research Institute. The method of *Agrobacterium*-mediated transformation of rice was adopted from Toki *et al.* (2006), except for using 6 mg/L glufosinate as selection agent.

PCR and Southern Blot

Rice total genomic DNA was isolated from leaves of T_1 glufosinate-resistant and non-transgenic control plants. The primers to detect *Epsps* gene were E1 (5'-CGCCAAGTCTTTGTGGGTGT-3') and E2 (5'-GTCCACGGTGACAGGGTTCT-3') that amplified a 1.3 kb fragment. The primers to detect *Bar* gene were B1 (5'-CACCATCGTCAACCACTACATCG-3') and B2 (5'-TAAATCTCGGTGACGGGCAGGAC-3') that amplified a 0.48 kb fragment. The PCR was performed on the program: denaturation at 95ºC for 3 minutes; then 30 cycles at 95ºC for 45 seconds, 55ºC (*Epsps*)/58ºC (*Bar*) for 1 minute, 72ºC for 1 minute; and final extension at 72ºC for 10 minutes.

Rice total genomic DNA (about 20 μg) was digested with *Hin*dIII overnight at 37 °C, and separated on a 1.0% TAE agarose gel, then transferred onto nylon membrane by capillary action. Prehybridization and hybridization were performed on the standard procedure (Sambrook and Russell, 2001). The probe for Southern blot was prepared by labeling the 1.3 kb fragment of *Epsps* gene with digoxygenin (DIG) by PCR using the same primers as above. The protocol of washing and detecting recommended by the DIG nucleic acid detection kit I (LabKit, China) was adopted.

Real Time Quantitative PCR

The total RNA of rice leaf was extracted by the Trizol reagent (Invitrogen, USA) and quantified by the ND-100 spectrophotometer (NanoDrop Technologies, Wilmington DE USA). The RNA samples digested by DNase I (Frementas, Vilnius, Lithuania) were reverse transcribed into first strand cDNA

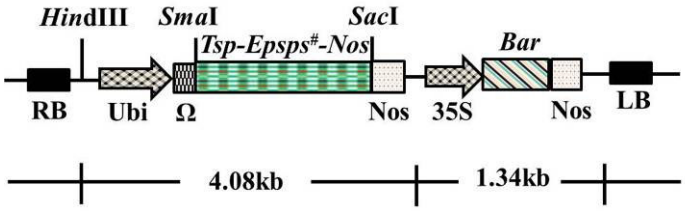

Figure 1. T-DNA region of plasmid pC3300-Epsps. The expression cassette of pC3300-Epsps contains the optimized *Epsps* gene drived by maize ubiquitin promoter and the *Bar* gene derived by CaMV35S promoter. RB and LB are the right- and left-border sequences of the T-DNA region, respectively. Abbreviations: 35S: CaMV35S promoter; Ubi: Maize ubiquitin promoter; Nos: Terminator of nopaline synthase gene; Ω: TMV translational enhancer sequence; *Bar*: Bialaphos resistance gene; *Tsp-Epsps-Nos*: The synthetic fused sequence.

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using the oligo$(dT)_{18}$ primers and MMLV reverse transcriptase (Frementas). The real-time PCR amplification was carried out using 25 μL amplification mixtures containing 12.5 μL 2×SYBR Green premix Ex Taq™ (TakaRa, Dalian, China), equivalent to 100 ng cDNA and 0.2 μM primers. The primers for ubiquitin gene used as a reference gene were Ubq-F (5'-AACCAGCTGAGGCCCAAGA-3') and Ubq-R (5'-ACGATTGATTTAACCAGTCCATGA-3'). The primers for *Epsps* gene were Epq-F (5'-TATGGGCTTGGTCGGAGTCTAC-3') and Epq-R (5'-GTGATGGGAGTGGGAGTCTTG-3'). Reactions were run on the ABI PRISM 7900 HT detection system (Applied Biosystems, Foster City, CA USA). Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Relative Quantification of EPSPS Protein

Approximately 10 mg of rice leaves was homogenized by grinding with 250 μL of extraction/dilution buffer supplied by enzyme linked immunosorbent assay (ELISA) kit (EnviroLogix, USA). After quiescence for 30 minutes at room temperature, a 50 μL aliquot of the supernatant was transferred to the well of ELISA kit (AP010), and the EPSPS protein was detected and the relative content of EPSPS protein in leaf was calculated based on the standard curve provided by the company.

Herbicide Resistance Aanalysis

The herbicide resistance of seed at germination stage was detected by placing the sterilized mature seeds on 1/2 MS regeneration medium containing 0, 6, 12, and 18 mg L^{-1} glufosinate, or 0, 34, 136, and 170 mg L^{-1} glyphosate, respectively, or glufosinate and glyphosate simultaneously, and incubating for 7 days under 24ºC in light and 19ºC in dark alternatively. The herbicide resistance of plant at seeding stage was determined by spraying the overground parts of plant with 0, 0.375, and 0.750 g glufosinate m^{-2} (i. e., 0, 100 and 200 mL of 450 mg glufosinate L^{-1} over 0.12 m^2 rectangle basin which included 100 plants), or with 0, 0.833, and 3.332 g glyphosate m^{-2} (i. e., , 0, 100 and 400 mL of 1000 mg glyphosate L^{-1} over 0.12 m^2 rectangle basin which included 100 plants), or with 0.375 g glufosinate m^{-2} and 0.833 g glyphosate m^{-2} simultaneously. The results were observed after 7 days.

Pollen Fertility and Agronomic Trait Analysis of Transgenic Rice

The receptor 7001S is a PGMS line; the sterility of pollen is mainly regulated by photoperiod and is more or less affected by air temperature (Peng *et al*., 2008). Generally, it retains complete male sterility when day length is longer than 14 hours during the young panicle development and converts to partial or complete male fertility when day length is shorter than 14 hours. However, a low temperature (21-23°C) can promote the conversion of sterility-to-fertility under long-day conditions (Zhou *et al*., 2012). The tested transgenic lines of T_2 generation were planted in 30-cm-diameter pots; each line having 10 pots×3 plants/pot. When the young panicle developed to the stamen and pistil primordium formation stage, half of the plants ,i.e.15 plants, of each line still grew under natural long-day and high-temperature environment, while the other half were moved to a pool and irrigated with 21ºC water for about 5 weeks until the plant heading. The depth of the water was about 25 cm above the soil surface. The anthers of three spikelets from the main panicle were stained by 1% I_2-KI solution and the morphology and number of the pollens isolated from anthers were observed under a microscope (Olympus BX51, Japan) with 100 times amplification. The pollen fertility rate was calculated as follows:

Pollen fertility rate (%)= Number of fertile pollen×100/(Number of fertile pollen+Number of sterile pollen).

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The main agronomic traits, including plant height, panicle length, effective panicles per plant, spikelets per panicle, filled grains per panicle, 1,000-grain weight, and seed setting were investigated at mature stage, using the following definitions:

Seed setting (%)= Filled grains per panicle/Spikelets per panicle×100.

Grain yield per plant (g)= Filled grains per panicle×Effective panicles per plant×1,000-Grain weight/1000. Data were processed by DPS 8.01.

RESULTS

Optimization of *Epsps* Gene

The optimized *Epsps* gene possesses the same length (1371 bp) and amino acid sequence as wild-type *Epsps* gene (Genebank No. AB209952.1). However, in the optimized sequence, the usage frequencies of rare codon ATA, TTA, CTA and GTA were reduced; the distributions of codon GCG, ACG, CCG, TCG and GGG were decreased in case of DNA methylation; one AT-rich sequence was eliminated; the sites of frequently-used restriction enzyme such as *Bam*HI, *Pst*I, *Nco*I were removed; and the mRNA secondary structure was optimized to ensure efficient translation of proteins. Finally, 163 nucleotides accounting for 11.89% of the total nucleotides and 138 codons accounting for 30.20% of the total codons were changed and its GC content was reduced from 65.62 to 59.94%. The codon distribution in optimized *Epsps* gene was close to that in rice and should be expressed more easily and efficiently than the original *Epsps* gene. The synthesized *Epsps* gene is shown in Figure 2.

Molecule Identification of Transformants

Via *Agrobacterium*-mediated transformation, 34 regenerated plants from 2652 calli were gained under glufosinate selection pressure and all of them were identified as true transgenic plants by PCR analysis (Figures 3-a and -b). Ten transgenic plants of vigorous growth were selected for Southern blot analysis, by which the integrations of *Epsps* gene in rice genome were confirmed (Figure 3-c). The copy numbers of *Epsps* gene in these plants varied from one to three, and all of these plants came from independent transformation events, except plant No. 4 and 6 (Figure 3-c).

Expression Analysis of the *Epsps* Gene

The transgenic lines No. 1, 2, 3, 4, 5, 7, 8, and 9 of T_1 generation, in which the expression of *Epsps* gene was 100-400 times higher than the non-transgenic plant (Figure 4-b), were selected for RT-PCR (Figure 4-a) and real-time PCR. A 150 bp sequence of *Epsps* gene was amplified from cDNA of all eight transformants (Figure 4-a). The EPSPS protein was detected in transgenic lines of T_1 generation by the CP4/EPSPS ELISA kit, whereas it was not detected in non-transgenic plant (Figure 4-c). According to the standard curve supplied by the company, the relative expression level of EPSPS protein in leaves of transgenic lines No. 1, 2, 3, 4, 5, 7, 8 and 9 were 7.83, 6.36, 7.75, 7.56, 7.24, 7.86, 9.02, and 8.31%, respectively (Figure 4-d). Comparison of Figure 4-b with 4-d shows that, for different transgenic lines, the relative expression level of EPSPS protein changed with the same trend as the mRNA expression level, with line No. 8 showing the highest and line No. 2 the lowest relative expression. All aforementioned results showed that the optimized *Epsps* gene was stably transcribed in T_1 transgenic plants.

Herbicide Resistance Analysis

The transgenic rice seeds of T_2 generation were germinated on 1/2MS medium containing glufosinate (Figure 5-a), glyphosate (Figure 5-b), or glufosinate and glyphosate simultaneously (Figure 5-c), but the non-transgenic seeds were germinated on 1/2MS medium without any herbicide. The results showed that the germinated seeds of transgenic T_2 generation were resistant to both glufosinate and

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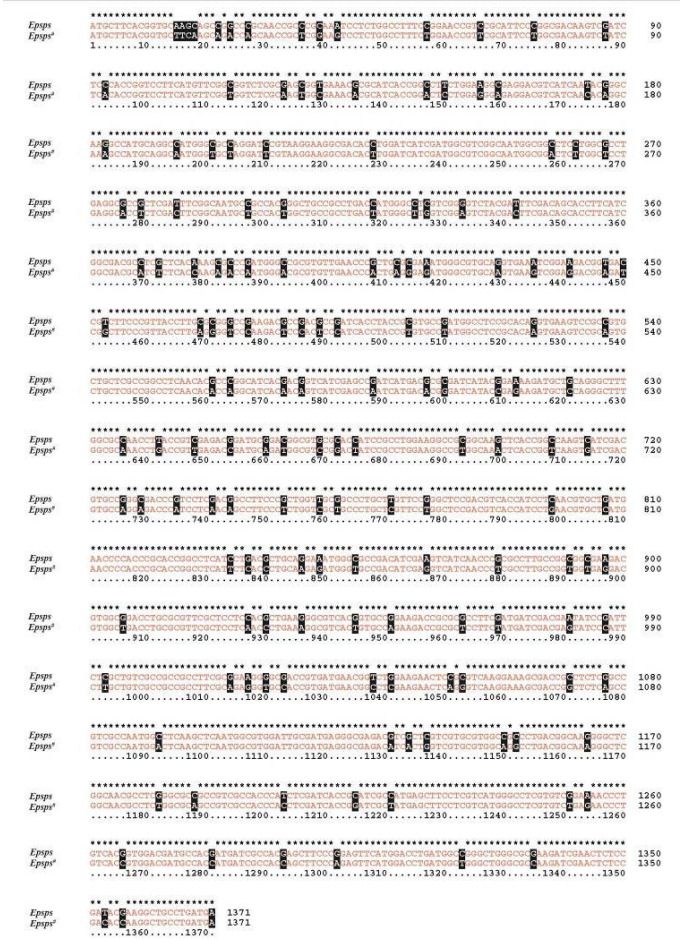

Figure 2. The DNA sequence comparison between the wild-type *Epsps* and optimized *Epsps*. The blank section indicated the modified nucleotides.

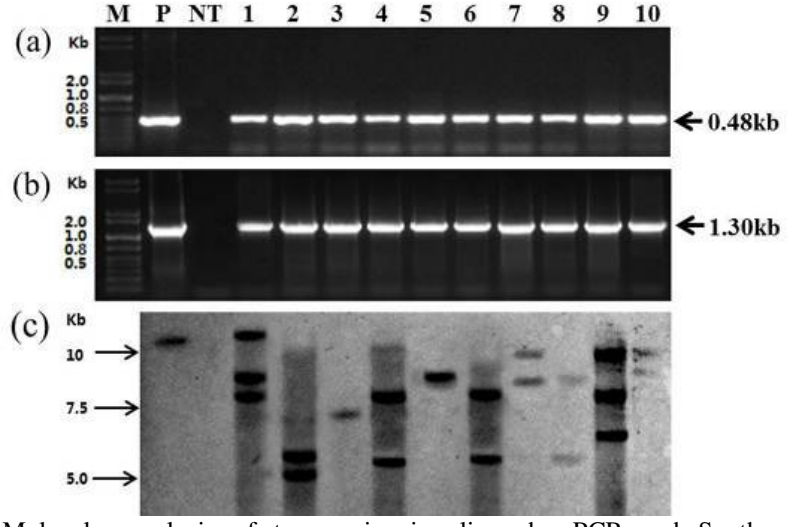

Figure 3. Molecular analysis of transgenic rice lines by PCR and Southern blot. PCR amplification of: **(a)** A 0.48 kb fragment of *Bar* gene; **(b)** A 1.30 kb fragment of *Epsps* gene in T_1 generation; **(c)** Southern blot analysis of *Epsps* gene in T_1 generation. Abbreviations: M: DNA molecular marker; P: Plasmid; NT: Non-transgenic control; Lane 1-10: Transgenic rice lines.

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glyphosate. The transgenic rice plants of T_2 generation at seedling stage grew normally after spraying 0.375 g glufosinate m^{-2}, but some of them died when sprayed with 0.750 g glufosinate m^{-2} (Figure 6-a); and they all survived after spraying 0.833 g glyphosate m^{-2} and still grew well after spraying with 3.332 g glyphosate m^{-2} (Figure 6-b). The results also showed that the transgenic rice plants could withstand 0.375 g glufosinate m^{-2} and 0.833 g glyphosate m^{-2} simultaneously (Figure 6-c).

Pollen Fertility and Agronomic Trait Analysis on T_2 Generation

The fertility alteration of seven transgenic lines of T_2 generation was studied. The pollens were all sterile and were not stained by I_2-KI solution when the transgenic plants grew under natural high-temperature and long-day environment. The pollens from the same lines became fertile and were stained by I_2-KI solution when the transgenic plants were irrigated with cold-water at 21ºC, and their pollen fertility rate ranged from 57.2 to 82.1% (Table 1). The main agronomic trait analysis showed that there were no significant differences between the transgenic PGMS line of T_2 generation and the non-transgenic control (Table 2). It was reported that the insertion of foreign genes impacted the pollen fertility of plants (Yasuor *et al*., 2006). But, in this study, the fertility alteration and main agronomic traits of the transgenic PGMS line had not been influenced by the insertion of foreign genes.

Table 1. Pollen fertility of transgenic rice lines of T_2 generation.

Line		Pollen fertility rate under natural environment (%)	Pollen fertility rate after cold water irrigation at 21ºC (%)[a]
Non-transgenic control		0	72.26±2.63
Transgenic rice lines	1	0	82.13±1.81
	2	0	69.61±8.07
	3	0	67.19±4.15
	4	0	71.24±5.96
	5	0	70.90±3.42
	7	0	57.20±5.32
	8	0	76.90±6.05

[a] There is no statistical difference between non-transgenic control and transgenic lines (P= 0.699).

Table 2. *T*-test of agronomic traits between transgenic rice lines of T_2 generation and non-transgenic control.

	Line	Plant height (cm)	Panicle length (cm)	Effective panicles number	Seed setting (%)	1,000-grain weight (g)	Grain yield per plant (g)
Lines after cold water irrigation	TL[a]	60.23±2.02	21.63±1.23	9.7±1.1	24.22±7.55	22.39±0.59	5.15±1.35
	NT[b]	62.21±4.15	20.89±1.50	10.4±3.5	18.94±7.45	22.64±0.68	4.46±1.03
	P	0.097	0.163	0.134	0.113	0.296	0.224
Lines under natural environment	TL	59.81±2.29	20.32±1.80	9.2±1.6	-	-	-
	NT	61.17±3.39	20.70±2.54	8.2±1.9	-	-	-
	P	0.167	0.592	0.157	-	-	-

[a]Transgenic rice lines, [b] Non-transgenic control.

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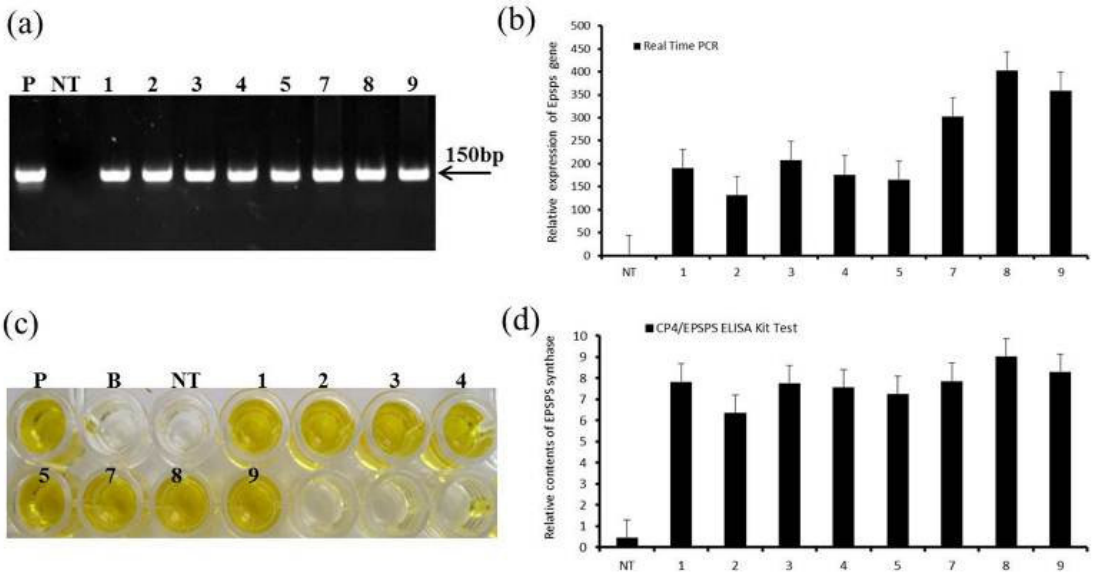

Figure 4. Expression analysis of *Epsps* gene in transgenic rice lines of T_1 generation. **(a)** RT-PCR; **(b)** Real-time PCR analysis of *Epsps* gene in T_1 generation; **(c)** EPSPS protein assay by ELISA kit in T_1 generation, and **(d)** relative expression level analysis of EPSPS protein detected by CP4/EPSPS ELISA kit in T_1 generation. Abbreviations: NT: Non-transgenic control; B: Blank control; P: Plasmid; 1-9: Transgenic rice lines.

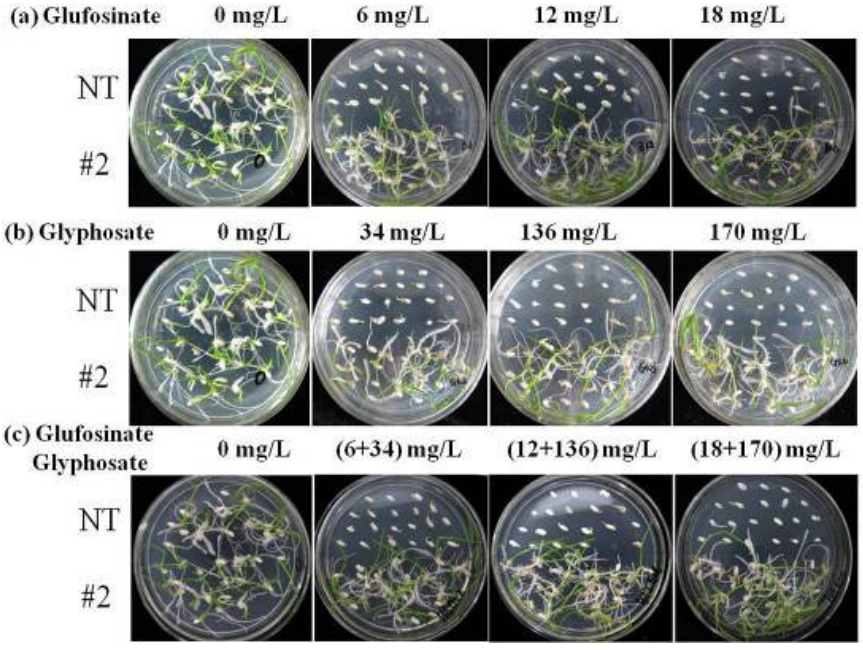

Figure 5. Glyphosate and glufosinate resistance analysis of transgenic rice seeds of T_2 generation. The seeds of transgenic line No. 2 and non-transgenic control were germinated on 1/2MS medium containing **(a)** 0, 6, 12 and 18 mg glufosinate L^{-1}, or **(b)** 0, 34, 136, and 170 mg glyphosate L^{-1}, or **(c)** glufosinate and glyphosate simultaneously. Abbreviations: NT: Non-transgenic rice seeds; #2: Seeds of transgenic line No.2.

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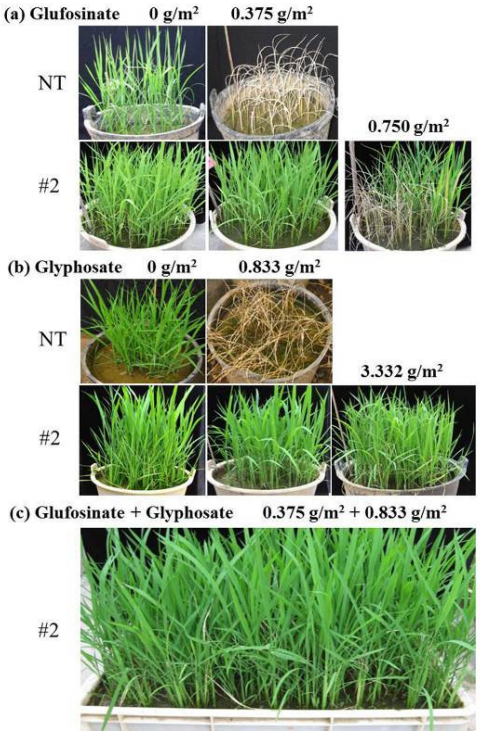

Figure 6. Glyphosate and glufosinate resistance analysis of transgenic rice plants of T_2 generation. The plants of transgenic line No. 2 and non-transgenic control were sprayed with **(a)** 0, 0.375 and 0.750 g glufosinate m^{-2}, or **(b)** 0, 0.833 and 3.332 g glyphosate m^{-2}, or **(c)** 0.375 g glufosinate m^{-2} and 0.833 g glyphosate m^{-2} simultaneously. Abbreviations: NT: Non-transgenic plants; #2: Plants of transgenic line No.2.

DISCUSSION

Optimizing codon usage and connecting appropriate location signal sequence to the exogenous gene are important strategies to increase the stability and cumulative amount of exogenous protein in receptors (Deng *et al*., 2003; Peng *et al*., 2006; Kim *et al*., 2009). The *Epsps* gene from bacteria is only expressed and accumulated in cytoplasm due to no chloroplast transit peptide sequence which can lead mature EPSPS protein to locate in chloroplast in plant cells (Della *et a1*., 1986; Smart and Amrhein, 1987). Barry *et al*. (1997) had optimized *Cp4-Epsps* gene according to the plant preferred codons and added *Arabidopsis* chloroplast transit peptide sequence *CTP4* to the 5' end of the gene. The obtained commercialized transgenic soybean could resist 0.899 g glyphosate m^{-2}. In the present study, the *Cp4-Epsps* gene was optimized according to the rice preferred codons and the tobacco chloroplast transit peptide sequence *TSP* was also added to its 5'end. The results showed that the transgenic rice with optimized *Epsps* gene could resist 3.332 g glyphosate m^{-2}, which was 2.7 times higher than that reported by Monsanto Company (Barry *et al*., 1997) and could well satisfy the demands for rice production.

The procedure of conventional hybrid seed production is complex; for example, while the sterile line and restorer line should be planted in one field, they should be sown, transplanted, and harvested separately. Furthermore, the hybrid seed could be easily contaminated by the seeds of restorer line (Kim *et al*., 2007). If the herbicide-resistant sterile line and herbicide-sensitive restorer line are used to produce hybrid seed, the restorer line can be killed by herbicide after pollination, but the male sterile line remains alive. Therefore, the herbicide-resistant sterile lines and herbicide-sensitive restorer lines can be mixed and directly sown in order to save the cost of separate sowing and transplanting. This will also improve the outcrossing due to uniform distribution of restorer lines in male sterile lines, and the hybrid seeds on male sterile lines can be harvested by combine harvester regardless of contamination of the restorer line (Xiao, 1997; Kim *et al*., 2007). Fu *et al*. (2001) transformed the PGMS line Pei'ai 64S with the *Bar* gene and preliminarily studied the mechanized production of hybrid seeds. In the present study, the herbicide-resistant gene *Bar* and *Epsps* were jointly transformed into the PGMS line 7001S, and then the transformed 7001S (named as EB7001S) and its hybrid combinations could resist both glufosinate and glyphosate. On the one hand, using the herbicide-resistant PGMS line and its hybrids is convenient for weeding in parent reproduction and hybrid rice production, since it reduces labor intensity and cost of weeding and decreases environmental pollution by disusing slowly degrading herbicides. On the other hand, it is convenient for mixed sowing

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of the herbicide-resistant sterile line with herbicide-sensitive restorer line in hybrid seed production by allowing mechanical harvest of hybrid seeds after spraying herbicide to kill herbicide-sensitive restorer lines after pollination, thereby decreasing the cost of hybrid seed production and increasing the outcrossing of female plants.

The mixtures of multiple selective herbicides have always been used to control various weeds in rice field. Overuse of herbicides year by year not only results in the environmental pollution but also has caused some weeds resistance to herbicides. For example, the common barnyard grass in rice field induced remarkable resistance to propanil, molinate, thiobencarb, and so on (Fischer *et al*., 2000). Since almost all plants do not have natural degradation or resistance mechanism to non-selective herbicides, it is very difficult to induce resistance. Up to now, the non-selective herbicides glyphosate and glufosinate have been used for more than 30 years, and it is reported that only a few of weeds have resistance to glyphosate (Nandula *et al*., 2005), but there is no report that the weeds are resistant to glufosinate. Therefore, it is significant to decrease the total amount of herbicides and the risk of weed resistance by developing new non-selective herbicide resistant transgenic crops. Furthermore, the transgenic crops resistant to both kinds of non-selective herbicides will further decrease the risk of weed resistance by alternate use of non-selective herbicides in the same fields. The GM corn resistant to two kinds of herbicides (glyphosate and glufosinate) has been commercially planted in USA (James, 2010). To the best of our knowledge, this study is the first report on developing the transgenic rice that is resistant to two kinds of herbicides.

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بهینه سازی ژن *Epsps* و تولید برنج تراریخته PGMS (نرعقیمی ژنی حساس به فتوپریود) مقاوم به دوعلف کش

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چکیده

کار برد متناوب علف کش های غیر انتخابی در سال های مختلف به منظور جلوگیری از در معرض قرار گرفتن مداوم گیاهان کاشته شده به فشار گزینشی علف کش، امکان مقاوم شدن علف های هرز به علف کش را کاهش می دهد. در این زمینه، استفاده از برنج نرعقیم شده ژنی حساس به فتوپریود (PGMS) به علف کش مقاوم باشد، ماشینی کردن تولید بذر هیبرید و کنترل علفهای هرز را آسان میکند. به منظور تولید مقاوم به دو علف کش، با استفاده از تراریختگی با اگروباکتریوم، ژن بهینه سازی PGMS برنج تراریخته وارد شدند. توالی رمز گذاری 7001S (PGMS *japonica*) به بذر برنج *Bar* همراه با ژن *Epsps* شده *Epsps* بر اساس کاربرد رمزه ای ژن های برنج بهینه سازی شد و عملکرد ژن بهینه سازی شده *Epsps* ژن در پیوند با آنزیم ، و سنجش مقاومت EPSPS با تحلیل پی.سی.آر در زمان واقعی ، ایمنی سنجی از پروتئین به علف کش درتراریخته های مستقل حاصله اعتبار سنجی شد. بیشترین سطح نسبی بیان(تظاهر) پروتئین در مرحله جوانه زنی حد اقل T2٪ کل پروتئین محلول رسید. تراریخته های نسل 9.02 در برگ به ESPSP) در متر مربع را تحمل می کردند که این مقدار ۲/۷ برابر بیشتر از glyphosate۳/۳۳۲ گرم گلای فوسیت (و ژن *Epsps* آنچه بود که شرکت مونسانتو گزارش کرده است. تراریخته های دارای ژن بهینه سازی شده) هم در مرحله جوانه زنی مقاوم بودند. glufosinate به کاربرد همزمان گلای فوسیت و گلوفوسینات (Bar در این مورد، دز مقاومت معادل۰/۳۷۵ گرم گلوفوسینات در متر مربع و ۰/۸۳۳ گرم گلای فوسیت در متر مربع بود.

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