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Overexpression of a specific OsGSTL2 isoenzyme improves glyphosate and chlorsulfuron tolerance of transgenic rice plants

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Plant glutathione S-transferases (GSTs) have been a focus of attention because of their role in herbicide detoxification. *OsGSTL2* is a glutathione S-transferase, lambda class gene from rice. Transgenic rice plants overexpressing OsGSTL2 were generated from rice calli by the use of an *Agrobacterium* transformation system. The transgenic rice plants were screened by a combination of hygromycin resistance, polymerase chain reaction (PCR) and Southern blot analysis. Transgenic rice plants overexpressing *OsGSTL2* gene showed higher levels of *OsGSTL2* transcripts in the absence of any treatment compared to non-transformed rice plants. In the vegetative tissues of transgenic rice plants, the overexpression of the *OsGSTL2* increased levels of GST and glutathione peroxidase (GPX) activities, and reduced content of superoxide. Transgenic rice plants also had higher tolerance to glyphosate and chlorsulfuron, which often contaminates agricultural fields. These findings demonstrate the detoxification role of OsGSTL2 in the growth and development of rice plants. It should be possible to apply the present results to crop plants for developing herbicides tolerance and in limiting herbicides availability in the food chain.

Key words: Glutathione S-transferase, transgenic rice, overexpression, herbicide resistance.

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INTRODUCTION

Plants grow in a dynamic environment. Abiotic stresses such as water deficit, high temperature, salinity, cold, heavy metals, mechanical wounding, and exogenous chemicals often impose constraints on plant growth and development (Hasegawa et al., 2000; Sreenivasulu et al., 2007; Vij and Tyagi, 2007). Among the stress conditions of plants, the exogenous chemicals are a major limit on plant productivity worldwide. Plants have developed a sophisticated and highly complex network of interacting regulatory mechanisms to defend themselves against harmful chemical compounds during evolution. Plants actively detoxify endogenous and exogenous toxins using a three-phase detoxification system that involves cytochrome P450s, Glutathione *S*-transferases (GSTs, E.C.2.5.1.18) and ATP-binding cassette transporters (Hu et al., 2011).

GSTs are an ancient, ubiquitus and multifunctional protein family encoded by a large gene family found in bacteria, fungi, animals and plants (Frova, 2006). Based on the predicted amino acid sequences, GSTs in plants are divided into nine classes: phi, tau, theta, zeta, lambda, glutathione-dependent dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase (TCHQD), elongation factor 1 gamma

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(EF1G) and Microsomal-class GSTs (Basantani and Srivastava 2007; Jain et al., 2010; Hu et al., 2011).

Among these classes, Phi, Tau, Lambda and DHAR classes are plant specific (Edwards and Dixon, 2005). Plant GSTs have been a focus of attention because of their role in herbicide detoxification, which have been actively investigated during last decades (Chi et al., 2011). Intensive studies of plant GSTs are required because of the considerable agronomic potential of these enzymes with regard to herbicide selectivity, tolerance and environmental safety (Edwards and Dixon, 2005). So far, large numbers of GST genes have been identified or annotated from at least 17 plant species (Basantani and Srivastava, 2007; Conn et al., 2008; Chronopoulou and Labrou, 2009). The systematic analysis revealed at least 53 GST genes in *Arabdopsis*, 59 GST genes in rice, and 81 GST genes in *Populus trichocarpa* (Dixon et al., 2002a; Soranzo et al., 2004; Chi et al., 2011).

Some researchers suggested that GSTs are present at every stage of plant development from early embryogenesis to senescence and in all cellular organisms (Sari-Gorla et al., 1993; McGonigle et al., 2000; Soranzo et al., 2004). GSTs have been found to be differentially regulated by a wide variety of stimuli, including plant hormones, abiotic and biotic stresses and exhibit extensive functional diversification (Jain et al., 2010; Chi et al., 2011). GSTs contain a complex array of enzymes associated with the metabolic response of plant tissues to many environmental stresses (Akbulut and Cakir, 2010; Banerjee and Goswami, 2010; Gajewska and Skłodowska, 2010). Glutathione (GSH) is the tripeptide γ-glutamyl-cysteinyl-glycine and plays a central role in the processes of detoxification and redox buffering (Noctor and Foyer, 1998). The most essential biological functions of GSTs are involved with mediating the conjugation of toxic xenobiotics and electrophillic hydrophobic substrates with GSH and providing protection against oxidative burst spreading throughout plant tissues (Edwards et al., 2000; Dixon et al., 2002a; Axarli et al., 2009; Akbulut and Cakir, 2010; Gill and Tuteja, 2010). Catalytic functions of GSTs enzymes are connected with the phase II-mediated detoxification of a wide spectrum of xenobiotics and endogenous toxic substances. GSTs are also associated with limiting the level of oxidative stress by scavenging of reactive oxygen species (ROS) in plant tissues and play the crucial role in maintaining the redox homeostasis (Karavangeli et al., 2005; Halliwell, 2006; Mylona et al., 2007; Axarli yet al., 2009; Dalton et al., 2009; Sappl et al., 2009). Furthermore, plant GSTs are involved in non-catalytic binding of flavonoids, signal transduction, and participate in intermediary metabolism (Chronopoulou and Labrou, 2009).

The complete identification in a genome-wide level revealed the presence of at least 79 GST genes in the rice genome (Soranzo et al., 2004). Sequence analysis and the organization of putative motifs indicated the potential diverse functions of GST gene family members in rice. Microarray data analysis revealed tissue-/organ- and developmental stage-specific expression patterns of some rice GST genes. At least 31 GST genes showed response to plant hormones auxin and cytokinin, 20 to abiotic stress, 32 to arsenate stress, and 48 to biotic stress. Many of the GST genes in rice were commonly controlled by developmental processes, hormones, abiotic and biotic stresses (Jain et al., 2010).

The GSTLs are one of the smaller groupings within the GST superfamily in plants, but the several behaviors of GSTLs make them important targets for functional characterization. In particular, specific GSTLs are strongly upregulated in response to exposure to xenobiotic compounds including herbicides, herbicide safeners and pharmaceuticals (Hershey and Stoner, 1991; Dixon et al., 2002b; Theodoulou et al., 2003). As with other GST classes, this correlation provides circumstantial evidence for a role in stress tolerance (Dixon et al., 2011). The isoenzyme OsGSTL2 occurring in rice tissues belongs to the class lambda within the large and diverse family of GSTs. The molecular characterization and heterologous expression of this isoenzyme have been studied. OsGSTL2 gene showed response to chlorsulfuron, a selective herbicide that controls select broadleaf weeds and undesirable grasses. The OsGSTL2 protein has a specific activity of GST (Hu et al., 2011). The purpose of this study was to overexpress OsGSTL2 in rice and investigate the role of OsGSTL2 in protecting plants from the injury caused by herbicides.

MATERIALS AND METHODS

Construction of transgene vector and plant transformation

To construct OsGSTL2 overexpression vector, the *OsGSTL2* gene that had been cloned into pENTR/D-TOPO vector was cloned into the pHOS vector by LR reaction (Figure 1). The construct was confirmed by restriction digest analysis and then by sequencing. After transferred to *Agrobacterium tumefaciens* strain AGL0 by the freeze-thaw method (Höfgen and Willmitzer, 1988), the construct was used to transform *Oryza sativa cv*. Zhonghua 11 calli by the Agrobacterium-mediated co-cultivation method (Hu, 2008). Transgenic plants that rooted on hygromycin were transferred to vermiculite-mixed soil (rich soil: vermiculite=1:3, V/V) in small pots and grown at 28℃ under a 16/8 h light/dark photoperiod at an intensity of approximately 250 μE m^{-2} s^{-1}.

Confirmation of positive transgenic rice plants

The positive transgenic plants were selected by PCR and Southern blot analysis. The genomic DNA of transgenic rice plants and nontransformed rice plants was extracted using Plant Genomic DNA Extraction kit (VOK-Bio, Beijing, China) and was used as templates. Because *HPT* is closely adjacent to the target gene in the transformation constructs and not present in the nontransformed rice genome, it can been used to indicate the presence of the transgene. The primers hyg f (5'-ATGAAAAAGCCTGAACTCACC-3') and hyg r (5'-CCGGTCGGCATCTACTCT-3') were designed according to *HPT* gene sequence. Amplification was carried out by initial denaturation

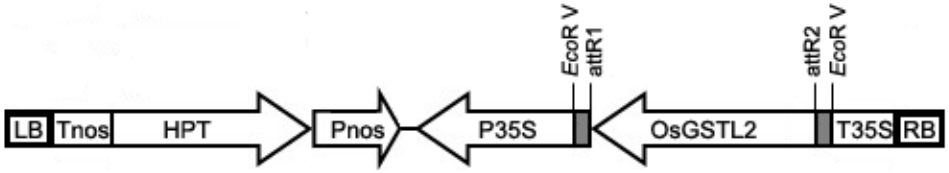

Figure 1. Structure of the OsGSTL2 overexpression vector used for rice transformation. LB, Left border; Tnos, nopaline synthase terminator; HPT, hygromycin phosphotransferase; Pnos, nopaline synthase promoter; P35S, CaMV 35S promoter; OsGSTL2, coding region of OsGSTL2; T35S, CaMV 35S terminator, *RB* right border.

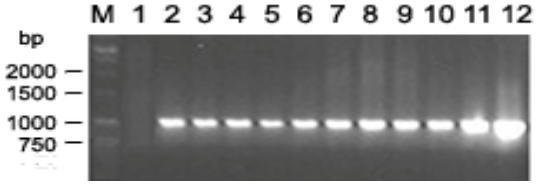

Figure 2. PCR analysis of transgenic rice plants. *Lane M,* DNA ladder; Lane 1, negative control nontransformed plants; Lane 2-11, independent transgenic lines; Lane 12. positive control OsGSTL2 overexpression vector.

at 94°C for 2 min followed by 35 cycles of 94°C denaturation for 1 min, 58°C annealing for 1 min, and 72°C elongation for 1 min. About 15 µg of genomic DNA of transgenic rice plants and nontransformed rice plant were digested by *Eco*RI, respectively. The digested genomic DNA was separated on a 0.8% agarose gel electrophoresis and was transferred to a Hybond nylon membrane (Amersham, Piscataway, USA). The DNA was hybridized with a labeled DNA probe, which was generated from *HPT* gene located within the T-DNA borders of pHOS vector and radiolabeled with [α-^{32}P]dCTP using the random primer system. Standard procedures of Southern blot analysis were performed (Sambrook et al., 1989).

Expression analysis of OsGSTL2 gene

Real-time PCR for analysis of *OsGSTL2* expression was performed using total RNA from rice plant tissues. Total RNA samples were isolated using the Trizol reagent (Gibco-BRL, USA) and subsequently treated with DNase I. The RNAs were reverse transcribed using the Superscript™ Ⅲ RNase H-Reverse Transcriptase kit (Invitrogen, USA). Real-time PCR DNA amplification and analysis was carried out as described by He et al. (2012). All reactions were performed with primers OsGSTL2 qf (5'-CGTTCAACAAAGCATCGTAC-3') and OsGST qr (5'-GCAAAAACTGTGGGTCCTGT-3') using the SsoFast™ EvaGreen® Supermix. The primers OsEF1α f (5'-AGGGATGGGTCAAAAGGATGC-3') and OsEF1αr (5'-GAGACAACACCGCCTGAATAGC-3') were designed and the expression of *OsGSTL2* gene was normalized to *OsEF1α* gene of rice.

Activity assay of enzyme and detection of superoxide

OsGSTL2 over-expression transgenic seedlings and non-transformed rice seedlings cultured for 15 days were used to assay GST activity and glutathione peroxidase (GPX) activity. Crude protein extracts were prepared from the rice plants. Protein concentration was determined using the Bradford method (Bradford, 1976). GST activity was measured spectrophotometrically (Habig et al., 1974; Takesawa et al., 2002). One unit of activity was defined as the amount of the enzyme that catalyzes the conversion of 1 µM 2, 4-dinitrochlorobenzene (CDNB) per minute at 25°C.

Using the GSH-PX Kit (NJBI, P. R. China) and following the manufacturer's instructions, GPX activity was determined with H_2O_2 as substrate, One unit of activity was defined as the amount of the enzyme that catalyzes the consumption of 1 µM H_2O_2 per minute. Transgenic seedlings and non-transformed rice seedlings cultured for a month were used to detect superoxide. Superoxide levels were visually detected with nitro blue tetrazolium (NBT) described previously (Yang et al., 2004).

Assay for herbicide tolerance of transgenic rice plants

To measure responses to herbicide, transgenic seedlings and non-transformed rice seedlings cultured for 12 days were treated with 100 µmol/L glyphosate and 0.02% chlorsulfuron for 24 h, respectively. The seedlings subsequently were transferred into vermiculite-mixed soil and grown as described previously.

RESULTS

Generation and confirmation of transgenic rice plants

We generated transgenic rice plants to test OsGSTL2 physiological function in rice. The plant expression vector was introduced to *Agrobacterium tumefaciens* AGL0, and transformed into *Oryza sativa cv*. Zhonghua 11 plants. Eleven transgenic lines were obtained. The transformants were verified by PCR analysis. The 1042 bp *HPT* gene fragments were detected in the selected independent lines and positive control OsGSTL2 overexpression vector, whereas were not found in non-transformed plants (Figure 2). Southern blot analysis was used to further confirm the presence of transgene and copy number of insertion. The genomic DNA of transgene rice lines and non-transformed rice plants were hybridized with the probe of radiolabeled *HPT* gene fragment. Because *HPT* is closely adjacent to the target gene *OsGSTL2* in the transformation constructs and not present in the wild-type rice genome, it should indicate the presence and copy number of the transgene. The result showed that the *HPT* hybridizing fragments were detected in all eleven of the independent transgenic lines

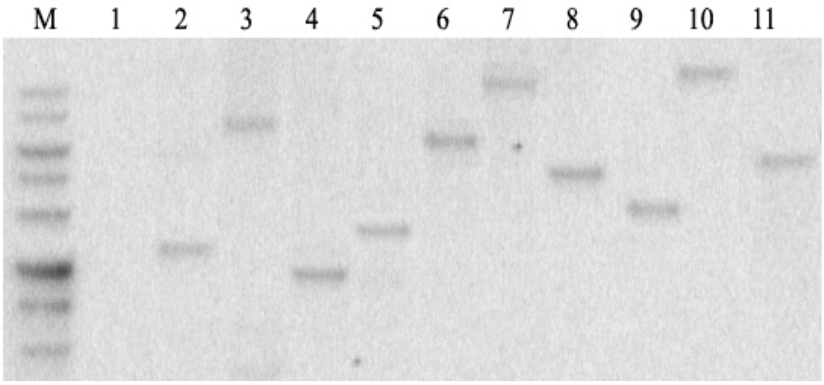

Figure 3. Southern blot analysis of transgenic rice. Lane M, DNA ladder; Lane 1, negative control nontransformed plants; Lane 2-11, independent transgenic lines.

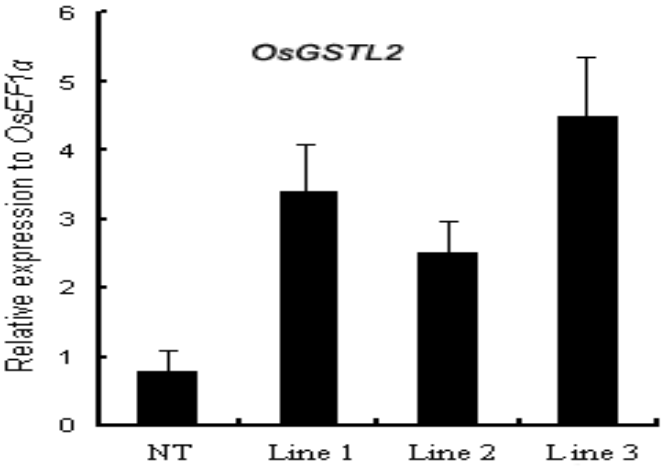

Figure 4. Real time RT-PCR analysis expression of OsGSTL2 in transgenic rice plant. NT, Non-transformed plants; Line 1-3, independent transgenic lines.

but were not found in non-transformed rice (Figure 3). Independent transgenic lines were selected for further analysis.

Expression of *OsGSTL2* in transgenic rice plants

To investigate the *in vivo* role of OsGSTL2 in rice plant, *OsGSTL2* expression was modulated by overexpressing OsGSTL2 in transgenic rice plants. The independent transgenic lines were selected based on PCR analysis. Real time RT-PCR revealed that expression level of *OsGSTL2* was higher in the transformants than in the non-transformed plant. The *OsGSTL2* transcripts in transgenic line 1, 2 and 3 were 4.29, 3.16 and 5.65 time of the non-transformed plants (Figure 4), respectively. The findings suggested *OsGSTL2* gene in transgenic ricer plants was overexpressed.

Transgenic rice has higher activities of GST and GPX

GST activity of crude extracts from the transgenic seedlings and non-transformed rice seedlings was measured with CDNB as the substrate. The transformants contained higher levels of GST activities than the non-transformed rice. The GST activity of line 1, 2 and 3 were 2.07, 1.79 and 2.40 time of the non-transformed plants, respectively (Figure 5a). GPX activities in the transgenic lines of OsGSTL2 were measured with H_2O_2 as the substrate. The GPX activity in transgenic lines also was higher than the non-transformed rice. The GPX activity of line 1, 2 and 3 were 1.61, 1.50 and 1.71 time of the non-transformed plants, respectively (Figure 5b). GPX activities were elevated in the transgenic rice, which could degrade superoxide levels.

Transgenic rice has lower superoxid*e*

Superoxide detection was performed during the vegetative growth phase of plants. The leaves of rice seedlings lines were stained with NBT to detect superoxide visually. The leaves of OsGSTL2 transgenic rice seedlings contain lower superoxide than those of non-transformed rice plants (Figure 6).

Transgenic rice has higher tolerance to herbicide

OsGSTL2 transgenic rice plants grew normally, which phenotypes were indistinguishable with Non-transformed rice plants. To examine the role of OsGSTL2 in plants, 12-day-old seedlings of OsGSTL2 transgenic rice plants and non-transformed rice plants were incubated with 100 µmol/L glyphosate and 0.02% chlorsulfuron for 24 h, respectively. After glyphosate treatment, the seedlings were also cultivated for 10 days; all the treated plants

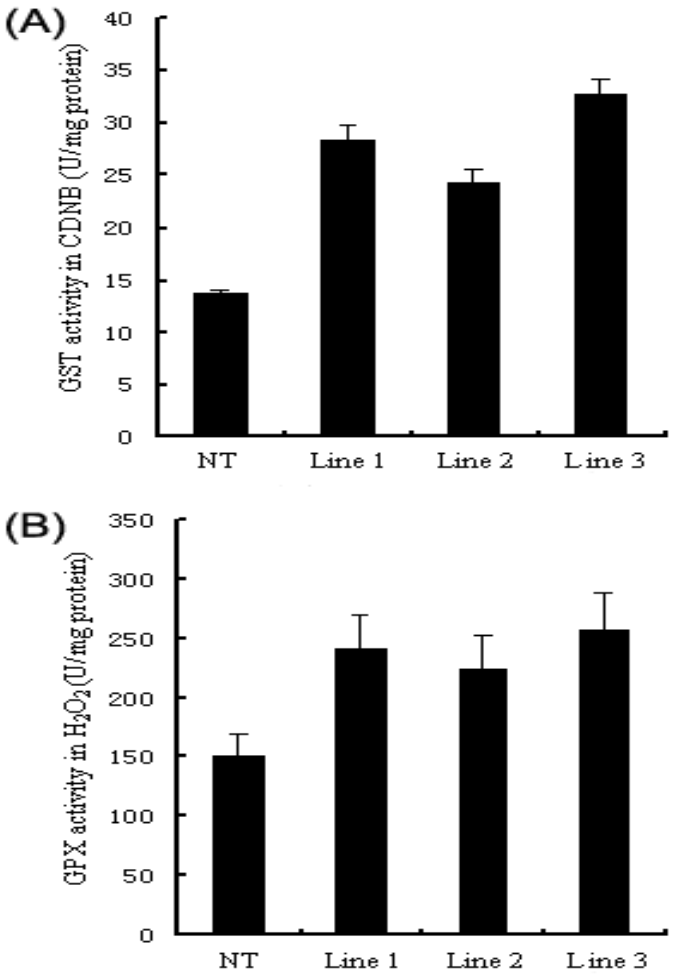

Figure 5. Activities of GST and GPX in transgenic rice plant. (A) GST activity; (B) GPX activity. NT, non-transformed plants; Line 1-3, independent transgenic lines.

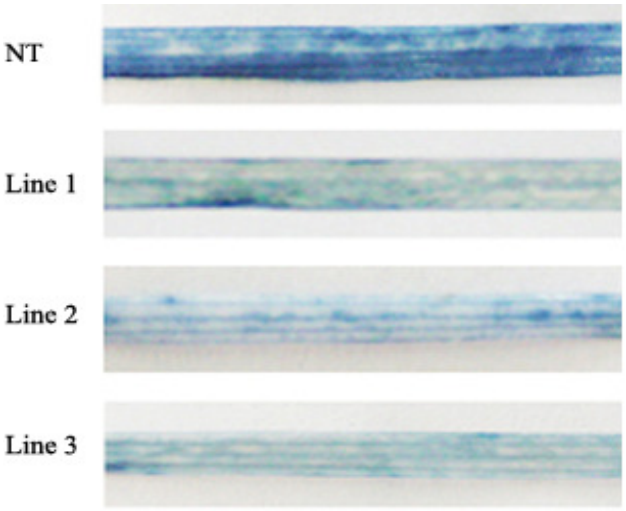

Figure 6. Detection of superoxide level by NBT staining in rice leaves. NT, Non-transformed plants; Line 1-3, independent transgenic lines.

grew slowly, but the non-transformed plants grew more slowly than the transgenic plants (Figure 7a). After chlorsulfuron treatment, the seedlings were also cultivated for 10 days, all the treated plants abnormality, grew slowly and yellow. However, the phenotypic difference of transgenic rice seedlings and non-transformed seedlings was obvious. The non-transformed rice plants showed more severe yellow and grew more slowly compared with the transgenic rice plants (Figure 7b). These results suggested that overexpression of *OsGSTL2* isoenzyme improves glyphosate and chlorsulfuron tolerance of transgenic rice plants.

DISCUSSION

Although plant GSTs have been discovered for more than 30 years, many GST genes have been isolated or annotated, only a small number of them have been functionally characterized (Chi et al., 2011; Dixon et al., 2002a). Thus, limited data are available on expression analysis and their functional divergence of the GST family. We know little about its members that are involved in biotic and abiotic stress-related biological processes. Some reports showed that plant GSTs might play important roles in herbicide resistance and detoxification (Coleman et al., 1997; Edwards et al., 2000).

It has been shown that tandem duplication represented the major mechanism for the subfamily expansion. There are three tandemly arranged segments on chromosome 3 of the rice genome, which showed high sequence similarity each other. They belong to lambda class GST. It has been postulated that these genes came from the same ancient gene, which may have evolved early in plant development by a gene duplication event. OsGSTL2 is located upstream of OsGSTL1 and downstream of OsGSTL3 (Hu et al., 2011). *OsGSTL2* transcripts were detected in the roots and leaves of seedling stage and tillering stage, and the roots, leaves and panicles of heading stage from rice plants, and the expression of *OsGSTL2* in rice roots is upregulated in response to exposure to chlorsulfuron (Hu et al., 2011).

GSTs are known to protect plants against oxidative stress induced by biotic and abiotic agents. Some GSTs also have secondary activities as glutathione peroxidase and can protect the cells/organisms from oxidative damage. GSTs can eliminate membrane lipid peroxides as well as products of oxidative DNA degradation by conjugating them with GSH (Berhane et al., 1994). Earlier workers have shown that transgenic plants which overexpressed plant GST/peroxidase showed enhanced tolerance to different abiotic stresses (Roxas et al., 1997, 2000; Dixon et al., 2003; George et al., 2010; Jha et al., 2011; Ji et al., 2010; Qi et al., 2010; Dixit et al., 2011; Zhang and Liu, 2011). Overexpression of some GSTs in plants improved herbicide tolerance. For example, transgenic wheat plants expressing maize GST-27 gene

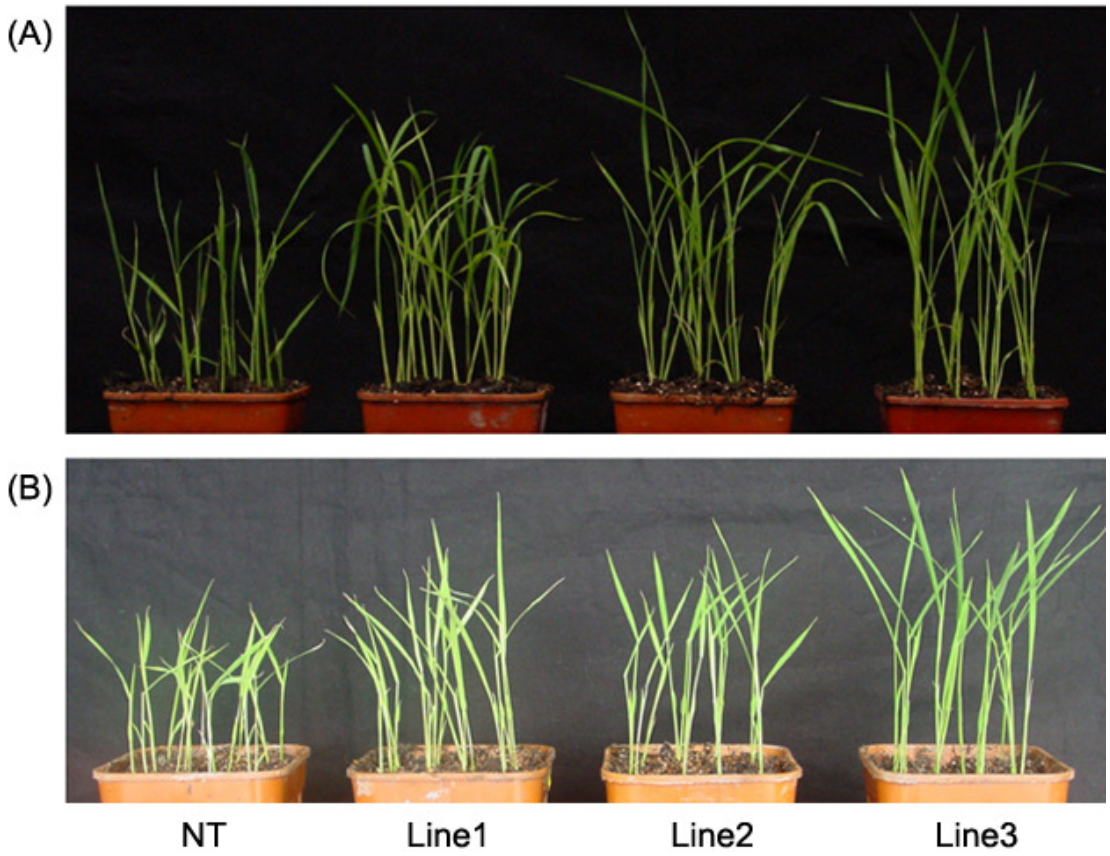

Figure 7. The effect of glyphosate and chlorsulfuron treatment in transgenic lines. (A) After 100 μmol/L glyphosate treatment for 24 h and then the seedling was cultivated for 10 days; (B) After 0.02% chlorsulfuron treatment for 24 h and then the seedling was cultivated for 10 day. NT, Non-transformed plants; Line 1-3, Independent transgenic lines.

were resistant to chloroacetanilide herbicide alachlor and dimethenamid and the thiocarbamate herbicide S-ethyldipropylthio-carbamate (Milligan et al., 2001). Transgenic tobacco plants expressing cotton Gst-cr1 gene had much higher levels of GST and GPX activities and showed an enhanced resistance to oxidative stress induced by a low concentration of methyl viologen (Yu et al., 2003).

Transgenic tobacco plants expressing maize glutathione *S*-transferase I showed substantially higher tolerance to alachlor compared to non-transgenic plants (Karavangeli et al., 2005). Overexpression of a specific soybean GmGSTU4 isoenzyme improves diphenyl ether and chloroacetanilide herbicide tolerance of transgenic tobacco plants (Benekos et al., 2010). In this present study, Transgenic rice plants overexpressing OsGSTL2 gene showed higher levels of OsGSTL2 in the absence of any treatment, increased levels of GST and GPX activities, and showed lower level of superoxide compared to wild type plants. Transgenic rice seedlings had higher tolerance to glyphosate and chlorsulfuron than non-transformed rice seedlings. In the changing scenario of increasing herbicides contamination in the environment due to altered agricultural practices and increasing anthropogenic activities, it is necessary to develop crop plants which can tolerate herbicides without accumulating them in edible parts. The transgenic plants detoxifying herbicide are potentially useful biotechnological tools for the development of phytoremediation system for the degradation of herbicide pollutants in agricultural fields. It should be possible to extend the present results to crop plants for developing herbicides tolerance and in limiting herbicides availability in the food chain.

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