Botanical Studies (2007) 48: 35-41.

Tissue and inducible expression of a rice glutathione transporter gene promoter in transgenic *Arabidopsis*

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(Received January 17, 2006; Accepted July 7, 2006)

ABSTRACT. The promoter of a rice glutathione transporter (*OsGT1*) was fused to the reporter gene *ß-glucuronidase* **(***GUS***)** and introduced into *Arabidopsis* to analyze promoter and expression patterns. Deletion of various length-promoter studies indicated that a 1246 bp fragment upstream of start codon (ATG) of *OsGT1* is sufficient to activate the expression of *GUS* gene. The GUS assays revealed that the *OsGT1* promoter is expressed in an organ and tissue-specific manner. GUS was being expressed in the root stele, the veins of cotyledons, the anthers and flower petals, but not in leaves or stems under normal growth conditions. *GUS* expression activated by the *OsGT1* promoter was strongly stimulated by NaCl and low temperature, moderately by abscisic acid and naphthaleneacetic acid, and not at all by salicylic acid and gibberellic acid. Expression was inhibited by cadmium but not by copper. The function of *OsGT1* in glutathione transport in normal or stress conditions is discussed.

Keywords: *Arabidopsis*; Flower; Glutathione transporter; Promoter; Rice; Root.

INTRODUCTION

Like the synthesis and cycling of glutathione (GSH), GSH transport systems also have important roles in sustaining the normal development of plants and protecting them from biotic and abiotic stresses (Foyer et al., 2001). GSH is not produced at equivalent rates by all tissues, or even by all cells within a tissue (May et al., 1998; Noctor et al., 2002). The trichomes on the stem and leaf surface of *Arabidopsis*, for example, show much higher expression of enzymes involved in the synthesis of cysteine and GSH; and have GSH contents 2-3 times higher than the surrounding basal and epidermal cells (Gutierrez-Alcala et al., 2000).

The GSSG (oxidized glutathione) and GS-conjugated transport systems on the plasma membrane associated with systemic transport have been previously reviewed (Foyer et al., 2001). The differential intercellular partitioning of the GSH metabolism has been observed in maize (Doulis et al., 1997), and in broad bean leaf tissues (Jamai et al., 1996). GSH is also a major means of transporting reduced sulfur over long distances, within both the xylem and the phloem (Rennenberg et al., 1979; Herschbach et al., 2000). Long distance GSH transport has also been implicated in different roles, including phloem-mediated shoot-to-root allocation of and demand-driven control of sulfate uptake and/or loading into the xylem stream by the roots in herbaceous plants (Lappartient and Touraine, 1997).

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Recently, two plant GSH transporters, *BjGT1* from *Brassica juncea* (Bogs et al., 2003) and *OsGT1* from *Oryza sativa* (Zhang et al., 2004), were shown to be able to promote transport of GSH, GSSG, and glutathione conjugates when they were used to complement yeast mutants with defects in GSH transport. However, the physiological functions and expression patterns of the rice glutathione transporter (*OsGT1*) (Zhang et al., 2004) were not studied. Here, *Arabidopsis* transgenic for the reporter gene *GUS* under the control of the *OsGT1* promoter was used to analyze the expression patterns of *OsGT1* at various developmental stages and after treatment with NaCl, heavy metals, abscisic acid (ABA), naphthalene acetic acid (NAA), salicylic acid (SA), gibberellin (GA), and low temperature.

MATERIALS AND METHODS

Construction of different deletion *OsGT1* promoter-*GUS* fusion

The promoter region of *OsGT1* was identified from the database of rice genome sequences: accession no. AP001168. The 2246 bp putative *OsGT1* promoter was amplified from genomic DNA of *Oryza sativa* cv., nipponbar and confirmed by sequencing. The sequence data from this article have been deposited at NCBI under accession number AY338469. To generate promoter deletion fragments of the putative *OsGT1* promoter, sets of primers were used to amplify different-length fragments of the promoter. The 2230-bp fragment P_{13} was from -2246 to -17 bp

before the start codon (ATG) of *OsGT1* cDNA (AF393848, A of the start codon was marked as +1), and was amplified using the forward primer HP_1 (5′-CCCAAGCTTATGGAACATGGGAAACAC-3′) and the reverse primer BP_{2213} (5′-CGCGGATCCAGCCTTCTCAGTTCTCAC-3′); the 989-bp fragment P_{56} (from position -1263 to -273 bp) was obtained using the forward primer HP_{985} (5′-CCCAAGCTTAAAATCTTTAGGAGCATA-3′) and the reverse primer BP_{1956} (5′-CGCGGATCCTCTAAATTACTGCCATCC-3′); the 1973-bp fragment P_{16} (from position -2246 to -273 bp) was obtained using HP_1 and BP_{1956} primers; and the 1246-bp fragment P_{53} (from position -1263 to -17 bp) was obtained using HP_{985} and BP_{2213} primers. The introduced *Hind* III site for HP_1 and HP_{985} primers and *BamH* I site for BP_{1956} and BP_{2213} primers were underlined. The four fragments of *OsGT1* promoter were cloned into the vector pBI 101 using the *Hind* III + *BamH* I sites (Clontech) to generate the promoter-GUS fusions (Figure 1A). The constructs were named P_{13}-GUS, P_{16}-GUS, P_{53}-GUS, and P_{56}-GUS, respectively, and they were checked by restriction digestion before transfer to *A. tumefaciens* (LBA4404) by the electroporation method.

Arabidopsis transformation

Transformation of *Arabidopsis thaliana* ecotype Columbia was carried out by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on the basis of hygromycin resistance. Four to ten *Arabidopsis* plants were transformed for each construct, and mature seeds were harvested. Hygromycin-resistant transformants were germinated on MS media supplemented with hygromycin and transplanted to pots. After a three-generation selection, the seedlings (T_3) were used for analyses.

Southern analysis

Total genomic DNA was isolated from young leaves of T_3 plants by the CTAB method (Murray and Thompson, 1980) for Southern blot. Southern analyses were performed following the standard protocol of Sambrook et al. (1989). Full-length of *OsGT1* promoter (P_{13}) was used as a probe for Southern blot.

GUS activity assays

GUS activity in transgenic plants harvested at different developmental stages or after different treatments was assessed as described by Jefferson (1987). Histochemical staining was carried out using X-Gluc as a substrate. For the fluorogenic assay, 4-methylumbelliferyl glucuronide (4MU) was used as a substrate (Jefferson, 1987). Four independent transgenic lines were tested for each construct. Protein content was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

Inducible treatments of transgenic *Arabidopsis*

T_3 seedlings of P_{53}-GUS were grown on sand in a 15 cm diameter pot, and watered with 1/4 MS liquid every 2 days at 22°C under a 16 h/d photoperiod. 14 day-old seedlings were used for treatments. One hundred milliliters of 100 μM abscisic acid (ABA), 100 μM naphthalene acetic acid (NAA), 100 μM salicylic acid (SA), 100 μM gibberellin (GA), 120 mM NaCl, 100 μM $CuSO_4$, 90 μM, and 250 μM $CdSO_4$ were added to the sand in each pot. 4°C treatments were conducted in the growth chamber in 1500 lux light. Twenty-four hours after the treatments, the roots were harvested, washed with distilled water, and deep-frozen before protein extraction. Fifteen plants from five pots were used for each treatment.

Transcripts of *OsGT1* in rice

Oryza sativa cv. IR64 was used to analyze the transcript of *OsGT1*. Leaves, roots and panicles (flowers) of IR64 at the heading stage under normal growth conditions in Guangzhou, China, were used to analyze the transcripts of *OsGT1*. For treatments, seeds of IR64 were germinated and grown in 1/2 MS liquid media for one week, before being treated with 100 μM SA, 100 μM ABA, 120 mM NaCl, 90 μM $CdSO_4$ and 4°C for 24 h under natural light. Total RNA was isolated with TaKaRa RNAiso Reagent (TaKaRa, China). DIG-labeled full-length anti-sense RNA probes of *OsGT1* were generated *in vitro* transcription from the cloned *OsGT1* cDNA in pGEM-T easy vector (Promega) by SP6 RNA polymerase following the protocols of the DIG Northern Starter Kit (Roche).

RESULTS AND DISCUSSION

Analysis of the *OsGT1* promoter

The 2246 bp putative promoter region of *OsGT1* was obtained by PCR, and it was analyzed for using *cis*-elements in the *cis*-acting regulatory DNA elements databases: http://www.dna.affrc.go.jp/sigscan/signal1.pl. (Higo et al., 1999) and http://oberon.rug.ac.be:8080/plantCARE/. Various putative *cis*-sequences were found in the 2.23-kb putative *OsGT1* promoter (see the note of NCBI GeneBank accession no. AY338469). Interestingly, the consensus sequences of nine pollen specific-expression *cis*-elements (POLLEN1LELAT52) and eighteen root specific-expression *cis*-elements (ROOTMOTIFTAPOX1) were found in the promoter (Figure 1A). Meanwhile, several other inducible *cis*-elements were also found, including light response elements, an enhancer-like element of anoxic specific inducibility (GC-motif), an ABA-responsive element (ABRE), an auxin-responsive element (AuxRR-core), a gibberellin-responsive element (P-box), an ethylene-responsive element (ERE), and a SA-responsive element (TCA-element). These *cis*-elements might contribute to the anther and root tissue-specific expressions and the inducible expressions of the transgenic plants on which we will be focused.

The *OsGT1* promoter confers tissue-specific expression in transgenic *Arabidopsis*

To investigate *OsGT1* promoter, four deleted promoter fragments were generated by PCR, and they (P_{13}, P_{16}, P_{53}

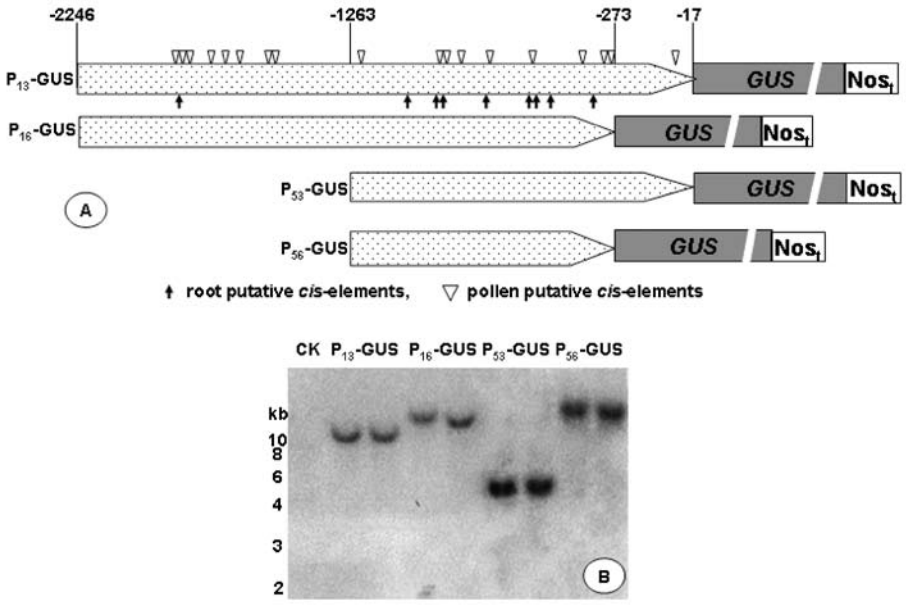

Figure 1. A, Schematic diagram of *OsGT1* promoter deletions fused to the *GUS* gene. Different length regions of the *OsGT1* promoter were amplified by PCR and fused to the *GUS* gene of the binary vector pBI 101. The negative numbers indicated the bp upstream from start codon of *OsGT1* (the A of start codon ATG of *OsGT1* was marked +1). B, Southern analysis of transgenic *Arabidopsis*. The genomic DNA of each construct was isolated from T_3 generation and digested with *Hind* III; the full-length of *OsGT1* promoter was used as a probe. No *Hind* III cut site is in the promoter. Un-transgenic *Arabidopsis* was as control (CK).

and P_{56}) were 2230, 1973, 1246 and 989 bp in length, respectively (Figure 1A). These fragments were inserted into the front of the *ß-glucuronidase* (*GUS*) gene in the vector pBI 101. *Arabidopsis* was transformed with these constructs. Four to ten transformed *Arabidopsis* lines were obtained for each construct. Southern analysis confirmed that all the constructs were integrated into the *Arabidopsis* genome (Figure 1B).

To understand the tissue and developmental expression of *GUS* activated by the *OsGT1* promoter, the transgenic *Arabidopsis* plants were grown on sterile 1/2 MS solid media at 22°C under 16 h light/day regimen. The seedlings were tested for GUS activity, using the histochemical stain method, at different developmental stages. The earliest GUS activity was observed in the cotyledons of 2-day-old seedlings of P_{53}-GUS (Figure 2 C1). Five days after germination, GUS expression was present in the roots and the cotyledons of all P_{13}-GUS (Figure 2 A2-A4) and P_{53}-GUS (Figure 2 C2-C4) transgenic *Arabidopsis*, but not in P_{16}-GUS (Figure 2 B1-B4) or P_{56}-GUS (Figure 2 D1-D4). Under these growth conditions, regardless of developmental stage, the GUS staining pattern of P_{13}-GUS and P_{53}-GUS was similar, suggesting that the 1246 bp region upstream of the *OsGT1* cDNA start codon (from -1263 to -17 bp in P_{53}-GUS; Figure 1A) was sufficient to drive the specific expression of *GUS* gene in roots and flowers. Furthermore, 10 of the 18 root-specific motifs and 8 of the 9 pollen specific motifs were located in this region. However, when the 273-bp region immediately upstream of the start codon of *OsGT1* was deleted (to create P_{16}-GUS and P_{56}-GUS), the promoter was no longer able to drive expression of the *GUS* gene in transgenic *Arabidopsis*. This observation indicates that the 256-bp region from -273 to -17 bp of the *OsGT1* promoter is essential to activating *GUS* expression. The eighteen root motifs (ATATTT, ROOTMOTIFTAPOX1) and nine pollen motifs (POLLEN1LELAT52) in the *OsGT1* promoter (see note of AY338469) could be involved in the root and anther specific-expression of *GUS*. POLLEN1LELAT52 motif was one of the two co-dependent regulatory elements responsible for pollen-specific activation of tomato *lat52* gene (Bate and Twell, 1998), and ROOTMOTIFTAPOX1 motif was found for the root specificity and strength of the *rol D* promoter in roots of tobacco (Elmayan and Tepfer, 1995). These results might further support the reports of Hertig et al. (1991) and Santamaria et al. (2001), where the ATATTT motif (ROOTMOTIFTAPOX1) was required for expression in root tissues. The role of these motifs in the *OsGT1* promoter needs to be further studied.

The transgenic *Arabidopsis* P_{13}-GUS and P_{53}-GUS showed an identical expression pattern in flowers, cotyledons and roots (data not shown). GUS staining occurred in the veins of cotyledons (Figure 3A). Flowers showed GUS-staining in the anthers and in the veins of the petals (Figure 3B), but not in the pistil. Root staining revealed GUS-activity throughout the main root though not in the root caps or root hairs (Figure 3A, C and E). Tissue slices of roots showed strong GUS-staining in the stele, but not in the cortex, epidermis, or endodermis (Figure 3D, E). These results indicated that the *OsGT1* promoter was ex-

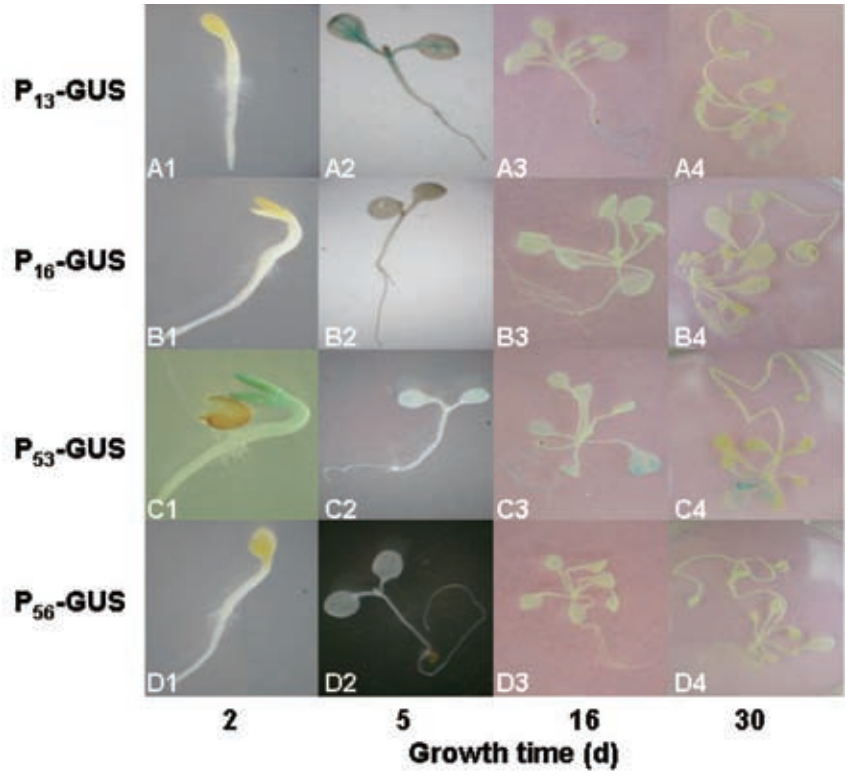

Figure 2. GUS staining patterns of transgenic *Arabidopsis* at different developmental stages. Plants were transformed with *OsGT1* promoter fragments fused to *GUS* gene. The transgenic *Arabidopsis* were grown on 1/2 MS solid media at 22° C under a 16 h/day photoperiod. Four independent transgenic lines were analyzed for each construct; the GUS staining of the different transgenic lines was similar within the same constructs.

pressed in a tissue- and organ-specific manner in the transgenic *Arabidopsis*.

To confirm the above transgenic results, rice IR64 was used to analyze the organ expression of *OsGT1* by Northern blot. Under normal growth conditions, *OsGT1* is expressed in roots and panicles (flowers) of rice, but not in the leaves (Figure 5, normal). These results suggested that the *OsGT1* promoter has a similar expression pattern in rice and *Arabidopsis*, and *OsGT1* is mainly expressed in the roots and flowers of rice.

The constitutive expression of *GUS* driven by *OsGT1* promoter in the stele of roots might indicate that *OsGT1* is involved in long distance GSH transport in the root stele. GSH's importance to root development was shown. The *Arabidopsis rml1* (root meristemless) mutant, which lacks an enzyme involved in GSH synthesis, abolishes cell division in the root but not in the shoot (Vernoux et al., 2000), indicating that GSH is essential for postembryonic root development in the root apical meristem after germination. The expression of *OsGT1* in cotyledon suggests that GSH transport takes place from cotyledons to the growing organs and that it may be important for

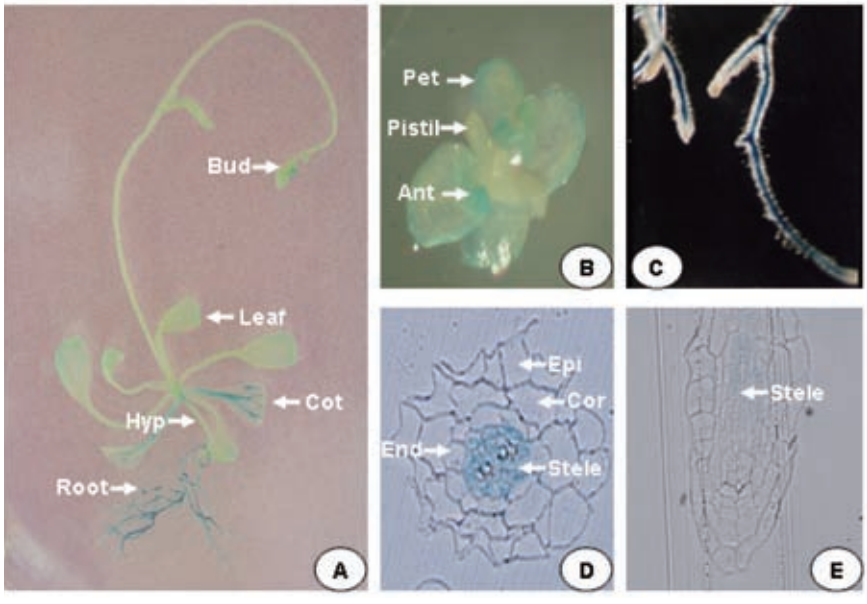

Figure 3. Histochemical localization of GUS activity in the transgenic *Arabidopsis* (P_{53}-GUS) transformed with *GUS* gene under the control of the 1246 bp *OsGT1* promoter region. A, whole plant; B, flower; C, whole root; D, cross section of a root; E, longitudinal section of a root. *Ant*, anther; *Cor*, cortex; *Cot*, cotyledon; *End*, endodermis; *Epi*, epidermis; *Hyp*, hypocotyl; *Pet*, petal.

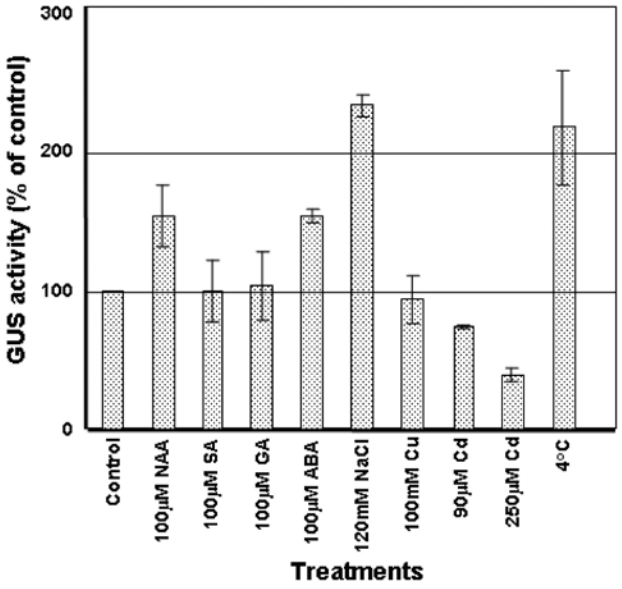

Figure 4. Effects of different treatments on the GUS activity in the P_{53}-GUS transgenic *Arabidopsis* line. P_{53}-GUS transgenic *Arabidopsis* were grown on sand at 22°C under a 16 h/day photoperiod. The 14-day-old seedlings were rinsed in the pots with 100 mL of 100 μM abscisic acid (ABA), 100 μM naphthalene acetic acid (NAA), 100 μM salicylic acid (SA), 100 μM gibberellin (GA), 120 mM NaCl, 100 μM $CuSO_4$ (Cu), 90 μM and 250 μM $CdSO_4$ (Cd), or treated with low temperature (4°C). After treatment for 24 h, roots were analyzed. All treatments were independently repeated twice.

the growth of roots and other organs. *OsGT1* promoter drives the GUS expression in the anthers of transgenic *Arabidopsis* (Figure 3A), and *OsGT1* expression was in panicles (flowers) of rice (Figure 5 Normal). These findings indirectly support the controlling role GSH has in *Arabidopsis* flowering (Ogawa et al., 2001), which is associated with the rate of GSH biosynthesis and/or the levels of GSH in *Arabidopsis*. Our results indicate the involvement of not only GSH synthesis but also of GSH transport in normal development of roots and flowering organs. *OsGT1* is grouped into the oligopeptide transporter family (OPT) (Zhang et al., 2004). The *AtOPT3* gene of the OPT family was also shown to be expressed in pollen and is essential for embryo development (Stacey et al., 2002). Together with our results, this indicates that GSH and other oligopeptides are transported into anthers and embryos.

Inducible expression of *GUS* driven by *OsGT1* promoter

Because GSH is involved in resistance of plants to biotic and abiotic stress (Noctor et al., 2002), the transgenic *Arabidopsis* (P_{53}-GUS) was used to check the response of the *OsGT1* promoter to environmental factors. GUS activities driven by P_{53}-GUS were approximately twofold up-regulated by NaCl and low temperature and approximately onefold up-regulated by ABA and NAA. GUS activity was down-regulated by high concentrations of cadmium, but unaffected by copper (Figure 4). These results indicate that GSH transport might be involved in protecting plants from saline and low temperature stresses. Given the induction by ABA, the response of *OsGT1* to saline stress and low temperature might be linked to ABA-dependent pathways. To confirm this result of the transgenic *Arabidopsis*, one-week-old rice seedlings were treated with NaCl, low temperature, ABA, SA, and $CdSO_4$. Transcripts of *OsGT1* in rice seedlings also could be strong up-regulated by NaCl, and down-regulated by Cd (Figure 5, treatment).

Although GSH is known to chelate heavy metals (Perrin and Watt, 1971), we found GUS activity directed by *OsGT1* promoter was inhibited by Cd (Figure 4). Bogs et al. (2003) also showed that Cd inhibited the expression of *BjGT1*. The amount of BjGT1 protein in *Brassica juncea* leaves is decreased by a 96 h exposure of the root system to Cd. However, we could not find the transcription response of *OsGT1* gene in rice plants to Cd (Figure 5). These results might indicate that OsGT1 is not involved in the detoxification of toxic metals. They confirm that OsGT1 is distinct from ABC transporters (Zhang et al., 2004), another family of GS-conjugate transporters (Rea et al., 1998), because the sequence of OsGT1 is not homologous with ABC transporters and lacks signature sequences (Walker motifs) characteristic of ABC transporters (Zhang et al., 2004). However, when the transgenic *Arabidopsis* were sprayed with the above chemicals, GUS staining could still not be detected in leaves and stems (data not shown).

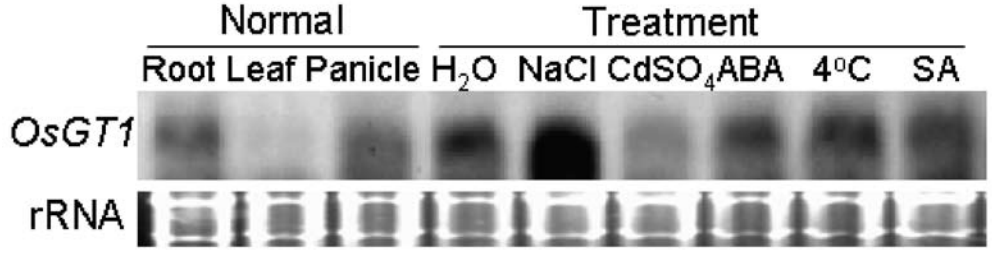

Figure 5. Expression of *OsGT1* in rice under normal growth condition and treatments. For normal, the roots, leaves and panicles of the heading *Oryza sativa* cv. IR64 were grown in field at Guangzhou, China. For treatment, one-week old IR64 seedlings were treated by 4°C, 120 mM NaCl, 90 μM $CdSO_4$, 100 μM salicylic acid (SA) and 100 μM ABA for 24 h. 20 μg of total RNA were loaded for Nothern analysis with *OsGT1* probe.

In conclusion, our data show that the heterogenous *OsGT1* promoter from a Monocotyledonous plant could drive tissue-specific expression and responses to environmental signals in the Dicotyledonous *Arabidopsis*, and *OsGT1* mainly expresses in roots and flowers of rice and might responses to the saline and cold stress.

Acknowledgement. We thank Serge Delrot of University of Poitiers, France for supplying vectors and MT Mei and CX Zhuang of Agriculture University of South China for Southern blots. This work was supported by the director foundation of South China Botanical Garden, Guangdong Science Foundation (06026070, 2006A20101004) National Science Fund (30670169) and "863" Project (2006AA10Z168).

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水稻 *OsGT1* 啟動子在轉基因擬南芥菜中的組織和誘導表達分析

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爲研究水稻穀胱甘肽轉運蛋白 (*OsGT1*) 基因在植物體內的表達譜，將該基因啓動子區 (AY338469) 與 *GUS* 報告基因融合後轉化擬南芥菜。結果表明：*OsGT1* 基因編碼區前 1246 bp 的啓動子區已足夠啓動 *GUS* 基因的表達，並且啓始密碼（ATG）前 257 bp 是必須具備的區域。*OsGT1* 啓動子啓動的 *GUS* 表達具有組織特異性，主要在根、子葉和花等器官的維管組織中表達。同時受外界鹽和低溫脅迫的誘導，以及激素 ABA 和 NAA 的誘導，然而 *OsGT1* 基因啓動子啓動的 GUS 活性被高濃度的重金屬 Cd 抑制。表明水稻 *OsGT1* 基因主要在根和花中表達，與水稻抗鹽與低溫脅迫相關。

關鍵詞：擬南芥菜；花；穀胱甘肽轉運蛋白；啟動子；水稻；根。