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Proteomic identification of an embryo-specific *1Cys-Prx* promoter and analysis of its activity in transgenic rice

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Proteomic analysis of a rice callus led to the identification of 10 abscisic acid (ABA)-induced proteins as putative products of the embryo-specific promoter candidates. 5′-flanking sequence of *1Cys-Prx*, a highly-induced protein gene, was cloned and analyzed. The transcription initiation site of *1Cys-Prx* maps 96 nucleotides upstream of the translation initiation codon and a TATA-box and putative seed-specific *cis*-acting elements, RYE and ABRE, are located 26, 115 and 124 bp upstream of the transcription site, respectively. β-glucuronidase (GUS) expression driven by the *1Cys-Pr*x promoters was strong in the embryo and aleurone layer and the activity reached up to 24.9 ± 3.3 and 40.5 ± 2.1 pmol (4 MU/min/μg protein) in transgenic rice seeds and calluses, respectively. The activity of the *1Cys-Pr*x promoters is much higher than that of the previously-identified embryo-specific promoters, and comparable to that of strong endosperm-specific promoters in rice. GUS expression driven by the *1Cys-Pr*x promoters has been increased by ABA treatment and rapidly induced by wounding in callus and at the leaf of the transgenic plants, respectively. Furthermore, ectopic expression of the GUS construct in *Arabidopsis* suggested that the *1Cys-Pr*x promoter also has strong activity in seeds of dicot plants.

1. Introduction

When plant cells and plants are used as bioreactors for plant-made pharmaceuticals (PMPs), enhancing gene expression levels is an important issue [1]. The use of a strong promoter whose expression is tissue specific and restricted to a particular developmental stage should be more effective and advantageous than the use of constitutive promoters. To achieve the successful engineering of genetically modified (GM) crops producing PMPs, it is necessary to introduce carefully designed genetic constructs with suitable promoters to drive specifically enhanced expression patterns of transgenes in plants. Thus, the identification of strong promoters to drive particular organ- or tissue-specific expression patterns is important for the development of efficient PMP production systems.

Rice seed has been used as a bioreactor for the production of recombinant proteins [2–4]. In a grain of rice, the aleurone layer and embryo contain most of the seed storage proteins (6–12% of dry weight), whereas the remaining endosperm is mostly starch [5]. The protein enriched outermost aleurone layer and embryo of dry seeds can be separated mechanically from the starch endosperm, which facilitates the design of cost-effective manufacturing processes for the pharmaceutical products. The biotechnological importance of the protein-enriched aleurone layer, embryo and embryo-derived callus as a platform of recombinant protein expression has been well recognized [1]. However, most of the strong seed-specific promoters identified in rice and other monocots are endosperm-specific and a small number of aleurone layer- and embryo-specific promoters have been characterized [6]. In this study, ABA-induced major proteins from embryo-derived rice callus were examined by 2-D and MALDI-TOF MS analyses to identify strong aleurone layer- and embryo-specific promoters in rice seeds. Among the 10 identified proteins, a *1Cys-Peroxiredoxin* (*1Cys-Prx*), exhibiting the strongest expression in the presence of ABA, was chosen and characterized for PMP production in plants.

2. Materials and methods

2.1. Rice callus suspension culture and ABA treatment

Embryo-derived rice (*Oryza sativa* L. cv. Dongjin) callus was induced from hulled seeds after sterilization on MS agar medium

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[7] supplemented with 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). After 20 d, embryonic calluses (ca. 1.0 mg) were transferred to 25 ml liquid medium containing MS basal salts, B5 vitamins, 2% sucrose and 2.0 mg/l 2,4-D (R2 medium) in a 100 ml Erlenmeyer flask and placed on a gyratory shaker (80 rpm) in the dark at 28 °C. Three-day-old suspension cells were mock-treated or treated for 48 h with 10 μM ABA.

2.2. Two-dimensional electrophoresis (2-D), in-gel digestion and protein identification

Total soluble protein was extracted from the rice suspension culture cells. Rice callus cells were ground in liquid nitrogen using a tissue homogenizer. Fine powder was dissolved in pre-chilled protein extraction buffer (50 mM Tris–Cl, pH 7.5, 1 mM ethylenediamine-tetra-acetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The soluble proteins were extracted by using the extraction buffer with vortex and separated by centrifugation at 12,000 rpm at 4 °C for 15 min. 2-D, in-gel digestion, and protein identification were performed according to previously reported methods [8].

2.3. 5′ RLM-race

5′ RLM-RACE was performed using the GeneRacer kit (Invitrogen, Carlsbad, CA) [9]. PCR was performed to amplify the resultant cDNAs using the GeneRacer 5′ primer and a primer consisting of bases downstream of the translation start site of the *1Cys-Prx* gene (1CP_R1: 5′-GGTACAGGAAGCTCAGCTTCACCTTCTTGT-3′). This was followed by nested PCR to eliminate the possibility of artifacts using the GeneRacer 5′ nested primer and *1Cys-Prx* primer (1CP_R2: 5′-ATGTTCAGCTGCTTGATGGCCTCG-3′). The RACE nested PCR products were cloned into the pGEM-T Easy vector (Promega, USA). DNAs obtained from the resultant colonies were analyzed by automatic sequencing.

2.4. Western blot analysis

Analyses of protein by western blotting using the polyclonal 1Cys-Prx antibody were essentially the same as those described previously [10].

2.5. Plasmid construction and the production of transgenic plants

The 5′-flanking regions of the 1Cys-Prx gene were isolated by PCR from rice (*Oryza sativa* L. cv. Dongjin) genomic DNA with the use of ExTaq DNA polymerase (Takara, Japan). To generate the appropriate fragments, the common 3′-end primer 1CP_C1 (5′-CATGGCAGAGCTCACAATCAGAGACAC-3′), which is specific for the AUG translation initiation codon, was combined with each of the 5′- end primers, 1CP_P1 (5′GAGCATCTCTATAACAGCACCA CATAAACCTAGG-3′), 1CP_P2 (5′-TCAAAGCTTATTACTATCTGAGCA TTCCCC-3′), and 1CP_P3 (5′-ACGCGTCGACGCATCTCAACGATGA TGCC-3′), which are specific for the sequences near the −1812, −1007, and −606 bp upstream regions of the transcription start site, respectively. The above primer sets introduced an *Eco*RI restriction site at the 5′ end and an *Nco*I at the 3′ end of the PCR products, resulting in each case in a respective truncated promoter region. Amplified PCR fragments were cloned into pGEM-T Easy vector (Promega, USA) and sequences were verified by automatic sequencing. The *Eco*RI and *Nco*I fragments of the promoter deletions were ligated in frame upstream of the gusA gene in a pCAMBIA1301 plasmid. The resultant plasmids were introduced into *Agrobacterium tumefaciens* strains LBA4404 and GV3101. Transgenic plants were produced by *Agrobacterium*-mediated transformation [11]. Transgenic plants were selected on media containing 50 mg/l of hygromycin. Regenerated rice plants were cultured aseptically in growth chambers with an irradiation (300 μmol m^{-2} s^{-1}) of white light at a cycle of 14 h light (28 °C)/10 h dark (25 °C) for acclimation and subsequently transferred to a greenhouse.

2.6. Southern blot analysis

Southern blot analysis of transgenic plants for T-DNA integration was performed using essentially the same procedures as described previously [12]. DNA fragments of the gusA gene in the pCAMBIA1301 plasmid were labeled with [^{32}P] dCTP and used as a hybridization probe.

2.7. Histochemical and fluorometric GUS assay

Histochemical GUS assays were performed by incubating samples in 1% 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) solution in 20 mM sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM EDTA, and 5 mM potassium ferrocyanide overnight at 37 °C. After staining, samples were fixed in ethanol/acetic acid (1:1) and then clarified with chloral hydrate in a modified Hoyers solution. Fluorometric measurements of GUS activity were carried out using 4-methyl umbelliferyl glucuronide (4-MUG), β-D-glucoronide hydrate (Fluka, BioChemika, Switzerland) as a substrate and employing a fluorescence spectrophotometer, SpectraMax_Gemini EM (Molecular Devices Corporation, Sunnyvale, CA, USA) [13]. Protein concentrations were determined following Bradford [14] and GUS enzyme activity was expressed in picomoles of 4-methylumbelliferone (MU) produced per min per μg protein.

3. Results and discussion

3.1. Identification of ABA-induced proteins from embryo-derived rice callus

Embryo-derived rice calluses were stimulated with ABA to investigate proteins that are highly expressed in the late stage of embryogenesis. Proteins from the control and ABA-treated calluses were separated by 2-D and their expression levels were compared after silver staining by PD Quest software (Fig. S1). Protein spots with significant differences (more than twofold) in abundance between the control and ABA-treated calluses were selected as differentially-expressed proteins. Among them, 10 protein spots that were highly increased by ABA-treatment were selected, excised from the silver stained gels and identified by MALDI-TOF MS analysis (Fig. S1 and Table S1). Spot 6 was highly induced by ABA treatment together with spot 5, and they were identified as 1Cys-peroxiredoxin (1Cys-Prx) containing thiol-dependent peroxidase activity [10,15].

3.2. Cloning the 5′-flanking region of the 1Cys-Prx gene and identifying putative cis-elements in the sequence

To investigate functional characteristics as a tissue-specific promoter and spatiotemporal patterns of reporter gene expression, the 5′-flanking region of *1Cys-Prx* containing 1920 bp upstream of the AUG translation initiation codon was isolated, cloned and sequenced. The transcription start site of *1Cys-Prx* was determined by RNA ligase-mediated rapid amplification of 5′ cDNA ends (5′ RLM-RACE) [9]. Direct cloning of the RLM-RACE products followed by sequencing demonstrated that the major mRNA cap site in the 5′-flanking region of *1Cys-Prx* is 96 nucleotides upstream of the AUG translation initiation codon (Fig. 1). Bioinformatic analysis of the sequence allowed the identification of conserved sequences for TATA- and CAAT-boxes within the promoters, as well as other *cis*-elements such as ABRE, ARE, CAT-box, CCGTCC-box, RYE and

W-box (Fig. 1 and Table S2). A putative TATA-box was positioned 26 bp upstream from the transcriptional initiation site. Among the *cis*-elements found in the 5′-flanking region of *1Cys-Prx*, RYE and ABRE are the closest, located at 89 and 98 bp upstream from the transcriptional initiation site, respectively. ABRE and RYE have been previously identified as *cis*-elements that are involved in embryo or seed development [16–18].

3.3. Spatial and temporal patterns of GUS expression driven by the 1Cys-Prx promoter

Based on the distribution of *cis*-regulatory elements identified by bioinformatic analysis in the 5′-flanking regions of *1Cys-Prx*, the 1814, 1007 and 606 bp regions upstream of the transcription start site were chosen as candidate promoter regions. These promoter regions together with the 96 bp 5′-UTR were amplified by PCR from a rice genomic DNA and designated as F1, F2 and F3, respectively (Figs. 1 and 2). To characterize expression patterns and promoter activities, isolated promoter regions containing the 5′-UTR were linked to the GUS reporter gene and introduced into rice via *Agrobacterium*-mediated transformation. Several representative T_3 transgenic plants for each T-DNA construct were chosen for the analyses of promoter properties.

To determine the temporal and spatial patterns of GUS expression by the three *1Cys-Prx* promoter regions, transgenic rice seeds were examined by histochemical staining (Fig. 2). GUS expression driven by the three *1Cys-Prx* promoter regions (F1, F2 and F3) was strong in the embryo and aleurone layer. However, a transgenic rice seed containing the GUS reporter gene driven by the CaMV 35S promoter showed much weaker GUS activity in the embryo and endosperm (Fig. 2). To investigate GUS expression by the *1Cys-Prx* promoters (F1, F2 and F3) during rice seed development, the transgenic rice seeds collected from stages 2–8 weeks after flowering (WAF) were longitudinally sectioned and stained with X-Gluc (Fig. 3A). GUS activity in seeds during development (2–8 WAF) was too strong to be compared visually by X-Gluc staining. However, western blot analysis of endogenous 1Cys-Prx using the polyclonal 1Cys-Prx antibody revealed that the expression of 1Cys-Prx in rice seeds reached its highest level at 3 WAF and this level was maintained to the maturation stage (8 WAF) (Fig. 3B). Histochemical GUS analysis of transgenic seeds during seed germination and seedling development was also performed to examine the temporal, spatial and hormonal regulation of GUS expression directed by *1Cys-Prx* promoters (F1, F2 and F3) (Fig. 3C). High GUS expression in the germinating transgenic seeds was maintained up to 1 day after imbibition (DAI); thereafter, it decreased gradually from 2 DAI, and weak GUS expression was observed in 10–15 days-old seedlings (Fig. 3C). Consistent with the GUS staining result, high expression levels of the endogenous 1Cys-Prx in germinating seeds was maintained up to 1 DAI, followed by a gradual decrease from 2 DAI, and small amounts of 1Cys-Prx were detected in 10–15 days-old seedlings in the absence of ABA in the growth medium (Fig. 3D). However, when seeds were grown on medium containing 10 μM ABA, they did not germinate and the expression of 1Cys-Prx remained at high steady-state levels (Fig. 3D).

3.4. Quantitative analysis of GUS expression driven by 1Cys-Prx promoter regions in transgenic rice seeds and calluses

To evaluate the potential strength of *1Cys-Prx* promoter regions, GUS expression levels in mature seeds from independent

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                                                                                                     GAATTCTTCC -1805
                                                                                                     F1
CTTCAACATTAACCAGAAGAAGATTTTGACTTTTAAGGGAATGGAGGACCTCCACAGGTCCTGCAAAACCCTATCTTTAACCCCTCCAAAACATAGGGCC -1705
TGGTAAAGGGATTTGGTAGTATAAGAGCCTGTCGATGTTTGATGTCGCGATTCCGGTATTTGCATAGTATGGGGATCGTTGGTACTATGATATACGCGAG -1605
ACTGAGGTAAAAGAGACGGAGACAGGGATTTTTATACAGGTTCGGGCCCCTGAATTGTCAGGTAATAACCCAACATCCTGTTGGCCGAAGCCGGTATTAC -1505
TCTTATTCACCATAATCACACTAGTACAATATTTGGGATAACCTATCAAACTGTTGTCGATATGGCGGTGTGAAGGTCTGACTCGTAGTCGATAACAGTG -1405
TAGCCTTCCTCCTCGAATCCGCATCCGGCGAGATCAGAGACAGCGCTATATCTCTCCTGCTGACAGTATCCGTAGGCACCGTAAGGGACTAGCCATGCCT -1305
ATCTCTGAAGTCGATATCTGGCGTCTTGTCTTGGCGTATGTCAACTTGTATGTTGTGGCTTCTGTTGTTCCATGTATGTTGTGGTGGGTGTCTTCCATGG -1205
TGGGTGTGTCCTCTCTCCTCTTAGGGGGTCTTGTATTTATACCCATAGGTGTCCCCTTGTCCAAGTAGAACTAGGGAAACCAATATGAATACAATCTAAG -1105
                                                                            ARE CAAT-box
TAGTCCTTGTCGTTTCCATGTAAAACTCTGGTTATCTTTTCTTATCCGGAACTCCTCCTATATCTGTAGGTTGCTTCCGTATAGGACATGGTATGTAGTG -1005
                                                                                                   F2
GGTCCTACCGAGATTTAGTCAACTACTATTAGGTATGTGATATCCATAACCCTGACAGAGCCAGACTTATCAGCTTCCAAAACATTTGATCTTTATCACC  -905
TGAAAGATGAATCTCCCCTACAAATTATGTAGTTCTTCCCATTCCTCCATCTCCCCTTCACTCAGAGATCTCCTCAACTCTATAACCCAGCTCCCTCAGG  -805
                   CAAT-box
ACCATAAGTTTGATGTACAGTTTTCTTCTGGTCTGCACAAATTTTGTAGATATATGGAAACTGAGTTTTTAGGGGTGTCTTTCCTATCCATACATCATCC  -705
CAGACTAGGATTGTTGACCCTCCACATTTTATTTCTTCCCACTTACATGTATTTATTTTTTATTCTTTGCTAATCGGATGCTATGTCAGTGAAACCAGGC  -605
              W-box                                                                             ARE  F3
ATCAATACTACCTCGGGATCTTTTTTTGGACGGTTTTATATAGTTAAGGTATTCCGGTTAGGAGACCTCAGACAGATTTCGTGTATAAATTGAGGGACGT  -505
                           CCGTCC-box                                                      CAAT-box
CTGGTGAACTTAGGGCCTGTTCACTTTGATGCCATTTTCAATCTTACCAAATTTTGGTAAAGTTGTCAAAAAAAATGTGGCTACATTTAGTTTGCTGCTA  -405
                                      CAAT-box
AATTTTGGTAACTATATAAGAAATCCTGCCAAAATTTTGACAAGTTATCAAAATTTTGGCCACTATAAATTTTGGTAATACCAAAATTTGGTAAGGTTTT  -305
                                                          CAT-box                   CAAT-box
TTTTTTCATCAAAGTGAATAGGCGCTTGGTCCTTTCTGAGCTAAACCGAGTCCAAATTCCAAAAGCTCTCCCGACATGGGCCGCCACGATCAGTTTCAGG  -205
                                                    CAAT-box
TGGCCGTGAGAAGATTCCAGAATGGACGGTCATGGGGATGGGGGAGGTCAACACGACTCACAAAGGTATAGTAGGAGGCGCGGCTGCCACGAGACGAGGT  -105
                       CCGTCC-box            W-box
ACGTGGCCCCCATGCAGCACGCCGTGGCGACACCTCTGCACACTGATCAACTCCACTCACCTCACTCCTCACCTATAAAAATGCTTCGATCTCACTCCAG    -5
 ABRE     RYE                                                            TATA-box
TCTCCAGTGCCTTCGTCTCGACACGTTTGCATTGCAGCAGCTCATAAGTTTCTTCTTCGTTTCTGCTCCCAGTGCTAAGGCAGCACAGTCGTTCGTCGCC    96
    +1
ATGCCAGGGCTCACCATCGGCGACACCGTCCCCAACCTGGAGCTGGACTCCACCCACGGCAAGATCCGCATCCACGACTTCGTCGGCGACACCTATGTCA   196
 M  P  G  L  T  I  G  D  T  V  P  N  L  E  L  D  S  T  H  G  K  I  R  I  H  D  F  V  G  D  T  Y  V    (33)
TCCTCTTCTCCCACCCCGgtatacacactacacactacactcgctcgattctcacattctcattcggtagtagtttgtgtggatcgatgtgcatccgatt   296
 I  L  F  S  H  P                                                                                     (39)
gattgattgaatgatccgtcgatcgatcag                                                                         326
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Fig. 1. Analysis of the *1Cys-Prx* promoter sequence and putative *cis*-acting elements. The 5′ flanking region of *1Cys-Prx* was analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The transcription start site (**C**) is denoted as +1. Several putative *cis*-elements, including the TATA-box, are boxed, CAAT-boxes are shaded, and the names of each are given under the elements. The start sites of *1Cys-Prx* 5′-flanking regions (F1: −1814 to 96, F2: −1007to 96, and F3: −606 to 96) used for promoter analysis are indicated with arrows. The first intron (gt–ag) of *1Cys-Prx* is shown after the first exon, including the 96 bp 5′-untranslational region (5′-UTR) and the 118 bp coding region.

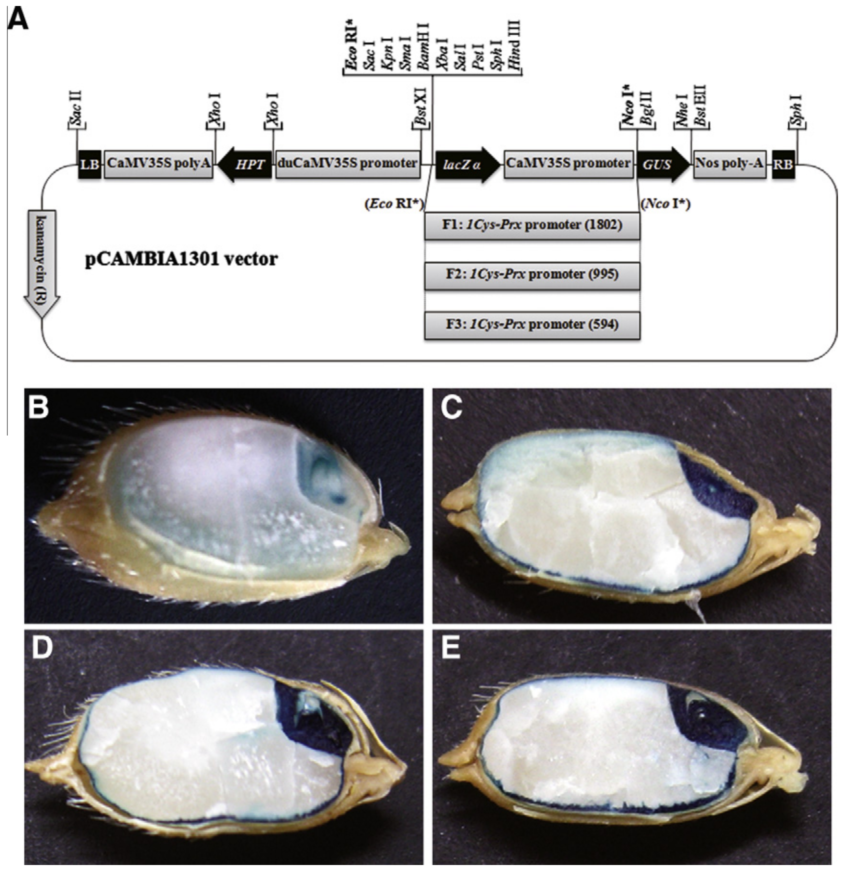

Fig. 2. Schematic diagrams of the *1Cys-Prx* promoter-gusA constructs and expression of GUS in transgenic rice seeds. (A) Three *1Cys-Prx* promoter regions (F1: 1814, F2: 1007, and F3: 606) were fused to the gusA gene in the plasmid pCAMBIA1301. *1Cys-Prx* promoter derivatives were cloned at *Eco*RI and *Nco*I sites in the pCAMBIA 1301 vector by replacing the CaMV35S promoter. The parenthesized numbers of each construct indicate distance from the transcription start site. Plasmid pCAMBIA1301 with CaMV35S promoter-gusA was used as a control. Distribution and intensity of GUS expression in seeds of representative transgenic rice lines carrying (B) CaMV35S, (C) F1, (D) F2, and (E) F3 *1Cys-Prx* promoter regions.

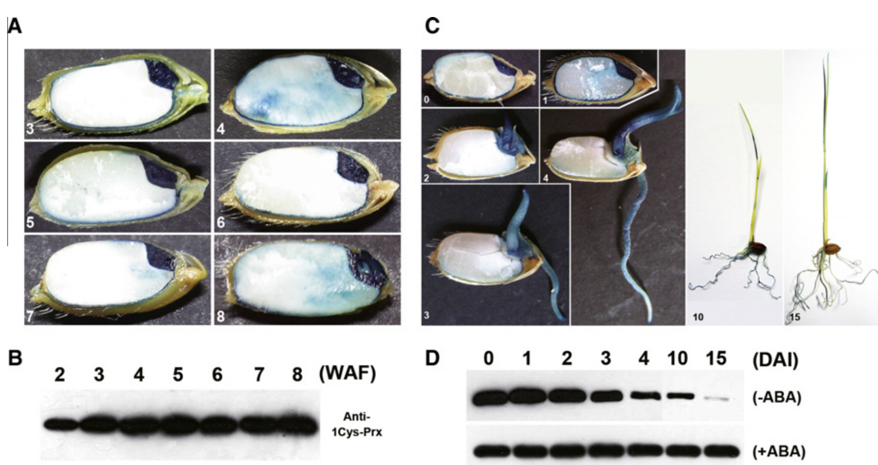

Fig. 3. Histochemical analysis of GUS expression and western blot analysis of 1Cys-Prx during seed maturation and germination. (A) Histochemical localization of GUS activity in the seeds of transgenic rice carrying the 1Cys-Prx promoter (F1). Seeds were harvested at 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, and 8 weeks after flowering (WAF). (B) Western blot analysis of 1Cys-Prx in the seeds of WT rice. Seeds were harvested at 2, 3, 4, 5, 6, 7 and 8 WAF. (C) Histochemical X-gluc staining of transgenic rice seedlings carrying the 1Cys-Prx promoter (F1). Seedlings were harvested at 0 d, 1 d, 2 d, 3 d, and 4 d after imbibition (DAI). (D) Western blot analysis of 1Cys-Prx in the seedlings of WT rice. WT rice seedlings untreated (−ABA) or treated (+ABA) with 10 μM ABA were harvested at 0, 1, 2, 3, 4, 10, and 15 DAI. A total of 10 μg of protein was separated for each by SDS–PAGE and the expression of 1Cys-Prx was examined by western blot analysis using 1Cys-Prx antibody.

single-copy T-DNA lines with each transgenic construct were determined. The average GUS activities for the *1Cys-Prx* promoter regions determined fluorometrically were 21.9 ± 1.8, 24.9 ± 3.3 and 23.4 ± 3.4 pmol (4 MU/min/μg protein) for 3, 4 and 4 independent lines of F1, F2 and F3, respectively (Fig. 4A). In a previous study, the GUS activities in the seeds of transgenic plants driven by *GluB-4*, 10 kDa prolamin, 16 kDa prolamin, *Glb-1*, 1.3 kb *GluB-1*, *GluB-2*, 13 kDa prolamin, *REG-2*, and *Ole18* promoters were

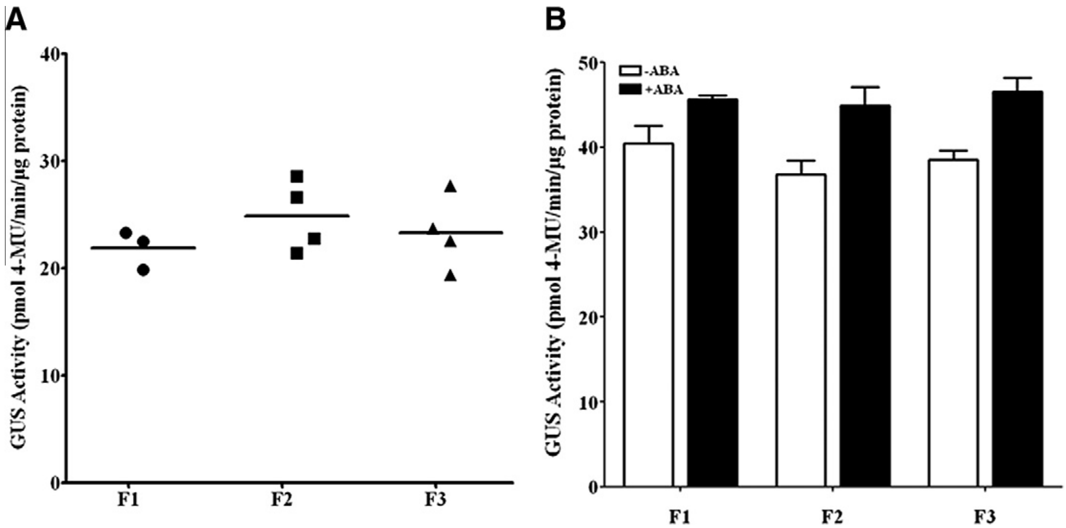

Fig. 4. Quantitative analysis of GUS activity in transgenic rice seeds and calluses directed by the 1Cys-Prx promoter regions F1, F2 and F3. (A) GUS activity in the seeds of representative transgenic rice lines of the 1Cys-Prx promoter regions F1, F2 and F3, were quantified. Each horizontal line represents an average GUS activity. (B) GUS activity in the calluses induced from the representative transgenic rice lines of the 1Cys-Prx promoter regions F1, F2 and F3, were quantified. White and black bars represent average GUS activity in the transgenic seeds in the absence or presence of ABA, respectively. GUS activity is expressed in pmol (4 MU/min/μg protein). Calluses induced from transgenic rice seeds were cultured in suspension for 2 weeks and treated with 10 μM ABA for 48 h in fresh media; others were subjected to a mock treatment.

44.8 ± 16.5, 38.8 ± 10.8, 27.1 ± 12.7, 28.6 ± 11.8, 2.1 ± 1.2, 5.5 ± 2.2, 7.4 ± 5.5, 2.4 ± 1.2, and 2 ± 4.6 pmol (4 MU/min/μg protein), respectively [6]. Since the GUS expressions under the control of *1Cys-Prx* promoter regions were specifically observed in the aleurone layer and embryo of rice grains (Fig. 2B), embryo-derived calluses were induced from the representative transgenic lines (F1-1, F2-3 and F3-1 containing a single copy of the GUS gene directed by the *1Cys-Prx* promoter regions, F1, F2 and F3, respectively) and subjected to further analysis. In the absence of ABA, GUS activities directed by the F1, F2, and F3 *1Cys-Prx* promoter regions in the embryo-derived calluses were 40.5 ± 2.1, 36.8 ± 1.6, and 38.5 ± 1.1 pmol (4 MU/min/μg protein), respectively (Fig. 4). However, when the calluses were treated with 10 μM ABA for 36 h, GUS activities directed by the F1, F2, and F3 *1Cys-Prx* promoter regions increased up to 45.7 ± 0.4, 44.9 ± 2.2, and 46.5 ± 1.6 pmol (4 MU/min/μg protein), respectively (Fig. 4). Thus, GUS activities directed by the F1, F2 and F3 *1Cys-Prx* promoter regions in the embryo-derived calluses are significantly higher than those of the previously-identified embryo-specific REG-2 and Ole18 promoters in whole seeds and are further increased by ABA treatment. Histochemical GUS assays of the embryo-derived calluses induced from the transgenic rice seeds with the *1Cys-Prx* promoter regions F1, F2 and F3 also showed strong X-Gluc staining (Fig. S2).

3.5. Activities of the 1Cys-Prx promoter in various tissues of the transgenic rice and ectopic expression of 1Cys-Prx promoter-GUS

Bioinformatic analysis of the *1Cys-Prx* promoter region revealed the presence of two W-box elements and the involvement of ABA in the wound response signaling pathway has also been demonstrated [19]. Therefore, wound-inducible GUS expression was evaluated by histochemical staining using the leaves of a transgenic rice line with *1Cys-Prx* promoter F1-GUS (Fig. S2). When wound stress was imposed on the leaves by cutting with scissors or by repeated pricking with a needle, strong GUS activity was promptly observed at the wound sites together with weak activity throughout the transgenic rice leaf, whereas no GUS expression was observed in the WT leaf (Fig. S2). The weak GUS activity throughout the transgenic rice leaf may be caused by the diffusion of GUS reaction products into the neighboring tissue through the phloem and xylem enclosed within the leaf bundle sheath (Fig. S2). Furthermore, when the dicotyledonous plant *Arabidopsis* was transformed with the GUS reporter gene driven by the *1Cys-Prx* promoter, the embryo of mature seeds exhibited strong GUS expression, indicating that the *1Cys-Prx* promoter functions not only in monocot seeds, but also in dicot seeds (Fig. S2). In summary, the seed-specific *1Cys-Prx* promoter used here showed strong activity with appropriate tissue-specific and temporal expression, which is compatible with biotechnological applications. The *1Cys-Prx* promoter reported here can serve as a useful component for a high level expression system in plants.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.120.

References

[1] S. Hellwig, J. Drossard, R.M. Twyman, R. Fischer, Plant cell cultures for the production of recombinant proteins, Nat. Biotechnol. 22 (2004) 1415–1422.
[2] T. Nochi, H. Takagi, Y. Yuki, L. Yang, T. Masumura, M. Mejima, U. Nakanishi, A. Matsumura, A. Uozumi, T. Hiroi, S. Morita, K. Tanaka, F. Takaiwa, H. Kiyono, Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination, Proc. Natl. Acad. Sci. USA 104 (2007) 10986–10991.
[3] Y. Fujiwara, Y. Aiki, L. Yang, F. Takaiwa, A. Kosaka, N.M. Tsuji, K. Shiraki, K. Sekikawa, Extraction and purification of human interleukin-10 from transgenic rice seeds, Protein Expr. Purif. 72 (2010) 125–130.
[4] S. Nandi, D. Yalda, S. Lu, Z. Nikolov, R. Misaki, K. Fujiyama, N. Huang, Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain, Transgenic Res. 14 (2005) 237–249.
[5] J. Glimn-Lacy, P.B. Kaufman, Seed structure and germination, in: J. Glimn-Lacy, P.B. Kaufman (Eds.), Botany Illustrated: Introduction to Plants, Major Groups, Flowering Plant Families, Springer Science, New York, 2006, p. 40.

[6] I.Q. Qu, F. Takaiwa, Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice, Plant Biotechnol. J. 2 (2004) 113–125.
[7] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497.
[8] Y.H. Chi, J.C. Moon, J.H. Park, H.S. Kim, I.S. Zulfugarov, W.I. Fanata, H.H. Jang, J.R. Lee, Y.M. Lee, S.T. Kim, Y.Y. Chung, C.O. Lim, J.Y. Kim, D.J. Yun, C.H. Lee, K.O. Lee, S.Y. Lee, Abnormal chloroplast development and growth inhibition in rice thioredoxin m knock-down plants, Plant Physiol. 148 (2008) 808–817.
[9] V. Volloch, B. Schweitzer, X. Zhang, S. Rits, Identification of negative-strand complements to cytochrome oxidase subunit III RNA in *Trypanosoma brucei*, Proc. Natl. Acad. Sci. USA 88 (1991) 10671–10675.
[10] K.O. Lee, H.H. Jang, B.G. Jung, Y.H. Chi, J.Y. Lee, Y.O. Choi, J.R. Lee, C.O. Lim, M.J. Cho, S.Y. Lee, Rice 1Cys-peroxiredoxin over-expressed in transgenic tobacco does not maintain dormancy but enhances antioxidant activity, FEBS Lett. 486 (2000) 103–106.
[11] Y. Hiei, T. Komari, T. Kubo, Transformation of rice mediated by *Agrobacterium tumefaciens*, Plant Mol. Biol. 35 (1997) 205–218.
[12] J.R. Lee, K.O. Lee, J.H. Park, J.Y. Yoo, J.S. Kang, H.S. Jeon, S.Y. Kim, Y.M. Lee, S.T. Kim, C.O. Lim, J.D. Bahk, M.J. Cho, S.Y. Lee, Molecular and functional characterization of a PEX14 cDNA from rice, Plant Sci. 166 (2004) 123–130.
[13] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, Embo J. 6 (1987) 3901–3907.
[14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[15] C. Haslekås, R.A. Stacy, V. Nygaard, F.A. Culianez-Macia, R.B. Aalen, The expression of a peroxiredoxin antioxidant gene, *AtPer1*, in Arabidopsis thaliana is seed-specific and related to dormancy, Plant Mol. Biol. 36 (1998) 833–845.
[16] M.J. Guiltinan, W.R. Marcotte Jr., R.S. Quatrano, A plant leucine zipper protein that recognizes an abscisic acid response element, Science 250 (1990) 267–271.
[17] Q. Shen, S.J. Uknes, T.H. Ho, Hormone response complex in a novel abscisic acid and cycloheximide-inducible barley gene, J. Biol. Chem. 268 (1993) 23652–23660.
[18] H. Baumlein, I. Nagy, R. Villarroel, D. Inze, U. Wobus, *Cis*-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue-specific expression of a legumin gene, Plant J. 2 (1992) 233–239.
[19] J. Leon, E. Rojo, J.J. Sanchez-Serrano, Wound signalling in plants, J. Exp. Bot. 52 (2001) 1–9.