Transgenic Res (2011) 20:153–163
DOI 10.1007/s11248-010-9400-y

ORIGINAL PAPER

Analysis of the *Wsi18*, a stress-inducible promoter that is active in the whole grain of transgenic rice

Nari Yi · Se-Jun Oh · Youn Shic Kim · Hyun-Jun Jang · Su-Hyun Park · Jin Seo Jeong · Sang Ik Song · Yang Do Choi · Ju-Kon Kim

Received: 3 February 2010 / Accepted: 21 April 2010 / Published online: 20 May 2010

Abstract There is currently a shortage of efficient promoters for stress-inducible gene expression, especially in monocotyledonous crops. Here, we report analysis of the rice *Wsi18* promoter, a member of the group 3 *Lea* family, in transgenic rice plants. The abundance of *Wsi18* mRNA increased in leaf tissues within 2 h of exposure to NaCl or abscisic acid (ABA) and within 6 h of exposure to drought, but there was no transcript increase in response to low-temperature conditions. *Wsi18* mRNA accumulated in the roots similarly to in the leaves, but at a faster rate. The promoter was linked to the *GFP* reporter gene, transformed into rice, and its activity was analyzed in transgenic plants at all stages of plant growth from calli, vegetative tissues, flowers, and to dry seeds, both before and after stress treatment. The activity of the promoter was significantly increased in the whole plant body, including flowers, on exposure of plants to stress conditions, with very low levels of basal activity in all tissues. Moreover, the promoter was found to be predominantly active in the whole grain, including endosperm, embryo, and aleurone layer during seed development. Together, we have identified and analyzed the *Wsi18* promoter and found a previously undescribed characteristic—a stress-inducible property in the whole plant body with activity in the whole grain during seed development.

Keywords GFP · Whole grain · Promoter · Stress-inducible · Transgenic rice · *Wsi18*

Nari Yi, Se-Jun Oh, and Youn Shic Kim contributed equally to the paper.

Electronic supplementary material The online version of this article (doi:10.1007/s11248-010-9400-y) contains supplementary material, which is available to authorized users.

N. Yi · S.-J. Oh · Y. S. Kim · H.-J. Jang · S.-H. Park · J. S. Jeong · S. I. Song · J.-K. Kim (✉)
School of Biotechnology and Environmental Engineering, Myongji University, Yongin 449-728, Korea
e-mail: jukon306@gmail.com

N. Yi · Y. D. Choi
School of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Korea

S.-J. Oh
Syngenta Seeds Co., Ltd, 100 Gongpyeong-dong, Seoul 110-702, Korea

Introduction

The abiotic stresses drought, high salinity, and low temperature pose a serious threat to the sustainability of crop yields. Approximately 20% crop yield is reduced worldwide because of these unfavorable growing conditions. To improve stress tolerance of crops, many genes including transcription factors have been tested in transgenic plants using either constitutive or stress-inducible promoters. Several constitutive promoters have been successfully used to make stress-tolerant transgenic crops. For example,

OsCDPK7 (Saijo et al. 2000) and *HVA1* (Xu et al. 1996) have been regulated in transgenic rice by the cauliflower mosaic virus (CaMV) *35S* promoter and the rice *Act1* promoter, respectively. The maize ubiquitin 1 (*Ubi1*) promoter has been used to drive the biosynthesis of trehalose in rice (Jang et al. 2003) and the expression of transcription factors, *CBF3/DREB1A*, *ABF3* (Oh et al. 2005a), and *HvCBF4* (Oh et al. 2007), resulting in increased tolerance of rice to abiotic stress. The *OsCc1* promoter (Jang et al. 2002) was effective in constitutively expressing a gene encoding a transcription factor with an AP2 domain, AP37, conferring tolerance of rice to drought stress (Oh et al. 2009). Those transgenic plants with strong constitutive expression of transcription factors, however, often suffer from undesirable phenotypes. For example, growth retardation and low reproductive yields (Nakashima et al. 2007) were observed for stress-tolerant transgenic rice with *Ubi1:OsNAC6*. Similarly, varying degrees of growth retardation, abnormal development, and reduced seed production were observed for transgenic *Arabidopsis* with *35S:DREB1A* (Liu et al. 1998; Kasuga et al. 1999), transgenic rice with *35S:Adc* (Capell et al. 1998), transgenic tomato with *35S:CBF1* (Hsieh et al. 2002), and transgenic tobacco with *35S:TPS1* (Romero et al. 1997). It is therefore desirable to generate transgenic plants that accumulate transgenic products only under stress conditions.

Several stress-inducible promoters have been isolated and characterized. These include the *kin1* and the *cor6.6* (Wang et al. 1995) promoters from *Arabidopsis*, the *Gmhsp17.6-L* (Lee and Schöffl 1996) promoter from soybean, the *Hahsp17.7 G4* (Coca et al. 1996) promoter from sunflower, the *Rab16A* (Rai et al. 2009; RoyChoudhury et al. 2007) and *OsABA*2 (Rai et al. 2009) promoters from rice, the *TaLTP1* promoter (Jang et al. 2004) from wheat, and the *Hva1* (Straub et al. 1994; Xiao and Xue 2001) promoter from barley. Despite such efforts to functionally characterize stress-inducible promoters, very few have been analyzed for their response to stresses through all the stages of plant growth. The Arabidopsis *Rd29A* promoter has been shown to be effective in all the tissues and stages of transgenic plants under stress conditions (Pellegrineschi et al. 2004; Yamaguchi-Shinozaki and Shinozaki 1993). Thus, there is currently a shortage of efficient promoters that act in a stress-inducible manner but with low background activity. In addition, stacking of several transgenes in a single transgenic plant requires a battery of different promoters, which may otherwise undergo the homology-dependent gene silencing that often occurs in transgenic plants with multi-copies of the same promoter (Lessard et al. 2002; Potenza et al. 2004).

During seed maturation in plants, expression of late embryogenesis abundant (*LEA*) protein genes is induced by ABA (Finkelstein and Rock 2002). Of nine groups of LEA proteins so far identified, the group 3 LEA family is relatively well characterized (Xiao et al. 2007; Xu et al. 1996). *Wsi18*, a member of the group 3 *Lea* family, is a stress-inducible rice gene (Joshee et al. 1998; Shobbar et al. 2008; Takahashi et al. 1994). Here, we analyzed the activity of the *Wsi18* promoter in transgenic rice plants at all stages of growth, including flowers and developing seeds. The promoter drives high levels of GFP expression in the whole body of plants including calli, leaves, roots, and flowers, on exposure of the plants to stress conditions. Moreover, the promoter was found to be predominantly active in the whole grain, including endosperm, embryos, and aleurone layers, during seed development, and so is a stress-inducible promoter with activity in the whole grain.

Materials and methods

Development of *Wsi18::GFP* transgenic rice plants

Rice (*Oryza. sativa* cv Nakdong) plants was used for isolation of the promoter and for plant transformation. Genomic DNA was prepared from leaves of 14-day-old rice plants using the DNAzol ES plant genomic DNA isolation reagent (Molecular Research Center, USA) according to the manufacturer's instructions. The *Wsi18* promoter region (1.8 kb) (GQ903792) was amplified by PCR using a forward primer (5′-AAG CTTGAGTCATAGGGAGA-3′) and a reverse primer (5′-AGTGATTCCAGCCAAGTTTGGATCC-3′). The PCR product was inserted into a plasmid, generating the construct *Wsi18::GFP* that contains the *GFP* coding region under the control of the *Wsi18* promoter and the potato *Pin II* 3′ region. The construct also contains the bacterial phosphinothricin acetyl transferase gene (*bar*), a selectable marker gene for rice transformation, fused between the *35S* promoter and the polyadenylation

regions of the nopaline synthase. These cassettes are flanked by a pair of 5′-matrix attachment regions of the chicken lysozyme gene for stable expression of the transgene (Oh et al. 2005b). The *Wsi18::GFP* plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating. Embryonic calli from the mature seeds of rice were transformed by co-cultivation (Hiei et al. 1994), selected with 7 mg/l phosphinothricin, and used to regenerate transgenic plants as previously described by Jang et al. (2002). Three independent homozygous T_4 transgenic lines were used for promoter analysis.

Stress treatments and RNA blot analysis

For RNA blot analysis, transgenic and non-transgenic (NT) rice (*Oryza. sativa* cv Nakdong) seeds were germinated in half-strength Murashige–Skoog (MS) solid medium (Murashige and Skoog, 1962) in a growth chamber in the dark at 28°C for three days followed by in the light at 28°C for one day, transplanted into soil, and grown in a glasshouse (16 h light/8 h dark cycle) at 28–30°C. Each plant was grown in a pot (5 × 5 × 6 cm^3; six plants per pot) filled with rice nursery soil (Bio-media, Kyeongju, Korea) for 14 days after germination. Stress-treatment was performed as previously described (Oh et al. 2005a, 2007). For drought stress, 14-day-old seedlings were pulled from the soil and air-dried under continuous light of approximately 900–1,000 μmol m^{-2} s^{-1}. For high-salinity and ABA treatment, 14-day-old seedlings were grown in a nutrient solution, 0.1% (*v*/*v*) hyponex (Hyponex, Busan, Korea), for two days and then transferred to fresh nutrient solution containing 400 mM NaCl or 100 μM ABA in the greenhouse under continuous light of approximately 900–1,000 μmol m^{-2} s^{-1}. For low-temperature stress treatment, 14-day-old seedlings were exposed to 4°C at a cold-chamber under continuous light of 150 μmol m^{-2} s^{-1}. Total RNA extraction and RNA blot analysis were performed as previously reported (Jang et al. 2002; Jeong et al. 2009). Full-length cDNAs were used as a probe for *Wsi18* (AK064074), *Lip5* (AB011368), *Dip1* (AY587109), and *RbcS* (AK121444).

Detection of GFP fluorescence in transgenic plants

For the dry and the maturing seeds, GFP fluorescence was detected by use of a luminescent-image analyzer (LAS 3000; Fuji, Tokyo, Japan), with 460 nm blue light and the GFP 510E filter set, and a stereomicroscope (SZX9-3122; Olympus, Tokyo, Japan). The stereomicroscope was equipped with an attachment for fluorescence observations, and images were captured using a C5060-ZOOM digital camera (Olympus, Tokyo, Japan). For detection of GFP fluorescence in calli, we grew transgenic (*Wsi18::GFP* and *OsCc1::GFP*) and nontransgenic control calli in 2N6 medium containing 7 mg/l phosphinothricin for one month, and transferred them into the modified 2N6 medium containing phytagel (10 g/l), 400 mM NaCl or 100 μM ABA. In the indicated time course, GFP fluorescence was detected by use of the luminescent-image analyzer (LAS 3000). For detection of GFP fluorescence in leaves and roots, transgenic and NT seeds were germinated on hormone-free MS medium in darkness for three days at 28°C and further incubated for 11 days under continuous light to allow leaf development. The 14-day-old seedlings were exposed to stress conditions: for drought, they were pulled from the soil and air-dried for 2 h under continuous light of approximately 1,000 μmol m^{-2} s^{-1}; for NaCl and ABA, they were transferred to nutrient solution containing 400 mM NaCl for 2 h or 100 μM ABA for 6 h under continuous light of approximately 1,000 μmol m^{-2} s^{-1} in the glasshouse. The tissues of 14-day-old seedlings were visualized and photographed using a Carl Zeiss (Jena, Germany) LSM410 CLSM with a standard filter set at the Korea Basic Science Institute (Daejoen, Korea). Pseudocolor, similar to the color observed with a fluorescence microscope, was added to the images by importing data collected in the green and red channels of the confocal microscope. Sections were taken along the optical axis and were projected into a single image. For detection of GFP fluorescence in a whole plant body, transgenic seeds were germinated and grown on hormone-free MS medium in darkness for 6 days at 28°C and the etiolated seedlings were exposed in the time course to stress conditions in the dark as described for 14-day-old-seedlings. GFP fluorescence was observed by using the luminescent-image analyzer (LAS 3000). For detection of GFP fluorescence in transgenic flowers, transgenic plants were grown to the stage of meiosis in pots and water was withheld, in a greenhouse, for 3 days. GFP fluorescence was detected in a whole spikelet and floral organs inside

a spikelet, before and after drought treatment, by use of the luminescent-image analyzer (LAS 3000).

GFP protein extraction and immunoblotting

Approximately 50 mg transgenic rice seeds was ground in liquid-N_2 with a mortar and pestle. Ground tissues were homogenized with 0.3 ml 50 mM Tris–HCl (pH 8.0) buffer solution, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 2 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin for 1 h at 4°C. After homogenization, the mixture was clarified by centrifugation in Eppendorf tubes for 5 min at 4°C. The protein concentration was determined by use of Bradford solution (Bio-Rad, Hercules, CA, USA). Protein extracts were separated on 10% SDS–polyacrylamide gels and transferred to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus (BioRad). In the immunoblot analysis, GFP was detected using an alkaline-phosphatase-conjugated secondary goat anti-rabbit antibody for recognition of bound anti-GFP primary antibodies (Clontech, Palo Alto, CA, USA).

Results

Stress-inducible expression and production of *Wsi18::GFP* transgenic rice plants

To measure transcript levels of *Wsi18* in rice (*Oryza sativa* cv Nakdong) under stress conditions, RNA gel-blot analysis was performed using total RNA from leaf and root tissues of 14-day-old plants that were exposed to drought, NaCl, ABA, and low temperature (Fig. 1). *Dip1*, *Lip5*, and *RbcS*, whose transcript levels are known to be increased or reduced in response to stress treatments, were included as controls to monitor when stress-induced damage occurred (Oh et al. 2005a, 2007). *Wsi18* mRNA abundance increased in leaf tissues within 2 h of exposure to NaCl or ABA and within 6 h of exposure to drought, but there was no transcript increase in response to low-temperature conditions. *Wsi18*

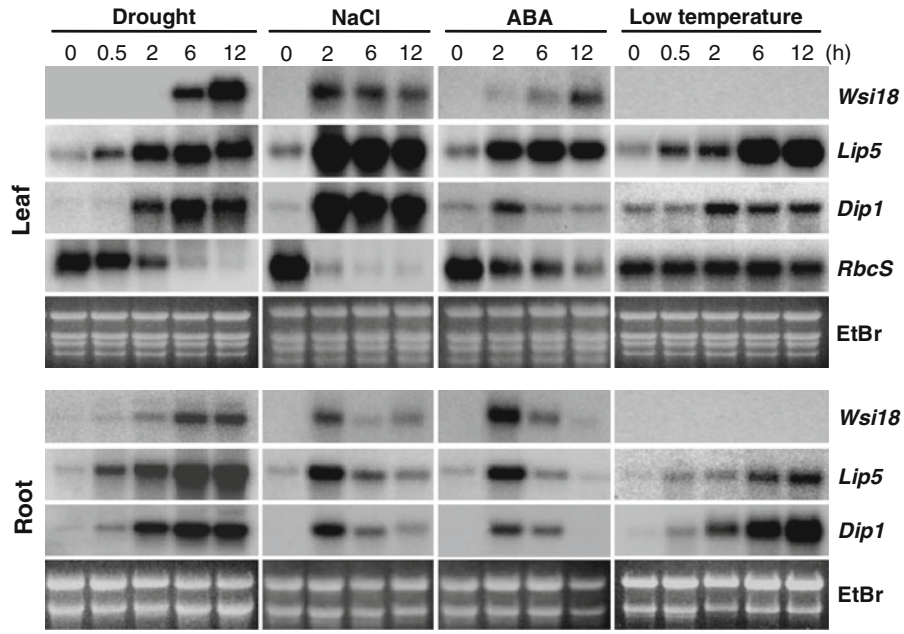

Fig. 1 Expression of the rice *Wsi18* gene in response to abiotic stress. Five micrograms of total RNA from the leaves and roots of 14-day-old plants exposed to drought, NaCl (400 mM), ABA (100 μM), and low temperature (4°C) for indicated time points, was blotted and hybridized to rice *Wsi18* (AK064074), *Lip5* (AB011368), *Dip1* (AY587109), and *RbcS* (AK121444) gene-specific probes. *Dip1*and *Lip5* were used as markers for up-regulation and *RbcS* for down-regulation on stress treatment (Oh et al. 2005a, 2007). Ethidium bromide (EtBr) staining of total RNA was used for equal loading of RNA

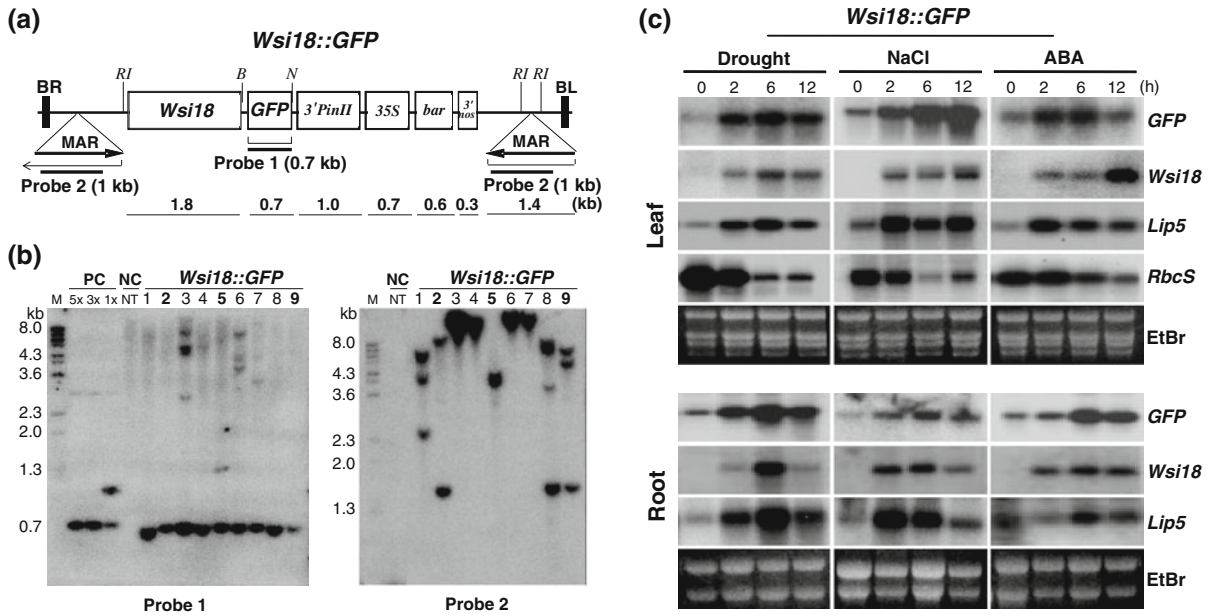

Fig. 2 Production of the *Wsi18::GFP* transgenic rice plants. **a** The construct for rice transformation *Wsi18::GFP* consists of the rice *Wsi18* promoter linked to the *GFP* coding region and the 3′-region of the potato proteinase inhibitor II gene (*3′PinII*), and a gene expression cassette that contains the *35S* promoter, the *bar* coding region, and the 3′-region of the nopaline synthase gene (*3′nos*). The entire expression cassette is flanked by the 5′-matrix attachment region (*MAR*) of the chicken lysozyme gene (Oh et al. 2005b). *Scale bars* representing the size of DNA fragments for each component are shown. **b** Genomic Southern blot analyses of *Wsi18::GFP* transgenic rice plants. Genomic DNAs from leaf tissues were digested with *Not*I, *Bam*HI, and *Eco*RI and hybridized with probes 1 and 2, respectively (shown in **a**). The DNA molecular size markers are indicated *on the left*. Transgenic lines (2, 5, and 9) that were analyzed in detail are shown in *bold letters*. *PC*, the plasmid DNA as a positive control; *NC*, non-transgenic rice plants as negative controls. **c** RNA blot analysis using total RNA from leaves and roots of the 14-day-old *Wsi18::GFP* plants (line 2 shown in **a**) exposed to stress conditions for indicated time points. The blots were hybridized with *Wsi18*, *Lip5*, *RbcS*, and *GFP* gene-specific probes. Ethidium bromide (EtBr) staining of total RNA was used for equal loading of RNA

mRNA accumulated in roots similarly to in leaves, but at a faster rate.

To investigate *Wsi18* promoter activity, the *Wsi18* promoter region was PCR-amplified from rice genomic DNA, and linked to the visible marker, the synthetic green fluorescent protein gene *GFP*, generating the construct *Wsi18::GFP* (Fig. 2a). The construct was transformed into rice by use of the *Agrobacterium*-mediated method (Hiei et al. 1994), which yielded 30 independent transgenic lines. All the transgenic lines were grown to maturity in a greenhouse. To determine transgene copy numbers and intactness of the *Wsi18::GFP* region in the transgenic plants, genomic Southern blots were performed using two probes, each specific to *GFP* and *Mar* sequences (Fig. 2b). We chose three independent transgenic lines with single (lines 2 and 5) or two (line 9) copies of the *Wsi18::GFP* construct that had not been rearranged in the *promoter:GFP* region. By self-pollinating and selecting of germinating transgenic seeds on phosphinothricin-containing MS media, we obtained T_3 homozygous lines and used these for further analysis.

To measure promoter activities in *Wsi18::GFP* plants under stress conditions, RNA gel-blot analysis was performed on total RNA from leaves and roots of 14-day-old *Wsi18::GFP* plants exposed to drought, NaCl, and ABA. *GFP* transcript levels were significantly increased after 2 h of exposure to drought, NaCl, and ABA in the leaves and the roots of the *Wsi18::GFP* plants (Fig. 2c and Supplementary Fig. 1), demonstrating the stress-inducible nature of the promoter. *GFP* transcripts were detected at low levels in the leaves and roots of transgenic plants at zero time of stress treatment and no endogenous *Wsi18* mRNA was detected under the same conditions. The different background levels may reflect different sensitivities of probes for *GFP* and *Wsi18* transcripts.

Wsi18 is a stress-inducible promoter in the whole plant, including flowers

To examine *Wsi18* promoter activity in a the whole plant under stress conditions, we analyzed *Wsi18::GFP* plants in comparison with *OsCc1:GFP* plants, transgenic rice plants with a constitutive promoter *OsCc1*: *GFP* construct (Jang et al. 2002), and non-transgenic (NT) plants in a time course of stress treatments. We treated calli, six-day-old etiolated, mature transgenic, and NT plants with drought, NaCl, and ABA (Fig. 3). For the whole vegetative plant, etiolated young seedlings, which are free of chlorophylls, were used for detection of GFP fluorescence in order to avoid interference from chlorophyll red fluorescence. Under normal growth conditions, no vegetative *Wsi18::GFP* and NT tissues showed any GFP fluorescence, with the exception of the elongating region of *Wsi18::GFP* roots, which showed a little background GFP fluorescence. Upon exposure to drought conditions, the activity of the *Wsi18* promoter was significantly enhanced in calli (Fig. 3a), the whole body of etiolated seedlings (Fig. 3b), and the mature leaves and mature roots of *Wsi18::GFP* plants (Fig. 3c

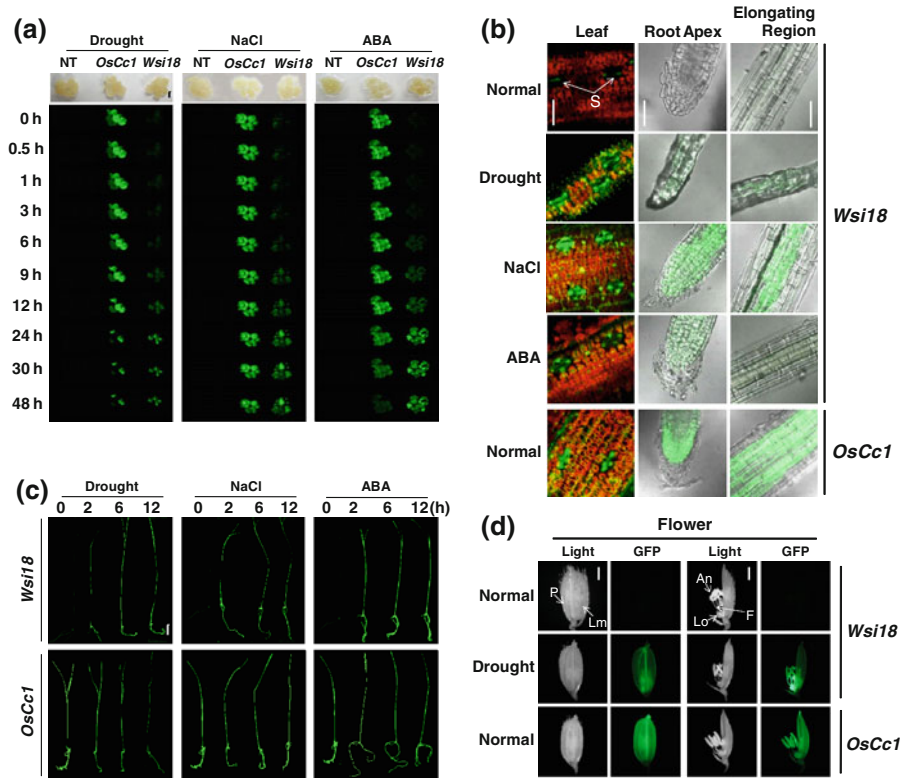

Fig. 3 Activity of the *Wsi18* promoter in different tissues and growth stages of transgenic rice plants (line 2 shown in Fig. 2b). **a** GFP fluorescence in transgenic calli. GFP fluorescence was detected in calli exposed to drought, NaCl (400 mM) and ABA (100 μM) for the indicated time points using a luminescent-image analyzer. *NT*, non-transgenic calli; *OsCc1*, calli induced from the *OsCc1::GFP* transgenic seeds (Jang et al. 2002); *Wsi18*, calli induced from the *Wsi18::GFP* transgenic seeds. *Bar* = 1 mm. **b** GFP fluorescence in whole bodies of the *Wsi18::GFP* and *OsCc1::GFP* plants. GFP fluorescence was detected in six-day-old etiolated plants exposed to drought, NaCl (400 mM), and ABA (100 μM) for the indicated time points using a luminescent-image analyzer. *Bar* = 1 cm. **c** Confocal microscopic images of GFP fluorescence in leaf and root tissues of transgenic plants. GFP fluorescence was detected in a leaf, a root apex, and a elongating region of a root of the *Wsi18::GFP* and *OsCc1::GFP* plants before (normal) and after exposure to drought, NaCl (400 mM), and ABA (100 μM) for 2 to 6 h. The images collected in the *green* and i channels of the confocal microscope were merged. *Bars* = 1 mm. *S*, stomata. **d** GFP fluorescence in transgenic flowers. GFP fluorescence was detected, by use of a stereomicroscope, in a whole spikelet (*left panel*) and floral organs inside a spikelet (*right panel*) at the stage of meiosis before (Normal) and after drought treatment. For drought treatment, water was withheld from plants in pots, in a greenhouse, for three days. *An*, anther; *F*, filament; *Lm*, lemma; *Lo*, lodicule; *P*, palea. *Bars* = 1 mm

and Supplementary Fig. 2). GFP fluorescence was particularly obvious in stomata and mesophyll cells of a leaf, and in the root cap and vascular cylinder of the root (Fig. 3c). In contrast, levels of GFP fluorescence in the corresponding tissues of the *OsCc1::GFP* plants remained constitutive before and after stress treatments. After 12 h or prolonged exposure of *OsCc1::GFP* calli to drought conditions, GFP fluorescence started to decrease, because of shrinkage of calli upon drying. Interestingly, GFP fluorescence was lower in *OsCc1::GFP* calli after 24 h of exposure to ABA by the time the *Wsi18* promoter became active, suggesting repression of the *OsCc1* promoter by prolonged exposure to ABA. We also examined GFP fluorescence in *Wsi18::GFP* flowers at the stage of meiosis under drought conditions (Fig. 3d). Under normal growth conditions, as in the vegetative tissues, no GFP fluorescence was detected in *Wsi18::GFP* flowers. After drought stress for three days, the *Wsi18::GFP* flowers showed a high level of GFP fluorescence in floral organs. Overall, activity of the *Wsi18* promoter was significantly enhanced by exposure to stress conditions in the whole plant, including flowers.

Wsi18 promoter is active in the whole grain during seed development

During seed maturation, expression of *LEA* genes is induced by a plant stress hormone, ABA (Finkelstein and Rock 2002). Expression of the *Wsi18* gene, a member of the group 3 *Lea* family, was expected to be induced in drying seeds as a whole. It remains elusive, however, whether it is induced in the embryos, endosperm, and/or aleurone layers of drying seeds. GFP fluorescence was detected at varying intensities, depending on transgenic lines, in dehulled whole dry *Wsi18::GFP* seeds including embryos and aleurone layers (Fig. 4a). Analysis of the longitudinal and cross-sectional slices of dry seeds, by use of a stereomicroscope, revealed that GFP fluorescence was clearly detected in the *Wsi18::GFP* endosperm and in its embryos and aleurone layers (Fig. 4b). In the *OsCc1::GFP* seeds, in contrast, GFP fluorescence was detected in embryos only (Fig. 4b). We examined GFP fluorescence of the *Wsi18::GFP* seeds in the time course of development after pollination under more stringent conditions (Fig. 4c). GFP fluorescence was particularly evident in embryos and aleurone layers on the dorsal side of the seeds under stringent conditions. Levels of GFP fluorescence steadily increased in the seeds, peaked at five to six weeks after pollination, and then remained relatively constant until seven weeks when seeds fully matured. To verify the increase in activity of the *Wsi18* promoter during seed development, GFP protein levels were measured by immunoblot experiments using total soluble proteins from developing seeds. Consistent with GFP fluorescence, protein levels increased in the *Wsi18::GFP* seeds as they matured (Fig. 4c). These results indicate that the *Wsi18* promoter is active in the whole grain, which drives expression of transgene in endosperm and in embryos and aleurone layers during seed development.

Discussion

Late embryogenesis abundant (LEA) proteins were initially characterized in mature cotton (*Gossypium hirsutum*) seeds (Galau et al. 1986). Later, many LEA proteins were identified in bacteria (Stacy and Aalen 1998), nematodes (Gal et al. 2004), mosses (Kamisugi and Cuming 2005), and in plants. During plant seed maturation, *LEA* gene expression is induced by ABA (Finkelstein and Rock 2002). Some of these *LEA* genes are induced in vegetative tissues of plants in response to dehydration, high salt, or exogenous ABA (Bies-Ethève et al. 2008; Joshee et al. 1998). Overexpression of some *LEA* genes in plants resulted in enhanced tolerance of abiotic stresses (Liu and Zheng 2005; Xu et al. 1996). All these previous observations suggest that *LEA* genes are important in plants, not only in seeds under normal conditions, but also in vegetative tissues under stress conditions. In this study, we analyzed the activity of the *Wsi18* gene promoter in transgenic rice plants during all stages of plant growth and seed development. The promoter drives high levels of GFP expression in the whole body of plants including calli, leaves, roots, and flowers on exposure of plants to drought and high salinity conditions. Moreover, it is active in a whole rice grain and the *Wsi18::GFP* seeds contain GFP not only in embryos and aleurone layers, but also in the endosperm. Up to now, activities of promoters derived from the *LEA* gene family have not been detected in endosperm. The *HVA1* promoter from barley, a member of the group 3 LEA family, was active in

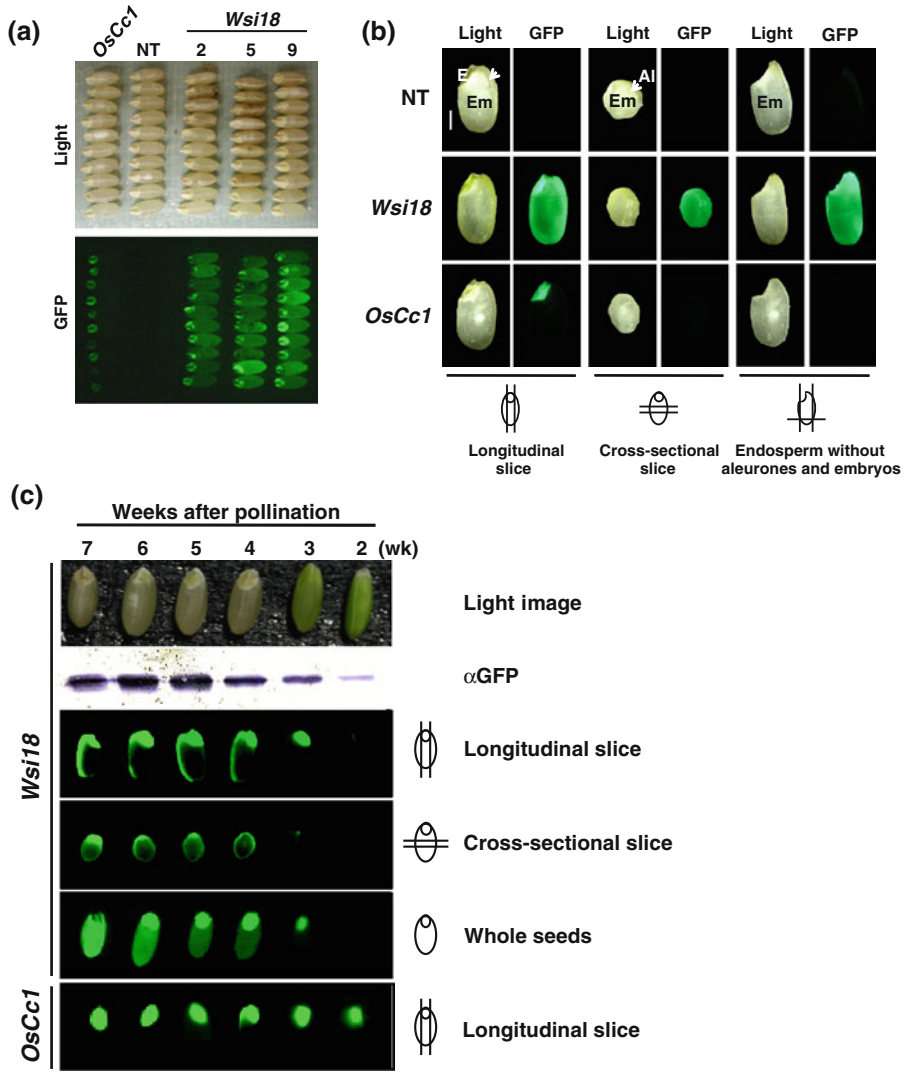

Fig. 4 Activity of the *Wsi18* promoter in developing and dry seeds. **a** GFP fluorescence was detected in dehulled dry seeds by use of a luminescent-image analyzer. **b** GFP fluorescence was detected in hand-cut longitudinal and cross-sectional slices of dry seeds by use of a stereomicroscope. *E*, embryo; *Em*, endosperm; *Al*, aleurone layer. *Bar* = 2 mm. **c** Increase in levels of GFP fluorescence and proteins during seed maturation. GFP fluorescence was detected in the developing seeds of the *Wsi18::GFP* plants at the indicated weeks after pollination, by use of a luminescent-image analyzer. *αGFP* represents the antibody used in immune-blot analysis of GFP protein in the *Wsi18::GFP* seeds. *NT*, non-transgenic seeds; *OsCc1*, *OsCc1::GFP* transgenic seeds; *Wsi18*, *Wsi18::GFP* transgenic seeds. *Bars* = 2 mm

aleurone layers of transgenic barley (Straub et al. 1994), and in the leaves and roots of transgenic barley after exposure to stress conditions (Xiao and Xue 2001). The rice *Rab16A* promoter (Ono et al. 1996), a member of the group 2 LEA family, had similar activity in seed embryos and in vegetative tissues under stress conditions. The wheat *Em* promoter, a member of the group 1 LEA family, was only active in the embryos and aleurone layers of transgenic barley, rice (Furtado and Henry 2005), and tobacco (Marcotte et al. 1989). In our *Wsi18::GFP* seeds, we could clearly detect GFP fluorescence in endosperm, as evidenced by analyzing longitudinal and cross-sectional slices of dry seeds lacking embryos and aleurone layers (Fig 4b). As shown in Fig. 4c, where GFP fluorescence was observed under more stringent conditions, our *Wsi18* promoter is predominantly more active in embryos and aleurone layers than in

endosperm. It might well be that the *Wsi18* promoter is active during early endosperm development and that the fluorescence is because of a stable GFP protein in otherwise metabolically inactive tissue.

Expression levels of the endogenous *Wsi18* gene (Fig. 1) and its promoter activity (Figs. 2b and 3) in *Oryza sativa* cv Nakdong were both elevated in the whole plant at the vegetative stage by the plant stress hormone, ABA. Interestingly, the ABA-dependent expression of our *Wsi18* gene is consistent with that of *Wsi18j* from *Oryza sativa* cv Jarrah (Xiao and Xue 2001), but not with *Wsi18* from *Oryza sativa* cv IR36 (Joshee et al. 1998). Database search for nucleotide sequences covering the *Wsi18* promoter regions led to identification of ABRC (ABA-responsive complex) elements in three japonica types (Nakdong, Jarrah and Nipponbare) and one indica type (IR36) (Table 1). Other motifs including the DRE/CRT-core motif (GCCGAC) and G-box (CACGTG) (Joshee et al. 1998) are all conserved in the promoter sequences except for the ABRC motif. Multiple alignments of the promoter sequences and expression patterns of the *Wsi18* genes reported in the literature revealed that one mismatched base pair in inter-element spacer regions of the CE (coupling element) and the ABRE (ABA-responsive element) (Shen et al. 2004) seems to determine the ABA-responsive expression of the gene (Table 1). More specifically, two cultivars that are induced by ABA contain a perfect match in the spacer regions; whereas one cultivar that is not induced by ABA carries a single base-pair deletion (Table 1). We speculate that the absence of ABA inducibility in the IR36 cultivar *Wsi18* promoter may be because of a 1-bp deletion in the spacer region. The presence of a CE specifies ABA responsiveness in combination with an ABRE. In the promoter of barley *HVA22* gene, a CE1 (TGCCACCGG) is necessary for high-level ABA induction together with ABRE3 (GCCACGTACA), and replacement of either of these sequences abolished ABA responsiveness (Shen and Ho 1995). Shen et al. (2004) also demonstrated that in the ABRC3 complex of the barley *HVA1*, the CE3 and ABRE2 are next to each other, and the distance between the elements is essential for such an ABA response. In our *Wsi18* promoter, the CE3 element lies immediately upstream of ABRE2 element, just like in the barley *HVA1*, another member of the group 3 LEA gene family. The inter-element spacer between the two elements in our *Wsi18* (5′-C**C**CCCT-3′) differs in composition from that of the barley *HVA1* (5′-C**T**C CCT-3′) (Table 1), but is identical in length. These observations suggest that the specific response to ABA of our *Wsi18* promoter in rice is mediated by the length of the inter-element spacer in addition to the presence of the CE3 and ABRE2 core sequences. Recently, Ross and Shen (2006) also reported the importance of the inter-element spacing of *HVA1*-like ABA responsive rice promoters.

Cereal seeds can be used as an ideal platform for production of various compounds, for example proteins, industrial enzymes and pharmaceuticals (Barro et al. 1997; Cahoon and Shanklin 2000; Shintani and DellaPenna 1998; Takaiwa et al. 2007). Seed-specific promoters are essential components of the engineering and have received more attentions. Qu and Takaiwa (2004) analyzed activities of various seed promoters in transgenic rice—endosperm-specific

Table 1 Comparison of inter-element spacer regions between CE3 and ABRE2 core sequences of rice *Wsi18* and barley *HVA1* promoters

Cultivar	Gene	CE3 and ABRE2 complex	Subspecies	ABA response	Sequence	Reference
Nakdong	*Wsi18*	A**ACGCGTGTC**CCCCCT**ACGT**GGCC	Japonica	Induction	GQ903792	This study
Jarrah	*Wsi18j*	A**ACGCGTGTC**CCCCCT**ACGT**GGCC	Japonica	Induction	AF246702 (99%)	Xiao and Xue (2001)
Nipponbare	*Wsi18*	A**ACGCGTGTC**CCCCCT**ACGT**GGCC	Japonica	N/A	AP003381 (99%)	N/A
IR36	*Wsi18*	A**ACGCGTGTC**-CCCCT**ACGT**GGCg	Indica	No induction	AB001682 (97%)	Joshee et al. (1998)
Himalaya	*HVA1*	A**ACGCGTGTC**CTCCCT**ACGT**GGCG	Vulgare	Induction	X78205	Ross and Shen (2006)

N/A not available

Bold-face letters denote CE3 and ABRE2 core sequences; underlined letters denote inter-element spacer region sequences

promoters such as *glutelin* and *prolamin*, embryo and aleurone layer-specific promoters such as *REG-2*, *Ole18* and *β-conglycinin*, and the whole seed-specific promoter *AGPase* small subunit. The *Wsi18*, a stress-inducible promoter with activity in the whole grain will increase the potential to express foreign genes in transgenic cereal crops for the bioreactors and for study of seed development.

Acknowledgments This work was supported by the Ministry of Education, Science and Technology, Korea, through the Crop Functional Genomics Center (CG2111 to J.-K.K), and by the Rural Development Administration, through the Biogreen21 Program (PJ007149 to J.-K.K. and S.I.S.).

References

Barro F, Rooke L, Békés F, Gras P, Tatham AS, Fido R, Lazzeri PA, Shewry PR, Barceló P (1997) Transformation of wheat with high molecular weight subunit genes results in improved functional properties. Nature Biotechnol 15: 1295–1299

Bies-Ethève N, Gaubier-Comella P, Debures A, Lasserre E, Jobet E, Raynal M, Cooke R, Delseny M (2008) Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*. Plant Mol Biol 67:107–124

Cahoon EB, Shanklin J (2000) Substrate-dependent mutant complementation to select fatty acid desaturase variants for metabolic engineering of plant seed oils. Proc Natl Acad Sci USA 97:12350–12355

Capell T, Escobar C, Liu H, Burtin D, Lepri O, Christou P (1998) Over-expression of the oat arginine decarboxylase cDNA in transgenic rice (*Oryza sativa* L.) affects normal development patterns in vitro and results in putrescine accumulation in transgenic plants. Theor Appl Genet 97: 246–254

Coca MA, Almoguera C, Thomas TL, Jordano J (1996) Differential regulation of small heat-shock genes in plants: analysis of a water-stress-inducible and developmentally activated sunflower promoter. Plant Mol Biol 31:863–876

Finkelstein RR, Rock CD (2002) Abscisic acid biosynthesis and response. In: Meyerowitz E, Somerville C (eds) *The Arabidopsis book*. Rockville: American Society of Plant Biologists. doi:10.1199/tab.0058. http://www.aspb.org/publications/arabidopsis/

Furtado A, Henry RJ (2005) The wheat *Em* promoter drives reporter gene expression in embryo and aleurone tissue of transgenic barley and rice. Plant Biotechnol J 3:421–434

Gal TZ, Glazer I, Koltai H (2004) An LEA group 3 family member is involved in survival of *C.* elegans during exposure to stress. FEBS Letters 577:21–26

Galau GA, Hughes DW, Dure L (1986) Abscisic acid induction of cloned cotton late embryogenesis-abundant (*Lea*) mRNAs. Plant Mol Biol 7:155–170

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hsieh TH, Lee JT, Yang PT, Chiu LH, Yy Charng, Wang YC, Chan MT (2002) Heterology expression of the Arabidopsis *C-repeat/dehydration response element binding factor 1* gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiol 129: 1086–1094

Jang IC, Choi WB, Lee KH, Song SI, Nahm BH, Kim JK (2002) High-level and ubiquitous expression of the rice cytochrome c gene *OcCc1* and its promoter activity in transgenic plants provides a useful promoter for transgenesis of monocots. Plant Physiol 129:1473–1481

Jang IC, Oh SJ, Seo JS, Choi WB, Song SI, Kim CH, Kim YS, Seo HS, Choi YD, Nahm BH, Kim JK (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress-tolerance without stunting growth. Plant Physiol 131:516–524

Jang CS, Lee HJ, Chang SJ, Seo YW (2004) Expression and promoter analysis of the *TaLTP1* gene induced by drought and salt stress in wheat (*Triticum aestivum* L.). Plant Sci 167:995–1001

Jeong JS, Park YT, Jung H, Park SH, Kim JK (2009) Rice NAC proteins act as homodimers and heterodimers. Plant Biotechnol Rep 3:127–134

Joshee N, Kisaka H, Kitagawa Y (1998) Isolation and characterization of a water stress-specific genomic gene, *pwsi18*, from rice. Plant Cell Physiol 39:64–72

Kamisugi Y, Cuming AC (2005) The evolution of the abscisic acid-response in land plants: comparative analysis of group 1 *LEA* gene expression in moss and cereals. Plant Mol Biol 59:723–737

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 17:287–291

Lee JH, Schöffl F (1996) An *Hsp70* antisense gene affects the expression of HSP70/HSC70, the regulation of HSF, and the acquisition of thermotolerance in transgenic *Arabidopsis thaliana*. Mol Gen Genet 252:11–19

Lessard PA, Kulaveerasingam H, York GM, Strong A, Sinskey AJ (2002) Manipulating gene expression for the metabolic engineering of plants. Metab Eng 4:67–79

Liu Y, Zheng Y (2005) PM2, a group 3 LEA protein from soybean, and its 22- mer repeating region confer salt tolerance in *Escherichia coli*. Biochem Biophys Res Commun 331:325–332

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10:1391–1406

Marcotte WRJ, Russell SH, Quatrano RS (1989) Abscisic acid-responsive sequences from the *Em* gene of wheat. Plant Cell 1:969–976

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nakashima K, Tran LSP, Nguyen DV, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH, Kim JK (2005a) Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol 138:341–351

Oh SJ, Jeong JS, Kim EH, Yi NR, Yi SI, Jang IC, Kim YS, Suh SC, Nahm BH, Kim JK (2005b) Matrix attachment region from the chicken lysozyme locus reduces variability in transgene expression and confers copy number-dependence in transgenic rice plants. Plant Cell Rep 24:145–154

Oh SJ, Kwon CW, Choi DW, Song SI, Kim JK (2007) Expression of barley *HvCBF4* enhances tolerance to abiotic stress in transgenic rice. Plant Biotechnol J 5:646–656

Oh SJ, Kim YS, Kwon CW, Park HK, Jeong JS, Kim JK (2009) Overexpression of the transcription factor *AP37* in rice improves grain yield under drought conditions. Plant Physiol 150:1368–1379

Ono A, Izawa T, Chua NH, Shimamoto K (1996) The *rab16B* promoter of rice contains two distinct abscisic acid-responsive elements. Plant Physiol 112:483–491

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. Genome 47:493–500

Potenza C, Aleman L, Sengupta-Gopalan C (2004) Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. In Vitro Cell Dev Biol 40:1–22

Qu LQ, Takaiwa F (2004) Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. Plant Biotechnol J 2:113–125

Rai M, He C, Wu RJ (2009) Comparative functional analysis of three abiotic stress-inducible promoters in transgenic rice. Transgenic Res 18:787–799

Romero C, Bellés JM, Vayá JL, Serrano R, Culiáñez-Macià FA (1997) Expression of the yeast *trehalose-6-phosphate synthase* gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta 201:293–297

Ross C, Shen QJ (2006) Computational prediction and experimental verification of HVA1-like abscisic acid responsive promoters. Plant Mol Biol 62:233–246

RoyChoudhury A, Roy C, Sengupta DN (2007) Transgenic tobacco plants overexpressing the heterologous *lea* gene *Rab16A* from rice during high salt and water deficit display enhanced tolerance to salinity stress. Plant Cell Rep 26:1839–1859

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J 23:319–327

Shen Q, Ho THD (1995) Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a nove1 *cis*-acting element. Plant Cell 7:295–307

Shen QJ, Casaretto JA, Zhang P, Ho THD (2004) Functional definition of ABA-response complexes: the promoter units necessary and sufficient for ABA induction of gene expression in barley (*Hordeum vulgare* L.). Plant Mol Biol 54:111–124

Shintani D, DellaPenna D (1998) Elevating the vitamin E content of plants through metabolic engineering. Science 282:2098–2100

Shobbar ZS, Oane R, Gamuyao R, Palma JD, Malboobi MA, Karimzadeh G, Javaran MJ, Bennett J (2008) Abscisic acid regulates gene expression in cortical fiber cells and silica cells of rice shoots. New Phytol 178:68–79

Stacy RAP, Aalen RB (1998) Identification of sequence homology between the internal hydrophilic repeated motifs of Group 1 late-embryogenesis-abundant proteins in plants and hydrophilic repeats of the general stress protein GsiB of *Bacillus subtilis*. Planta 206:476–478

Straub PF, Shen Q, Ho THD (1994) Structure and promoter analysis of an ABA- and stress-regulated barley gene, *HVA1*. Plant Mol Biol 26:617–630

Takahashi R, Joshee N, Kitagawa Y (1994) Induction of chilling resistance by water stress, and cDNA sequence analysis and expression of water stress-regulated genes in rice. Plant Mol Biol 26:339–352

Takaiwa F, Takagi H, Hirose S, Wakasa Y (2007) Endosperm tissues is good platform for artificial recombinant proteins in transgenic rice. Plant Biotechnol J 5:84–92

Wang H, Datla R, Georges F, Loewen M, Cutler AJ (1995) Promoters from *kinl* and *cor6.6*, two homologous *Arabidopsis thaliana* genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. Plant Mol Biol 28:605–617

Xiao FH, Xue GP (2001) Analysis of the promoter activity of late embryogenesis abundant protein genes in barley seedling under conditions of water deficit. Plant Cell Rep 20:667–673

Xiao B, Huang Y, Tang N, Xiong L (2007) Over-expression of a *LEA* gene in rice improves drought resistance under the field conditions. Theor Appl Genet 115:35–46

Xu D, Duan X, Wang B, Hong B, Ho THD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol 110:249–257

Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol Gen Genet 236:331–340