

Analysis of the promoter activity of a wheat dehydrin gene (DHN-5) under various stress conditions

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Abstract

A wheat dehydrin gene (DHN-5) is inducible by either treatment with abscisic acid (ABA) or by abiotic stress such as drought and salinity. To further investigate the regulation of the gene, a 1,128-bp genomic fragment upstream of the DHN-5 translated sequence has been isolated, cloned, and designated as the "PrDHN-5" promoter. Sequence analysis of PrDHN-5 revealed the presence of cis-regulatory elements which could be required for abiotic stress and ABA responsiveness. The PrDHN-5 promoter was fused to the β -glucuronidase (*gusA*) gene and the resulting construct was transferred into *Arabidopsis thaliana* (heterologous dicotyledonous system) and wheat (homologous monocot system). Histochemical assays of stably transformed *Arabidopsis* plants showed that PrDHN-5 is active in this heterologous system. The accumulation of *gusA* transcripts was up-regulated by salt, osmotic and ABA treatment. Histochemical assay in wheat embryos showed a staining in callus and younger leaves treated with various stress. Altogether, these results show that PrDHN-5 is an abiotic-stress-inducible promoter.

Keywords: Abiotic stresses; *Arabidopsis*; Gus-activity; PrDHN-5 promoter; Transgenic plants; Wheat.

Abbreviations: ABA_abscisic acid; ABREs_ABA responsive cis-element; Dhn_dehydrin; *gusA* β -glucuronidase; LEA_late embryogenesis abundant; PrDHN5_DHN5 promoter; Rab_response to ABA.

Introduction

Plants can be subjected to harsh environments all through their life cycle, and environmental stresses such as drought, salinity, and low temperature severely limit plant productivity worldwide. Important advances have been made in understanding the transcriptional changes induced by environmental constraints and in identifying the signalling proteins and transcription factors which regulate stress-induced gene expression (Mazzucotelli et al., 2008). These findings supported use of the transgenic approach as one strategy for improving the tolerance of plants to single or multiple abiotic stresses. With that objective, promoters affording strong constitutive expression, for example the cauliflower mosaic virus 35S promoter, the rice *act1* gene promoter, and the maize ubiquitin gene promoter (Odell et al., 1985; McElroy et al., 1990; Christensen et al., 1992; Cornejo et al., 1993) have been widely used to express genes conferring abiotic stress tolerance. However, it has been shown that high constitutive expression of the foreign gene may be detrimental to the host plant, with independent reports of increased sterility, retarded development, abnormal morphology, yield penalty, altered grain composition, or transgene silencing (Sinha et al., 1993; Kurek et al., 2002; Xu et al., 2006; Kanneganti and Gupta, 2008). The use of a strong, tissue-specific, or inducible promoter to restrict gene expression to the required tissue, at a particular development stage and/or in response to a stress may solve this type of problem (Karim et al., 2007; Pino et al., 2007). Until now, numerous inducible promoters have been isolated from a

wide variety of organisms. Among these, the biotic and abiotic stress-inducible *rd29A* promoter has been widely used to minimize the otherwise negative effect on plant growth of transgene expression in plants such as *A. thaliana*, tobacco, wheat, sugarcane and potato (Sun and Chen, 2002; Kasuga et al., 2004; Gao et al., 2005; Zhang et al., 2005; Behnam et al., 2006; Wu et al., 2008). Therefore, it is essential that these transgenes are switched on only during stress conditions under the control of drought-inducible promoters. In order to develop drought-inducible expression systems, it is essential to evaluate the inducibility, promoter strength and timing of induction of the selected promoter regions of the drought-inducible genes. Accordingly, promoters from highly expressed genes under dehydration conditions, such as *LEA* genes (late embryogenesis abundant), are potentially good candidates. The expression of most of these genes in vegetative tissues is up-regulated in conditions of water deficit, salinity and low temperature as well as by the application of exogenous ABA. Many proteins accumulated in response to dehydration caused by drought, salt or during seed development belong to the group 2 *LEA/RAB/dehydrin* family. Many *LEA* genes from small grain cereals, such as barley *HVA1* (Straub et al., 1994), rice *ws18* (Joshee et al., 1998), barley dehydrin (*Dhn*) (Choi et al., 1999) and rice *Rab* (response to ABA) (Yamaguchi-Shinozaki et al., 1989), have been isolated and the promoter function of many of these genes were characterized. Although *LEA* proteins probably play an important role in seed development and/or

germination, it is the response of *LEA* genes to ABA which has attracted the most attention. ABA is known to play an important role in several aspects of seed development including suppression of precocious germination, induction of accumulation of reserve proteins, acquisition of desiccation tolerance and induction of dormancy (Black, 1991; Hilhorst, 1995). Some of these effects clearly involve regulation of gene expression, and an increasingly large number of ABA responsive genes have been isolated (Thomas et al., 1991; Chandler and Robertson, 1994; Hull et al., 1993). Functional analysis of the promoters of ABA responsive genes has led to the identification of ABA responsive cis-element (ABREs) (Thomas, 1993; Giraudat et al., 1994). ABREs identified in plant promoters generally contain an ACGT core sequence characteristic of G-boxes, which are the recognition sequences of leucine zipper (bZIP) transcription factors. Cloning of transcription factors which specifically recognise ABREs has shown that they are indeed bZIP proteins, and has allowed the sequence requirements of high affinity binding to be analysed *in vitro* (Williams et al., 1992; Isawa et al., 1993). ABA is perceived by soluble ABA receptors, named PYR/PYL/RCAR and offers an elegant and unprecedented mechanism to control phosphorylation signalling cascades in a ligand-dependent manner (Santiago et al., 2012). The PYR/PYL/RCAR receptor family consists of 14 members participating in an ABA receptor. Signal complex that responds to the hormone by activating the transcription of ABA-responsive genes (Klingler et al., 2010). The wheat dehydrin gene (*DHN-5*) is induced during late embryogenesis when ABA levels are high, and it is also water and salt stress inducible in vegetative tissues (Brini et al., 2007a). In this work, we isolated and characterized the promoter sequence of the wheat dehydrin gene (*DHN-5*) and we analyzed its activity in heterologous and homologous systems under various stress conditions.

Results

Isolation and *in silico* analysis of the *PrDHN-5* promoter

A 1128 bp genomic DNA fragment upstream of the *DHN-5* gene, including 76 bp of the 5'-UTR, was isolated from wheat by the HE-TAIL-PCR method. During tertiary cycling, two major specific bands were amplified using G3/R2 and G4/R2 primers but no amplification was detected with R2/R2 primers (Fig. 1). The cloned 1,128-bp fragment generated with the G3/R2 primers (*PrDHN-5*) was sequenced (Fig. 2). Analysis of this sequence by Proscan software (version 1.7, HU<http://www-bimas.cit.nih.gov/cgi-bin/molbio/proscanUH>) showed the presence of a putative transcription start site (TSS: +1) and a TATA box at position -35 (relative to TSS) (Fig. 2). These findings were consistent with the regular features of eukaryotic promoters (Ke et al., 1997). Blast search of *PrDHN-5* against the plant promoter databases PLACE and PlantCARE also predicted the presence of some cis-regulatory elements related to tissue-specific gene expression (root specific), abiotic (dehydration, salt, and light induction), biotic (fungal elicitor), and hormone (ABA, gibberellins, auxins) stress responses (Table 1). In addition, several potential binding sites for transcription factors such as MYB, MYC, Dof and WRKY were also identified (Table 1).

Generation of transgenic *Arabidopsis* with the *PrDHN-5-gusA* construct

To precisely define the expression pattern of the *PrDHN-5-gusA* fusion, pCAMBIA1391Z-*PrDHN-5-gusA* T-DNA was

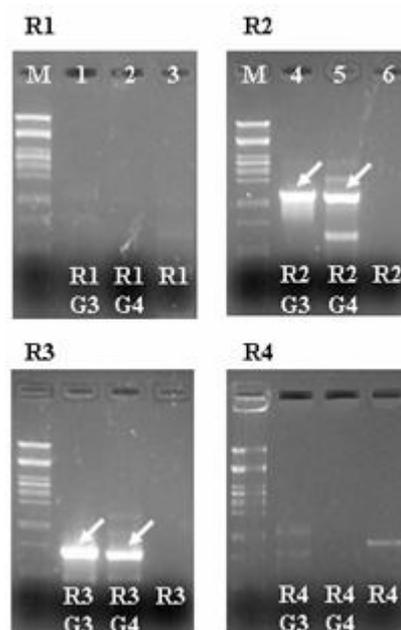


Fig 1. Agarose gel analysis of tertiary HE-TAIL-PCR products using four different degenerated primers (R1, R2, R3, R4) to isolate the promoter region of the wheat *DHN5* gene. The gene specific and random primers used in every reaction are marked at the bottom. M, is lambda *PstI* molecular weight marker.

introduced by *A. tumefaciens* transformation in several independent transgenic *Arabidopsis* lines (Fig. 3). T-DNA integration and *gusA* gene transcription were confirmed in four transgenic events by PCR (Fig. 3B). When β -glucuronidase activity was monitored by histochemical staining in these transgenic events, comparable patterns of *gusA* gene expression were observed in the T2 generation. Accumulation of β -glucuronidase activity and *gusA* transcript were monitored by histochemical staining and RT-PCR, respectively (Fig. 4).

PrDHN-5 is an organ specific and stress-inducible promoter in *Arabidopsis* transgenic plants

It has been previously reported that expression of the *DHN-5* gene is induced by abiotic and hormonal stresses in wheat (Brini et al., 2007a). Here, β -glucuronidase (*GUS*) expression was examined under control, NaCl, mannitol or ABA treatment in transgenic *Arabidopsis* harboring the *PrDHN-5-gusA* construct (Fig. 3A). Histochemical staining of whole plant seedlings grown under control conditions did not enable detection of any *GUS* activity (Fig. 4A). In contrast, *GUS* activity was observed in leaves and in roots of transgenic lines (Fig. 4A). The intensity of staining was clearly affected by the type of stress, with deeper blue staining of tissues treated with ABA. In addition, the most intense *GUS* staining was observed in roots and leaves, compared to stem parts (Fig. 4A). These results indicate that *PrDHN-5* is an organ-specific promoter and the activity of the *PrDHN-5* promoter was shown to be strongly enhanced by abiotic stresses (Fig. 4A). Stress treatment has led to an intense *GUS* staining in roots and leaves compared with weaker *GUS* activity under control conditions in similar tissues (Fig. 4A). These findings are in agreement with the presence of predicted cis-regulatory elements related to abiotic stress responses (Table 1).

Table 1 Putative *cis*-acting elements present in the PrDHN-5 promoter of the DHN-5 gene.

Function/responsive	Putative cis-elements /consensus	Position ^a	References
ABA responsive elements	ABRE	+115, +171, -273, -275	PlantCare
Salicylic acid response element	TCA-element	- 830	PlantCare
Light-responsive elements	Sp1	-99, -165, -194,	PlantCare
	G-Box	+275, -146, -171, -259	PlantCare
	ACE	-857	PlantCare
Anaerobic induced gene elements	GC-motif	-164	PlantCare
Fungal elicitor Responsive element	Box-W1	+680	PlantCare
Giberrelin- responsive element	P-Box	-1080, -1083	PlantCare
Auxin- responsive element	TGA-element	-830	PlantCare
Tissue specific Expression elements	GATABOX	-425, -712, -978 +172, +363, +423	Lam and Chua (1989)
	CACTFTPPCA1	-175, -208, - +85, +413, +859	Gowik et al. (2004)
Core promoter element around -30 of transcription start	TATABOX5	-802	Tjaden et al. (1995)
Transcription factor binding sites	DOFCOREZM	-118, -256, -770 +235, +1156, +1200	Yanagisawa and Schmidt (1999)
	MYBCORE	-538, -617, +354 +1069	Urao et al. (1993)
	MYBCOREATCYCB1	-1069, +617	Planchais et al. (2002)
	WRKY71OS	-178, -522, -899 -1149, +77	Zhang et al. (2004)

^a Position of the *cis*-elements upstream ATG; +, positive strand; -, minus strand.

Accumulation of GUS transcripts in relation to stress treatment in Arabidopsis

To further ascertain the induced activity of the PrDHN-5 promoter, RT-PCR was used to detect the presence of gusA transcripts in transgenic lines under various stress conditions (Fig. 4B). Accumulation of gusA transcripts was both detected and found up-regulated by NaCl, mannitol and ABA treatment (Fig. 4B). These results are in complete agreement with those of the histochemical staining assays. Our results have demonstrated that the PrDHN-5 promoter directs transcription of the gusA gene which is induced by abiotic and hormonal stress in roots and leaves but not in stems.

Generation of transgenic wheat seedlings overexpressing the PrDHN-5-gusA construct

The promoter constructs PrDHN-5-gusA and Ubiquitin-1-gusA were used for transformation of freshly excised embryos. Initially, embryogenic callus shoots and other regenerating tissues were sacrificed at 4-8 weeks after *Agrobacterium* inoculation in order to test for GUS activity. The frequency of GUS-stained structures ranged from 4% to 20%, thereby confirming successful *uidA* gene integration and the development of some regenerative tissue. GUS-positive plantlets were considered to be putative positive transgenics and were transferred to soil when a good root

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AAACAGGGAGTGATCCCTTGTAAATTTGCATGAAGATCATAGAAATATACTCGTAGCTCA -1068
TACCTAAATATTGTGCTGACAGCGCACTACTCCAAAATTTAATCAATAAAACATGCCTT -1008
CAAT-box CAAT-box
TTGAGAGAAGCATAACAGACATAGATCTAGATCCTTGAGCTGCTGTAGTTGGATAGTGTG -948
ACCGGGCGGTTAGTGGTAGATTGAGTATGCCCTCACACAGAGGCGCTGCTGCAAAAAGAC -888
AAAATCGAGGCACTGCTTTCAACAAAATDAGATCCCCATCTGGATTTCAAGTGAAGACGACGC -828
CAAT-box
TCCATTGGTACATGATTGAGAGGGATCAATGAAATTTACTAACCTATTTCCGCTGTGG -768
CAAT-box
AGTGTACAGAAGAGGGAGAGAGAGAGAGAGAAAGAGAGAATACTGATTTTCGTTGAAGAT -708
CTGATATCGGTTCTTGAGCTCGCTGGACCTTAACAATGAACCCAGGTGGCTGCACTGGTA -648
CAAT-box
CAAAATCTGGCAAGGGGAAGAACAAGATCTGGATAGGTGAGGTCAAAATTGCTGCATTTAA -588
CAAT-box
CAGCACTGCAAGGATTGTTCTGCAATGTGCCAGCAGTCCCATCATCGCACTGAATCCTGT -528
CAAT-box
CCTTCTATTTTGAATCCAACGGCCATGTATCGCTGGAGCATCTGGTGACCCAGTTTGCTC -468
GTTTCACCAGATACATTTCTCATATGAGCACCTCCGAGAGACTGAGTCGGCATATCATCTT -408
GAGATTTACGAACCCCGTAGGCGCTCGTCTCGACGAAACGCTCTCCTTCACTGAA -348
AGCGCACCCCTAAAATCCTGAATAATTCAGGAATAATGCGAGCACCAAAATTCAAATC -288
TTGGTGGGTTGAAGATACCACTGTCCACTTGGTTCGCAACGAGGAGCTAATCTGTCAAGT -228
CACCTTCCAGAACAGTCAATCGCTACCTGGCGCCACCGGCACGTTTCAGGATCTCGGCG -168
ABRE-box
CGTGACACGCTCCCGGCTATCCGTACGCCCCACGCCGACCAATGCGCCTCCCGACTCACC -108
CAAT-box
ACGCATGCACACCTACCCCCGGCTCAGTAGCCACCTGGCCTGCCACATCCGTTACAAGGC -48
ABRE-box GC-motif
CTGTGCTACGGTATAAATTCCACCCGGCAATGCACTTCCCTGCACACAACSACTCACC 13
TATA-box
AGSTTTCTGTGACCAAGACAAAAGCTAAAAGCCACAACCAAGTCCAGTTTAGGAGGCA 73
AAGATG

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Fig 2. Nucleotide sequence of the PrDHN-5 promoter with the UTR (76 bp) of the DHN-5 gene. Numbers indicate the position relative to the transcription start site +1. The putative TATA box, CAAT box and other important putative cis-elements are boxed and labelled.

system had been established. Molecular analysis of the plants that were identified by GUS expression was done by PCR-positive for *uidA*. Histochemical GUS activity was observed in embryogenic callus and young seedling plants to determine how the promoter is regulated in vegetative tissue.

PrDHN-5 is salt-inducible promoter in wheat transgenic plants

To further investigate the time course of PrDHN-5/GUS expression, histochemical staining in the transgenic wheat was determined. GUS activity in mature embryos and in leaf seedlings treated with NaCl was higher compared to GUS activity in non-transformed seedlings that remained undetectable throughout the experiment (Fig. 5 and 6). Transgenic plants grown under normal conditions displayed no GUS activity in the leaves. These results indicate that the promoter PrDHN-5 is inducible by salt stress.

Discussion

The over-accumulation of stress regulators by using strong constitutive promoters has, in many cases, improved stress tolerance (Kasuga et al., 1999; Hsieh et al., 2002; Ito et al.,

2006). Nevertheless, this enhanced stress tolerance is sometimes conferred at the expense of plant development and growth (Sinha et al., 1993; Kurek et al., 2002; Xu et al., 2006; Kanneganti and Gupta, 2008). The use of stress inducible promoters is expected to be optimized for driving candidate abiotic stress tolerance genes. Here, the isolation of a novel promoter, PrDHN-5, from wheat was described.

Sequence analysis revealed the presence of potential abiotic and biotic stress responsive cis-elements, transcription factor-binding sites such as MYB, MYC, Dof, and WRKY and several tissue-specific expression and light-responsive elements (Table 1). The presence of these cis-elements related to various stresses is consistent with our previous finding that DHN-5 is induced not only by various abiotic stresses (NaCl, osmotic, and drought) but also by ABA (Brini et al., 2007a, b). In the same way, in-silico analysis of the region upstream of the maize *rab17* gene revealed the presence of various cis-acting regulatory elements involved in abiotic stress-responsive gene expression (Busk et al., 1997). The *rab17* promoter has five putative ABREs of which four are active in embryos but only three are active in leaves. In addition, protein binding in vivo on the active ABREs is weaker in leaves than in embryos (Busk and Pagès, 1998). The promoter PrDHN-5 contains also a conserved MYB protein recognition sequence (PyAACTG) Myb-related regulatory genes have been isolated from several plant species, one example being the regulatory C1 gene of maize. Urao et al. (1993) isolated a myb-related gene of *Arabidopsis* (*Atmyb2*) which is induced by ABA, NaCl and drought stress, and which binds to conserved MYB recognition sites in plant and viral promoters. As an initial step towards understanding regulatory mechanisms controlling DHN-5 gene expression, the expression pattern of PrDHN-5 was investigated using a *gusA* reporter gene system in transgenic *Arabidopsis* and wheat plants grown under control or stressed conditions. Histochemical staining revealed that the monocotyledonous PrDHN-5 promoter is active in the heterologous transgenic *Arabidopsis* system. During the past two decades some examples of monocot promoters that are

Table 2. The primers used in the HE-TAIL PCR method and in RT-PCR.

Primer	Sequence
<i>G1</i>	5'-TCGGAAGACGAGCTGGAGCTGGAGCTG-3'
<i>G2</i>	5'-CGGTCTTGTGCTCTCCCCTGTGG-3'
<i>G3</i>	5'-CGGAAACGGGTTGCCGTACTIONCGTCG-3'
<i>G4</i>	5'-TTGTCGTGCTGCCCTTGGAACTCCATCTTTGCCTCC-3'
<i>R1</i>	5'-NGTCGASWGANAWGAA-3'
<i>R2</i>	5'-GTNCGASWCANAWGTT-3'
<i>R3</i>	5'-WGTGNAGWANCANAGA-3'
<i>R4</i>	5'-NCAGTWSCTNTSCTT-3'
<i>GusAF</i>	5'-ACGCGCTATCAGCTCTTTAATC-3'
<i>GusAR</i>	5'-CTCCTACCGTACCTCGCATTAC-3'
<i>Act1F</i>	5'-GGC GAT GAA GCT CAA TCCAAA CG-3'
<i>Act1R</i>	5'-GGT CAC GAC CAG CAA GAT CAAGAC G-3'

also functional in dicots have been reported (Colot et al., 1987; Scherthner et al., 1988; Matzke et al., 1990; Quattrocchio et al., 1990; Liu et al., 2003; Iwamoto et al., 2004; Tittarelli et al., 2007). Histochemical staining revealed the ability of the PrDHN-5 promoter to direct β -glucuronidase expression with multiple-abiotic-stress inducible patterns. Indeed, in plants grown under control conditions, GUS activity was not detected in the different plant tissues tested. However, this activity increased substantially with NaCl, mannitol or ABA treatment. RT-PCR analysis of steady-state *gusA* mRNA levels in transgenic *Arabidopsis* revealed that reporter gene transcription under the control of PrDHN-5 was highly induced by the abiotic stresses tested in this study. It was suggested that there are ABA-independent and ABA-responsive signal transduction pathways between the initial signal for water stress and the gene expression (Yamaguchi-Shinozaki et al., 1992). We propose that the expression pattern controlled by the promoter of PrDHN-5 involves the ABA-dependant pathway. However, it is known that plants react to stress by increased ABA production in roots and leaves. Thus, it might be possible that the increased β -glucuronidase expression in transgenic *Arabidopsis* and wheat occurs due to the increased ABA production under osmotic stress (high NaCl or mannitol concentration). Since ABA is mainly produced in roots and leaf tissues this might also explain the higher levels of staining in these tissues. Additional experiments are needed to confirm if the promoter Pr-DHN-5 operated through ABA pathways.

Materials and Methods

Plant materials

Arabidopsis thaliana ecotype columbia was used in all gene transformation experiments. Seeds were surface sterilized in 10% bleach for 15 min followed by 3 to 4 rinses with sterile water. Sterilized seeds were grown on half Murashige and Skoog medium (BRL) in the presence of 1 % sucrose and 0.8 % agar. Plated seeds were incubated at 4°C for 48 h and then maintained under a 16 h d⁻¹ photoperiod at 24°C. A Tunisian cultivar of durum wheat (*Triticum turgidum* L. subsp. durum), Om Rabia3, was supplied by INRAT, Laboratoire de physiologie Végétale (Tunis, Tunisia). Seeds of this line were used for the isolation of the promoter region of DHN-5 gene and for genetic transformation.

Cloning of PrDHN-5 by HE-TAIL-PCR method

The flanking region of DHN-5 gene was isolated by use of the high-efficiency thermal asymmetric interlaced (HE-

TAIL) PCR method as described by Michiels et al. (2003a). PCR reactions were carried out with genomic DNA, extracted from wheat leaves (CTAB method, Michiels et al., 2003b), as template, four gene-specific reverse primers (*G1* (27 nucleotides), *G2* (23 nucleotides), *G3* (25 nucleotides), and *G4* (36 nucleotides)) designed close to the DHN-5 UTR sequence, and four arbitrary degenerate primers (*R1*, *R2*, *R3* and *R4*) (Table 2). Three rounds of PCR were performed on a Perkin-Elmer 9600 thermal cycler using the product of the previous PCR as template for the next. Thermal conditions and reaction mixture were used as described for the HE-TAIL-PCR method by Michiels et al. (2003a). Products of the tertiary PCR, with the control (Rn-Rn), were loaded on a 1% agarose/EtBr gel to determine appropriate product lengths. Target products were defined as fragments that are absent in the control reaction (Rn-Rn) and with similar amplification lengths in reactions *G3*-Rn and *G4*-Rn (taking the small length difference caused by the specific primers *G3* and *G4* into consideration). Target bands were cut from the agarose gel and purified by use of the spin column DNA gel extraction kit (Qiagen, Germantown, USA). The purified bands were ligated into the pGEMT-easy vector (Promega) to obtain the recombinant plasmid pGEM-PrDHN-5. Then, plasmid DNA was transformed into *E. coli* DH5 α by the heat-shock method. Colony PCR was used to validate positive clones. A total promoter "PrDHN-5" sequence of 1,128 bp was obtained by end sequencing (ABI 3100 automatic DNA sequencer, Applied Biosystems) of the positive clones (five different clones) using T7 and SP6 primers. The search for putative cis-elements in the PrDHN-5 promoter sequence was carried out using the databases PLACE (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) and Plantcare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) (Higo et al., 1999; Lescot et al., 2002).

Construction of PrDHN-5/GUS fusion

The promoter fragment was released by *HindIII/BamHI* from pGEM-PrDHN-5 vector and then cloned upstream of the *gusA* gene into the pCAMBIA1391Z vector (Cambia, Canberra, Australia) digested with the same enzymes. The construct pCAMBIA1391Z-PrDHN-5-*gusA* generated, the pCAMBIA1301-CaMV35S-*gusA* (used as a positive control for genetic transformation of *Arabidopsis thaliana*) and the pCAMBIA1391Z-Ubiquitin-1-*gusA* (used as a positive control for genetic transformation of wheat) were then mobilized into *Agrobacterium tumefaciens* GV3101 strain by the freeze-thaw transformation method (Chen et al., 1994).

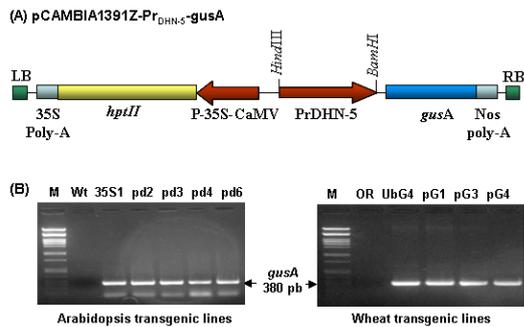


Fig 3. Schematic representation of the PrDHN-5/GUS constructs and PCR analysis of transgenic Arabidopsis and wheat transformed with the PrDHN-5-gusA construct. (A) T-DNA schematic map of the binary vector pCAMBIA1391Z-PrDHN-5gusA, used for Arabidopsis and wheat transformation. (B) PCR analysis of T₂ transgenic Arabidopsis lines (pd2, pd3, pd4, and pd6), transgenic wheat line (pG1, pG3 and pG4), 35S1 and UbG4 were positive transgenic lines of Arabidopsis and wheat with P-35S-CaMV and P-Ubiquitin-1 respectively and untransformed plant (Wt for Arabidopsis and OR for wheat). PCR product corresponds to the amplification of the gusA gene.

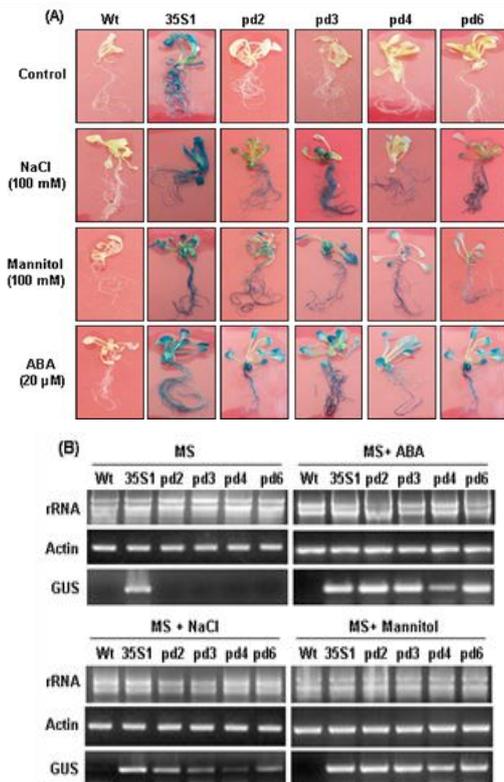


Fig 4. Histochemical GUS activity and RT-PCR analysis in transgenic *Arabidopsis* plants carrying the PrDHN-5-gusA construct in response to various abiotic stresses. (A) T₂ transgenic seedlings grown in MS medium and challenged with various abiotic stresses (100 mM NaCl, 100 mM mannitol, or 20 μM ABA) for 72 h. (B) RT-PCR analysis of transgenic *Arabidopsis* plants under control and stressed conditions. A specific PCR product of 0.5 kb was detected in 4 PrDHN-5 (lanes 3–6) transgenic lines. Wt: Non-transformed plants; (35S1): transgenic *Arabidopsis* plants with PrCaMV35S. A 380 bp Actin fragment was amplified by RT-PCR as an internal control.

Plant transformation

For *Arabidopsis* transformation, *Agrobacterium*-mediated transformation was performed via the floral dipping technique (Clough and Bent, 1998). Transgenic plants (T₀) were selected on MS agar medium containing 15 mg L⁻¹ hygromycin. The transgenic plants (T₀) were transplanted into soil and allowed to self-fertilize to produce the T₁ and then the T₂ seed generations. Integration of the T-DNA and gusA expression in T₂ homozygous plants was ascertained by PCR. The homozygous T₂ generations of gusA transgenic *Arabidopsis* plants, either under the control of the PrDHN-5 or 35S promoters, were used in subsequent assays. The WT *Arabidopsis* and 35S-gusA transgenic plants were used as negative and positive controls, respectively. For wheat transformation, *Agrobacterium tumefaciens* strain GV3101 was used in this experiment. The culture was incubated overnight at 27–29°C with shaking (250 rpm), and when the culture was at log phase or immediately thereafter, cells were collected by centrifugation at 4.5 g for 10 min and then re-suspended in inoculation medium with or without 200 μM acetosyringone (Wu et al., 2003). The inoculation density of *Agrobacterium* (OD₆₀₀) ranged from 1.0 to 2.0. Freshly isolated mature embryos were pre-cultured on co-cultivation medium for 0.5–1.0 h, and then immersed in *Agrobacterium* suspension for 3 h in the dark. Excess bacteria were removed and the explants transferred, scutella-side-up, without blotting, to fresh co-cultivation medium. Co-cultivation was carried out in darkness at 24–25°C. The media used for inoculation, co-cultivation and induction were based on Cheng et al. (1997). After 3 days of co-cultivation in darkness, explants were transferred to an induction medium containing 160 mg L⁻¹ of the antibiotic Timentin. All subsequent media plates contained Timentin at this concentration. Explants were maintained intact on the induction medium for 3–4 weeks, after which they were transferred to R_{DZ} medium (R medium plus 5 mg L⁻¹ zeatin and 0.1 mg L⁻¹ 2,4-dichlorophenoxyacetic acid) (Wu et al., 2003), and moved to light. After a further 3–4 weeks, regenerating tissues were transferred to R_{ppT} medium (R medium containing 2–4 mg L⁻¹ L-phosphinothricin) (Wu et al., 2003). Approximately 3–4 weeks later, shoots or plantlets which survived the first round of selection were transferred to a second round and, if necessary, a third round of selection. Plants showing resistance to PPT were subjected to a polymerase chain reaction (PCR) analysis. Positive plants were transferred to soil in a containment glasshouse.

Treatment of transgenic plants

The effects of abiotic stress on gusA transcripts accumulation and GUS activity were monitored by use of seeds of homozygous transgenic lines of *Arabidopsis* (35SgusA, PrDHN-5-gusA) and WT plants. Experiments involving NaCl, mannitol or ABA treatments were performed on 10-day-old seedlings grown on MS basal medium supplemented with 15 mg L⁻¹ hygromycin. These seedlings were incubated for 72 h with MS liquid medium containing 100 mM NaCl, 100 mM Mannitol or 20 μM ABA. After each treatment, plants were harvested for analysis by histochemical GUS staining or to extract RNA. For transgenic wheat in greenhouse assays, 14-day-old seedlings were transplanted into soil pots (20 cm × 20 cm) and left to grow under normal conditions by irrigating them daily until they reached the reproductive stage. At this stage a set of plants was subjected to salt stress (irrigated with a 100 mM NaCl solution). Leaf

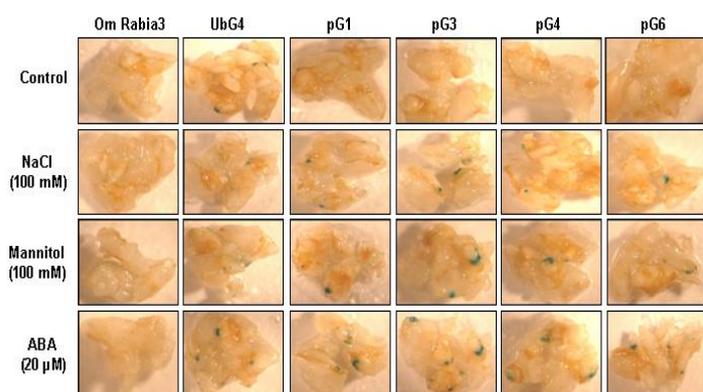


Fig 5. GUS activity of transgenic wheat callus lines carrying the PrDHN-5-gusA construct in response to various abiotic stresses (100 mM NaCl, 100 mM mannitol, or 20 μ M ABA). Om Rabia3: non transformed callus; UbG4: callus transformed with the promoter Ubiquitin-1 of maize; pG1, 3, 4, 6: callus transformed with the PrDHN-5.

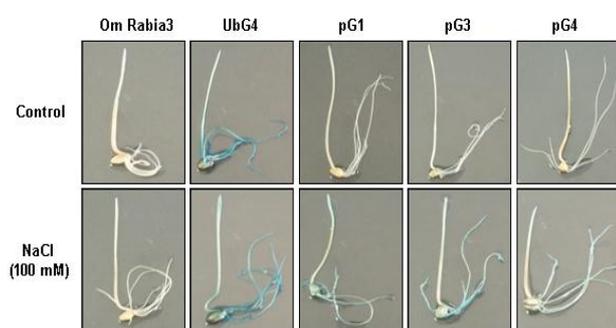


Fig 6. Histochemical GUS activity of transgenic wheat younger plants carrying the PrDHN-5-gusA construct in response to salt stress. Om Rabia3: non transformed callus; UbG4: callus transformed with the promoter Ubiquitin-1 of maize; pG1, 3, 4, 6: callus transformed with the PrDHN-5.

samples were collected from control and salt-stressed (two days after beginning the stress) plants for histochemical GUS staining.

Histochemical GUS staining

GUS activity was assayed histochemically by incubating tissue sections or seedlings under vacuum infiltration in 50 mM Na_2HPO_4 buffer (pH 7.0), 0.5 mM $\text{K}_3(\text{Fe}[\text{CN}]_6)$, 0.5 mM $\text{K}_4(\text{Fe}[\text{CN}]_6)$, 0.1% Triton X-100, and 1 mg/l X-Gluc (5-bromo-4-chloro-3-indolyl β -D-glucuronide cyclohexylammonium salt) for several minutes and then overnight at 37°C (Jefferson et al., 1987). The pigments and chlorophyll were removed by soaking the plant tissues for several hours in 70% ethanol. These stained tissues were photographed with a Leica binocular microscope. Transgenic plants driven by the CaMV35S or Ubiquitin-1 promoters and wild type plants were the respective positive and negative controls.

gusA expression analysis by semi-quantitative PCR

Transcription levels of the gusA gene under the control of the PrDHN-5 promoter were assessed by RT-PCR in T₂ homozygous transgenic *Arabidopsis* plants treated for 72 h with NaCl, mannitol or ABA. Total RNA was isolated by the

Trizol method (*Invitrogen*) from 100 mg of plant seedlings materials according to the manufacturer's recommendations. RNA quality and concentration were determined by gel electrophoresis (1%, RNase-free) and measuring OD with a spectrophotometer (260/280 nm), respectively. First strand cDNA synthesis was performed on 2 μ g total RNA treated with DNase I (Promega) using SuperScript II reverse transcriptase (*Invitrogen*) and oligo-dT₁₈, according to the supplier's recommendation. Primer pairs were designed to amplify fragments of gusA and the housekeeping actin gene. The primers used for RT-PCR were: GusAF, GusAR, Act1F and Act1R (Table 2). The PCR product (10 μ l) was separated on 1% agarose gel to visualize the intensity of gene amplification. To ensure reproducibility the experiment was repeated three times.

Conclusion

We have isolated the PrDHN-5 promoter from wheat controlling expression of the DHN-5 gene encoding for a dehydrin protein (Brini et al., 2007a). Our main results demonstrated that PrDHN-5 is an abiotic stress-inducible and ABA dependant promoter in the transgenic *Arabidopsis* and wheat plants. These results will lead to more interest in the PrDHN-5 promoter, because it could be an attractive candidate promoter for transgenic crop plants. Moreover, the PrDHN-5 may be an effective and desirable promoter for controlling stress tolerance candidate genes in terms of driving low constitutive transgene expression under normal conditions and high induction in response to ABA, salt, and osmotic stress. For these reasons using the PrDHN-5 promoter could avoid potential harmful effects related to an overexpression of the target gene under control of constitutive promoters in transgenic plants.

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