# Analysis of the Stress-inducible Promoter of *TdDHN8/WCOR410* from Wheat Using Transient Expression Assays

by

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A thesis submitted for partial fulfillment of the requirements of the Masters of Biotechnology (Plant Biotechnology)

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# DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text[1].

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# Preface

The master research project went through eight months from February 5th to September 27th, and has successfully been completed as we planned at the beginning. The research was mainly focusing on the analysis of a new wheat promoter, pTdDHN8/WCOR410, which was originally presumed as a drought-inducible promoter. As a backup research plan, we also tested nine lines of the T1 transgenic barley plants transformed with the pRab17-*GUS* fusion under 250mM salt stress.

We spent nearly five months to optimize the experimental conditions of transient expression assay using cell suspension cultures that are rarely used for the analysis of inducible promoter in plants. The effort included: 1) which plant tissue was optimum to characterize the activity and inducibility of the *TdDHN8/WCOR410* promoter; 2) how to minimize the factors that affected the transformation efficiency in cell suspension cultures via particle bombardment; 3) how to precisely induce the osmotic stress in the growth medium. Finally, we efficiently optimized the experiment conditions, paving the way to further dissect the *TdDHN8/WCOR410* promoter activity using transient expression assay in cell suspension cultures. In term of the backup research, we got four transgenic plants that were qualified using GUS staining assay, and they have been transplanted into soil for T2 seeds.

In the present thesis, the revised version of literature review, which has been examined by Dr. Andrew Jacobs, Dr. Oliver Cotsaftis, and Prof. John Randles on March, is present in the first part. The second part is the revised manuscript of the *TdDHN8/WCOR410* promoter analysis according to the format of *The Plant Journal*. The final version of my master thesis was revised based on the critical suggestions by Prof. Peter Langridge, Dr. Oliver Cotsaftis, and Dr. Bujun Shi. Although some big progress was made in last few months, yet we recognize that more hard work is still needed to address the problem of the big variation of transformation efficiency in cell suspension cultures via particle bombardment, and extend our findings in the thesis in the next few months. At the end of my master study, I thank those lovely persons who help me for the master research in plant genomics center. The big gratitude are also given to our program coordinator Dr. Amanda Able for her assistance during my two-year master study in The University of Adelaide, and my supervisors Dr. Sergiy Lopato and Dr. Serik Eliby for their kindness and patience in my master research project.

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# Glossary

## Promoter Regulatory Elements

G-box, ubiquitous regulatory element.

ABRE, ABA-responsive element.

CE, coupling element.

ABRC, ABA-responsive element/coupling-element.

MYBR, MYB recognition sequence.

MYCR, MYC recognition sequence.

DRE, drought-responsive element.

CRT, C-repeat.

LTRE, low temperature-responsive element.

SRE, SA-responsive element.

HSE, heat-shock element.

RSRE, rapid-stress-responsive element.

ICEr, inducer of CBF expression recognition sequence.

NACR, NAC recognition sequence.

## Transcription Factors That Bind to Promoter Regulatory Elements

DREB/CBF, dehydration-responsive element binding protein/ C-repeat-binding factor.

AP2/ERF, APETALA2/ethylene-responsive factor.

bZIP, a family of transcription factors with basic region and Leu-zipper motif.

MYB, a family of transcription factors with Trp cluster motif.

MYC, a family of transcription factors with basic-helix loop-helix (bHLH) and Leu-zipper motif.

HD-ZIP, homeodomain-leucine zipper protein.

NAC, NAM, ATAF, and CUC transcription factor.

## Others

LEA, late embryogenesis abundant protein.

ROS, reactive oxidative species.

ABA, abscisic acid.

SA, salicylic acid.

LUC, luciferase.

GUS, β-glucuronidase.

GFP, green fluorescent protein.

# Introduction

Drought, high salinity, and cold that affect or even retard the crop growth, are the three main types of abiotic stress accounting for the huge loss of cereal yield in the world. It was estimated that at least fifty-percent cereal yield in average can be damaged by the three types of abiotic stress in comparison with the possible maximum yield under ideal growth conditions (Bray *et al.*, 2000). Therefore, improving abiotic stress-tolerance in plants has its agricultural importance for the increase of cereal production and the huge economic importance. Of the strategies for such a purpose, the efficient one is to introduce functional genes that are involved in the improvement of abiotic stress tolerance. The first group of genes encodes the proteins and enzymes that mediate the synthesis of many protective osmoprotectants on the maintenance of the cell viability, including late embryogenesis abundant (LEA) proteins, dehydrins, reactive oxidative species (ROS)-scavenging proteins, proline, sugar, and etc. The effort of overexpression of these genes does enhance the abiotic-stress tolerance such as the increasing survivability of plants and the improvement of plant biomass production (see reviews, Bajaj *et al.*, 1999; Vinocur and Altman, 2005; Umezama *et al.*, 2006). The second group is the regulatory genes encoding transcription factors through interacting with the *cis*-acting elements in the promoter regions to alter the pattern of genes expression. Overexpression of drought responsive-element/C-repeat binding factor (DREB1/CBF) in Arabidopsis and rice was found that it significantly improves plants survivability under osmotic stress (see review Umezama *et al.*, 2006). In addition, engineering of some signal transduction factors in the upstream of transcription factors showed better performance for the improvement of stress tolerance in plants, yet the signaling process is not fully understood (see review Umezama *et al.*, 2006). Although the overproduction of osmotic metabolites improves plant tolerance for survival under stressful conditions, this kind of effort always sacrifices some agronomic traits during the process (Sreenivasulu *et al.*, 2004). On the other hand, growth retardation was always found by constitutively expressing the transcription

factors under unstressed conditions (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Haake *et al.*, 2002; Lee *et al.*, 2005; James *et al.*, 2008).

However, recent evidence indicates that inducible promoters can be used to drive transgenes expression in an ideal temporal and spatial fashion. In comparison with constitutive promoter, this type of promoter only switches on genes transcription when the internal or external stimuli appear, this strategy thus can provide better potential for the improvement of abiotic stress tolerance in plants. To date, the research on the promoter of LEA-protein-like *RD29A* gene in Arabidopsis shows important advances. They discovered that the expression of DREB1A/CBF3 with the inducible *RD29A* promoter reflects no discernible effects on Arabidopsis and wheat plants growth while providing an even greater tolerance to several stress conditions than do overexpression of DREB1A/CBF3 with 35S promoter (Kasuga*, et al.*, 1999; Pellegrineschi *et al.*, 2004). Similarly, another report also showed no deleterious effects on the transgenic bahiagrass plants transformed with the inducible-promoter HVA1-*DREB1A/CBF3* fusion (James *et al.*, 2008). Therefore, the main effort of this article is: 1) to review recent advances of promoter regulatory-elements involved in abiotic stress; 2) to analyze the strategies for promoter activity analysis.

# 1. What is a gene promoter?

A promoter is roughly defined as a cluster of functional DNA sequences located around the gene(s), and is desirable to initiate gene transcription and to regulate the frequency of transcription (Allison, 2007). Those regulatory DNA sequences are termed as *cis*-regulatory elements including core promoter elements, proximal promoter elements, and long-range regulatory elements. The core element is a necessary and functional DNA sequence in every promoter region that serves as a recognition site for RNA polymerase II and other subunits to initiate the transcription. Some of the known core elements contain the TATA box, the initiator element, and the downstream promoter element. A promoter will lose its regulatory ability when

adjusting the orientation or the distance between the core element and the transcription start site. By contrast, an enhancer element, which is a type of long-range regulatory elements, can still but not always have abilities to activate the gene expression. In different types of promoters, variations of enhancer elements always exist in the component, the number, the orientation relative to the transcription start site, and the distance between them (Allison, 2007). Another type of long-range regulatory elements is the insulator that has two distinct functions originally discovered in *Drosophila*: some insulators may function as boundary marker between regions of heterochromatin and euchromatin, or may function as blocking enhancer activity to regulate the appropriate expression of neighbor genes (Burgess-Beusse *et al.*, 2002).

Based on the nature of promoters, they are divided into three types: constitutive promoters, tissue-specific promoters activated at specific developmental stages or tissues, and inducible promoters excited by internal or external signals. The interest of inducible promoter system is typically increasing, not only because it allows expression of some genes in their native patterns for the precise understandings of genes' functions, but also because such system allows plants "self-regulating" the expression of transgenes under unpredicted environmental conditions (Reynolds, 1999). Unfortunately, in some cases, the ectopic expression (leaky expression) and pleiotopic expression are two main possible problems to affect the application of inducible promoters (Aoyama, 1999), suggesting the importance of ideal inducible promoters collection from the genomic database. Optionally, accompanying with the deep understanding of functions of regulatory elements in promoters, synthesizing promoters could provide another choice on control of the ideal pattern of transgenes expression in field conditions (Venter, 2007).

## 2. *cis*-Acting elements in response to abiotic stress

The promoter regulates gene expression by those long-regulatory elements either enhancing (enhancers) or repressing (repressors) the transcription efficiency. Many such kinds of *cis*-acting elements were discovered in the response to one specific or several environmental signals, such

as ABA-responsive element (ABRE) responsive to ABA treatment and drought-responsive element/C-repeat (DRE/CRT) in response to high salinity, dehydration, and low temperature.

## 2.1 *cis*-Acting elements in response to ABA

The ABRE motif was originally identified from the *EM1A* gene (*Triticum aestivum*) promoter as PyACGTGGC (Guiltinan *et al.*, 1990), where Py refers to the nucleotide base pyrimidine. ABRE is now known as an enhancer in the promoter regions of a large amount of stress-inducible genes. Interestingly, ABRE shares the same core sequence, ACGT, with G-box which is another type of *cis*-acting element responding to various stressful conditions such as osmotic stress, UV light, and intense light (Table 1). In addition, one single ABRE linked with a 35S core promoter was not sufficient for the reporter gene expression to ABA treatment in transient expression assay (Guiltinan*, et al.*, 1990; Skriver *et al.*, 1991). Thus, these results indicated that ABRE and G-box might share similar functions under some stressful conditions. Meanwhile, they beg the question whether ABRE is a partial component to confer the promoter activity and meanwhile requires another component to distinguish its hallmark with G-box. The answer remained in vain until the coupling element (CE) was identified to play such a role (Table 1). In transient expression experiments, the short promoter fragments of *HVA1* and *HVA22* genes encoding LEA protein in barley aleurone layers were found to confer a high level of ABA induction only when both ABRE and CE (ABRC) appear at the same time, and one of them is not sufficient to excite the promoter activity (Shen and Ho, 1999). ABRE and ABRC were both found prevailing in the promoter regions of ~1000 ABA- or abiotic-stress-responsive genes (Kaplan *et al.*, 2006), it thus obscures the difference of their functions.

Slightly different from ABRE with the core sequence, ACGT, the other type of ABA-induced *cis*-acting element is denoted as MYBR and MYCR (Table1). In the native promoters, they act as weak enhancers responsible for the dehydration- and ABA-inducible expression of the *RD22* gene (Abe *et al.*, 1997). MYBR and MYCR as well as ABRE are two types of significant

components involved in the ABA-dependent pathways in sensitive to various abiotic stress and ABA treatment (Shinozaki and Yamaguchi-Shinozaki, 1997).

## 2.2 *cis*-Acting elements in response to water deficit

Another group of *cis*-acting elements is sensitive to dehydration, high salinity, and/or cold, whereas they can still activate the promoter activity in the absence of ABA. This kind of *cis*-acting elements is termed as DRE/CRT derived from the promoter of Arabidopsis *RD29A/COR78/LTI78* gene in responsive to high salinity, drought, and low temperature (Table 1, Yamaguchi-Shinozaki and Shinozaki, 2006), and low temperature-responsive element (LTRE) originally identified in *BN115* gene promoter from winter *Brassica napus* typically responding to low temperature (Table 1, Jiang*, et al.*, 1996). Furthermore, it was noticed that ABRE and DRE both exist in the *RD29A* promoter region and act as functional elements under osmotic stress: DRE serves as a rapid-responsive *cis*-acting element in the first twenty minutes, and ABRE exhibits a slow induction of gene expression in the continuing hours (Yamaguchi-Shinozaki and Shinozaki, 1994). This discovery indicated that two types of ABA-regulated pathways are involved in the abiotic stress: ABA-dependent pathway relying on the variation of ABA concentration and ABA-independent pathway in response to water deficit through ABA signal transductions (Shinozaki and Yamaguchi-Shinozaki, 1997).

## 2.3 Other *cis*-acting elements involved in abiotic stress

Besides ABA hormone-induced *cis*-acting elements, recent research indicate that jasmonic-acid-(JA), salicylic-acid- (SA), and ethylene-responsive elements impart large groups of gene expression during both biotic and abiotic stress (Fujita *et al.*, 2006). For example, SA-responsive element (TGACG) required activating pathogen-defense genes was also found responsive to ROS known as signals reacting to various abiotic stresses (Table 1, Garreton *et al.*, 2002). Another popular *cis*-acting element is the heat-shock element (HSE) typically responding to high temperature. However, a HSE from *APX1* gene promoter in Arabidopsis was also discovered in

responsive to drought, salt, and cold stress (Table 1, Storozhenko*, et al.*, 1998). In addition, a novel rapid-stress-responsive element (RSRE) was demonstrated that five minutes was a sufficient time for the element to perceive stressful signals to initiate transient genes transcription against both biotic and abiotic stress (Table 1, Walley*, et al.*, 2007).

However, the functions of one *cis*-acting element are argued with the progress of understandings on promoters. From four microarray analysis, several stress-related *cis*-acting elements were found to exist in both up-regulated genes and down-regulated genes (Seki *et al.*, 2002; Kaplan*, et al.*, 2006; Walley*, et al.*, 2007; Huang *et al.*, 2008), suggesting the dual role of ABRE, MYBR, MYCR, and DRE/CRT, which may perform as an enhancer in one group of gene promoters as well as act as a repressor in another group. Furthermore, those *cis*-acting elements that play roles on the crosstalk between several stresses are more interesting in the future. Secondly, the ABA-regulated pathways are challenged when other phytohormone-responsive elements were also found responsible for abiotic stress-inducible gene expression under stressful conditions (e.g. SA), which makes the mechanisms of abiotic stress tolerance in plants more complex.

**Table 1.** *cis*-Acting elements that were identified in response to abiotic stress

| ***cis* element** | **Sequence** | **Gene** | **Stress Condition*** | **References** |
|---|---|---|---|---|
| G-box | CACGTG | *CHS15* | ABA | Loake *et al.*, 1992 |
| ABRE | PyACGTGGC | *EM1A* | Water deficit, ABA | Guiltinan*, et al.*, 1990 |
| CE1 | TGCCACCGG | *HVA1* | ABA | Shen and Ho, 1995 |
| CE3 | ACGCGTGCCTC | *HVA22* | ABA | Shen and Ho, 1995 |
| MYBR | TGGTTAG | *RD22* | Water deficit, ABA | Abe*, et al.*, 1997 |
| MYCR | CACATG | *RD22* | Water deficit, ABA | Abe*, et al.*, 1997 |
| DRE | TACCGACAT | *RD29A* | Water deficit | Yamaguchi-Shinozaki and Shinozaki, 1994 |
| CRT | GGCCGACAT | *COR15A* | Cold | Baker *et al.*, 1994 |

| | | | | |
|---|---|---|---|---|
| LTRE | GGCCGACGT | *BN115* | Cold | Jiang *et al.*, 1996 |
| SRE | TGACG | *GNT35* | SA | Garreton *et al.,* 2002 |
| HSE | GTGGGCCCTCC | *APX1* | Water deficit, heat | Storozhenko *et al.*, 1998 |
| RSRE | CGCGTT | *RWR* | Water deficit | Walley *et al.*, 2007 |
| ICEr1 | GGACACATGTCAGA | *CBF2/DREB1C* | Cold | Zarka *et al.*, 2003 |
| ICEr2 | ACTCCG | *CBF2/DREB1C* | Cold | Zarka*, et al.*, 2003 |
| NACR | ACACGCATGT | *ERD1* | Water deficit | Tran *et al.*, 2004 |

* This table mainly summarizes those stress conditions associated with drought, high salinity, and cold or freezing, which are also characterized by water deficit.

# 3. Transcription-factors-binding initiates transcription

A transcription factor is a protein that binds to specific sequences of DNA and thereby controls transcription of genetic information from DNA to RNA (Latchman, 1997). To date, large groups of transcription factor families in plants have been identified as AP2/ERF, bZIP, MYB and MYC, zinc-finger proteins, HD-ZIP, NAC, and etc. On the whole, the research on transcription factors is an indispensable part for the study of regulatory-elements, in order to fully dissect the promoter activity. We will not discuss details of transcription factor in this review. Several great literature reviewed transcription factor advances in other places (Bartels and Sunkar, 2005; Umezawa *et al.*, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006).

# 4. Strategy to decipher the functions of a promoter

*cis*-Acting element research belongs to the reverse genetics realm. The better understanding of *cis*-acting element functions can help us precisely interpret the regulated activities of promoters. On the other hand, using those known *cis*-acting elements sequences we can "pray" those responding transcription factors (Lopato *et al.*, 2006), the decorating proteins, and the eliciting signals, and finally draw the full map of circuits of abiotic stress-tolerance in plants. However, it should be noted that the component, the number, the location of *cis*-acting elements in one

promoter region and the sequence of one same element can vary in different promoters (Table 2). The varying *cis*-acting element matrix in promoters may be used to differentiate promoter activities in plant programming process. In the meantime, the tiny variation of the sequence of one same *cis*-acting element is a possible signal to avoid the wrong recognition by similar transcription factors and wrong gene expression thereof. Therefore, the mapping of *cis*-acting elements in gene promoter region is desired to decipher promoter activities, theoretically should be conducted for each single gene promoter.

**Table 2.** Specific interaction analysis of drought-responsive element/C-repeat (DRE/CRT) and responsible transcription factor (TF)

| **Sequence of DRE/CRT** | **Gene** | **Specific binding TF** | **Plant species** | **References** |
|---|---|---|---|---|
| GGCCGACA/GT | *COR15A* | DREB1B/CBF1 | Arabidopsis | Stockinger *et al.*, 1997 |
| TACCGACAT | *BN115* | BNCBF5 | *Brassica napus* | Gao *et al.*, 2002 |
| TGGCCGAC | *BN28* | BNCBF17 | *Brassica napus* | Gao *et al.*, 2002 |
| ACCGAC | *RAB17* | ZmDREB1 and ZmDREB2 | Maize | Kizis and Pagès, 2002 |
| TTGCCGACAT | *HVA1* | HvCBF1 | Barley | Xue, 2002 |
| A/GCCGACNT | *RD29A* | DREB1A/CBF3 and DREB2A | Arabidopsis | Liu et al., 1998, Maruyama *et al.*, 2004 and Sakuma *et al.*, 2006 |

## 4.1. Transient expression assay is an efficient strategy to assess the functions of *cis*-acting elements

Insofar, every paper to characterize the promoter activity utilized the transient expression system *in planta* by either *Agrobacterium*-mediated or biolistic-mediated transformation. In this approach, the regulatory region of interest (e.g. a partial promoter fragment or an enhancer) and a reporter gene are constructed into a plasmid, and then transformed into plant materials for short-time incubation. By screening the expression level of reporter gene termed as qualitative and quantitative assay, we thus determine the component, location, intensity, and even timing of *cis*-acting elements in the promoter. This approach can work efficiently because anything that originally affects the expression of natural gene would also change the expression of reporter gene (Allison, 2007). Three reporter genes now available to analyse the plant promoter activity are summarized in Table 3. In this review, we mainly discussed the strategy using biolistics-mediated transformation for the promoter activity analysis.

**Table 3.** A comparison of the application of *LUC*, *GUS*, *GFP* reporter genes for promoter activity analysis

| **Reporter gene** | **Species** | **Product** | **Use** |
|---|---|---|---|
| *LUC* | *Photinus pyralis* (firefly) | Luciferase | Highly sensitive reporter enzyme that oxidizes luciferin and generates a bioluminescent product. It is quite useful to analyse those promoters responding to rapid transcriptional initiation. Critical experiment conditions needed to quantify the gene products. |
| *GUS* | *E. coli* | β-glucuronidase | Generally used reporter in plant systems; hydrolyzes colorless glucuronides like X-gluc to yield colored products for qualitative and quantitative assay of any plant tissue. |
| *GFP* | *Aequorea victoria* (jellyfish) | Green fluorescent protein | A reporter that fluoresces on irradiation; compared with GUS reporter, it has the advantages to be used as a real-time reporter gene and to reflect promoter activity in living cells. |

X-gluc, 5-bromo-4-chloro- β-D-glucuronide.

Since the "gene gun" is invented, especially the PDS 1000/He biolistics system by Bio-Rad, it has been broadly applied to transform organisms either to conduct transient expression assay or to generate new plant species (e.g. Bt maize by Monsanto). The big advantage of biolistics is simple and fast. The whole experiment procedure only includes DNA coating, material preparation, transformants culture, and identification of the reporter gene expression. Moreover, biolistics-mediated transformation can be used in any type of tissues ranging from chloroplasts, protoplasts, cell suspension cultures, callus, embryos, seeds, and seedlings (Yamaguchi-Shinozaki and Shinozaki, 1994; Shen and Ho, 1995; Busk *et al.*, 1997; Joshee *et al.*, 1998; Xiao and Xue, 2001; Kizis and Pagès, 2002; Xue, 2002; James *et al.*, 2008). Especially useful for some monocotyledonous plant species like wheat that is not efficient using *Agrobacterium*-mediated transformation. However, a factor for the high price to conduct one experiment is that the gold used in the transient expression experiment cannot be recycled for further use. Other cheap coating material such as tungsten and glass could be the option, but the transformation efficiency for plant tissues is relative worse (Sanford *et al.*, 1992).

From the result of initial transient expression assay, we can utilize general information associated with the activity of a full-length promoter to determine whether and how the truncated promoter and reporter gene linker-scanning analysis should be conducted. During the process, one necessary and desirable work should be implemented to predict and assess the possible matrix of *cis*-acting elements in the promoter region using bioinformatics data (e.g. PLACE; Prestridge, 1991; Higo *et al.*, 1999). Based on the critically computer-generated mapping, in one place, we can briefly predict the possible activities of the new promoter and possible results from future experiments; in the second place, appropriate primers thus can be designed without disrupting the possible *cis*-acting elements in the promoter region. However, to avoid the latent deviations, it should be taken in consideration that the predicted mapping must be validated by further experimental evidence.

*cis*-Acting element mapping is generally divided into three steps based on the resolution required: 100 bp mapping, 10 bp mapping, and 1 bp mapping. The purpose of preliminary mapping by 5' and/or 3' deletion analysis is used to roughly locate the sites of *cis*-acting elements, and to assess activities of candidate *cis*-acting elements in the promoter (Figure 1). The second step mapping is focusing on the interesting element attained from the first step. Before further manipulations, one question is how to extract the precise information of expected *cis*-acting element in the deleted promoter fragments. Hence, a more precise location of the element sequence needs to be determined for such a purpose; there are several pragmatic tools herein that can be used: DNA footprinting, gel shift assay, or chromatin immunoprecipitation (Galas and Schmitz, 1978; Garner and Revzin, 1981; Breiling and Orlando, 2005). When attaining the sequence of *cis*-acting element, further transient expression experiments then could be conducted to determine activities by analyzing the copy number effect, the orientation effect, the distance effect, and even the interactions of two *cis*-acting elements (Figure 1). The objective of 1 bp mapping is to look for the critical base responsible for activities of one specific *cis*-acting element for the *cis*-acting element through, for example, mutating consequent 1 base of the sequence of the *cis*-acting element (Figure 1).

Transient expression assay serves several advantages to collect the preliminary data on the matrix and activity of a promoter by qualitative and quantitative assay. The results from transient expression assay determine whether and how stable transformation should be carried out for further promoter activity analysis. However, transgenic plants transformed with the full-length promoter or promoter fragments by stable transformation must be conducted to calibrate and validate the activities of a promoter attained from the transient expression assay. That is because the abnormal manipulations in transient expression assay to reflect the promoter activities may produce the wrong information.

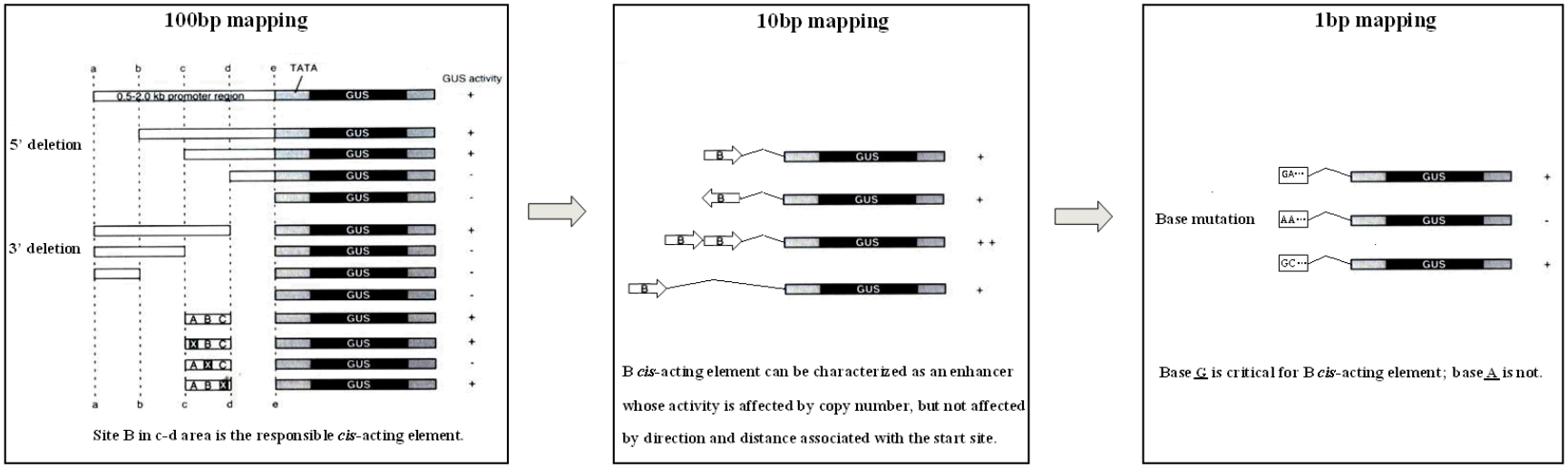


**Figure 1.** A mapping strategy to decipher the activities of a promoter using transient expression assay. A whole mapping procedure can be divided into three steps based on the mapping resolution: 100 bp mapping, 10 bp mapping, and 1 bp mapping. By identifying the reporter gene product (e.g. GUS) activity, we are able to find the *cis*-acting element of interest for further analysis. a) *cis*-Acting elements termed as either enhancers or repressors normally reside in the upstream region of transcription start site. After logic truncating the sequence, e.g. 100 bp per time, the candidate *cis*-acting elements will be finally located in the promoter region. However, the example in the figure is not always the case; the cropped length per time largely depends on the predicted mapping and experimental evidence. b) If one *cis*-acting element is identified as a new enhancer or repressor whose general length is approx. 10 bp, this step thus will focus on deciphering properties of the new *cis*-acting element such as the direction effect, the copy number effect, and the distance effect. If it is not a new *cis*-acting element, we can neglect this step and directly move onto the third step. c) It is an optional strategy to analyse the specificity of one *cis*-acting element. The particular nature of one *cis*-acting element is not only dependent on the core sequence (e.g. ABRE characterized by core ACGT) but also relies on one or two critical flanking base (s) responsible for the specific binding of one or several transcription factor(s).

## 4.2 Stable transformation is desired to validate the promoter activities

Stable transformation is a necessary and reliable step to estimate the activities of a promoter or functional *cis*-acting elements induced by internal or external signals through both qualitative and quantitative analysis. A basic procedure to attain successful transgenic lines can follow eight steps:

1) Construct plasmids with desired promoter fragments;
2) Transform plant tissues with constructed plasmids;
3) Select transformed explants with antibiotic reagent;
4) Regenerate transformed explants;
5) Transplant transgenic plants (T0) into soil in a glasshouse;
6) Harvest the seeds (T1) of transgenic plants;
7) Plant the T1 transgenic lines in selection media and harvest the seeds of the successful transgenic plants;
8) Use the homozygous T2 transgenic plants for reporter gene expression analysis.

T0 and T1 transgenic lines are seldom used for qualitative and quantitative assay, because the abnormal reporter gene expression often occurred in T0 transgenic lines (they are regenerated from the damaged tissues), and the abnormal reporter gene expression and segregation events occur in T1 lines (Nakashima and Yamaguchi-Shinozaki, 2002). After careful selection, homozygous T2 transgenic lines are more credible for qualitative and quantitative analysis. Furthermore, in order to precisely assess the promoter activity, apart from histochemistry staining assay, Northern blotting needs to be conducted in case the "leaky" events (i.e. some promoters can direct expression even in the absence of the inducing signals, Slater *et al.*, 2008).

## Concluding comments

1. The potential to improve plant abiotic-stress tolerance by overexpressing abiotic-stress genes was demonstrated in several papers. However, it is not always efficient to apply constitutive promoters for overexpression of abiotic-tolerance proteins and transcription factors, because the retarded phenotypes of plants under normal growth conditions are always found with this overexpressing strategy. Application of stress inducible promoters however provides the potential to address such problems.
2. *cis*-Acting elements and transcription proteins are both quite improtant regulatory factors that can affect the activities of promoters. The discovery of ABRE and DRE as the two key *cis*-acting elements in drought-inducible promoters can be further used to identify and characterize these types of promoters, or to "pray" new transcription factors using yeast one hybrid system (Lopato *et al.*, 2006).
3. Methods to characterize stress inducible promoters are well described in the literature. Two plant systems such as transient expression assay and stable transformation assay with promoter-reporter gene constructs can provide the qualitative and quantitative evidence for promoter activity analysis.
4. Very few stress inducible promoters were cloned and characterized for maize, rice and barley, and almost nothing is achieved for wheat. Successful development of plant biotechnology aiming to increase drought tolerance of agriculturally important varieties needs a collection of different and well characterized stress inducible promoters from cereals.

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# Manuscript

# Analysis of the stress-inducible promoter of *TdDhn8/WCOR410* from wheat using transient expression assays

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**Summary**

**The full-length gene and promoter sequences of *TdDHN8/WCOR410* were isolated from a *Triticum durum* BAC library. Transient expression assay in cell suspension cultures was used to characterize the activity and inducibility of the 2685 bp promoter of *TdDHN8/WCOR410* and to map functional DRE/CRT elements. It was demonstrated that the *TdDHN8/WCOR410* promoter has a weak basal activity in the absence of stress and can be induced by osmotic stress. The full-length *TdDHN8/WCOR410* promoter and six promoter fragments deleted from the 5' end were linked to the *GUS* reporter genes and co-transformed with either pUbi-*TaDREB3* or pUbi-*HvDREB9* into cell suspension cultures via particle bombardment. Both transcription factors are able to activate the *TdDHN8/WCOR410* promoter. However, they specifically bind to different DRE/CRT elements and provide different levels of promoter activation. TaDREB3 weakly activates the *TdDHN8/WCOR410* promoter through binding to the DNA sequence, TTCCGGCCGACACGCT, which is present in both *TdDHN8/WCOR410* and *HvDHN8* promoters. The *TdDHN8/WCOR410* promoter has the DRE/CRT element proximal to the TATA box, which is absent in the promoter of the homologous *HvDHN8* gene and can be highly transactivated by HvDREB9 but not by TaDREB3.**

**Keywords: cell suspension cultures, transient expression assay, transcription factor, *cis*-acting element, DRE/CRT**

## Introduction

One of the major groups of late embryogenesis-abundant (LEA) proteins is a subfamily of genes which encode dehydrins (Close *et al*., 1989). The products of these genes are constitutively expressed in mature embryos and, in some cases, in endosperm, and can be activated in other plant tissues by several forms of osmotic stress such as drought, salt and cold stress (Mundy *et al*., 1990). Because the level of expression of these genes under stress conditions is very high, promoters of dehydrins are potentially good candidates for the stress-inducible expression of genes in transgenic plants (Xiao and Xue, 2001). These promoters are also a good source of stress responsive *cis*-acting elements for the isolation of stress related transcription factors using the yeast one-hybrid (Y1H) system (Lopato *et al*., 2006).

The *WCOR410* gene from wheat was originally identified as a gene encoding an acidic LEA protein, and the gene expression accumulates to equal levels in root, crown and leaf tissues of freezing tolerant gramineae during cold acclimation (Danyluk *et al*., 1994). Later it was confirmed by the quantitative expression analysis that the *WCOR410* gene expression is highest for low temperature tolerant and lowest for tender genotypes (Ganeshan *et al*., 2008). The peak of transcript levels in crown and leaf tissues of wheat (*Triticum aestivum* L.) was observed on the second day of cold acclimation (Ganeshan *et al*., 2008). Electron microscope analysis of immunolocalised WCOR410 protein revealed that it is accumulated in close vicinity of the plasma membrane of cells in the sensitive vascular transition area where freeze-induced dehydration is likely to be most severe (Danyluk *et al*., 1998). This finding suggests a protective function of the protein on the cell

membrane under freezing and/or dehydration conditions. The *WCOR410* gene was expressed in transgenic strawberry at a level comparable to that in wheat after cold acclimation (Houde *et al*., 2004). Some improvement in freezing tolerance of the transgenic strawberry was observed after cold acclimation, but no improvement was detected in the absence of acclimation. This suggests the need of another protein(s) induced by acclimation for the activation of *WCOR410* (Houde *et al*., 2004). The closest homologues of *WCOR410* from other plants are *AtCOR47* from Arabidopsis (Lin *et al*., 1990), *HvDHN8* from barley (*paf93*, Grossi *et al*., 1992) and *OsDHN1* from rice (Lee *et al*., 2005). The expression of *AtCOR47* is induced by drought, cold, and ABA treatment (Welin *et al*., 1994), and the expression of *HvDHN8* showed a similar pattern (Choi *et al*., 1999). However, the expression of *OsDHN1* is induced by cold, high salinity, ABA treatment, and most strongly by drought (Lee *et al*., 2005).

A group of transcriptional activators, called drought-responsive element (DRE) binding proteins (DREBs) or C-repeat (CRT) binding factors (CBFs), can regulate the expression of drought-related genes by binding a CCGAC core motif (Stockinger *et al*., 1997; Gao *et al*., 2002; Kizis and Pagès, 2002; Xue, 2002; Maruyama *et al*., 2004; Sakuma *et al*., 2006). Since many DREB/CBF transcription factors are targeted to bind to a specific DNA sequence, it can be used for the functional analysis of drought-inducible promoters. Overexpression of Arabidopsis *DREB1B/CBF1* up-regulates the gene expression of *OsDHN1* in transgenic rice plants (Lee *et al*., 2004), suggesting the activation of the *OsDHN1* promoter through the DRE/CRT element(s). This finding is consistent with the up-regulation of the *DHN8* gene in transgenic bahiagrass plants (*Paspalum notatum* Flugge cv. 'Argentine') transformed with a CaMV35S-*HsDREB1A* fusion (James *et al*., 2008).

However, *cis*-acting elements responsible for the activation of *WCOR410* and *WCOR410*-like genes from other plants have not been reported.

In the present study, we optimized the conditions of transient expression assay using cell suspension cultures of wheat (*Triticum monoccocum* L.). The transient assay was used to characterize the activity and inducibility of *TdDHN8/WCOR410* promoter. The full-length sequence of the promoter was isolated from a *T. durum* BAC library. We demonstrated that the *TdDHN8/WCOR410* promoter has weak basal activity in the absence of stress and can be induced by osmotic stress. In addition, transient expression of two DREB/CBF type transcription factors, TaDREB3 and HvDREB9, was used to identify the functional DRE/CRT elements in the *TdDHN8/WCOR410* promoter region.

**Results**

*Optimization of transient expression assay in wheat cell suspension cultures*

To optimize the osmotic stress conditions, several different concentrations of mannitol were added to growth media 1-2 h before bombardment. The same media was also used during the transformation procedure and 24 h growth culture after bombardment, until the GUS detection assay. The 2685 bp *TdDHN8/WCOR410* promoter-*GUS* fusion was used for cell suspension cultures transformation, and the maize polyubiquitin promoter-*GUS* construct was used as a control for the basal level of *GUS* gene expression. It was found that transformation efficiency is strongly affected by different concentrations of mannitol in the growth media used for the pre-incubation, transformation and post-incubation growth of cell suspension cultures. *GUS* gene expression driven by the non-osmotic-stress induced,

constitutive polyubiquitin promoter was four fold stronger in the growth medium with 500mM mannitol, than that in without mannitol (Figure 1Aa and Ba). It was also found that concentrations of mannitol higher than 500mM negatively influence transformation efficiency (Figure 1Aa and Ba). For the *TdDHN8/WCOR410* promoter-*GUS* construct, the difference between 0 and 500mM mannitol in the growth media led to about nine fold difference in the number of GUS foci (Figure 1Bb). This increase can be accounted for by two components: increased transformation efficiency, and promoter activation by osmotic stress (Figure 1Ba and Bb). To minimise the first component, the conditions of transient expression assay were changed: cell suspension cultures were pre-incubated for 2 h in the growth medium containing 150mM sucrose, and were then bombarded either with the pUbi-*GUS* or the pTdDHN8-*GUS* construct; transformed cells remained on 150mM sucrose medium for 2 h and were then transferred to the growth medium containing 500mM mannitol to initiate promoter induction. Although these conditions of the experiment strongly decreased the number of GUS foci, the transformation efficiency was successfully neutralised and no activation of the polyubiquitin promoter by 500mM mannitol was detected (Figure 2A). However, the activity of the *TdDHN8/WCOR410* promoter increased 2.5 fold (Figure 2B).

To further optimize the experimental conditions, 150mM sucrose was replaced with 300mM sucrose in the growth medium during transformation to provide higher transformation efficiency. The final version of the experimental procedure for the transient expression assay in cell suspension cultures is presented in Figure 3.

*Selection of DREB transcription factors, as activators of the TdDHN8/WCOR410 promoter*

The *WCOR410*-like promoters from rice and barley can be activated by stable overexpression of some DREB factors (Lee *et al.*, 2004; James *et al.*, 2008; Morran *et al.*, unpublished), suggesting the possibility of promoter activation through such type of *cis*-acting elements. We decided to test whether some of DREB factors from our laboratory collection can activate this promoter. Firstly, the computer-predicted mapping of DRE/CRT element and low-temperature-responsive element (LTRE) was generated using PLACE software (Prestridge, 1991; Higo *et al.*, 1999; http://www.dna.affrc.go.jp/PLACE/signalup.html). Eleven DRE/CRT/LTRE elements were identified in the 2685 bp promoter region (Figure 4).

To select the candidate DREBs from ten available transcription factors, the mixture of equal amount of each pUbi-*TF* plasmid and the pTdDHN8-*GUS* plasmid was used to co-transform the wheat embryos via particle bombardment (data not shown). Although the efficiency of transformation was very low, we were still able to select four candidate DREBs for the next round of selection. These were ZmDREB2, TaDREB2, TaDREB3, and HvDREB9. The second round of selection was carried out by co-transformation of the cell suspension cultures with each of these four pUbi-*TF* constructs and the pTdDHN8-*GUS* construct (Figure 5A). An increase of GUS expression from eight to two hundred foci in three independent experiments was activated by ZmDREB2 (data not shown). No activation of the *TdDHN8/WCOR410* promoter by TaDREB2 was detected. TaDREB3 transactivated twice as much of the GUS expression driven by the *TdDHN8/WCOR410* promoter. HvDREB9 demonstrated the strongest induction of the *TdDHN8/WCOR410*

promoter: the GUS expression increased fifteen fold in relation to the basal GUS expression (Figure 5B).

Two DREBs were selected for further work: TaDREB3 (Gene bank no. ABC86564) was originally isolated from wheat grain (Lopato *et al*., 2006). Its involvement in both drought and freezing stress response has been recently demonstrated using transgenic barley and wheat plants (Morran *et al*., unpublished). HvDREB9 was isolated from the Y1H cDNA library using a DRE element as the bait sequence. The Y1H cDNA library was prepared from the freezing-tolerant barley variety subjected to cold/freezing stress (Pillman *et al*., unpublished).

*Mapping of functional DRE/CRT elements in the TdDHN8/WCOR410 promoter using co-bombardment and transient expression assay*

Preliminary results showed that the *TdDHN8/WCOR410* promoter is inducible by osmotic stress (500mM mannitol). Furthermore, Northern blot analysis of *WCOR410* gene expression indicated that the promoter contains *cis*-acting element(s) responsive to drought, high salinity, and low temperature (Danyluk *et al.*, 1994). The up-regulation of *WCOR410*-like genes in transgenic plants with constitutive overexpression of DREB factors, suggested that DRE/CRT elements are involved in the activation of *WCOR410*-like gene promoters (Lee *et al.*, 2004; James *et al.*, 2008; Morran *et al.*, unpublished). To map the functional DRE/CRT elements, the full-length *TdDHN8/WCOR410* promoter and six deletions from 5' end promoter fragments were fused to the *GUS* reporter genes (Figure 6A and 6B), and the resulted constructs were co-transformed with either the pUbi-*TaDREB3* or pUbi-*HvDREB9* construct via particle bombardment. Based on the computer-generated mapping

of *cis*-acting elements (Figure 4), -1872, -945, -556, -417, -299, and -230 deletions of the promoter were generated. Each of these deletions, except for deletion -945, was designed to decrease the number of potential DRE/CRT elements one by one thus making easy identification of functional elements (Figure 6A and Figure 6B).

It was demonstrated that TaDREB3 is involved in the activation of the *TdDHN8/WCOR410* promoter. The full-length promoter resulted in two-fold increase of activity over the basal level in the presence of TaDREB3 (Figure 6A). Cell cultures transformed with –1872, -945, -556, -417, and -299 promoter deletions showed similar levels of GUS expression (2.1- to 2.9-fold increase). However, the -230 promoter deletion could not activate the reporter gene (Figure 6A), indicating that the promoter is probably regulated by TaDREB3 through the DRE/CRT element located between -299 and -230.

Several repeats of the core sequence (GCCGAC) and the core sequence with five adjacent base pairs from each side (TTCCG**GCCGAC**ACGCT-) were used as baits for screening Y1H cDNA libraries from unstressed wheat grain. Four different transcription factors containing the AP2-domain were isolated with the 6 bp core element, but only one of them, OsBIERF (Oryza sativa benzothiadiazole-induced ethylene responsive factor (ERF))-like transcription factor (Cao *et al*., 2006), was isolated with the 16 bp element, and hence is considered to be the most specific element (data not shown).

HvDREB9 provides stronger activation of the *TdDHN8/WCOR410* promoter than TaDREB3. The number of GUS foci obtained for the full-length promoter transactivated by HvDREB9 was 5 fold higher than that in the negative control (Figure 6B). The GUS activities of cell cultures transformed with the full-length sequence and six deletions of the promoter maintained a similar level of epression, ranging from 5.9- to 12.6-fold increase in

relation to the basal level. However, nearly two hundred GUS foci were still presented in cell cultures transformed with the -230 promoter deletion. Thus the single putative DRE/CRT located very close to the predicted TATA box seemed to provide a functional *cis*-acting element, which could be efficiently utilized for the promoter activation by HvDREB9, but not by TaDREB3 (Figure 6A and 6B). The sequence of this element, TCCGG**ATCGAC**CTCCT, has the core sequence (ATCGAC), which differs from the element (GCCGAC) specific for TaDREB3 at two base pairs.

*Comparison of the HvDHN8 and TdDHN8/WCOR410 promoter activations by TaDREB3 and HvDREB9*

The sequence alignment showed a high level of conservation of the *TdDHN8/WCOR410* promoter and the previously published *HvDHN8* promoter (Gene Bank Accession No. AF043093) (Figure 7). Four predicted *cis*-acting elements were found to be identical in sequence and position in both promoters. These include three DRE/CRT/LTRE elements (with a core element RYCGAC) potentially inducible by water deficiency, and one MYC recognition sequence (CANNTG), which is potentially responsive to ABA. However, the DRE/CRT element proximal to the TATA box is present only in the *TdDHN8/WCOR410* promoter, but was not identified in the *HvDHN8* promoter (Figure 7). The high identity of the promoter sequences of *TdDHN8/WCOR410* and *HvDHN8* and the similar distribution of *cis*-acting elements in their promoter regions suggested that the *TdDHN8/WCOR410* promoter and *HvDHN8* promoter might display a similar response to abiotic stresses. To test this suggestion, the 2685 bp *TdDHN8/WCOR410* promoter and the 1276 bp *HvDHN8* promoter fused to the *GUS* reporter genes were transactivated with each of TaDREB3 and

HvDREB9 (Figure 8). Both promoters were activated. The GUS expression driven by the *TdDHN8/WCOR410* promoter increased three fold as a result of activation by TaDREB3 and eighteen fold by HvDREB9 relative to the basal level of GUS expression. The GUS expression in cell cultures co-transformed with *HvDHN8* promoter increased seven fold as a result of activation by TaDREB3 and ten fold by HvDREB9. However, the basal level of the *HvDHN8* promoter activity as well as an absolute value of the induced activity was lower than that of the *TdDHN8/WCOR410* promoter. Unlike the situation for the *TdDHN8/WCOR410* promoter, there was no pronounced difference in the levels of the *HvDHN8* promoter activation with TaDREB3 and HvDREB9 transcription factors. This result can be explained by the presence of HvDREB9 specific DRE/CRT element proximal to the TATA box in the *TdDHN8/WCOR410* promoter, and the absence of such an element in the *HvDHN8* promoter (Figure 7).

**Discussion**

There are several advantages in application of cell suspension cultures for assaying promoter activity. Compared with transformation of plant embryos and seedlings, it is easier to produce sufficient amount of material. The efficiency of transformation of cell cultures is much higher than for other plant tissues tested including leaf and embryo (data not shown). Initially, the protocol of Sanford *et al.* (1992) was followed where cell suspension cultures were incubated before and during transformation in the media containing osmotic stress agents. Surprisingly, preliminary results indicated that the high osmotic growth media applied for the pre-incubation of cell suspension cultures before

transformation strongly influence the transformation efficiency and perturbed the analysis of promoter inducibility (Figure 1). To address this problem, the growth media with 150 or 300mM sucrose was used for the pre-incubation and transformation of cell suspension cultures to provide the same level of transformation efficiency across different treatments, and the growth medium containing 500mM mannitol was used after transformation to induce the *TdDHN8/WCOR410* promoter. As a result of this work, a simple protocol for the characterization of stress inducible promoters using a transient expression assay in cell suspension cultures has been developed (Figure 3).

Mannitol was applied to analyse the inducibility of the *TdDHN8/WCOR410* promoter. This is commonly used to induce osmotic stress. The *TdDHN8/WCOR410* promoter has a weak basal activity in cell cultures, and is moderately induced (2-2.5 fold) by osmotic stress (Figure 2). These results are in a good agreement with the results of Northern blot analysis for the *WCOR410* gene expression, which is weakly expressed in wheat leaves, crowns, and roots in the absence of stress, and is strongly induced by low temperature, high salinity, and dehydration (Danyluk *et al.*, 1994).

Computer prediction of *cis*-acting elements identifies eleven potential DRE/CRT/LTRE elements distributed in the 2685 bp promoter region (Figure 4). Many other elements like MYBR, MYCR and ABRE, which are known to be involved in osmotic stress and ABA induction of promoters, were also identified (Figure 4). However, because it was known that the *WCOR410*-like promoters are activated in transgenic plants by constitutively overexpressing DREB/CBF transcription factors (Lee *et al.*, 2004; James *et al.*, 2008; Morran *et al.*, unpublished). The *TdDHN8/WCOR410* promoter was evaluated to see if it can be activated by any of ten DREBs isolated in our laboratory. After two co-

bombardment experiments, two transcription factors, TaDREB3 and HvDREB9, were selected for further work. Both activated the *TdDHN8/WCOR410* promoter albeit with different efficiency: 2 and 18 folds, respectively (Figure 5B).

TaDREB3 and HvDREB9 were used for the 5' promoter deletion analysis to search for functional DRE/CRT elements in the *TdDHN8/WCOR410* promoter region. One functional *cis*-acting element located between -299 and -230 was successfully mapped using TaDREB3 (Figure 6A). The core sequence of this *cis*-acting element, GCCGAC, belongs to the RYCGAC elements known to be involved in response to low temperature (Xue, 2002), suggesting that this DRE/CRT element and the interacting TaDREB3 could be involved in cold regulation. This DRE/CRT element (core GCCGAC) was also partially transactivated by HvDREB9 (Figure 6B), which was isolated from cold/freezing stress induced material and is also expected to be a cold stress-related transcription factor (Pillman *et al.*, unpublished). However, -230 promoter deletion still showed strong activity (Figure 6A and 6B), suggesting that the only remaining DRE/CRT element (core ATCGAC) in this region was recognized by HvDREB9. In addition, this *cis*-acting element is specific for the *TdDHN8/WCOR410* promoter and is absent in the *HvDHN8* promoter (Figure 7). Activation of the *HvDHN8* promoter with HvDREB9 was less efficient than that of *TdDHN8/WCOR410* promoter (Figure 8). Therefore, the results suggest the following:

1) The DRE/CRT element (core ATCGAC) located on the -230 promoter deletion is a main functional *cis*-acting element, but some other DRE/CRT elements may also be utilized by transcription factors with different binding specificity;

2) TaDREB3 and HvDREB9 transcription factors have different DNA-binding specificity: TaDREB3 specifically binds to the DRE/CRT element (core GCCGAC) located between -

290 and -230, and HvDREB9 binds to the DRE/CRT element (core ATCGAC) located on the -230 promoter deletion. An even shorter deletion, -141, without the single putative DRE/CRT, is currently being undertaken to further confirm these conclusions.

Using the TaDREB3 specific DRE/CRT element as a bait sequence in the Y1H screen of the cDNA library prepared from the unstressed wheat grain, an OsBIERF-like transcription factor was isolated (data not shown). OsBIERF genes belong to the ERF subfamily of transcription factors containing the AP2-domain. They were shown to have a moderate level of expression in the absence of stress and to be strongly induced by cold, salt and drought stress (Cao *et al*., 2006). The new wheat transcription factor may regulate both the basal level of activity of the *TdDHN8/WCOR410* promoter in the absence of stress and the strong inducibility of the promoter under different environmental stresses. Furthermore, the OsBIERF transcription factor was also found to be up-regulated by wounding (Cao *et al*., 2006). The OsBIERF-like transcription factor from wheat may provide an explanation for the accumulation of WCOR410 mRNA in wounded wheat shoots (Danyluk *et al*., 1994).

The level of GUS expression from the *HvDHN8* promoter was increased by transient up-regulation of two cold/drought stress- related transcription factors, TaDREB3 and HvDREB9. The *HvDHN8* promoter showed low basal GUS expression in the negative control and seven- and ten-fold increase in activity upon co-expression with the TaDREB3 and HvDREB9 transcription factors, respectively. This result correlates with data previously that the *HvDHN8* gene expression (barley cv. Dicktoo) is transiently up-regulated on dehydration and cold stress, but shows a low level of expression in unstressed conditions (Choi *et al*., 1999). However, there is contradictory data in the literature on the *HvDHN8* promoter activity. In two reports, the *HvDHN8* gene expression (*paf93*, barley cv.

Georgie) was not observed in well-watered plant leaves, but was rapidly induced by dehydration (Grossi *et al*., 1992 and 1995). In another report, the *HvDHN8* promoter (barley cv. Tallon) was characterized as a constitutive promoter that has stronger activity than the rice *Act1* promoter (Xiao and Xue 2001). These results may indicate that the activity of *HvDHN8* promoter in different genotype backgrounds may vary under different environmental stresses, but the reasons of these differences are not clear.

In conclusion, an optimized protocol of transient expression assays using cell suspension cultures was developed to analyse the activity of stress-inducible promoters. Two DREB factors, TaDREB3 and HvDREB9, were selected and successfully used to map the functional DRE/CRT elements in the *TdDHN8/WCOR410* promoter region.

**Experimental procedures**

*Promoter cloning and plasmid construction*

The full length coding region of *WCOR410* cDNA (Gene bank accession no. L29152) was isolated by PCR using a cDNA library from the spike of drought stressed wheat (*Triticum aestivum* L. cv. Chinese spring). This region was used to probe a BAC library prepared from the genomic DNA of *T. durum* cv. Langdon (Cenci *et al*., 2003) using Southern blot hybridisation as described elsewhere (Sambrook *et al*., 1989). Plasmid DNA from two BAC clones (#583G18 and #661E9), which strongly hybridised with the probe, was isolated using a Large Construct Kit (QIAGEN). The *T. durum* homolog of *WCOR410* was identified by PCR using the BAC DNA as templates and primers derived from the ends of

the coding region of *WCOR410* cDNA. Both clones gave identical PCR products; #661E9 was used in further work. The *TdPR60* promoter sequence was first identified on the BAC clone (#661E9) by consecutive sequencing reactions. As a result of 'walking' along the DNA, about 3100 bp of sequence upstream from the *TdPR60* translation start codon was obtained. This sequence was subsequently used to design forward and reverse primers for the isolation of the promoter segment. A 2685 bp fragment of promoter with a full-length 5'-untranslated region of *TdPR60* was amplified by PCR using AccuPrime™ Pfx DNA polymerase (Invitrogen) from DNA of BAC clone #661E9 as a template. This sequence was cloned into the pENTR-D-TOPO vector (Invitrogen), and the cloned insert was then verified by sequencing and subcloning into the pMDC164 vector (Curtis and Grossniklaus, 2003) using recombination cloning. The resulting construct was designated pTdDHN8-*GUS*. Selectable marker genes conferred hygromycin resistance in plants and kanamycin resistance in bacteria. The construct of pHvDHN8-*GUS* was kindly provided by K. Pillman, and the pUbi-*GUS* construct was kindly provided by S. Eliby. The resulting binary vector was used in transient expression assay.

*TdDHN8/WCOR410* promoter deletions were generated by PCR using AccuPrime™ Pfx DNA polymerase (Invitrogen) and the full length *TdDHN8/WCOR410* promoter as a template; the map of computer-predicted *cis*-acting elements in the *TdDHN8/WCOR410* promoter region (Figure 4) was used to design forward and reverse primers. Information on primers is summarised in the Table 1. Promoter deletions were verified by sequencing and cloned into the pMDC164 vectors and used in transient expression assay, as described above.

Coding regions of ten different DRE/CRT binding transcription factors were cloned into the pENTR-D-TOPO vector (Invitrogen); the cloned insert was verified by sequencing and subcloned into the pUbi vector (pMDC32 derivative with maize ubiquitin promoter instead of 2x35S). In the same time, pUbi-*GFP* plasmids were constructed, and the mixture of 5 µl pUbi-*GFP* plasmids and 5 µl pTdDHN8-*GUS* plasmids were then used as a negative control in transient expression assay.

*Transient expression assay using cell suspension cultures*

Cell suspension cultures of *T. monoccocum* L., were cultured with 100 ml 1/2MS and 2 mg/l 2,4-D liquid medium in the dark room, 25°C, and was subcultured weekly. The cell suspension cultures were harvested on sieves in a laminar flow hood. 3-4 ml material was gently spread as a 3.5 cm circle on the growth medium containing 300mM sucrose over one layer of filter paper, 2 h prior to bombardment.

Concentrations of every plasmid sample were measured with NanoDrop ND-1000 Spetro and were adjusted to 0.5 µg/µl. 5 µl of plasmids containing transcription factor coding sequence and 5 µl of plasmids containing promoter fragments were mixed and precipitated with 1 µl NaAc (3 M, pH 4.8) and 15 µl 70% isopropanol, then centrifuged in 1.5 ml Eppendorf tubes at 13,200 rpm, 4°C for 15 min. The pellets were gently washed twice with 75% ethanol and dried in a laminar flow hood. The purified DNA mixture dissolved in 10 µl MilliQ water was used for gold (0.6 µm) coating according to the protocol of Sanford *et al*. (1992).

Particle bombardment was performed utilizing the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). The plate loaded with the pre-incubated cell suspension

cultures was carefully placed under the launch point. Bombardment conditions were 1100 psi, with a 15 mm distance from the macrocarrier launch point to the stopping screen and a 60 mm distance from the stopping screen to a target plant material. The distance between the rupture disk and the launch point of the macrocarrier was 12 mm. The pre-incubated cells were bombarded on growth medium containing 300mM sucrose, except where otherwise indicated. The transformed cells remained on 300mM growth medium for 2 h and then were transferred on treatment growth media. The transformed cells were grown in the dark chamber at room temperature for 24 h.

Preparation of GUS staining solution was described previously (Li *et al*., 2008), and 20% (v/v) methanol was added in the GUS staining solution just before use. The filter paper containing the transformed cells was transferred to a new Petri dish; 1.3 ml GUS staining solution was carefully pipetted under the filter paper so as not to disturb the cell circle. The cells were placed into 37°C chamber for overnight incubation. GUS activity was determined by counting the number of blue cells with the aid of a stereomicroscope Leica DC 300F.

*Transient expression assay using wheat embryos*

Wheat (*T. aestivum* L. cv. Bobwhite) embryos were transformed using biolistic bombardment according to the following protocol. Immature seeds of wheat were surface-sterilized by immersing into 70% ethanol for 2 min, followed by incubation in 1% sodium hypochlorite solution with shaking at 125 rpm for 20 min and follwoed by three washes in distilled water. Before bombardment, immature embryos (1.0-1.5 mm in length, semitransparent) were pre-treated for 4 h on MS2 medium supplemented with 100 g/l

sucrose. Embryos (50/plate) were then placed in the centre of a plate to form a circle with a diameter of 10 mm. The transformation of embryos followed the same bombardment conditions described above. The transformed embryos were cultured in the dark chamber at room temperature for 24 h. Then the transformed embryos were collected in a 1.5 ml tube, 1 ml GUS staining solution was added, and infiltrated in a vacuum chamber for 2-3 minutes before placing them in the 37°C chamber for overnight incubation. GUS activity was determined by counting the number of blue cells with the aid of a stereomicroscope Leica DC 300F.

*Y1H screen analysis*

Y1H reporter constructs were prepared by the cloning of tandems containing three to four repeats of the 6 bp core *cis*-acting element, -GCCGAC-, and the 16 bp *cis*-acting element, -TTCCGGCCGACACGCT-, for the TaDREB3 transcription factor into pINT1-HIS3NB vector. The primers used for the *cis*-acting elements preparation are shown in the Table 1. The resulting fragments were cloned into the *Spe*I-*Not*I sites of the pINT1-HIS3NB vector. The Y1H screen was carried out as described by Lopato *et al.* (2006).

*Statistical analysis*

Statistical analysis was performed using the ANOVA-procedure of GenStat 9.0. Standard error is shown in figures as horizontal or vertical bars ($P<0.05$). The transformation efficiency (TE) was calculated: TE= number of expressed GUS foci on each plate/ maximum number of GUS foci driven by the polyubiquitin promoter (3800 GUS foci).

**Acknowledgements**

We thank Prof. U. Grossniklaus for providing us with the collection of pMDC vectors, K. Pillman for providing the HvDREB9 and pHvDhn8-*GUS* construct, Dr. A. Ismagul and O. Nguyen for the preparation of wheat cell suspension cultures and embryos, Dr. Natalia Tihomirov for the isolation of *TdDHN8/WCOR410* promoter, and Dr. T. Pyvovarenko for the preparation of the bait strains and the Y1H screen. Thanks are especially given to N. Bazanova for the preparation of the full-length T*dDHN8/WCOR410* promoter and the ten pUbi-*TF* constructs, and my supervisors S. Lopato and S. Eliby for their communications on the manuscript. This work was supported by the Australian Centre for Plant Functional Genomics and Masters of Plant Biotechnology project (Plant SC 7229A WT& SC7229BWT extended research project) of The University of Adelaide.

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**Table 1.** Summary of primers and oligonucleotides used for the construction of expression plasmids

| Primers used for the generation of the deleted *TdDHN8/WCOR410* promoter fragments | |
|---|---|
| Primers | Sequence |
| Forward primer D1 | CACCTATGAGAGCCTTCAAGAC |
| Forward primer D2 | CACCAGTTTAATCAGTCCGCTCAC |
| Forward primer D3 | CACCGATCACTTAATCAATCGGTC |
| Forward primer D4 | CACCACGCCTCGGTGAGCGTAACTAC |
| Forward primer D5 | CACCTCCACTGGCTCACGCGCTG |
| Forward primer D6 | CACCGTCCTTCTTTCCTGCTTG |
| Reverse primer | GATCGAGATCGATCGGTGCAG |
| Oligonucleotides used for the generation of bait sequences in Y1H screen | |
| Oligonucleotide | Sequence |
| Forward primer for the core element | GGCCGCGCCGACGCCGACGCCGACGCCGACGCCGACA |
| Reverse primer for the core element | CTAGTGTCGGCGTCGGCGTCGGCGTCGGCGTCGGCGC |
| Forward primer for the extended element | GGCCGCTTCCGGCCGACACGCTTTCCGGCCGACACGCT TTCCGGCCGACACGCTA |
| Reverse primer for the extended element | CTAGTAGCGTGTCGGCCGGAAAGCGTGTCGGCCGGAA AGCGTGTCGGCCGGAAGC |

**Figure legends**

**Figure 1.** Analysis of the effect of different concentrations of mannitol in the growth media on transformation efficiency in cell suspension cultures. **A.** Comparison of the GUS expression levels controlled by the constitutive polyubiquitin promoter (a) and inducible *TdDHN8/WCOR410* promoter (b). The cell suspension cultures remained on the same growth media during and after the transformation via particle bombardment. **B.** Quantification of the effect of the concentration of mannitol in the growth medium on the transformation efficiency. Two repeats were conducted for each treatment. Error bar = standard deviations (SD).

**Figure 2.** Induction of the *TdDHN8/WCOR410* promoter by 500mM mannitol treatment after particle bombardment. The pre-incubated cell suspension cultures on the growth medium containing 150mM sucrose were bombarded with the pUbi-*GUS* construct (A) and pTdDHN8-*GUS* construct (B), respectively. Transformed cells remained on 150mM sucrose growth medium for 2 h and then were transferred to the growth medium containing 500mM mannitol to initiate the osmotic-stress induction. Two repeats were conducted for each treatment. Error bar= standard deviations (SD).

**Figure 3.** Schematic representation of the transient expression assay in cell suspension cultures.

**Figure 4.** Identification of the potential *cis*-acting elements in the *TdDHN8/WCOR410* promoter using computer software. The prediction was completed based on the PLACE online program (version 30.0). The initiation codons (ATG) and TATA boxes are highlighted. Numbers indicate the nucleotide positions used for the 5' deletion analysis of the *TdDHN8/WCOR410* promoter. Transcription initiation sites that were determined by primer extension are marked by arrowheads. The predicted *cis*-acting elements are underlined. The symbols in the figure are used to represent different *cis*-acting elements as follows: empty triangle (Δ), MYB recognition sequence; empty pentagon (⏢), MYC recognition sequence; empty square (□), ABA-responsive element (ABRE); solid oval (●),

drought-responsive element/C-repeat (DRE/CRT); and solid moon-like shape (◖), low temperature-responsive element (LTRE).

**Figure 5.** Selection of candidate transcription factors by transactivation of the *TdDHN8/WCOR410* promoter in transient expression assay. A. Constructs used for the selection of candidate DREB factors. Potential transcription factors (effectors) were constitutively expressed under the control of the maize polyubiquitin promoter. *GFP* reporter gene linked to the maize polyubiquitin promoter was used as an empty effector in the negative control (NC). *GUS* reporter gene linked to the *TdDHN8/WCOR410* promoter was used to detect the activity of the promoter. B. Analysis of the activity of the *TdDHN8/WCOR410* promoter by co-bombardment with effectors. The mixture of pUbi-*GFP* and pTdDHN8-*GUS* constructs was used for the negative control. Three repeats were conducted for each treatment. Error bar= standard deviations (SD).

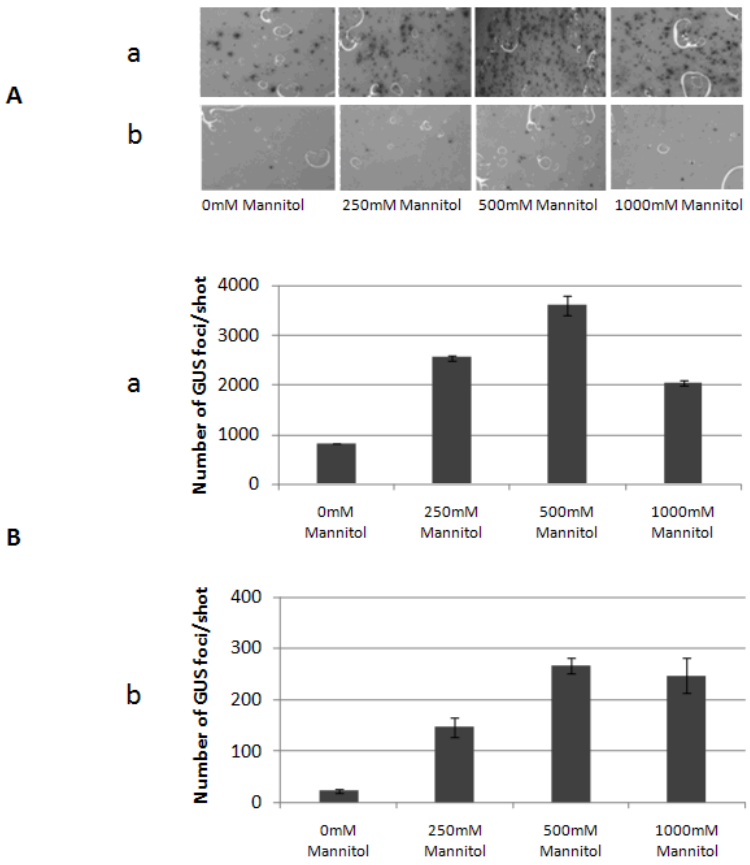
**Figure 6.** Identification of functional DRE/CRT elements by the 5' deletion analysis of the *TdDHN8/WCOR410* promoter by transactivation of *GUS* reporter gene in transient expression assay. The full-length *TdDHN8/WCOR410* promoter and six deleted from 5' end fragments were linked to the *GUS* reporter genes and co-transformed with either pUbi-*TaDREB3* (A) or pUbi-*HvDREB9* (B) into cell suspension cultures via particle bombardment. Schematic representation of 5' end deletions of the promoter fused to *GUS* genes is shown in the left part of the figure: asterisk (*), predicted DRE site; solid rectangular (■), TATA box. Negative control (basal level of promoter activity) is shown in the right part as an empty box. Three repeats (A) and six repeats (B) were conducted for each treatment. Error bar= standard deviations (SD).

**Figure 7.** Sequence alignment of the promoter regions of *HvDHN8* and *TdDHN8/WCOR410*. The 657 bp 5' promoter sequence of *HvDHN8* was aligned with the 647 bp 5' promoter sequence of *TdDHN8/WCOR410*. Slashes indicate the identical nucleotides in the two promoter regions. Numbers indicate the nucleotide positions used for

the 5' deletion analysis of the *TdDHN8/WCOR410* promoter. Transcription initiation sites that were determined by primer extension are marked by arrowheads. The translation initiation codons (ATG) and TATA boxes are shaded. The conserved *cis*-acting elements are enclosed in boxes. The non-conserved *cis*-acting elements are underlined. DRE/CRT represents the drought-responsive element/C-repeat. MYBR and MYCR represent the MYB recognized sequence and MYC recognized sequence, respectively.

**Figure 8.** Comparison of transactivation of the *TdDHN8/WCOR410* and *HvDHN8* promoters by TaDREB3 and HvDREB9 transcription factors. The mixture of the pUbi-*GFP* construct with either the pTdDHN8-*GUS* construct or pHvDHN8-*GUS* construct was used for the negative control. Three repeats were conducted for each treatment. Error bar= standard deviations (SD).

**Figure 1.**


A
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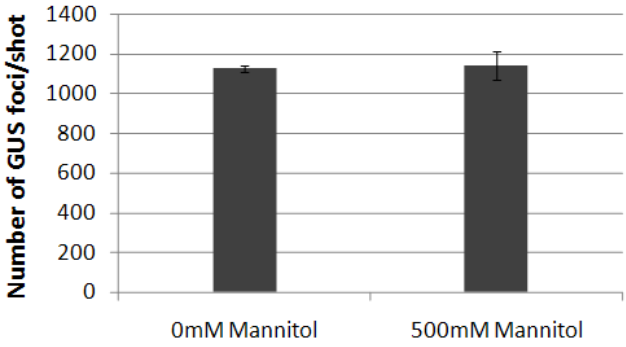
Number of GUS foci/shot
4000
3000
2000
1000
0
0mM Mannitol
250mM Mannitol
500mM Mannitol
1000mM Mannitol
b
Number of GUS foci/shot
400
300
200
100
0
0mM Mannitol
250mM Mannitol
500mM Mannitol
1000mM Mannitol

**Figure 2.**

A


Number of GUS foci/shot
1400
1200
1000
800
600
400
200
0
0mM Mannitol
500mM Mannitol

B

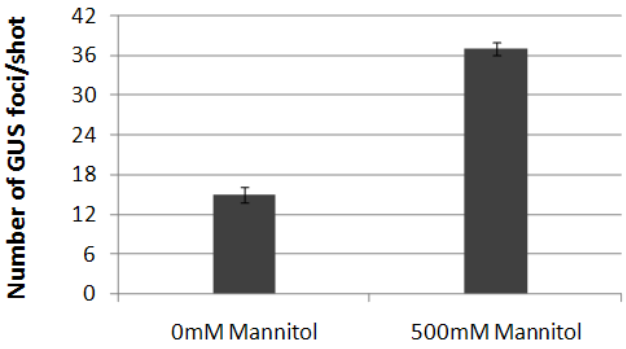

Number of GUS foci/shot
42
36
30
24
18
12
6
0
0mM Mannitol
500mM Mannitol

**Figure 3.**

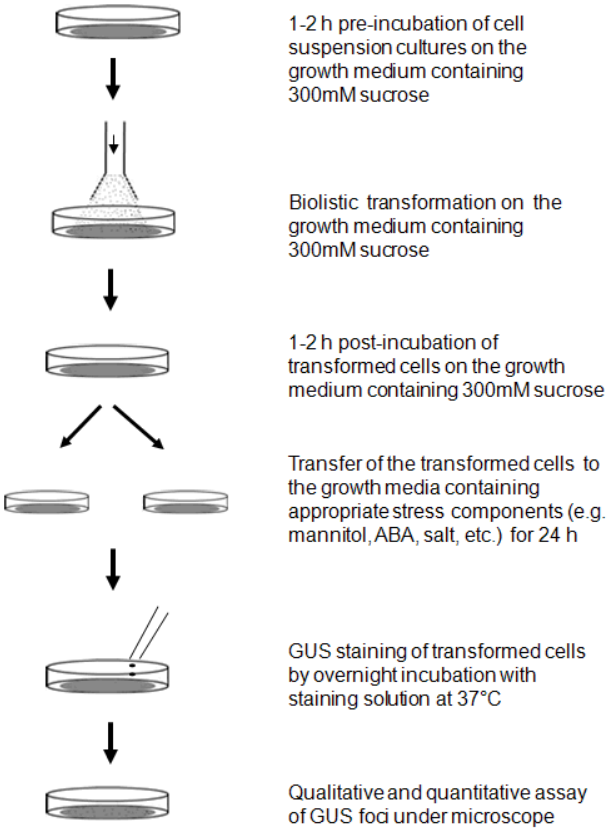

1-2 h pre-incubation of cell suspension cultures on the growth medium containing 300mM sucrose
Biolistic transformation on the growth medium containing 300mM sucrose
1-2 h post-incubation of transformed cells on the growth medium containing 300mM sucrose
Transfer of the transformed cells to the growth media containing appropriate stress components (e.g. mannitol, ABA, salt, etc.) for 24 h
GUS staining of transformed cells by overnight incubation with staining solution at 37°C
Qualitative and quantitative assay of GUS foci under microscope

**Figure 4.**

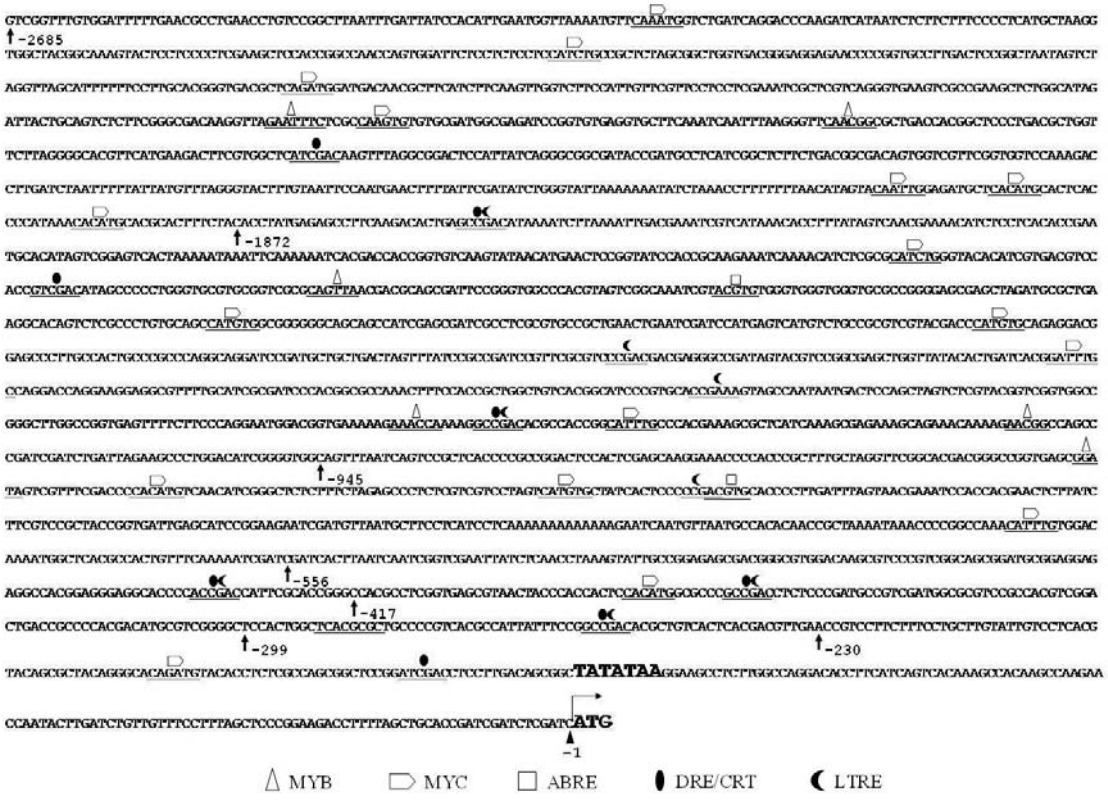

-2685
-1872
-945
-556
-417
-299
-230
TATATAA
ATG
-1
MYB
MYC
ABRE
DRE/CRT
LTRE

**Figure 5.**

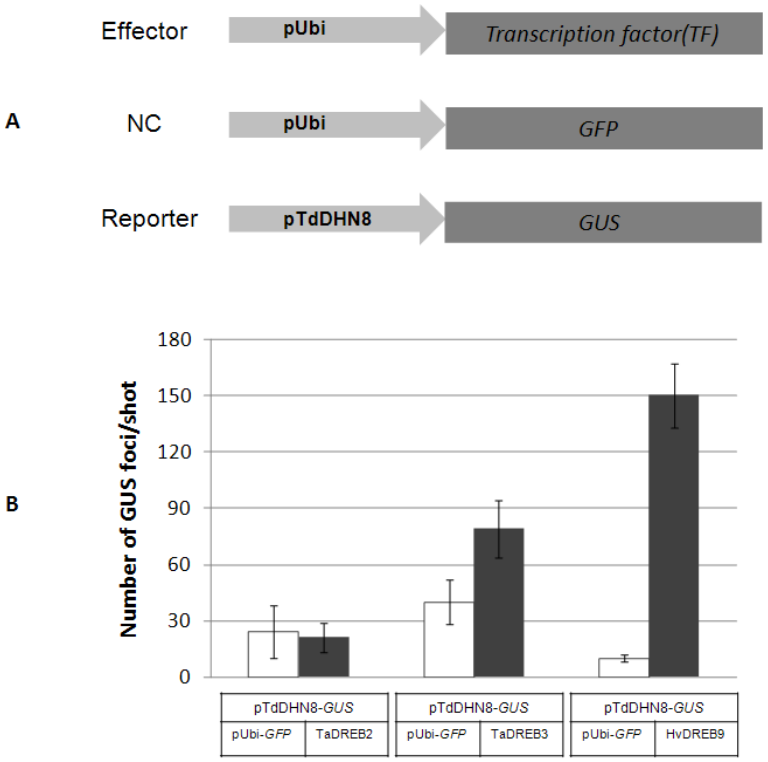

A
Effector
pUbi
Transcription factor(TF)
NC
pUbi
GFP
Reporter
pTdDHN8
GUS
B
Number of GUS foci/shot
180
150
120
90
60
30
0
pTdDHN8-GUS
pUbi-GFP
TaDREB2
pTdDHN8-GUS
pUbi-GFP
TaDREB3
pTdDHN8-GUS
pUbi-GFP
HvDREB9

**Figure 6.**

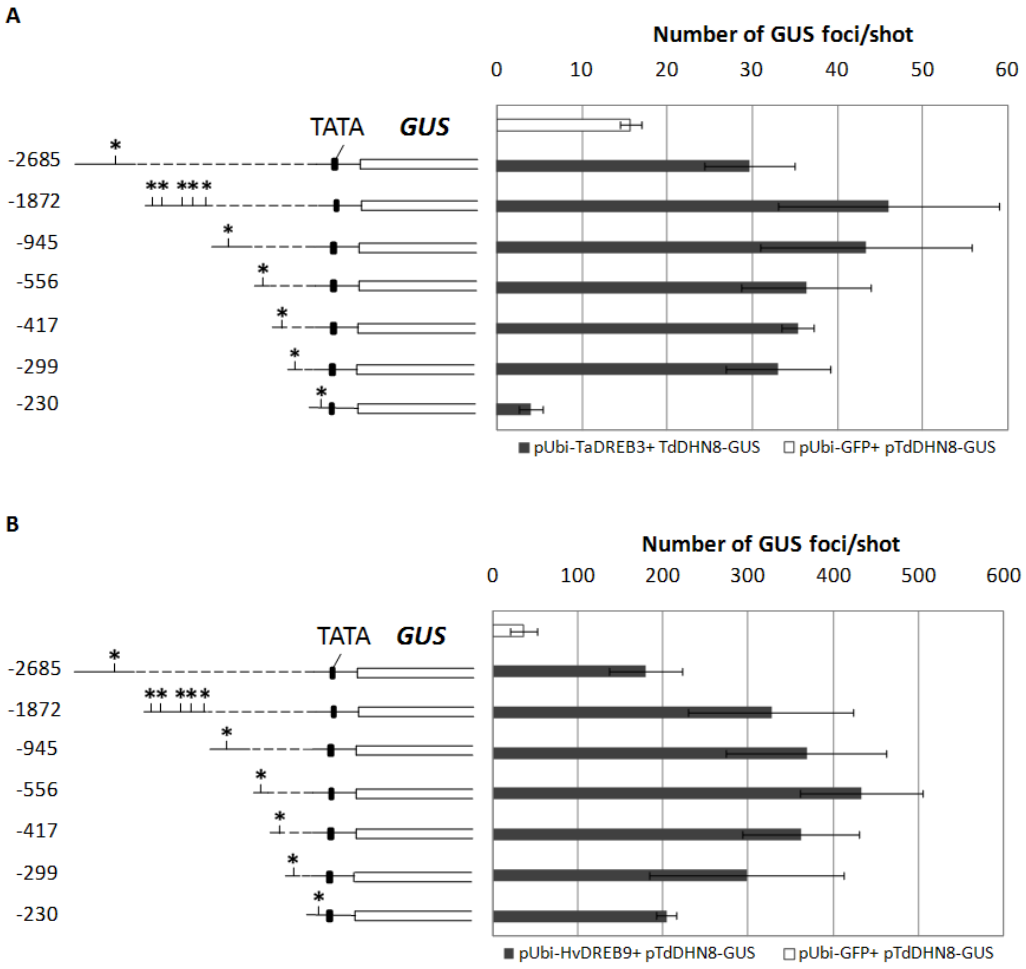

A
Number of GUS foci/shot
0
10
20
30
40
50
60
TATA
GUS
-2685
-1872
-945
-556
-417
-299
-230
pUbi-TaDREB3+ TdDHN8-GUS
pUbi-GFP+ pTdDHN8-GUS
B
Number of GUS foci/shot
0
100
200
300
400
500
600
TATA
GUS
-2685
-1872
-945
-556
-417
-299
-230
pUbi-HvDREB9+ pTdDHN8-GUS
pUbi-GFP+ pTdDHN8-GUS

**Figure 7.**

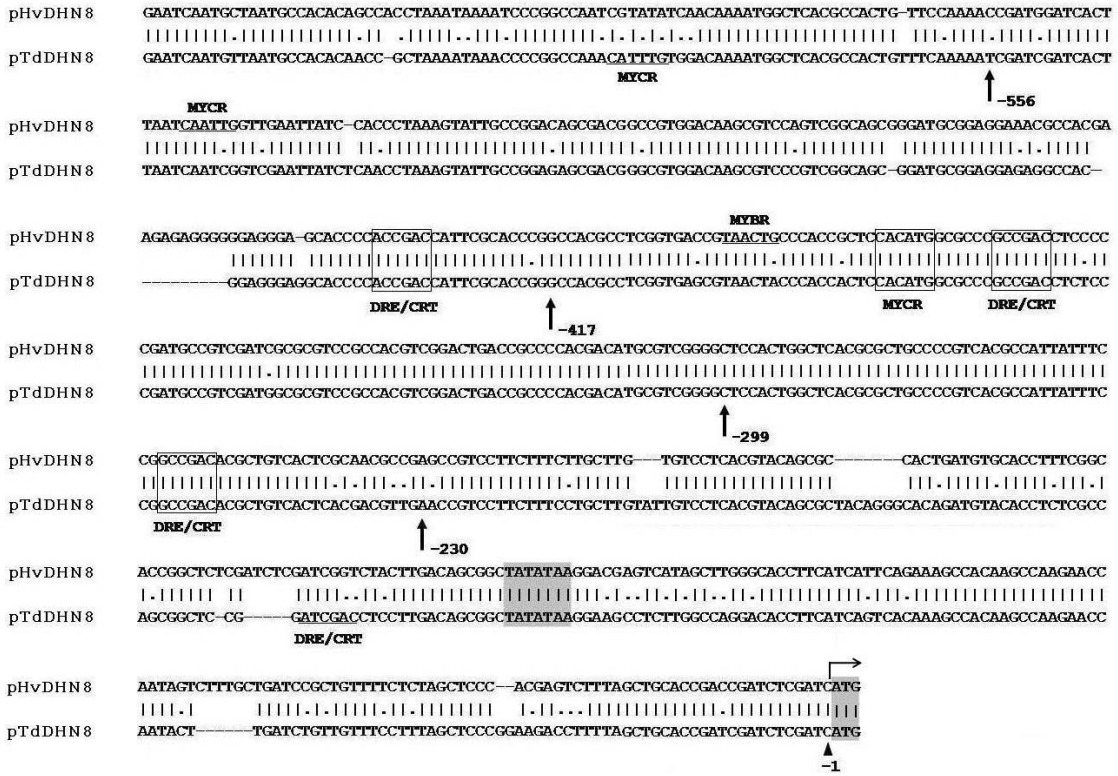
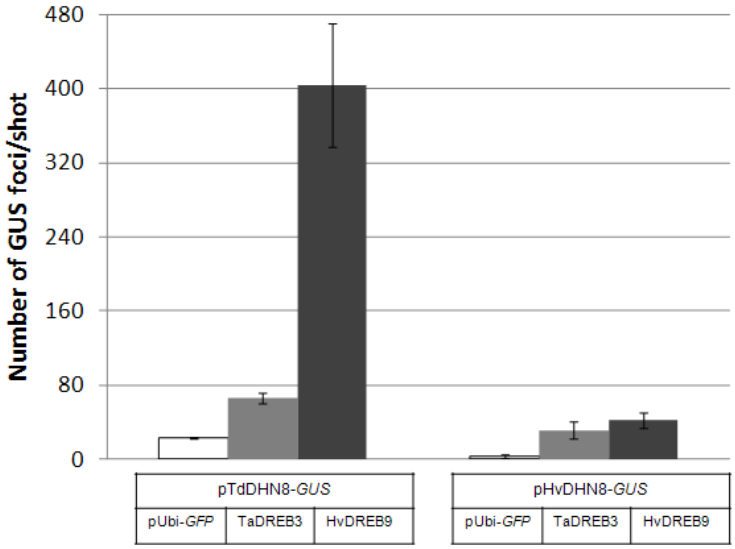


**Figure 8.**


Number of GUS foci/shot
480
400
320
240
160
80
0
pTdDHN8-GUS
pUbi-GFP
TaDREB3
HvDREB9
pHvDHN8-GUS
pUbi-GFP
TaDREB3
HvDREB9