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Molecular genetics studies of bZIP transcription factor, TRAB1, and MYB transcription factor, ME97, on the Viviparous 1 promoter in Zea mays

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Molecular genetics studies of bZIP transcription factor, TRAB1, and MYB transcription factor, ME97, on the *Viviparous 1* promoter in *Zea mays*

by

Joonbae Seo

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Program of Study Committee:
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To my parents and wife
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CHAPTER 1. GENERAL INTRODUCTION

Cereal grains are the major food source for human as well as livestock and poultry. Therefore, it is important to understand the regulatory networks controlling the complex developmental and metabolic processes of cereal grains. In cereals, seed development starts with a double fertilization, which generates a diploid zygote and a triploid endosperm. In the maturation phase of seed development, the embryo and aleurone layer of the endosperm acquire desiccation tolerance and enter developmental arrest, while the starchy endosperm undergoes program cell death. Blocks in the maturation program produce a viviparous phenotype, where seeds germinate precociously. The inadequate establishment of seed dormancy can lead to preharvest germination, a condition reminiscent of vivipary that results in severe agronomic losses (Neil et al., 1987; Bewley, 1997; Koornneef et al., 2002).

Seed maturation, is controlled by a network of transcription factors interacting with ABA signaling pathways. In this process, the *Viviparous1* (*Vp1*) gene, encoding a B3 transcription factor, acts as both a transcriptional activator to promote maturation by regulating ABA-inducible gene expression, and a repressor to inhibit germination during seed development (Bobb et al., 1995; Hoecker et al., 1995, 1999; Cao et al., 2007). Maize *vp1* mutants disrupt ABA sensitivity, causing kernels to fail to withstand desiccation and undergo precocious germination on the ear. Mutants also fail to activate *CI* expression, resulting in colorless kernels in the endosperm (McCarty et al., 1989; McCarty et al., 1991). ABA also functions in adapting to various environmental stresses, such as drought, salt, and cold stress. Drought
conditions trigger the biosynthesis of ABA, and the increased ABA level induces various ABA-responsive genes, such as drought-inducible genes (Skriver and Mundy, 1990).

Even though the regulation of Vp1 expression is key to this important process, how the regulatory networks of seed development control Vp1 expression is not well studied. Detailed studies of the maize Vp1 promoter sequence identified five conserved regions, including a predicted ABRE and an element resembling CE1, and a predicted MYB-binding site (MBSI) (Sandelin et al., 2004; Cao, 2005; Cao et al., 2007). ABA or osmotic stress treatment regulated the interaction of embryo nuclear proteins with the ABRE (Cao et al., 2007).

bZIP transcription factors that bind ABREs have been identified from different plants (Hobo et al., 1999; Finkelstein and Lynch, 2000; Casaretto and Ho, 2003; Zou et al., 2007). bZIP transcription factors transactivate the expression of downstream genes, and regulate stress responses, seed maturation and germination, flower development and ABA signaling (Uno et al., 2000; Casaretto and Ho, 2003). In addition, ABI3, the Arabidopsis ortholog of VP1 (Giraudat et al., 1992), interacts with ABI5 and homologous bZIP transcription factors to regulate ABA-inducible gene expression (Hobo et al., 1999; Lopez-Molina et al., 2002; Casaretto and Ho, 2003).

A rather large MYB family, reported from different plants, plays a variety of key roles in the regulation of gene expression, and is also related to transcriptional responses to hormones during seed development and germination. For example, the maize C1 gene regulates the expression of genes that are involved in the biosynthesis of anthocyanin pigments in the aleurone (Cone et al., 1986; Paz-Ares et al., 1986; Paz-Ares et al., 1987; Hattori et al., 1992). Interestingly, the MYC and MYB consensus sequences were reported in
the rd22 promoter, which do not contain any typical ABRE recognition site (Yamaguchi-Shinozaki and Shinozaki, 1993). However, both AtMYC2 and AtMYB2 genes are induced by drought and ABA treatment, suggesting that AtMYB2 may regulate cooperatively with AtMYC2 in another regulatory system other than the ABRE-bZIP regulatory system in the ABA signaling pathway in vegetative tissues and seeds under drought and salt stress (Iwasaki et al., 1995; Abe et al., 1997; Abe et al., 2003).

The main objective of this research is to understand the genetic control of seed maturation. Since Vp1 is the most upstream known factor in maize seed maturation, investigating the factors that regulate Vp1 expression will provide important information on this aspect of seed development. This also has implications for the specification of endosperm cell fate during grain development. In this thesis, the maize bZIP transcription factor, ZmTRAB1, and Myb transcription factor, ME97, are identified as proteins that respectively bind to an ABRE and MBSI in the Vp1 promoter.

1.1 Thesis Organization

The thesis is organized in the format consisting of two chapters preceded by a General Introduction and followed by a General Conclusion. The chapters are formatted according to the requirements of ‘Plant Physiology’. Chapter 2 is “The maize bZIP transcription factor, ZmTRAB1, binds to an abscisic acid response element (ABRE) in the Vp1 promoter”. Chapter 3 is “Isolation of a Myb transcription factor that interacts with a predicted Myb
recognition sequence of the Vp1 promoter of maize”. Joonbae Seo was the primary investigator for this work under the supervision of Dr. Philip W. Becraft and is the first author for both chapters.

1.2 Literature Cited

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15: 63-78


CHAPTER 2. THE MAIZE BZIP TRANSCRIPTION FACTOR, ZMTRAB1, BINDS TO AN ABSCISIC ACID RESPONSE ELEMENT (ABRE) IN THE VP1 PROMOTER

2.1 Abstract

The maize VP1 promoter contains a putative ABA response element (ABRE). The bZIP transcription factor, TRAB1, binds ABREs in seed maturation related gene promoters of other cereals. Database searches, multiple sequence alignment and phylogenetic analysis show that ZmTRAB1 is a putative TRAB1 ortholog in maize. ZmTRAB1 undergoes alternative splicing and two splicing variant cDNAs, designated ZmTRAB1-1, and ZmTRAB1-2 were isolated. As illustrated by gel mobility shift assays, the proteins encoded by each splicing variant could specifically bind to the ABRE in vitro. In spite of a high conserved bZIP domain, the transactivation activity of ZmTRAB1-1 was higher than that of ZmTRAB1-2 in transient assays. The relative GUS activity of a Vp1::GUS reporter cobombarded with ZmTRAB1-2 into maize embryos increased with exogenous ABA, while that of ZmTRAB1-1 showed a statistically insignificant induction by ABA. Taken together, these results suggest that ZmTRAB1 variants are embryo expressed transcription factors that function in ABA signaling.
2.2 Keywords

Abscisic acid · Abscisic acid response element · bZIP transcription factor · ABI5 · TRAB1 · Alternative splicing

2.3 Abbreviations

ABA: Abscisic acid · ABRE: Abscisic acid response element · GST: Glutathione S-transferase · GUS: β-Glucuronidase

2.4 Introduction

The unfavorable control of seed dormancy and germination transition causes severe agronomic loss via pre-harvest sprouting. The phytohormone abscisic acid (ABA) functions as an important regulator during this transition by triggering seed dormancy and inhibiting seed germination. ABA also functions in adapting to various environmental stresses, such as drought, salt, and cold stress. ABA deficient or insensitive mutants fail to undergo quiescence and precociously germinate, accompanied by a decreased expression of maturation genes (Bewley, 1997; Koornneef et al., 2002).

The Vp1 gene encodes a B3 domain transcription factor that is a key regulator of late embryogenesis and seed maturation in maize (McCarty et al., 1991). VP1 activates the C1
gene, a transcriptional regulator of anthocyanin synthesis genes, by directly binding to a Sph/Ry element. It also activates ABA inducible genes such as Em, by indirectly binding to ABREs (ABA responsive elements) (McCarty et al., 1989; Suzuki et al., 1997; Hobo et al., 1999). Maize vp1 mutant kernels are ABA insensitive, fail to acquire desiccation tolerance, and undergo precocious germination on the ear (McCarty et al., 1989; McCarty et al., 1991).

The orthologous ABI3 gene of Arabidopsis is required for the accumulation of storage proteins and the acquisition of desiccation tolerance (Finkelstein, 1993). Its mutant similarly inhibits seed maturation and the mutant phenotype can be complemented by the maize Vp1 (Giraudat et al., 1992; Suzuki et al., 2001). VP1 acts as both a transcriptional activator with ABA to promote maturation and a repressor to repress germination during seed development (Bobb et al., 1995; Hoecker et al., 1995, 1999). Maize Vp1 is induced by ABA, high salinity and osmoticum during mid-maturation phase of maize embryogenesis (Cao et al., 2007).

ABI3 and its orthologs interact with ABI5 and its homologs to regulate ABA-inducible gene expression (Hobo et al., 1999; Lopez-Molina et al., 2002; Casaretto and Ho, 2003).

Detailed studies of the maize Vp1 promoter sequence identified five conserved regions, including a predicted ABRE with ACGT core sequence and an element resembling CE1 (Sandelin et al., 2004; Cao et al., 2007). This predicted ABRE was also identified at position -77 to -84 (GCCACGTG), which is similar to rice OsEM promoter (ACGTGKC) (Hattori et al., 2002). ABA or osmotic stress treatment regulated the interaction of embryo nuclear proteins with the ABRE (Cao et al., 2007).

bZIP transcription factors can bind to ABREs, transactivate downstream gene expression, and regulate stress responses, seed maturation and germination, flower development and ABA signaling (Uno et al., 2000; Casaretto and Ho, 2003). Many bZIP
transcription factors that bind ABREs have been identified from different plants such as Arabidopsis ABI5, barley HvABI5, and rice OsTRAB1 and OsABI5 (Hobo et al., 1999; Finkelstein and Lynch, 2000; Casaretto and Ho, 2003; Zou et al., 2007). OsTRAB1 bound to both the ABRE and CE3 in the Osem promoter, and functioned synergistically with ABA and OsVP1, the rice VP1 orthologue, to transactivate this promoter (Hobo et al., 1999). Rice OsABI5 regulates rice fertility and adaptive stress response (Zou et al., 2008). The Arabidopsis AtABI5 gene is required for seed dormancy, maturation and stress responses and overexpression of AtABI5 causes hypersensitivity to ABA (Brocard et al., 2002). The barley HvABI5 is required for ABA-induced expression of the HVA1 and HVA22 genes (Casaretto and Ho, 2003).

Intron splicing is a crucial feature of post-transcriptional gene expression and a complex cellular process in eukaryotes (Lorkovic et al., 2000; Kazan, 2003). Alternative splicing has the potential to generate diverse gene products from a locus (Stamm et al., 2005; Li et al., 2006). Splicing variants can alter many properties of the protein such as protein structure, tissue specificity, intracellular localization, binding properties or interactions with other proteins (Stamm et al., 2005). In the present study, we identified two alternative splicing products of ZmTRAB1 in maize. We also investigated the function of splicing products of ZmTRAB1 in ABA signaling by testing the interactions of the alternative variants to ABRE in vitro, and their effects on ABA sensitivity with in vivo transient assays.
2.5 Materials and methods

2.5.1 Plant materials

The embryos used in electrophoretic mobility shift assay (EMSA) and transient expression assay were collected from B73 or W22 grown in the field under Iowa weather conditions, frozen and stored at -50 °C.

2.5.2 Sequence analysis

The Transcript Assemblies sequence (TA190185_4577) was predicted by the TIGR Maize Database (Chan et al., 2006) and the Open Reading Frame (ORF) was predicted by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The analysis of multiple sequence alignments is performed using ClustalW of the SDSC Biology Workbench (http://workebch.sdsc.edu/) with a BLOSUM matrix. The consensus and shading were conducted using the program BOXSHADE of the SDSC Biology Workbench with a cut-off of 50%. A Phylogenetic analysis was performed using MEGA version 4 (Kumar et al., 2008). Several programs were used to analyze the predicted structure and localization of the ZmTRAB1-1 and ZmTRAB1-2 proteins, including MultiLoc/TargetLoc (Hoglund et al., 2006), various tools at ExPASy (http://www.expasy.ch/), and BLAST (Altschul and Lipman, 1990).
2.5.3 Filter lift hybridization

DNA fragments in the flanking regions of the bZIP domain of ZmTRAB1 were amplified from a B73 embryo cDNA library by PCR, using TRAB1_SCREEN primers (Table 1). Radiolabeled probes were prepared from the amplified cDNA fragment by random primed labeling using $^{32}$P-dCTP. Recombinant phage (6.1 x $10^6$ pfu/µl) were plated at a density of 5 x $10^4$ pfu per 150-mm plate and grown for 6-8 hr at 37 °C. Nitrocellulose membranes were placed on top of the plate for 10 min, and then the membranes were transferred to denaturing, neutralization, and wash solutions in turn for 5min, 5min and 10 min, respectively. The DNA was UV cross-linked using a Stratalinker™ (Stratagene).

To hybridize the filters with the radioactive ZmTRAB1 probe, filters were prehybridized at 42°C in 120 mL of prehybridization solution (mix 60 mL of Formamide, 30 mL of 20 X SSPE, 4.8 mL of 50 X Denhardts, 0.6 mL of 20 % SDS, and dH2O to 120 mL. Add 2.4 mL of sheared, denatured salmon sperm DNA (10 mg/ml) to every 120 mL of solution). After 3 hr, prehybridization buffer was discarded and fresh hybridization solution containing denatured $^{32}$P-labeled probe was added. Hybridization was performed overnight at the same temperature. After hybridization, filters were washed for 10 min at room temperature using wash solution I (2X SSC, 0.2 % SDS), for 30 min at 65 °C using wash solution I, and for 30 min at 65°C using wash solution II (1X SSC, 0.1% SDS). The washed filters were wrapped in saran wrap and exposed to X-ray film. Positive plaques were identified by autoradiography.
2.5.4 Expression and purification of Glutathione S-Transferase fusion proteins

1,041-bp and 1,071-bp EcoRI-XhoI fragments, containing the complete coding derived from ZmTRAB1 splicing variants, were separately cloned onto the pGEX-4T-3 vector, and then recombinant plasmids were transformed into *E. coli* Rosetta™ cells (Invitrogen). The *E. coli* cells were grown in 100 mL LB medium containing 100 µg/mL ampicillin at 37 °C. When cultures had reached an absorbance of 0.8 at 600 nm, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and the cells were incubated further for 3 hr. Cells were pelleted and suspended in 5 mL of ice-cold 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3). The cells were then sonicated three times for 10 sec each on ice and centrifuged at 10,000 g for 10 min. Three mL of Glutathione-Sepharose™ 4B (Amersham Bioscience) was added into the supernatant and incubated with rotation for 4 hr at 4 °C. After washing the Sepharose beads three times with 1X PBS, the fusion proteins were eluted from the beads by incubation with rotation for 5 min with elution buffer. The eluted solution was aliquotted and stored at -80 °C. Protein concentrations were determined by the Thermo Scientific NanoDrop™ 1000 Spectrophotometer at 280 nm.
2.5.5 Electrophoretic mobility shift assays (EMSA)

DNA probes were generated by filling in the 3’ end overhangs of complementary oligonucleotides with $^{32}$P-dCTP (PerkinElmer) and the Klenow fragment of DNA polymerase I (Promega). Labeled probes were purified using a QIAquick® Nucleotide removal kit (Qiagen). Binding reactions (25 µl) containing 0.4 picomole of radiolabeled probe, 50 ng of sonicated pBlueScript II SK (Stratagene) and 2 µg recombinant proteins were incubated in 1 X binding buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 2.5 mM DTT, 1.25 µg BSA, 0.05 % v/v NP-40, 10 % glycerol) at room temperature for 20 min. Electrophoresis was conducted on a non-denaturing 4.5 % polyacrylamide gel in 0.5 x TBE (45 mM Tris-borate, 0.5 mM EDTA pH 8.0) buffer with constant 70 V for 4 hr at 4 °C. The gels were vacuum dried for 2 hr at 70 °C, and autoradiographed. For the competition assays, unlabeled competitors were incubated with recombinant proteins in 1 X binding buffer on ice for 10 min. After adding the radioactive probe, the binding reactions were further incubated for 20 min at room temperature before electrophoresis was performed, as described.

2.5.6 Transient transformation of developing maize embryos

To make an effecter construct with ZmTRAB1 under the control of the CaMV35S promoter, the original GFP fragment of pJ4GFP-XB (Igarashi et al., 2001) was replaced by the 1,041-bp or 1,071-bp XbaI-XhoI fragments of ZmTRAB1. 0.4 µg of 35S::ZmTRAB1-1 or
35S::ZmTRAB1-2 effector plasmid was mixed with 4 µg of Vp1::GUS reporter plasmid (Cao, et al., 2007), and 0.4 µg of ubiquitin::luciferase (ubi::LUC) (Christensen and Quail, 1996) as an internal standard, and precipitated onto 0.6 mg of 1 micron gold particles (BIO-RAD) and resuspended in 10 µl ethanol. As a control, 0.4 µg of pJ4GFP-XB (35S::GFP) was coprecipitated with the 4 µg of Vp1::GUS reporter and 0.4 µg of ubi::LUC.

Developing embryos were collected from B73 or W22 seeds and placed on half-concentration MS agar plates (Murashige and Skoog, 1962) with 1% sucrose. 10 µl of the gold particle suspension was dried on each macrocarrier disc and used to bombard 20 embryos per plate using a PDS-1000/He Biolistic Particle Delivery System (BIO-RAD) at 900 psi. Co-bombarded samples were divided in two and incubated with gentle shaking for 20 hr in MS liquid medium with 1 % sucrose, with or without 10 µM ABA. The samples were frozen in liquid nitrogen and stored at -50 °C. To assay GUS and luciferase activities, ground frozen samples were homogenized in 250 µl 1X CCLR (Cell Culture Lysis Reagent; Promega) and the supernatants were collected by centrifuging for 10 min 10,000 g at 4 °C. Assays were conducted with the Luciferase Assay System (Promega) on 20 µl of the supernatant, following the manufacturer’s instructions, and measured on a Sirius Luminometer (Berthold Detection Systems GmbH). Fluorometric MUG (4-methyumbelliferyl-b-D-glucuronide) assays were conducted by adding 100 µl protein extracts into pre-warmed 300 µl 1mM MUG in 1X CCLR with 20 % methanol at 37 °C. After the reaction mixtures were incubated for 2 hr at 37 °C, 100 µl was added to 100 µl 0.2 M Na₂CO₃. The GUS activities were measured on a Synergy HT plate reader (Bio-Tek Inc.). The relative GUS activities were normalized by dividing with the LUC assay values.
2.6 Results

2.6.1 Features and phylogenetic relationships of the maize bZIP transcription factor, ZmTRAB1

As demonstrated by EMSA experiments, nuclear proteins extracted from maize embryos and aleurone cells bind to the ABRE in the *Vp1* promoter, and the ABRE appears involved in the regulation of *Vp1* expression by exogenous ABA (Cao et al., 2007). Because ABI5-related proteins are important for ABA-regulation of seed genes containing an ABRE (Kim et al., 2002), we sought to identify gene(s) related to the ABI5 subfamily in maize. An extensive search was performed using amino acid sequences of ABI5 subfamily members, including *Arabidopsis thaliana* AtABI5 (GeneBank accession number AAD21438), barley (*Hordeum vulgare*) HvABI5 (GeneBank accession number AY150676), rice (*Oryza sativa*) OsTRAB1 (GeneBank accession number BAA83740), OsABI5 (GeneBank accession number EF199631) and wheat (*Triticum aestivum*) TmABI5 (GeneBank accession number AB238933.1). A 1,852 bp Transcript Assemblies sequence (TA190185_4577) was predicted by the TIGR Maize Database (Chan et al., 2006) and its longest ORF (1,071 bp) was adopted as the putative ORF, designated as ZmTRAB1. To address the relationships among the ABI5 subfamily members, the deduced amino acid sequence of ZmTRAB1 was determined and a multiple sequence alignment was conducted by using ClustalW (Thompson et al., 1994). This putative ZmTRAB1 shows five regions of high amino acid sequence homology with ABI5 subfamily members from different plants (single-underlined; Fig. 1). OsTRAB1 has
the highest conservation to ZmTRAB1 with 71% identity and 77% similarities (Fig. 1, 2). In addition, these family members share great similarity in the bZIP domain, including the basic region and the four highly conserved leucine residues forming the leucine zipper domain (double-underlined; Fig. 1). Three unique N-terminal conserved motifs containing potential phosphorylation sites are highly conserved (Casaretto, 2003; Kagaya, 2002; Fig. 1). The asterisks denote Ser residues in the second conserved region of OsTRAB1, which is phosphorylated in response to ABA in vivo, and is critical to OsTRAB1 activation binding to the ABRE (Kagaya et al., 2002; Kobayashi et al., 2005). This phosphorylation site is also highly conserved in ZmTRAB1 and suggests that phosphorylation events may play an important role in regulating ZmTRAB1 function(s) in response to ABA (Fig. 1).

A phylogenetic analysis was conducted using MEGA version 4 (Kumar et al., 2008). The six proteins fell into two major clusters (Fig. 2). The first contained two ABI5 proteins from wheat and rice. The second contained two subclades, one with AtABI5 and the other with HvABI5, ZmTRAB1, and OsTRAB1 (Fig. 2). OsTRAB1 was the most closely related to ZmTRAB1 (Fig. 2). The results of multiple sequence alignments and phylogenetic analysis support that ZmTRAB1 is a member of the ABI5 family of bZIP transcription factors and therefore could have a similar function in seed development.
2.6.2 Isolation of ZmTRAB1 coding sequence (CDS) from maize embryo cDNA libraries

To isolate ZmTRAB1 cDNAs, a library prepared from B73 maize 20 DAP embryos was used as a template for polymerase chain reaction (PCR) using TRAB1_SCREEN primers flanking of the bZIP domain region (Table 1). 364 bp amplification products were cloned into the pGEM-T Easy vector (Promega) and sequenced at the Iowa State University DNA facility. The cDNA libraries were then screened by filter lift hybridization using radiolabeled 364 bp PCR product probes. $7 \times 10^5$ plaques were screened and six positive clones were isolated. In vivo excision was performed and the isolated plasmids were sequenced. Two types of putative ZmTRAB1 cDNAs were isolated: two clones, designated ZmTRAB1-1, encoded 346 amino acids, and the rest, designated ZmTRAB1-2, encoded 356 amino acids. To get full lengths cDNAs, the 5’ end regions of ZmTRAB1 were amplified by PCR using TRAB1_FULL FR and TRAB1_SCREEN RP primers (Table 1) and the same 20 DAP embryo cDNA library as a template. Amplification products were inserted into pGEM-T Easy vector and sequenced. These amplified regions overlapped with 3’ end regions of the two different splicing variants of the ZmTRAB1 isolated by filter lift hybridization. These overlapping regions were combined by ligating at a BstEII restriction site contained within the regions.

These cDNAs represent alternatively spliced transcripts, as has been reported for rice OsABI5 (Zou et al., 2007). Both have identical nucleotide sequences except for the additional exon in ZmTRAB1-2, and both show nucleotide sequence identity through exonic regions to a single BAC sequence (GeneBank accession number AC205488) identified by
BLAST searching of the maize genome (Altschul and Lipman, 1990). This indicates that ZmTRAB1-1 and ZmTRAB1-2 are produced from a single ZmTRAB1 gene by alternative splicing (Fig. 3). The two ZmTRAB1 transcripts have the same start codon and share an identical bZIP domain. The extra 10 amino acids of ZmTRAB1-2 were located behind the leucine zipper, near the C-terminus. Both ZmTRAB1-1 and ZmTRAB1-2 proteins were predicted to localize to the nucleus by MultiLog and PSORT (Nakai and Horton, 1999), which is consistent with the localization pattern of OsTRAB1-GFP fusion protein in rice protoplast cells (Kagaya et al., 2002).

2.6.3 Analysis of the ABRE binding activity of the recombinant proteins of ZmTRAB1 in vitro

The two different full-length coding sequences were cloned as GST fusions in the pGEX-4T3 vector and transformed into *E. coli* Rosetta™ cells (Invitrogen). Recombinant ZmTRAB1-1 and ZmTRAB1-2 fusion proteins and GST (empty vector) were purified and had molecular masses of 65.43, 66.69 and 29 kDa, respectively (Figure 4; lane 1, 2, and 4) which is consistent with predicted sizes.

Sequence analysis showed that the *Vp1* promoter contains a conserved ABRE sequence (Cao et al., 2007). As illustrated by EMSA, embryo nuclear proteins bound to the ABRE but not ABRE-M3, containing three point mutations in the core binding site (ACGTG to AAAGG), and binding was dependent on ABA (Cao, 2007; Table 2). To examine whether the ZmTRAB1-1 and ZmTRAB1-2 recombinant proteins have DNA binding activity to the
ABRE in the \textit{Vp1} promoter, we performed an EMSA with the recombinant proteins and the ABRE probe (Table 2; Fig. 5). As shown in Figure 5, recombinant proteins of ZmTRAB1-1 and ZmTRAB1-2 both bound to the ABRE, but GST protein alone did not. The DNA binding activity of ZmTRAB1-1 to the ABRE was reduced by the addition of 5- and 10-fold excess unlabeled competitor of wild type ABRE oligonucleotide, but not by a 10-fold excess of ABRE-M3 competitors (Fig. 5a; lane 6). In the case of ZmTRAB1-2, the DNA binding activity to the ABRE was reduced by the addition of 10-, 50-, and 100-fold excess unlabeled competitor, but only partially competed away by 50-fold excess ABRE-M3 competitor, as shown in Figure 5b. These results indicate that both purified fusion proteins can bind specifically to the \textit{Vp1} ABRE sequence \textit{in vitro}.

\textbf{2.6.4 Transient assays of transcriptional activity of ZmTRAB1 variants on the \textit{Vp1} promoter}

To determine whether ZmTRAB1 proteins can activate transcription through the ABRE \textit{in vivo}, transient transformation of maize embryos was performed. The Vp1::GUS promoter fusion (Cao et al., 2007) was used as a reporter. Effectors consisted of the ZmTRAB1-1 or ZmTRAB1-2 cDNAs driven by CaMV35S, with 35S::GFP serving as a control, and ubi::LUC as an internal standard (Fig. 6a). After bombardment, half the samples were incubated in hormone-free liquid MS medium with 1% sucrose and the other half were incubated in the presence of 10 \mu M ABA. As seen in Figure 6b, cobombardment with the ZmTRAB1-1 construct increased GUS activity compared to the GFP control. The absolute
increase in GUS activity in the presence of exogenous ABA was slightly higher with ZmTRAB1-1 than without, although the relative fold increase was lower (Fig. 6b). In the case of ZmTRAB1-2, basal GUS activity was lower than the control in the absence of ABA. Although GUS expression levels in the presence of ABA were similar to controls, the 2X-fold induction by ABA was significantly higher than the other treatments (p-value <0.0001), whereas ABA inducibility with ZmTRAB1-1 overexpression is not significantly different from controls (p-value = 0.1026). Thus, overexpression of ZmTRAB1-2 appears to result in enhanced ABA inducibility of the Vp1 promoter.

2.7 Discussion

bZIP transcription factors that bind ABREs have been identified from different plants (Hobo et al., 1999; Finkelstein and Lynch, 2000; Casaretto and Ho, 2003; Zou et al., 2007). ZmTRAB1 displays high similarities in conserved functional domains, suggesting it may have functional similarities to ABA-related bZIP genes during plant development and stress responses. One of the common features of the ABA-related bZIP subfamily is shared similarity of three conserved N-terminal potential phosphorylation sites (Kagaya et al., 2002; Casaretto and Ho, 2003). Phosphorylation of the bZIP protein mediates ABA-induced transcription. The calcium-dependent protein kinases, CPK4 and CPK11, phosphorylate ABA-responsive transcription factors, such as ABF1 and ABF4, to mediate ABA signaling (Choi, 2000; Uno, 2000; Furihata, 2006; Fujii, 2007; Zhu, 2007). OsTRAB1 is phosphorylated at the Ser46 residue in response to ABA (Kagaya et al., 2002). But in the
Arabidopsis, rice and wheat ABI5 homologs, this Ser residue is replaced by a Cys (Fig. 1, indicated by an asterisk). Therefore, this Ser residue may be critical for ZmTRAB1 to mediate ABA signals by phosphorylation, and the mechanisms for mediating ABA signals via ABI5 and TRAB1 may be different.

Two alternative splicing variants of ZmTRAB1 were isolated. ZmTRAB1-2 contains an additional 10 amino acids inserted C-terminal to the bZIP domain, compared to ZmTRAB1-1. EMSA shows that the ZmTRAB1-1 and ZmTRAB1-2 recombinant proteins have specific DNA binding activities to the Vp1 ABRE *in vitro* (Fig. 5). This potentially corresponds to the EMSA activity detected in nuclear proteins extracted from maize 20 DAP embryos (Cao et al., 2007). It is worth noting the different transactivation activity between ZmTRAB-1 and ZmTRAB1-2. Although its relative GUS activity was weak, ZmTRAB1-2 was more highly inducible by exogenous ABA, suggesting the additional 10 amino acids of ZmTRAB1-2 may have functional consequences for gene regulation. On the other hand, ZmTRAB1-1 might elevate relative Vp1 promoter activity, but exhibit weaker induction by ABA. Whether the alternative splicing products may be associated with specific biological roles remains to be tested. Similar results were previously described with the rice OsABI5-1 and OsABI5-2 splicing products, which showed differential transactivation activity in yeast cells (Tamaoki et al., 1995; Zou et al., 2007).

In summary, we reported the isolation and characterization of two splicing variants of the maize gene ZmTRAB1, which encodes putative bZIP transcription factors, ZmTRAB1-1 and ZmTRAB1-2. Both variants are embryo expressed transcription factors, which may have similar and/or distinct physiological functions. ZmTRAB1-2 mediated slightly higher ABA-induction of a Vp1::GUS reporter gene in transient transformation of maize embryos, while
ZmTRAB1-1 showed higher basal transcriptional activation. To better understand the functional relationship between the ZmTRAB1 variants, further study is required.

2.8 Acknowledgments

We thank Becraft lab members for critical reading of the manuscript. This work was supported by funds from USDA-NRI 2006-01163.

2.9 References


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Finkelstein RR (1993) Abscisic acid-insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an Arabidopsis late embryogenesis-abundant (lea) gene. Mol Gen Genet 238: 401-408


Hobo T, Asada M, Kowyama Y, Hattori T (1999) ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J 19: 679-689


2.10 Table and figure

Table 1. Primers used in PCR analysis. FP: Forward primer, RP: reverse primer

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAB1_SCREEN</td>
<td>FP 5’- CAAGAGCGATGAGGATCTGTCATC - 3’</td>
</tr>
<tr>
<td></td>
<td>RP 5’ - CCGATCAACAACAAACTCAGTTACC - 3’</td>
</tr>
<tr>
<td>TRAB1_FULL</td>
<td>FP 5’ - GAGGCGATGGATCTCAACGAATGC - 3’</td>
</tr>
<tr>
<td></td>
<td>RP 5’ - CGCTCCACAACCTTCTCCACACC - 3’</td>
</tr>
</tbody>
</table>

Table 2. The oligonucleotides sequence used in the gel mobility shift assay

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotides sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABRE</td>
<td>5’- TCGGTCG<strong>GCCACGTG</strong>TGTCACGCAGCCGCA - 3’</td>
</tr>
<tr>
<td>ABRE-M3</td>
<td>5’- TCGGTCG<strong>GCCAAAGG</strong>TGTCACGCAGCCGCA - 3’</td>
</tr>
</tbody>
</table>
Figure 1
Sequence alignments of the ZmTRAB1 amino acid sequence with the sequences of other ABI5-like bZIP domain proteins from *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, Barley (*Hordeum vulgare*) and Wheat (*Triticum aestivum*). The identical residues are shaded in black and similar residues are shaded in light gray. The basic domain and leucine repeats of the bZIP domain are double-underlined and conserved regions are single-underlined. The Ser
residue in the protein kinase C phosphorylation signature is indicated by asterisks and potential phosphorylation sites are indicated by dots.
Figure 2
The phylogenetic analysis of ABI5 subfamily genes from *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Hordeum vulgare*, and *Triticum aestivum*. The multiple sequence alignment used is the same as in Fig. 1. The phylogenetic analysis was carried out by the neighbor-joining method (Saitou and Nei, 1987) using MEGA version 4 (Kumar et al., 2008). Each node indicates bootstrap values above 50% and 1,000 bootstrap replicates were calculated.
Figure 3
Alternative splicing variants of the ZmTRAB1 gene. a. Amino acid alignment of predicted proteins encoded by the two splicing variants. The additional 10 amino acids of ZmTRAB1-2 were located after the leucine zipper at the C-terminus. b. Schematic of ZmTRAB1 genomic organization represented with exons (boxes) and introns (lines) by EnCyklon® (http://www.encyklon.net). Red lines above the boxes represent the basic DNA-binding domain and leucine zipper.
Figure 4

Expression of ZmTRAB1-1 and ZmTRAB1-2 recombinant proteins in *E. coli*. Purified fusion proteins GST-ZmTRAB1-1 (lane 1), GST-ZmTRAB1-2 (lane 2), and GST alone (lane 4) were analyzed by SDS-PAGE with EZ-Run™ Pre-Stained Rec Protein Ladder (Fisher BioReagents) (lane 3).
Figure 5

Binding activity of purified fusion ZmTRAB1 proteins to the Vp1 ABRE in vitro. The ZmTRAB1-1 and ZmTRAB1-2 proteins specifically bind to the ABRE probe. a, b Each
binding reaction contained 0.4 picomole radioactive ABRE probe with or without 2 µg purified protein in 1 X binding buffer. The ABRE was bound by purified proteins causing two major retarded bands. The retarded bands could be competed away with excess unlabeled ABRE competitor, but be weakly competed by unlabeled ABRE-M3 competitor. The unlabeled competitors were added as 5- or 10-fold excess of radioactively labeled ABRE in ZmTRAB1-1 and as 10-, 50-, 100-fold excess of radioactively labeled ABRE in ZmTRAB1-2 binding assays. ABRE is indicated by underline and ABRE-3M is indicated by asterisks.
**Figure 6**

*a.* Schemes of gene constructs for transient assays. Blue squares represent the CDS of genes. GUS reporter expression is driven by the *Vp1* promoter while, expression of effector genes
were driven by the CaMV35S promoter. A ubiquitin::luciferase (ubi::LUC) construct was included as an internal standard. b. Relative transcriptional activity of the ZmTRAB1 variants, and the induction by ABA, on the Vp1::GUS reporter. Graphical representation of relative GUS / LUC activity of the Vp1::GUS reporter co-transformed with overexpressing ZmTRAB1-1, ZmTRAB1-2 or GFP as a control. White and black bars represent activities in the absence and presence of 10μM exogenous ABA, respectively. Each bar represents the back-transformation mean values after log mean transformations of at least six repetitions, except ZmTRAB1-1, which had three repetitions. The GUS and Luciferase activity were measured separately. The relative ratios were obtained by dividing GUS activity by Luciferase activity.
CHAPTER 3. ISOLATION OF A MYB TRANSCRIPTION FACTOR THAT INTERACTS WITH A PREDICTED MYB RECOGNITION SEQUENCE OF THE VP1 PROMOTER OF MAIZE

3.1 Abstract

The maize *Vp1* promoter sequence includes a predicted Myb-binding site I (MBSI), TAACTG, to which Myb transcription factors bind in other promoters. A candidate, designated ME97, was isolated in a yeast one-hybrid screen and contained the C-terminal region of a Myb-like protein. As illustrated by gel mobility shift assays, the partial ME97 protein could specifically bind to the MBSI *in vitro*. The reporter activity of the wild type *Vp1* promoter was significantly higher than that of a mutant reporter, changing the MBSI-core sequence AA to CC, both with and without ABA treatment. Taken together, these results suggest that ME97 is embryo expressed transcription factor that may regulate expression of the *Vp1* gene through binding to the MBSI.

3.2 Keywords

Abscisic acid · Electrophoretic mobility shift assays · MYB-binding site I · Yeast one-hybrid · Electrophoretic mobility shift assays
3.3 Abbreviations

ABA: Abscisic acid • EMSA: Electrophoretic mobility shift assays • GST: Glutathione S-transferase • GUS: β-Glucuronidase • MBSI: MYB-binding site I

3.4 Introduction

The phytohormone abscisic acid (ABA) functions as an important mediator during plant development and various environmental stresses. Drought conditions trigger the biosynthesis of ABA, and the increased ABA level induces various ABA-responsive genes, such as drought-inducible genes (Skriver and Mundy, 1990). ABA also mediates seed development by triggering seed dormancy and inhibiting seed germination. ABA deficient or insensitive maize mutants fail to undergo quiescence and precociously germinate, accompanied by a decreased expression of maturation genes (Bewley, 1997; Koornneef et al., 2002). The maize Vp1 gene encodes a B3 domain transcription factor that is a key regulator of late embryogenesis, seed maturation. VP1 activates ABA inducible genes, such as Em, by indirectly binding to ABREs (ABA responsive elements), and in late stages of aleurone differentiation, induces anthocyanin synthesis through the activation of CI expression (McCarty et al., 1989; McCarty et al., 1991; Suzuki et al., 1997; Hobo et al., 1999). VP1 also functions to repress germination associated gene expression during seed development (Bobb et al., 1995; Hoecker et al., 1995, 1999; Cao et al., 2007). Maize vp1 mutant kernels are ABA insensitive, undergo precocious germination on the ear, fail to withstand desiccation, and
lack anthocyanin due to lack of C1 expression in the aleurone (McCarty et al., 1989; McCarty et al., 1991). The orthologous ABI3 gene of Arabidopsis is required for the accumulation of storage proteins and the acquisition of desiccation tolerance (Finkelstein, 1993), whereas its mutant inhibits seed maturation, and the mutant phenotype can be complemented by the maize Vp1 (Giraudat et al., 1992; Suzuki et al., 2001).

Although a considerable amount of information is available on the functions of the Vp1 and Abi3 genes, less is understood about their regulation. Maize Vp1 expression is induced by ABA, and osmoticum during mid-maturation embryogenesis phase (Bobb et al., 1995; Hoecker et al., 1995, 1999; Cao et al., 2007). Several genes that regulate the transcription of the Arabidopsis Abi3 gene are known, but the molecular mechanisms by which this regulation occurs are unknown (To et al., 2006). In maize, upstream genes have not yet been identified. Detailed studies of the maize Vp1 promoter sequence identified five potential transcription factor binding regions, including a predicted MYB-binding site I (MBSI), TAACTG, to which MYB transcription factors bind (Luscher and Eisenman, 1990; Grotewold et al., 1991; Cao, 2005; Cao et al., 2007). This predicted MBSI was confirmed to have maize embryo nuclear protein binding activities by EMSA (Cao, 2005).

A large MYB family, reported from different plants, plays a variety of key roles in the regulation of gene expression, and in transcriptional responses to hormones during seed development and germination. For example, the maize C1 gene is an ABA-responsive gene that regulates the expression of genes involved in the biosynthesis of the anthocyanin pigments in the aleurone (Cone et al., 1986; Paz-Ares et al., 1986; Paz-Ares et al., 1987; Hattori et al., 1992). AtMYB2 is typical R2R3-MYB transcription factor, and can transactivate a promoter containing the MBSI (Urao et al., 1996; Abe et al., 1997). In
addition, AtMYB2 is induced by response to water stress, hypoxia, high salt stress, and
treatment with ABA, and regulates the stress-induced ADH1 and rd22 gene expression
(Shinozaki et al., 1992; Urao et al., 1993; Hoeren et al., 1998; Abe et al., 2003). Interestingly,
MYC and MYB consensus binding site sequences were reported in the rd22 promoter but no
typical ABRE (Yamaguchi-Shinozaki and Shinozaki, 1993). Both AtMYC2 and AtMYB2
genes are induced by drought and ABA treatment, suggesting that AtMYB2 may regulate
cooperatively with AtMYC2 in another regulatory system other than the ABRE-bZIP
regulatory system in the ABA signaling pathway in vegetative tissues and seeds under
drought and salt stress (Iwasaki et al., 1995; Abe et al., 1997; Abe et al., 2003).

In the present study, we isolated a candidate Myb transcription factor, ME97, by a
yeast one-hybrid screen. We demonstrated that the C-terminal region of recombinant ME97
protein, expressed in Escherichia coli, bound to the conserved MBSI of the Vp1 promoter in
vitro. We also investigated the functions of the MBSI, and of ME97 as a transcription factor,
with in vivo transient assays using a Vp1::GUS reporter.
3.5 Materials and methods

3.5.1 Plant materials

The embryos used in transient expression assays were collected from B73 or W22 grown in the field under Iowa weather conditions. Following microprojectile bombardment, embryos were cultured 24 hours, then frozen and stored at -50 °C.

3.5.2 Yeast one-hybrid screen

The 34 bp oligonucleotide containing the MBSI cis-element from the Vp1 promoter was synthesized, attaching EcoRI and XbaI adaptors at its 5´ and 3´ ends. Five tandem repeats of the synthetic oligonucleotide were ligated together and cloned into the corresponding restriction enzyme sites of the pHISi and pUC19 plasmids. With EcoRI and SalI restriction sites, the insert containing five tandem copies from PUC19 was cloned into the pLacZi plasmid. The described pLacZi and pHISi plasmid constructs were sequentially transformed into the yeast strain YM4271 to obtain the double-reporter yeast strain. A cDNA library was constructed in the lambda HybriZapII vector (Stratagene) from 20 days after pollination (DAP) embryos, creating fusions of the cDNA fragments to the yeast GAL4 activation domain in the pGAD424 vector. The cDNAs were transformed into the dual-reporter yeast strain and the transformants were placed under histidine selection. Those that were able to
grow in the absence of histidine and in the presence of 40 mM 3-AT were selected and tested for activation of the lacZ reporter. The His-positive yeast colonies were grown overnight on 1mm filter paper at 30 °C for the lacZ activity test. The filter containing the yeast colonies was frozen in liquid N2 for 10 sec and placed on a 3 mm filter paper soaked with 5 ml Z buffer (0.06 M Na$_2$HPO$_4$, 0.04 M NaH$_2$PO$_4$, 0.01 M KCl, MgCl$_2$) with 13.5 μl beta-mercaptoethanol plus X-gal (20 mg/mL in dimethylformamide) added fresh before use.

### 3.5.3 Sequence analysis

The Vp1 promoter region was analyzed by Plant Cis-acting Regulatory DNA Elements (PLACE) (Higo et al., 1999) and PlantCARE (Lescot et al., 2002). Positive yeast 1-hybrid clones were sequenced and used in BLAST searches (Altschul and Lipman, 1990) and the predicted subcellular localizations of the encoded proteins determined by MultiLoc/TargetLoc (Hoglund et al., 2006).

### 3.5.4 Expression and purification of recombinant ME97 proteins

The C-terminus was generated by cloning an 848-bp EcoRI-XhoI fragment from ME97 coding sequence (CDS) into the pGEX-4T-3 vector. The full-length protein was generated by cloning the 1,243-bp CDS into the same vector, and the recombinant plasmids were transformed into _E. coli_ Rosetta$^\text{TM}$ cells (Invitrogen). The _E. coli_ cells were grown in 100 mL
LB medium containing 100 µg/mL ampicillin at 37°C. When cultures had reached an absorbance of 0.8 at 600 nm, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1.0 mM, and the cells were incubated further for 3 hr. Cells were pelleted and suspended in 5 mL of ice-cold 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3). The cells were then sonicated three times for 10 sec each on ice and centrifuged at 10,000 g for 10 min. Three mL of Glutathione-Sepharose™ 4B (Amersham Bioscience) was added into the supernatant and incubated with rotation for 4 hr at 4 °C. After washing the Sepharose beads three times with 1X PBS, the fusion proteins were eluted from the beads by incubation with rotation for 5 min with elution buffer. The eluted solution was aliquotted and stored at -80 °C.

### 3.5.5 Electrophoretic mobility shift assays (EMSA)

DNA probes were generated by filling in the 3´ end overhangs of complementary oligonucleotides with ^32^P-dCTP (PerkinElmer) and the Klenow fragment of DNA polymerase I (Promega). Labeled probes were subsequently purified using a QIAquick® Nucleotide removal kit (Qiagen). Binding reactions (25 µL) containing 0.4 picomole of radiolabeled probe, 50 ng of sonicated pBlueScript II SK (Stratagene) and recombinant proteins were incubated in 1 X binding buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 2.5 mM DTT, 1.25 µg BSA, 0.05 % v/v NP-40, 10 % glycerol) at room temperature for 20 min. Electrophoresis was conducted on a non-denaturing 4.5 % polyacrylamide gel in 0.5 x TBE (45 mM Tris-borate, 0.5 mM EDTA pH 8.0) buffer with constant 70 V for 4 hr at
4 °C. The gels were vacuum dried for 2 hr at 70 °C, and autoradiographed. For the competition assays, unlabeled competitors were incubated with recombinant proteins in 1 X binding buffer on ice for 10 min. After adding the radioactive probe, the binding reactions were further incubated for 20 min at room temperature before electrophoresis was performed, as described.

### 3.5.6 Transient expression assays in cultured maize embryos

To make an effector construct with ME97 under the control of the CaMV35S promoter, the original GFP fragment of pJ4GFP-XB (Igarashi et al., 2001) was replaced by a 1,243-bp XbaI-XhoI fragment of ME97. Vp1m::GUS containing the mutant MBSIM8 (TAACCTGT to TCCCTGT) was made by the single-tube ‘megaprimer’ PCR method (Ke and Madison, 1997). With Vp1::GUS as a template, the first PCR was performed with MBSIM8 FR and MEGA RP primers (Table 2) by subjecting the reaction mixture to 25 cycles of amplification conditions: 94 °C for 40 sec, 42 °C for 1 min and 72 °C for 40 sec with a final extension step for 5 min at 72 °C. After completion of the first PCR, designated ‘megaprimer’, the second PCR was continued asymmetrically by the first PCR products as a template for five cycles of amplification using the following reaction conditions: 94 °C for 40 sec and 72 °C for 90 sec. Then, 100 pmol of MEGA FR primer was subsequently added, and the reaction was performed for additional 25 cycles with the same two-step temperature profile.

0.4 µg of 35S::ME97 effector plasmid was mixed with 4 µg of the wild type Vp1::GUS reporter plasmid (Cao, et al., 2007) or the mutant Vp1m::GUS reporter plasmid,
and 0.4 µg of ubiquitin::luciferase (ubi::LUC) (Christensen and Quail, 1996) as an internal standard, and precipitated onto 0.6 mg of 1 micron gold particles (BIO-RAD) and resuspended in 10 µl ethanol. As a control, 0.4 µg of pJ4GFP-XB (35S::GFP) was coprecipitated with the 4 µg of VP1::GUS reporter and 0.4 µg of ubi::LUC.

Developing embryos were collected from B73 or W22 seeds and placed on half-concentration MS agar plates (Murashige and Skoog, 1962) with 1% sucrose. 10 µl of the gold particle suspension was dried on each macrocarrier disc and used to bombard 20 embryos per plate using a PDS-1000/He Biolistic Particle Delivery System (BIO-RAD) at 900 psi. Co-bombardeed samples were divided in two and incubated with gentle shaking in MS liquid medium with 1% sucrose with or without 10 µM ABA for 20 hr in the dark at room temperature. The samples were frozen in liquid nitrogen and stored at -50 °C. To assay GUS and luciferase activities, frozen samples were ground, homogenized in 250 µl 1X CCLR (Cell Culture Lysis Reagent; Promega) and the supernatant was collected by centrifuging for 10 min 10,000 g at 4 °C. Assays were conducted with the Luciferase Assay System (Promega) on 20 µl of the supernatant, following the manufacturer’s instructions, and measured on a Sirius Luminometer (Berthold Detection Systems GmbH). Fluorometric MUG (4-methyumbelliferyl-b-D-glucuronide) assays were conducted by adding 100 µl protein extracts into pre-warmed 300 µl 1mM MUG in 1X CCLR with 20 % methanol at 37 °C. After the reaction mixtures were incubated for 2 hr at 37 °C, the 100 µl was added to 100 µl 0.2 M Na2CO3. The GUS activities were measured on a Synergy HT plate reader (Bio-Tek Inc.). The relative GUS activities were normalized by dividing with the LUC assay values.
3.6 Results

3.6.1 Isolation and analysis of ME97, a MYB-like protein

A potential MBSI in the 5’ UTR, 10 bp downstream of the major transcription start site in the $Vp1$ promoter, had a retarded band with 20 DAP embryo nuclear protein by EMSA (Cao, 2005). We performed a yeast one-hybrid screen to isolate cDNA clones that encode proteins, which bind to the MBSI in the $Vp1$ promoter. The double-reporter yeast strain containing five tandem repeats of the MBSI upstream of the His3 or lacZ reporters, failed to grow on selective medium. A cDNA library was constructed from 20 DAP embryos using lambda HybriZapII which fused the cDNA fragments to the yeast GAL4 activation domain in the pGAD424 vector. The reporter yeast strain was transformed with the cDNA library and plated on selective medium. Approximately $3.7 \times 10^6$ yeast transformants were screened and selected colonies were subsequently re-grown and assayed for β-galactosidase activity using an X-gal filter assay (Fig. 1). 26 clones grew on His selection and showed increased lacZ activity (Fig. 1). The isolated cDNA inserts were sequenced and analyzed by BLAST (Altschul and Lipman, 1990). One clone, designated ME97, was a partial of the sequence represented by TA192876_4577, which encoded a 413 amino acid protein that contained a highly conserved MYB-like domain (Fig. 2b). The isolated clone encoded the 191 C-terminal amino acids. The ME97 protein was predicted to be nuclear localized by MultiLoc (Hoglund et al., 2006). Because the MBSI is a predicted MYB binding site, this clone was pursued further.
3.6.2 The C-terminal region of ME97 specifically binds to the MBSI cis-element

in vitro

A GST fusion with the partial ME97 CDS was expressed and purified from E. coli Rosetta™ cells (Invitrogen). The fusion protein and GST protein have molecular masses of 50.43 and 29 kDa, respectively, which are consistent with the SDS-PAGE analysis shown in Figure 3b. Sequence analysis showed that the Vp1 promoter fragment used for EMSA contained two copies of an unnamed motif (CTCC), with the predicted MBSI (TAACTG) between them (Lescot et al., 2002; Cao et al., 2007). Two mutant oligonucleotides were prepared; MBSIM8 has the MBSI mutated from TAACTG to TCCCTG, and MBSIM9 consists of MBSI8 with the unnamed motifs mutated from CTCC to AGAA (Table 2). As illustrated by EMSA, the MBSI wild type oligonucleotide bound to nuclear extract proteins (Cao, 2005). To examine whether the partial ME97 recombinant protein has DNA binding activity to the MBSI in the Vp1 promoter, we performed an EMSA (Fig. 3; Table 2). To analyze the specificity of the DNA binding activities, we used MBSI wild type and MBSIM8 and MBSIM9 mutated forms in the assays (Table 2). The recombinant protein of partial ME97 bound to radiolabeled MBSI (Fig. 3b; lane 2), but not to radiolabeled probes MBSIM8 (data not shown) and MBSIM9 (Fig. 3b; lane 6). The DNA binding activity of partial ME97 to MBSI was competed away by the addition of 10-fold excess unlabeled competitor of MBSI, but the binding activity was less efficiently competed away by unlabeled MBSI8 and MBSI9 competitors, as shown in Fig. 3b; lane 3, 4, and 5. These results indicate that the binding
activity of the ME97 C-terminal region is specific and requires the AAC-core sequence of MBSI in vitro (Fig. 3b), and are consistent with nuclear extract proteins from maize 20 DAP embryos binding to the MBSI (Urao et al., 1993; Cao, 2005; Cao et al., 2007). Intriguingly, the C-terminus, which binds the MBSI, does not include the predicted MYB-like domain included in the full-length protein.

3.6.3 Transient assays of transcriptional activity of ME97 on the Vp1 promoter

To determine whether ME97 protein regulates Vp1 expression via the MBSI in vivo, transient assays were performed by microprojectile bombardment of developing maize embryos. To isolate full-length ME97 cDNAs, a library prepared from B73 maize 20 DAP embryos was used as a template for polymerase chain reaction (PCR) using ME97_FULL_FP and ME97_FULL_RP primers (Table 2). The fusion protein has molecular masses of 74.77 kDa which is consistent with the SDS-PAGE analysis shown in Figure 4. Effector constructs containing the full-length ME97 CDS under the control of the CaMV35S promoter were cobombarded with the wild type Vp1::GUS reporter or Vp1m::GUS containing a mutated MBSIM8. Ubi::LUC was included as an internal standard (Fig. 5). After bombardment, half the sample was incubated in hormone free liquid MS medium with 1 % sucrose and the other half was cultured in the presence of 10 μM exogenous ABA. As seen in Figure 6, the wild type Vp1::GUS expression was induced by 10 μM exogenous ABA in the cobombardments with both ME97 (p-value < 0.0001) and GFP (p-value = 0.0021). In Vp1m::GUS, containing MBSIM8, relative GUS activity was also induced by ABA (p-value = 0.0015) with the
ME97 construct. However, the overall activity of Vp1m::GUS was significantly lower than the wild type VpI promoter (p-value = 0.0004) indicating that the MBSI element is functionally important for VpI transcriptional regulation. However, at this point, we cannot conclusively say whether ME97 functions in the in vivo regulation of VpI.

3.7 Discussion

The MBSI in the VpI promoter region was predicted by PLACE and PlantCARE (Higo et al., 1999; Lescot et al., 2002), and this site was reported to function as a cis-acting element in the drought-induced expression of the rd22 gene (Yamaguchi-Shinozaki and Shinozaki, 1993; Abe et al., 1997). Myb-related proteins are involved in the transcriptional regulation of many genes in higher plants and are important in responses to hormones, such as ABA (Martin and Paz-Ares, 1997). Myb proteins often heterodimerize with Myc proteins (Abe et al., 2003). EMSA results show that the C-terminal ME97 recombinant protein has DNA binding activity to MBSI in vitro, and are also consistent with a previous observation of EMSA with the Arabidopsis MYB homolog gene binding to MBSI (Shinozaki et al., 1992; Urao et al., 1993; Hoeren et al., 1998; Abe et al., 2003). We identified a clone for a protein, ME97, which binds the MBSI in a yeast one-hybrid screen. The ME97 gene codes for a protein containing a MYB-like domain. Intriguingly, the partial sequence contained in the clone does not contain the Myb-like domain that originally drew our attention to this clone. No previously described motif is contained in the C-terminus that shows DNA-binding activity.
We analyzed the expression of a Vp1::GUS reporter in transient gene expression assays. Vp1m::GUS, containing a mutant MBSI, significantly reduced expression of the VpI promoter, suggesting this element is important for the regulation of VpI transcription. Both the wild type Vp1::GUS and Vp1m::GUS showed significant ABA induction of relative GUS activity, with mutant expression lower than wild type in both conditions, suggesting that this element is not likely involved in the ABA regulation of VpI expression.

In summary, a cDNA from developing embryos encodes a protein that binds the MBSI from the VpI promoter. A mutant MBSI that eliminates binding of ME97 also decreases the transcriptional activity of the VpI promoter, consistent with ME97 functioning as a transcriptional activator through this element. However, direct evidence of an in vivo function for ME97 in regulating VpI expression during seed development is currently lacking. To test whether the binding activity of ME97 to MBSI corresponds to that detected by EMSA of embryo nuclear extract proteins (Cao, 2005), we are currently generating ME97 antibodies to use in super-shift assays. Isolating mutations in the endogenous ME97 gene or suppressing ME97 function through RNAi will also be informative.

3.8 Acknowledgments

We thank Becraft lab members for critical reading of the manuscript. This work was supported by funds from USDA-NRI 2006-01163.
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*Proc Natl Acad Sci U S A* 97: 11632-11637


### 3.10 Table and figure

Table 3. Oligonucleotides used in yeast one-hybrid system. **FP**: Forward primer, **RP**: reverse primer

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotides sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBSI</td>
<td>5’-CTCCTCAGTAACTGCACCCACCTCCACCTAGG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AGGTGTGGAGGTGGGTGCAGTTACTGAGGAGCC-3’</td>
</tr>
<tr>
<td>EcoRI_adaptor</td>
<td>5’-GGCCGAATTCCG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GAATTCGCG-3’</td>
</tr>
<tr>
<td>XbaI_adaptor</td>
<td>5’-TCTAGACCG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CCGGTCTAGACC-3’</td>
</tr>
</tbody>
</table>
Table 4. Oligonucleotides used in PCR analysis. FP: Forward primer, RP: reverse primer

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotides sequence</th>
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<tr>
<td>MBSIM8</td>
<td>5’-CTCCTCAGTCCCTGCACCCACCTCCACACCTAGG-3’</td>
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<tr>
<td>MMSIM9</td>
<td>5’-CTGCACCCACCTCCACACCTAGG-3’</td>
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<tr>
<td>MEGA</td>
<td>FP 5’-TCTCGCATGCCTGTGGTTCACGCACGCTCGGCTCGC-3’</td>
</tr>
<tr>
<td></td>
<td>RP 5’-CGCGTCTAGATGTCTGTGTCTGCTG-3’</td>
</tr>
<tr>
<td>ME97_FULL</td>
<td>FP 5’-GAGGCGATGGATCTCAACGAATG-3’</td>
</tr>
<tr>
<td></td>
<td>RP 5’-CGCTCCACAACCTTCTCCACACC-3’</td>
</tr>
</tbody>
</table>
**Figure 1**

Growth and color selection of yeast one-hybrid system.

The dual reporter yeast strain failed to grow in the medium lacking histidine and containing 40 mM-3AT, but ME97 conferred the ability of cells to grow under selection. β-galactosidase filter assays were performed and ME97 formed blue color faster than YM4271 or the dual reporter as controls.
Figure 2
Bioinformatic analyses of the putative CDS of ME97.

a. Schematic of ME97 genomic organization represented with exons (boxes) and introns (lines). Boxes represent positions of the conserved regions and lines above the boxes represent the MYB_LIKE domain. The putative full length gene represented by TA192876_4577 (Chan et al., 2006), as well as the partial sequence included in the one-hybrid clone are shown. b. Domain predictions of the ME97 protein.
Figure 3
Expression of the C-terminal domain of ME97 recombinant protein in *E. coli* Rosetta™ cells (Invitrogen) and binding activity of purified fusion ME97 protein to MBSI *in vitro*.

a. Sequences of oligonucleotides used in the EMSAs. b. Purified GST-ME97 fusion protein (lanes 4, 5, and 6), and GST alone (lane 3) were analyzed by SDS-PAGE with EZ-Run™ Pre-Stained Rec Protein Ladder (Fisher BioReagents) (lane 2). c. EMSA shows the protein
specifically binds to the MBSI probe. The purified ME97 protein bound to MBSI causing one major retarded band but the protein could not bind radiolabeled MBSIM9. The retarded MBSI band could be competed away with 10-fold excess unlabeled MBSI competitors, but unlabeled MBSIM8 or MBSIM9 competitors were less effective.
Figure 4

Expression of full length of ME97 recombinant protein in E. coli *Rosetta*. Purified GST-ME97 fusion protein (lane 3 and 4), and GST alone (lane 2) were analyzed by SDS-PAGE with EZ-Run™ Pre-Stained *Rec* Protein Ladder (Fisher BioReagents) (lane 1).
Figure 5
Schemes of gene constructs for transient assays. Blue squares represent the CDS of genes. Reporters consist of GUS, driven by the Vp1 promoter or the mutant of VpIm promoter containing MBSIM8. Expression of effecter genes are driven by the CaMV35S promoter.
Figure 6
Relative activity of the Vp1::GUS or Vp1m::GUS reporter. Schematic representation of relative GUS / LUC activity of the Vp1::GUS or Vp1m::GUS reporter transiently transformed with overexpressing ME97. White and black bars represent activities in the absence and presence of exogenous 10μL ABA, respectively. Each bar represents the back-transformation mean values after log mean values of at least six repetitions. The GUS and Luciferase activity were measured separately and the relative ratios obtained by dividing GUS activity by Luciferase activity.
CHAPTER 4. GENERAL CONCLUSION

We reported that two putative transcription factors bind the \( Vp1 \) promoter and potentially regulate expression in developing embryos of maize. The \( Vp1 \) promoter was previously shown to contain an ABRE, which is typically bound by bZIP transcription factors related to ABI5. cDNAs for splicing variants of the maize \( ZmTRAB1 \) gene, encoding bZIP transcription factors, were isolated by filter lift hybridization from a developing embryo library. The \( Vp1 \) promoter was also shown to contain an MBSI-like element and ME97, encoding a putative Myb transcription factor, was isolated by a yeast one-hybrid screen using the MBSI. EMSA showed that the recombinant proteins of ZmTRAB1 variants and the C-terminus of ME97 have specific DNA binding activities to the ABRE and MBSI in the \( Vp1 \) promoter in vitro, respectively. Transient expression assays suggested the splicing variants, ZmTRAB-1 and ZmTRAB1-2, may have different transactivation activity. This result suggests that the additional 10 amino acids in the C-terminus of the ZmTRAB1-2 bZIP domain may have functional consequences for gene regulation.

To better understand the regulation of \( Vp1 \) expression, further studies of the functional differences between the ZmTRAB1 variants, and confirmation of binding activity of ME97 to the MBSI by gel super-shift using ME97 antibody are required. It will be worth testing whether a putative phosphorylation site, Ser, is critical for ZmTRAB1 to mediate ABA signals. It will also be interesting to determine the similarities and differences in \( Vp1 \) regulation between embryos and aleurone cells.
Taken together, with the identification of these transcription factors and potential binding sites, we will provide a framework for further studying how VpI gene is regulated with ABA in late embryogenesis and seed maturation in maize, and regulates the C1 gene in aleurone cells in maize. The ZmTRAB1 variants and ME97 transcription regulators will provide and support one step further up the genetic hierarchy that regulates VpI in both embryo and aleurone cell differentiation.
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