Jour. Agri. Sci., Tokyo Univ. of Agric., 51 (3), 113-121 (2006)
東京農大農学集報, 51 (3), 113-121 (2006)

論　文
Articles

Possible Involvement of a bZIP Protein in the Repression of ABI3/VP1-mediated Chymotrypsin Inhibitor Gene Expression at the Late-seed Maturation in Winged Bean

By

Yoichi SAKATA*, Tomomasa KUWANO**, Sachiko TAKAOKA***, Noriko TAKASHIMA****, Teruaki TAJI*, Hiroshi TAKENAGA***** and Shigeo TANAKA*

(Received May 26, 2006/Accepted July 13, 2006)

Summary : ABI3/VP1 is an important transcription factor, which regulates the expression of many kinds of genes during plant embryo maturation via ABA responsive elements or conserved RY repeats. The RY repeats are frequently found in 5′ upstream regions of seed-specific genes. Through an extensive study of temporally- and spatially-regulated expression of a winged bean chymotrypsin inhibitor (*WCI*) gene, we have demonstrated that the RY repeat is necessary but not sufficient for the seed-specific expression. In this study, we have cloned cDNAs encoding an ABI3/VP1 like factor (WbABI3) and a bZIP DNA binding protein from winged bean to investigate the participation of ABI3/VP1 and bZIP-type transcription factors in *WCI* gene expression. The deduced protein sequence of the bZIP protein (WbZIP1) was highly homologous to ROM2, which is shown to be a repressor against ABI3/VP1-activated transcription of *MAT* class genes during late-seed maturation in French bean. Bacterial recombinant WbZIP1 protein was prepared and tested in a gel mobility shift assay to verify its binding to the promoter region of the *WCI-3* gene, which encodes a major WCI protein of winged bean. The recombinant WbZIP1 protein proved to show a high affinity for specific fragments containing 5′-ACGT-3′ sequences from the *WCI-3* promoter. Enhanced expression of the *WbZIP1* gene was observed during late-stage seed maturation after the transient expression of *WCI-3* and *WbABI3* in mid-stage seed maturation. These results suggest that WbABI3 and WbZIP1 may function antagonisitically to tune the level of *WCI* gene expression from mid- through late-stage seed maturation in winged bean.

Key words : ABI3/VP1, bZIP protein, chymotrypsin inhibitor, temporal gene regulation, winged bean

Introduction

Legumes are known as one of the foremost crops in the world because of their striking ability to accumulate large amounts of proteins in the seeds. Molecular studies on this special ability of legumes to store proteins will afford a better understanding of production and accumulation of proteins in plants, contributing toward the development of agriculture. A legume, winged bean [*Psophocarpus tetragonolobus* L. (DC.)], was found to accumulate a protein, chymotrypsin inhibitor (WCI), largely in the storage organs such as seeds and tuberous roots, although a small amount of WCI is detectable in the stem[1]. In addition, accumulation of WCI was observed only during mid- to late- stage maturation of the seed development[1]. To elucidate how storage organ-specific or maturation-specific genes are regulated in legumes, we have investigated the unique expression of the *WCI* gene in detail. As a result, we have shown that transcriptional regulation is an important step for the seed-specific expression of the *WCI-3* gene which encodes a major WCI protein, and that the pro-

* Department of Bioscience, Faculty of Applied Bio-Science, Tokyo University of Agriculture
** FUSHIMI Phamaceutical Co., Ltd.
*** Fuji Pharma Co., Ltd.
**** Gunma Prefectural Isesaki Koyo High School
***** Professor Emeritus, Tokyo University of Agriculture

moter resides within 1.0 kbp of the 5′ upstream region of the *WCI-3* gene[2,3].

Arabidopsis seeds of *abi3* mutants and maize seeds of *vp1* mutants show deficiencies in normal induction of maturation (*MAT*) and late embryogenesis abundant protein (*LEA*) genes, resulting in reduction of storage protein accumulation[4–7]. These mutant seeds are also non-dormant and lack desiccation tolerance[4,6]. Molecular cloning of *ABI3* and *VP1* genes revealed both genes were orthologous and encoded transcription regulators[7,8]. Ectopic expression of *ABI3* gene can induce several seed-specific genes in *Arabidopsis* leaves upon treatment with ABA[5]. Therefore ABI3/VP1 had been believed to be one of the most important regulators of seed maturation in dicots and monocots, respectively. Although the ingenious mechanism by which ABI3/VP1 factor regulates the transcription of *MAT* and *LEA* genes is still unclear, at least two independent *cis*-acting elements have been identified[9,10]. One is the ACGT-containing ABA responsive element (ABRE) for ABA-dependent transactivation by ABI3/VP1. The other is the sequence 5′-CATGCATG-3′, known as the RY repeat conserved in the 5′ upstream region of seed-specific genes[11]. Unlike the ABRE, the RY repeat is essential for ABA-independent transactivation by ABI3/VP1[10,12]. Our transgenic experiments demonstrated that the RY repeat was responsible for the increased expression of the *WCI-3* gene in the developing seeds[3], suggesting that the *WCI-3* gene expression might be regulated by ABI3/VP1 like factor via the RY repeat in winged bean.

The RY repeat was necessary but not sufficient for the activation of the *WCI-3* gene promoter[3], indicating the participation of other factor(s) for the full activation of *WCI-3* gene in seeds. In this point, it is noteworthy that involvement of basic leucine zipper (bZIP) proteins in the regulation of seed-specific gene expression has been demonstrated in several plants, including Opaque2 for 22-kDa zein genes in maize[13], SPA for prolamin genes in wheat[14], and ROM1 and ROM2 for the β-phaseolin gene in French bean[15,16]. Recently it has been reported that Opaque2-related bZIP proteins synergistically activate seed storage protein genes with ABI3 in *Arabidopsis* developing seeds[17]. Moreover, a rice bZIP type protein, TRAB1, was reported to interact physically with rice ABI3/VP1 factor, binding to the ABRE in the promoter of a *LEA* gene[18]. Also it has been ascertained that *Arabidopsis* ABI5, a regulator of certain *LEA* gene expression during seed maturation, is a member of the TRAB1-type bZIP family[19–21]. The *WCI-3* gene promoter has several 5′-ACGT-3′ core motifs, possible binding sites of plant bZIP proteins[22], suggesting that a bZIP protein may be involved in the transcription regulation of the *WCI* gene. These facts led us to investigate the relationship between the ABI3/VP1 factor and bZIP protein with regard to the mechanism of *WCI-3* gene regulation in winged bean.

Here we report the cloning and characterization of cDNAs encoding an ABI3/VP1 like factor and a bZIP protein from winged bean. We also discuss a general gene regulation mechanism during legume seed development, in which mid-stage maturation specific gene activation by ABI3/VP1 is repressed at late-stage maturation by the expression of bZIP transcription factor.

Materials and Methods

Cloning of *WbABI3* and *WbZIP1*

Total RNA was isolated from seeds of winged bean 35 days after flowering (DAF) by acid guanidium-phenol-chloroform method[23]. Poly(A)+RNA was selected by Poly(A) Tract (Promega, USA) according to the instruction manual. Poly(A)+RNA (500 ng) was subjected to the first strand cDNA synthesis. RNA was denatured at 65℃ for 10 min, annealed with Oligo (dT)-P7 primer (TOYOBO, Japan) at 30℃ for 10 min. The first strand was synthesized at 42℃ for 2 hr in a buffer supplied with M-MLV RTase (TOYOBO, Japan). The cDNA was purified by repeating a combination of dilution in 400 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and filtration through a Suprec-02 (Takara Shuzo, Japan) four times. The cDNA was diluted to a final volume of 50 μl with TE, and subjected to the 3′ rapid amplification of cDNA ends (3′RACE)[24].

Conserved amino acid sequences in the B3 domain of ABI3/VP1 (MEDIGTSRVWNMRY) and bZIP region (RK[Q/E/L]SNRESARR) were used for designing ABI3/VP1- and bZIP protein-specific degenerate primers, respectively. The sequences of primers used are ALF1 (5′-ATG GAR GAY ATH GGN AC-3′) and ALF2 (5′-GTN TGG AAY ATG MGN TA-3′) for ABI3/VP1, and ZIP1 (5′-WSI AAY MGI GAR WSY GC-3′) and ZIP2 (5′-GAR WSI GCI MGI WSI MG-3′) for the bZIP protein. Amplification was carried out by two nested PCR protocols using one specific adapter primer (P7 ; 5′-CGC CAG GGT TTT CCC AGT CAC GA-3′) and two degenerate primers. Primary PCR was performed with 1 μl of cDNA, 200 μM dNTP, 2 μM ALF1 or ZIP1 primer, 0.2 μM P7 adapter primer and 5U rTaq (Takara Shuzo, Japan) in a buffer supplied with rTaq. The reaction was first set up without P7 primer, and the second strand was extended at 94℃ for 4 min, 45℃ for 5 min, and 72 ℃ for 20 min. After adding P7 adapter primer, PCR was repeated for 35 cycles of 94℃ for 1 min, 45℃ for 2 min, and 72℃ for 2 min. The PCR product was purified and recovered in 20 μl of

TE by Suprec-02 as mentioned above. The secondary PCR was carried out with ALF2 or ZIP2 primer and P7 primer using 1 μl of the primary PCR product as a template under the same condition except annealing temperature of 46℃. The PCR products were cloned by pPCR-script Amp SK(+) cloning kit (Stratagene, USA) according to the instruction manual.

Several pools of the amplified cDNA library, constructed in lambdaZAPII (Stratagene, USA), were subjected to PCR using primers corresponding to the 5′ end sequence of the partial cDNA clone and pBluescript sequencing SK primer to amplify the 5′ upstream region of the cDNA clone. The PCR products were separated by agarose gel electrophoresis, the library pool containing the longest amplified DNA fragment was used for infection of *E. coli* strain XL-1 Blue MRF', and one-tenth titer of original pool was plated on each plate to make 10 plates for next screening. The lysate fractions from these plates were subjected to PCR again. This procedure was repeated until the number of phage plaques was reduced to 500. The final phage pool was subjected to plaque hybridization[25] using partial cDNAs as probes. The cDNA inserts were excised from positive phage clones by the ExAssist Helper/SOLR system (Stratagene, USA).

DNA sequence analysis

Sequence analyses of cloned DNA fragments were performed by dideoxy sequencing using the AmpliTaq FS sequencing kit (Perkin Elmer, USA) and an ABI 373S autosequencer (Perkin Elmer, USA). Homology search of the sequence was done by BLAST program[26]. Alignments of amino acid sequences are performed by T-coffee program[27]

Southern- and RNA- gel blot analysis

Total DNA was prepared from leaves of winged bean using cetyltrimethyl ammonium bromide as described[28] and purified by CsCl density gradient ultracentrifugation[25]. Ten μg of DNA was digested by restriction enzymes, resolved by agarose gel electrophoresis, and blotted onto Zeta probe nylon membrane (Bio-Rad, USA). The membrane blot was hybridized with the *WbABI3* or *WbZIP1* cDNA labeled with [$^{\alpha\text{-}32}$P]dCTP by Random Prime labeling kit (Amersham, USA) in a buffer containing 50% (V/V) formamide, 720 mM NaCl, 40 mM phosphate buffer (pH 7.4), 4 mM EDTA, 0.1% Ficol, 0.1% BSA, 0.1% polyvinylpyrrolidone, 1% SDS, and 100 μg/ml denatured salmon sperm DNA at 42℃ for 16–24 hr. After hybridization, the membrane was subjected to subsequent wash in 2xSSC (1xSSC ; 150 mM NaCl, 15 mM trisodium citrate) containing 0.1% SDS for 30 min at room temperature, 0.1xSSC containing 0.1% SDS for 30 min at room temperature, and 0.1xSSC containing 0.1% SDS for 30 min at 55℃.

Total RNA was isolated from leaves and seeds of winged bean by acid guanidium-phenol-chloroform method[23]. Each total RNA (30 μg) was separated by formaldehyde-denaturing agarose gel electrophoresis, and transferred onto GeneScreenPlus (DuPont, USA). Prehybridization was performed in a buffer containing 50% formamide, 1% SDS, 10% dextransulfate, 1M NaCl, and 100 μg/ml denatured salmon sperm DNA at 42℃ for 2 hr. After prehybridization, P^{32}-labeled cDNA was added to the prehybridization solution to perform hybridization at 42℃ for 16–24 hr. The membrane was washed once in 2xSSC for 5 min at room temperature, twice in 2xSSC containing 1%SDS for 30 min at 60℃, and once in 0.1xSSC for 30 min at room temperature. The membranes were analyzed by BAS3000 (Fuji Photo Film, Japan).

Gel mobility shift assay

A cDNA fragment from +791 to poly(A) corresponding to the C-terminal region of WbZIP1 was amplified by PCR. The amplified fragment was sequenced to confirm accurate amplification and subcloned into pET-15b vector (Novagen, Germany). This construct was used to transform *Escherichia coli* strain BL21 (DE3)/pLysS. The transformant was pre-cultured overnight in LB medium containing 50 μg/ml ampicillin at 25℃. The culture was diluted ten-fold with the fresh LB medium and cultured for 1 hr at 25℃, then IPTG was added to a final concentration of 1 mM to induce the production of the recombinant WbZIP1 protein. After a 3-hr culture at 25℃, cells were harvested, resuspended in ice-cold sonication buffer (20 mM sodium phosphate, 10 mM imidazol, 500 mM NaCl, pH 7.4), and sonicated. The cell debris was spun down, and the supernatant was applied to a HisTrap Chelating column (Amersham Pharmacia Biotech, USA), according to the manufacture's instruction. WbZIP1 protein was eluted from the column with 300 mM imidazol. The purity of recombinant WbZIP1 was confirmed as a single band by Coomassie blue staining after SDS-PAGE. The binding reaction was carried out in a solution containing 2 mM sodium phosphate, 50 mM NaCl, 0.025% (W/V) BSA and 25 mg/ml poly(dI-dC) : poly(dI-dC) with 3fmol of P^{32}-labeled probe. The protein-DNA complex was separated by 3.6% polyacrylamide gel in 0.5x Tris-borate buffer (44.5 mM Tris-base, 44.5 mM boric acid, 1.1 mM EDTA). After electrophoresis, the gel was dried and analyzed by BAS3000.

Results and Discussion

Cloning of winged bean *ABI3/VP1*-like factor *WbABI3*

To gain a clearer understanding of transcription regulation of the *WCI-3* gene via the RY repeat, we searched for a cDNA encoding the winged bean ortholog of ABI3/VP1. The cloning was performed using a 3′ RACE method with cDNAs as the templates prepared from mid-maturation stage seeds of winged bean. The degenerate primers were designed from the conserved amino acid sequence in B3 domain of ABI3/VP1, which was reported to be the RY repeat binding domain[29]. After the secondary 3′ RACE, we detected a DNA fragment of appropriate size that was about 500 bp in length (data not shown). Sequencing analysis and homology search of this fragment convinced us that the fragment is a partial sequence of a novel winged bean ABI3/VP1 like factor (WbABI3 ; accession number AB164426). To obtain the whole sequence information of *WbABI3* gene, the cDNA library was screened by plaque hybridization using the *WbABI3* partial sequence as a probe. The longest cDNA clone was 2525 bp in length and encoded 751 amino acids as the open reading frame started with an ATG (Fig. 1A). In comparison with known ABI3/VP1 factors from various plant species, WbABI3 presented the highest similarity to PvALF, a French bean ABI3/VP1 like factor[30]. WbABI3 was identical to PvALF at an extremely high level of 80% through the entire amino acid sequence, while the identities with ABI3 and VP1 were 49% and 40%, respectively. Especially, extensive homology was observed between the B3 domain of WbABI3 and those of PvALF, ABI3 and VP1 at 97%, 94% and 88%, respectively. It is noteworthy that the conserved domains of WbABI3 are almost identical to that of PvALF (Fig. 1B), which has proved to transactivate seed-specific gene promoters via the RY repeats[31]. These results strongly suggested that WbABI3 is an ABI3/VP1 ortholog that regulates the transcription activity of seed maturation specific *WCI* gene, via the RY repeat in winged bean.

A

```
MDCEVELQGGDLHAGVITEANPVGFGTMEDSHALAVSERDMWLNSDQDEF    50
LGVNDASMFYADFPPLPDFPCMSSSSSSSSAPPLPAKTMACSTTTTTTSS   100
SSSSSSWVMLRSDVEEDAEKNHCNHYMHDQLDATALSSTASMEISQQHNP   150
DPALGGTVGECMEDVMDTFGYMELLESNDFFDPASIFQEDNEDPLVEFGT   200
LEEQVPLHDEQHAMVHQKGKADEEDHQVPVCEEIHGEEEGGDGVGVVDDE   250
MSNVFLEWLKSNKDSVSANDLRNVKLKKATIESAARRLGGGKEAMKQLLK   300
LILEWVQTSHLQNKRRKENSGSISTVLQGQFQDPSVQNTHTGSFAPEPNS   350
CFNNQTPWLSPQPFGTDQNPLMVPSQQFPQPMVGYVGDPYTSGAASNNIT   400
ATHNHNNNPYQPGAEQYHMLESAHSWPHSQFNVASHYGQSFGENGLFPHG   450
GFGGYGNNQYPYQFFHGPGDRLMRLGPSATKEARKKRMARQRRFLSHHRN   500
HNGNHQQNQGNDPHATLGGDNCTNVVAAPHANHAANWMYWQAMTAGVAGT   550
LGPVVPAEPPAGQPVVDRSTIHTQNCHQSRVASDRRQGWKPEKNLRFLLQ   600
KVLKQSDVGSLGRIVLPKKEAETHLPELEARDGISITMEDIGTSRVWNMR   650
YRYWPNNKSRMYLLENTGDFVRANGLQEGDFIVIYSDVKCGKYMIRGVKV   700
RQQGVKPETKKGGKSQKNQHGTNASTTAGTAANNGTQSSTKPKAEKSSKL   750
I                                                     751
```

B

```
S/T rich region
WbABI3   PPLPDFPCMSSSSSSSSAP-PLPAKTMACSTTTTTT---SSSSSSSS
PvALF    PPLPDFPCMSSSSSSSSAA-PLPLKTTTCSTTTTATTATSSSSSSSS  38/46
ABI3     PTLPDFPCMSSSSSSSTSPAPVNAIVSSASSSSAAS---SSTSSAAS  21/44
VP1      PSLPDFPCLSSPSSSTFSS----------------------NSSSNSS  16/26
         *.******:**.***: :.                    .:** :*

B1
WbABI3   VFLEWLKSNKDSVSANDLRNVKLKKATIESAARRLGGGKEAMKQLLKLILEWVQTSHLQ
PvALF    VFLEWLKSNKDSVSANDLRNVKLKKATIESAAKRLGGGKEAMKQLLKLILEWVQTSHLQ  58/59
ABI3     VFLEWLKNNKETVSAEDLRKVKIKKATIESAARRLGGGKEAMKQLLKLILEWVQTNHLQ  52/59
VP1      FFMEWLTSNRENISAEDLRGIRLRRSTIEAAAARLGGGRQGTMQLLKLILTWVQNHHLQ  35/59
         .*:***..*::.:**:*** :::::::***:** *****::.  ******* ***. ***

B2
WbABI3   LGPSATKEARKKRMARQRRFLSH
PvALF    LGPSATKEARKKRMARQRRFLSH  23/23
ABI3     LCSSATKEARKKRMARQRRFLSH  21/23
VP1      VEASATKEARKKRMARQRRLSCL  16/23
         : .****************; .

B3
WbABI3   DRRQGWKPEKNLRFLLQKVLKQSDVGSLGRIVLPKKEAETHLPELEARDGISITMEDIGTSR
PvALF    DRRQGWKPEKNLRFLVQKVLKQSDVGKLGEIVLPKKEAETHLPELEARDGISITMEDIGTSR
ABI3     DRRQGWKPEKNLRFLLQKVLKQSDVGNLGRIVLPKKEAETHLPELEARDGISLAMEDIGTSR
VP1      DKRQGAKADKNLRFLLQKVLKQSDVGSLGRIVLPKKEAEVHLPELKTRDGISIPMEDIGTSR
         *:*** *.:******:**********.**.*********.*****::*****:.********

WbABI3   VWNMRYRYWPNNKSRMYLLENTGDFVRANGLQEGDFIVIYSDVKCGKYMIRGVKVRQ
PvALF    VWNMRYRYWPNNKSRMYLLENTGDFVRANGLQEGDFIVIYSDVKCGKYMIRGVKVRQ  116/119
ABI3     VWNMRYRFWPNNKSRMYLLENTGDFVKTNGLQEGDFIVIYSDVKCGKYLIRGVKVRQ  112/119
VP1      VWNMRYRFWPNNKSRMYLLENTGEFVRSNELQEGDFIVIYSDVKSGKYLIRGVKVRP  104/119
         *******:***************:**::* **************.***:*******
```

Fig. 1 The deduced amino acid sequence of *WbABI3*. (A) An N-terminal serine domain and three basic domains (B1-B3) conserved in ABI3/VP1 like factors are shaded and underlined, respectively. (B) Sequence comparison of the conserved domains between WbABI3, PvALF (French bean), ABI3 (*Arabidopsis*) and VP1 (maize). Alignments were performed by T-coffee program. The numbers of identical amino acids/total amino acids in each domain of PvALF, ABI3 and VP1 to WbABI3 are indicated.

A winged bean bZIP protein WbZIP1 belongs to a repressor type bZIP family

To isolate cDNA clones encoding bZIP proteins expressed in developing seeds of winged bean, we used 2 sets of degenerate primers corresponding to a highly conserved basic DNA binding domain in N-terminal side of the leucine zipper region of plant bZIP proteins for 3′ RACE method. Although various species of DNA fragments were amplified in the primary PCR, three DNA fragments of 0.8, 1.0 and 1.1 kbp, were specifically amplified in the secondary PCR (data not shown). These fragments were cloned and sequenced. The protein encoded by the 0.8 kbp fragment started with ESARR which is a part of the conserved amino acid sequence, suggesting that the 0.8 kb fragment encodes a bZIP protein. We isolated a longer clone of the cDNA from the winged bean cDNA library using the 0.8 kb fragment as a probe. This cDNA was 1.6 kb in length, and the longest open reading frame started with ATG, sug-

```
MGNSEEGKSIKTGSPSSPATTDQTNQPSIHVYPDWAAMQYYGPRVNIPPY   50
FNSAVASGHAPHPYMWGSPQAMMPPYGPPYAAFYSHGGVYTHPAVAIGPH  100
PHGQGVPSPPAAGTPSSVESPTKLSGNTDQGLMKKLKGFDGLAMSIGNCN  150
AESAERGAENRLSQSADTEGSSDGSDGNTAGANKMRRKRSREGTPTTDGE  200
GKTETQEGSVSKETASSRKIMPATPASVAGNLVGPIVSSGMTTALELRNP  250
STVHSKANNTSAPQPCAVVPSEAWLQNERELKRERRKQSNRESARRSRLR  300
KQAETEELARKVEMLSTENVSLKSEITQLTESSEQMRMENSAIREKLRNT  350
QLGQTEEILLTSIDSKRGATPVSTENLLSRVNNSSSNDRTAENENDFCEN  400
KPNSGAKLHQLLDANPRADAVAAG                             424
```

Fig. 2 The deduced amino acid sequence of *WbZIP1*. The basic region and the leucine zipper region are underlined and boxed, respectively. The leucine residues in the zipper region are marked with dots. The fifth zipper is not leucine but methionine. Three amino acids which are different from those of ROM2 in the basic- and zipper region are shown by double underline.

gesting that it encodes the total sequence of the protein (Fig. 2). The cDNA encoded 424 amino acids, and the expected molecular mass is 45kDa. A basic region and a leucine zipper domain characteristic of bZIP protein are well conserved in the deduced amino acid sequence. The zipper region of the winged bean bZIP protein (WbZIP1 ; accession number AB164427) contains five leucines and one methionine (Fig. 2). The homology search by BLAST revealed that the deduced amino acid sequence encoded by the clone is highly homologous to French bean bZIP protein ROM2, which is reported to repress a PvALF-dependent activation of *MAT* genes in late-maturation stage seeds of French bean[15]. Because the amino acid identity between the winged bean bZIP protein and ROM2 is 89% through their entire protein sequence (data not shown), and only three amino acids are different from ROM2 in the basic zipper region (located between 277 and 343 in Fig. 2), WbZIP1 might be orthologous with the repressor protein ROM2.

Gene organization of *WbABI3* and *WbZIP1* in winged bean genome

Southern blot analysis of winged bean DNA digested with *Eco*RI, *Hin*dIII or *Bam*HI using the *WbABI3* cDNA as a probe resulted in single bands, indicated WbABI3 is encoded by a single copy gene (Fig. 3A). As far as we know, genes encoding ABI3/VP1 and their homologues in angiosperms are single copy in their genomes. This pattern is also conserved in winged bean.

The gene organization of *WbZIP1* was also predicted as a single copy like those of ROM2 in French bean, because only a single band was detected when *Eco*RI- or *Hin*dIII-digested DNA was probed by *WbZIP1* cDNA (Fig. 3B).

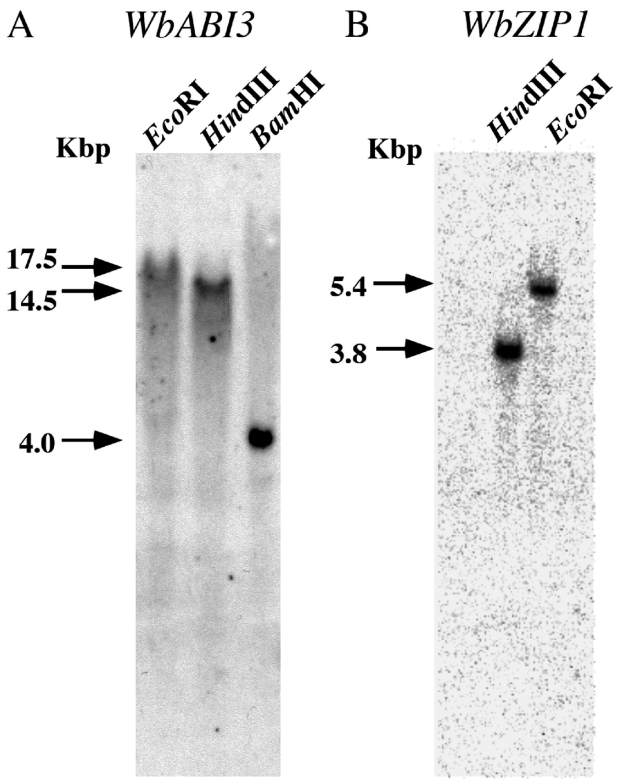

Fig. 3 Southern blot analysis of winged bean DNA with *WbABI3* and *WbZIP1* probe. Ten μg of winged bean DNA was digested with *Eco*RI, *Hin*dIII or *Bam*HI for *WbABI3,* and *Hin*dIII or *Eco*RI for *WbZIP1* probe. The digested DNAs were separated by electrophoresis. DNA fragments were transferred onto a nylon membrane and the filter was subjected to hybridization using the labeled *WbABI3* cDNA or *WbZIP1* cDNA probes. The estimated fragment length of the signals is shown on the left side.

Binding ability of WbZIP1 to the *WCI* gene promoter

Because *WbZIP1* was cloned by the homology-based strategy, it was unclear whether WbZIP1 is involved in the *WCI-3* gene regulation. To address the question whether WbZIP1 is a candidate for a transcription factor of the *WCI-3* gene, we investigated the binding ability of WbZIP1 to the *WCI-3* gene promoter. The His-tagged C-terminal region including the bZIP domain of WbZIP1 was expressed in *E. coli,* and purified by a nickel column. The purified protein was used for gel mobility shift assays. Although the *WCI-3* gene promoter contains several 5′-ACGT-3′ core motifs (Fig. 4A), these motifs do not exactly match with the proposed binding motif of ROM2, which is 5′-GCCACG/CTCAG/AYY-3′[15]. Therefore, the region between −882 and −91 relative to the transcription initiation site of the *WCI-3* gene, which was shown to contain *cis*-

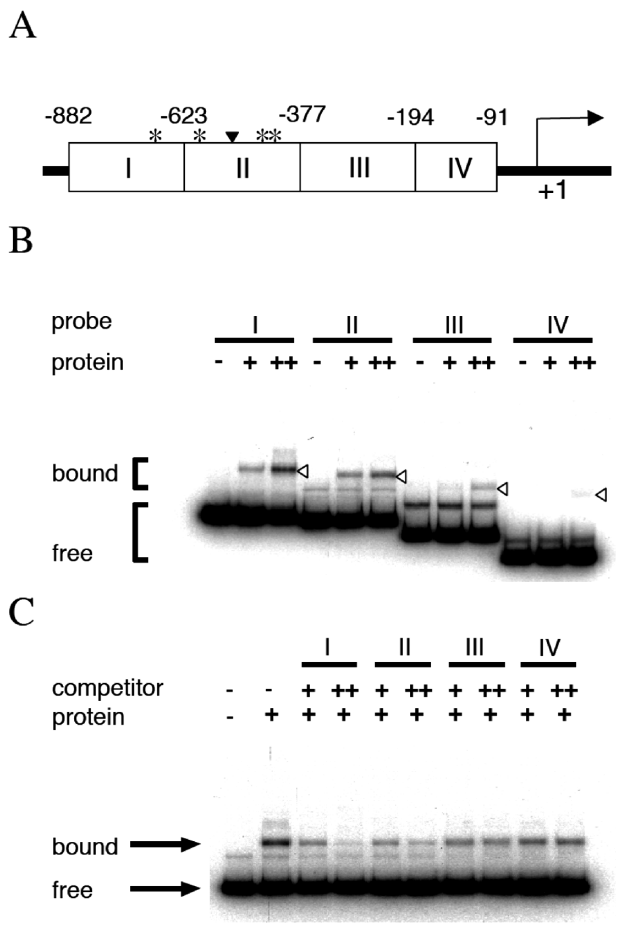

Fig. 4 DNA binding activity of WbZIP1 to the *WCI-3* gene promoter. A) Schematic representation of the *WCI-3* gene promoter from -882 to -91 used in the study. The fragments I-IV from -882 to -623 (I), -622 to -377 (II), -376 to -194 (III), and -193 to -91 (IV) were generated by PCR. Numbers are shown relative to the transcription initiation site (shown by arrow). ACGT motifs are shown by asterisks. The RY sequence in the region II is shown by a filled triangle. B) Gel mobility shift assay with P^{32}-labeled DNA fragments (I-IV) of the *WCI-3* promoter region and recombinant WbZIP1 protein. - ; no protein, + and ++ ; 5 and 10 ng of recombinant protein. The bound bands are indicated by open triangles. C) Competition experiments of gel mobility shift assay using the DNA fragment II as a labeled probe. Each DNA fragment was added to the binding reaction mixture in 100- or 200-molar excess to the probe.

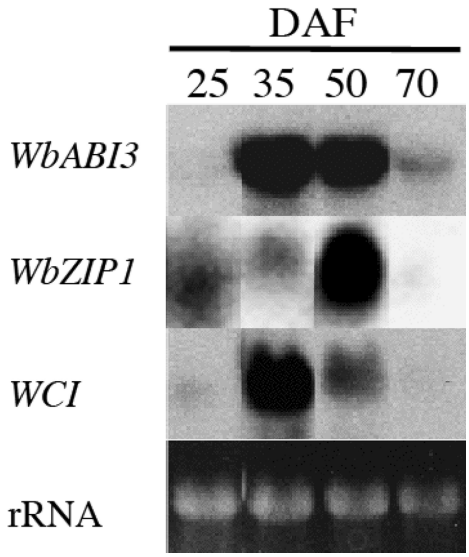

Fig. 5 RNA gel blot analyses of *WbABI3, WbZIP1* and *WCI-3* mRNA expression during the seed development. Total RNAs were isolated from seeds at various developing stages. Ten μg of each RNA was separated by electrophoresis in a denaturing agarose gel and transferred onto a nylon membrane. The filter was subjected to the hybridization experiment with the labeled *WbABI3, WbZIP1 or WCI-3* cDNA. An ethidium bromide-stained gel image of rRNA was shown as a standard of comparison with the RNA amount loaded in each lane. DAF ; days after flowering.

elements for transcription in seeds[3], was divided into four segments (DNA fragments I-IV, Fig. 4A), and these were used as probes for gel mobility shift assay. The labeled DNA fragments were separately incubated with WbZIP1 protein and analyzed by polyacrylamide gel electrophoresis (Fig. 4B). When the amount of WbZIP1 protein was increased, retarded bands were observed in each probe, however, the DNA fragments I and II seemed to have higher binding affinity to the WbZIP1. To confirm this, we performed a competition assay in which 100 or 200 fold-molar excess of each unlabeled fragment (I, II, II, or IV) was added to the binding mixture of the labeled fragment II and WbZIP1 (Fig. 4C). Competition effect was observed when the fragment I or II was used as a competitor, whereas the fragments III and IV had no competition effect even at 200 fold-molar excess. These results indicated that WbZIP1 binds to the *WCI-3* gene promoter in a sequence-specific manner. This also agreed well with the fact that the putative binding sequences (5′-ACGT-3′) were observed only in the fragments I and II (Fig. 4A).

Expression analysis of *WbABI3, WbZIP1* and *WCI*

Since the B3 domain of WbABI3 is almost the same as that of PvALF which activates *MAT* gene promoters via the RY repeats, and WbZIP1 was also shown to bind to the promoter region of *WCI-3* gene, it is suggested that WbABI3 and WbZIP1 are involved in the

regulation of *WCI-3* gene expression during the seed development of winged bean. To gain a deeper insight into the roles of WbABI3 and WbZIP1, we examined their expression pattern in the developing seeds of winged bean (Fig. 5). We reported in the previous paper[1] that the transcription activation of *WCI-3* gene occurred transiently at the mid-maturation stage of seeds. To put it more precisely, transcripts of the *WCI-3* gene started to accumulate at 30 days after flowering (DAF) and peaked in 40 DAF, then decreased to be a negligible amount at 60 DAF. Taking these results into account, we collected RNA samples for RNA blot analyses from seeds at the early-maturation stage (25 DAF), mid-maturation stage (35 DAF), late-maturation stage (50 DAF) and matured stage (70 DAF). The RNA blot analyses were performed with labeled cDNAs of *WbABI3, WbZIP1* and *WCI-3* as probes. As was observed in the previous report[1], the transient accumulation of *WCI* mRNA took place at 35 DAF, followed by a great reduction at 50 DAF. On the other hand, *WbABI3* mRNA was expressed from 35 DAF to 50 DAF at a high level. This agrees very well with previous reports that ABI3/VP1 regulates not only *MAT* genes expressed transiently at the mid-maturation stage, but also *LEA* genes expressed at the late-maturation stage[4–6,32–34]. Interestingly, enhanced accumulation of the *WbZIP1* mRNA was observed at 50 DAF, although weak accumulation was detected in the earlier stage of 25 DAF when the *WCI* and *WbABI3* mRNAs were undetectable. Thus, the study on mRNA expression also supports the probable repression of WbABI3-mediated activation of *WCI-3* gene by WbZIP1 at the late-maturation stage.

A bZIP protein named EEL has been reported in *Arabidopsis* to repress the transcriptional activity of *AtEm1* gene by competing a critical bZIP factor ABI5, that is essential to recruit ABI3 to the promoter, for the ABRE[35]. In the seeds of *Arabidopsis,* the maximum level of EEL expression was observed at the mid-maturation stage, suggesting that it prevents the precocious activation of the *AtEm1* gene by ABI3.

From the data showed here and the results from French bean PvALF[30] and ROM2[15], it seems to be a general system in legumes in which ROM2-related bZIP protein family actively represses the *MAT* genes activation by ABI3/VP1 at the late-stage maturation seeds. Plants seem to have worked out a strategy for utilizing bZIP transcription factors as repressors, as well as activators, to tune the level of ABI3/VP1-dependent gene expression during seed maturation. It will be interesting from the view of protein production in legumes whether disruption of target sequence of ROM2 family or repression of these bZIP proteins by RNAi will increase the accumulation of storage protein in seeds.

FUS3 and LEC2, other seed developmental regulators in *Arabidopsis,* have been shown also to encode B3 domain transcription factors[36,37]. FUS3 proved to interact directly with the RY repeat of legumin gene promoter. Furthermore, FUS3 can activate the promoter in concert with ABI3 via the RY repeat[38]. These data suggest that ABI3/VP1 is not an exclusive factor for RY repeat-mediated gene regulation. Further cloning and analysis of B3 domain transcription factors, such as FUS3 and LEC2, from winged bean would reveal the detail of regulation mechanism in *WCI* gene expression during the seed development.

Acknowledgements

We thank Dr. Gregg Clark (Washington University) for his critical reading of the manuscript. We also thank Dr. Shunsuke Yajima (Tokyo University of Agriculture) for his advice on the production of bacterial protein.

References

1) Habu, Y., Peyachoknagul, S., Umemoto, K., Sakata, Y. and Ohno, T. 1992. Structure and regulated expression of Kunitz chymotrypsin inhibitor genes in winged bean [*Psophocarpus tetragonolobus* (L.) DC.]. *J Biochem (Tokyo)* **111**, 249–258.
2) Sakata, Y., Fukushima, H., Habu, Y., Furuya, S., Naito, S. and Ohno, T. 1994. Transcriptional activities of a winged bean Kunitz chymotrypsin inhibitor gene promoter in stable and transient expression systems. *Biosci Biotechnol Biochem* **58**, 2104–2106.
3) Sakata, Y., Chiba, Y., Fukushima, H., Matsubara, N., Habu, Y., Naito, S. and Ohno, T. 1997. The RY sequence is necessary but not sufficient for the transcription activation of a winged bean chymotrypsin inhibitor gene in developing seeds. *Plant Mol Biol* **34**, 191–197.
4) Nambara, E., Keith, K., Mccourt, P. and Naito, S. 1995. A regulatory role for the *ABI3* gene in the establishment of embryo maturation in *Arabidopsis thaliana*. *Development* **121**, 629–636.
5) Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M. and Giraudat, J. 1994. Regulation of gene expression programs during Arabidopsis seed development : roles of the *ABI3* locus and of endogenous abscisic acid. *Plant Cell* **6**, 1567–1582.
6) Mccarty, D.R., Carson, C.B., Stinard, P.S. and Robertson, D.S. 1989. Molecular analysis of *viviparous-1* : An abscisic acid-insensitive mutant of maize. *Plant Cell* **1**, 523–532.
7) Mccarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I.K. 1991. The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**, 895–905.
8) Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. 1992. Isolation of the Arabidopsis *ABI3* gene by positional cloning. *Plant Cell* **4**, 1251–1261.
9) Ezcurra, I., Ellerstrom, M., Wycliffe, P., Stalberg, K.

and RASK, L. 1999. Interaction between composite elements in the *napA* promoter : both the B-box ABA-responsive complex and the RY/G complex are necessary for seed-specific expression. *Plant Mol Biol* **40**, 699–709.

10) EZCURRA, I., WYCLIFFE, P., NEHLIN, L., ELLERSTROM, M. and RASK, L. 2000. Transactivation of the Brassica napus napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements : B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. *Plant J* **24**, 57–66.

11) DICKINSON, C.D., EVANS, R.P. and NIELSEN, N.C. 1988. RY repeats are conserved in the 5'-flanking regions of legume seed-protein genes. *Nucleic Acids Res* **16**, 371.

12) HATTORI, T., VASIL, V., ROSENKRANS, L., HANNAH, L.C., MCCARTY, D.R. and VASIL, I.K. 1992. The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes Dev* **6**, 609–618.

13) SCHMIDT, R.J., KETUDAT, M., AUKERMAN, M.J. and HOSCHEK, G. 1992. Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD *zein* genes. *Plant Cell* **4**, 689–700.

14) ALBANI, D., HAMMOND-KOSACK, M.C., SMITH, C., CONLAN, S., COLOT, V., HOLDSWORTH, M., and BEVAN, M.W. 1997. The wheat transcriptional activator SPA : a seed-specific bZIP protein that recognizes the GCN4-like motif in the bifactorial endosperm box of prolamin genes. *Plant Cell* **9**, 171–184.

15) CHERN, M.S., BOBB, A.J. and BUSTOS, M.M. 1996. The regulator of MAT2 (ROM2) protein binds to early maturation promoters and represses PvALF-activated transcription. *Plant Cell* **8**, 305–321.

16) CHERN, M.S., EIBEN, H.G. and BUSTOS, M.M. 1996. The developmentally regulated bZIP factor ROM1 modulates transcription from lectin and storage protein genes in bean embryos. *Plant J* **10**, 135–148.

17) LARA, P., ONATE-SANCHEZ, L., ABRAHAM, Z., FERRANDIZ, C., DIAZ, I., CARBONERO, P. and VICENTE-CARBAJOSA, J. 2003. Synergistic activation of seed storage protein gene expression in Arabidopsis by ABI3 and two bZIPs related to OPAQUE2. *J Biol Chem* **278**, 21003–21011.

18) HOBO, T., KOWYAMA, Y. and HATTORI, T. 1999. A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. *Proc Natl Acad Sci U S A* **96**, 15348–15353.

19) FINKELSTEIN, R.R. and LYNCH, T.J. 2000. The Arabidopsis abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. *Plant Cell* **12**, 599–609.

20) CASARETTO, J. and HO, T.H. 2003. The transcription factors HvABI5 and HvVP1 are required for the abscisic acid induction of gene expression in barley aleurone cells. *Plant Cell* **15**, 271–284.

21) FINKELSTEIN, R.R., GAMPALA, S.S. and ROCK, C.D. 2002. Abscisic acid signaling in seeds and seedlings. *Plant Cell* **14** Suppl, S15–45.

22) IZAWA, T., FOSTER, R. and CHUA, N.H. 1993. Plant bZIP protein DNA binding specificity. *J Mol Biol* **230**, 1131–1144.

23) CHOMCZYNSKI, P. and SACCHI, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156–159.

24) FROHMAN, M.A., DUSH, M.K. and MARTIN, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts : amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci U S A* **85**, 8998–9002.

25) SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. 1989. *Molecular cloning : a laboratory manual.* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

26) ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. and LIPMAN, D.J. 1990. Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

27) NOTREDAME, C., HIGGINS, D.G. and HERINGA, J. 2000. T-Coffee : A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302**, 205–217.

28) WILSON, Z.A. 2000. *Arabidopsis : a practical approach.* (Oxford University Press, Oxford ; New York).

29) SUZUKI, M., KAO, C.Y. and MCCARTY, D.R. 1997. The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *Plant Cell* **9**, 799–807.

30) BOBB, A.J., EIBEN, H.G. and BUSTOS, M.M. 1995. PvAlf, an embryo-specific acidic transcriptional activator enhances gene expression from phaseolin and phytohemagglutinin promoters. *Plant J* **8**, 331–343.

31) BOBB, A.J., CHERN, M.S. and BUSTOS, M.M. 1997. Conserved RY-repeats mediate transactivation of seed-specific promoters by the developmental regulator PvALF. *Nucleic Acids Res* **25**, 641–647.

32) VASIL, V., MARCOTTE, W.R., JR., ROSENKRANS, L., COCCIOLONE, S.M., VASIL, I.K., QUATRANO, R.S. and MCCARTY, D.R. 1995. Overlap of Viviparous1 (VP1) and abscisic acid response elements in the *Em* promoter : G-box elements are sufficient but not necessary for VP1 transactivation. *Plant Cell* **7**, 1511–1518.

33) SUZUKI, M., KETTERLING, M.G., LI, Q.B. and MCCARTY, D.R. 2003. Viviparous1 alters global gene expression patterns through regulation of abscisic acid signaling. *Plant Physiol* **132**, 1664–1677.

34) NAKASHIMA, K., FUJITA, Y., KATSURA, K., MARUYAMA, K., NARUSAKA, Y., SEKI, M., SHINOZAKI, K. and YAMAGUCHI-SHINOZAKI, K. 2006. Transcriptional regulation of ABI3- and ABA-responsive genes including *RD29B* and *RD29A* in seeds, germinating embryos, and seedlings of Arabidopsis. *Plant Mol Biol* **60**, 51–68.

35) BENSMIHEN, S., RIPPA, S., LAMBERT, G., JUBLOT, D., PAUTOT, V., GRANIER, F., GIRAUDAT, J. and PARCY, F. 2002. The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis. *Plant Cell* **14**, 1391–1403.

36) LUERSSEN, H., KIRIK, V., HERRMANN, P. and MISERA, S. 1998. *FUSCA3* encodes a protein with a conserved VP1/AB13-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. *Plant J* **15**, 755–764.

37) STONE, S.L., KWONG, L.W., YEE, K.M., PELLETIER, J., LEPINIEC, L., FISCHER, R.L., GOLDBERG, R.B. and HARADA, J.J. 2001. *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. *Proc Natl Acad Sci U S A* **98**, 11806–11811.

38) REIDT, W., WOHLFARTH, T., ELLERSTROM, M., CZIHAL, A., TEWES, A., EZCURRA, I., RASK, L. and BAUMLEIN, H. 2000. Gene regulation during late embryogenesis : the RY motif of maturation-specific gene promoters is a direct target of the FUS3 gene product. *Plant J* **21**, 401–408.

シカクマメ種子成熟後期における ABI3/VP1 を介したキモトリプシンインヒビター遺伝子の発現抑制における bZIP 型転写因子の関与

坂田洋一*・桑野知昌**・高岡幸子***・髙島範子****・
太治輝昭*・武長　宏*****・田中重雄*

（平成 18 年 5 月 26 日受付/平成 18 年 7 月 13 日受理）

要約：ABI3/VP1 は ABA 応答配列あるいは保存された RY 配列を介して，植物の種子成熟において多くの遺伝子発現制御を行う重要な転写因子である。シカクマメ Kunitz 型キモトリプシンインヒビター（*WCI*）遺伝子の時期および器官特異的な遺伝子発現制御の解析から，我々は RY 配列が *WCI* 遺伝子の発現制御に必須ではあるが十分ではないことを示した。本研究では，シカクマメから ABI3/VP1 様のタンパク質（WbABI3）と bZIP 型転写因子をクローニングし，これらの因子が *WCI* 遺伝子の発現制御に関与しているか調査した。推定されるアミノ酸配列の解析から，この bZIP 型転写因子（WbZIP1）は，インゲンマメにおける ABI3/VP1 を介した種子タンパク質遺伝子の転写活性化を抑制する転写因子 ROM2 と高い相同性を示すことが明らかとなった。大腸菌を用いて発現させた組換え WbZIP タンパク質を用いたゲルシフト解析から，このタンパク質は *WCI-3* 遺伝子プロモーターの 5′-ACGT-3′を含む DNA 断片に結合することが示された。ノザンブロット解析を行なったところ，*WCI-3* と *WbABI3* の mRNA が一過的に蓄積する種子成熟中期の後に，*WbZIP1* mRNA の蓄積が増えることが明らかとなった。これらの結果から，WbABI3 と WbZIP1 はそれぞれ正の制御因子および負の制御因子として，*WCI* 遺伝子の種子成熟中期から後期にかけての一過的な発現を制御していることが考えられた。

キーワード：ABI3/VP1, bZIP 型転写因子，キモトリプシンインヒビター，シカクマメ，時期特異的遺伝子発現

* 東京農業大学応用生物科学部バイオサイエンス学科
** (株)伏見製薬所
*** 富士製薬工業株式会社
**** 群馬県立伊勢崎興陽高等学校
***** 東京農業大学名誉教授