Identification and Expression Analysis of Wheat Vacuolar Processing Enzymes (VPEs)

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ABSTRACT Vacuolar proteins are associated with various functions, such as germination and programmed cell death (PCD) activity. Precursors of most vacuolar proteins are synthesized in the rough endoplasmic reticulum (rough ER). They are transported to the vacuoles, and converted into the mature forms. Vacuolar processing enzyme (VPE) is responsible for maturation of vacuolar proteins. Intensive studies revealed that VPE-mediated system is important for vacuolar functions in plant. However, the role and mechanism of VPE still remain to be elucidated, especially in wheat. Here, we isolated three putative wheat VPE genes which were designated as *TaVPE2*, 3 and 4. To investigate the expression of *TaVPEs*, RT-PCR was conducted using RNAs extracted from caryopsis, pericarp, leaf and/or stem tissues at seed development stage and leaf, stem and/or root of seedling with phytohormone or abiotic stress treatment. Transcript accumulation of *TaVPEs* including *TaVPE1* was displayed in caryopsis during seed development stage. *TaVPE1*, *TaVPE2* and *TaVPE3* were expressed in root under abiotic stress whereas they showed no or very weak expression pattern in other tissue. Promoter region of *TaVPEs* contain many environmental-associated and tissue-specific *cis*-elements. In fact, VPEs sensitively responded to certain treatment condition. *TaVPEs* belong to seed type VPE in phylogenetic grouping. However, expression pattern of *TaVPEs* is different from other seed type VPEs. The classification into vegetative and seed type VPE according to gene expression patterns cannot be applied to *TaVPEs*.

Keywords Vacuolar processing enzyme, Wheat, Promoter analysis, Grain development

INTRODUCTION

The vacuole is an enclosed compartment that is widely distributed in most plant organisms. Particularly, vacuole is well developed in higher plants compared with other organism. The central vacuole in plant cells performs diverse functions such as waste storage, turgor pressure maintenance, protein storage, and balancing between biogenesis and degradation. It was examined that plant cells contain two functionally distinct vacuolar compartments, lytic vacuole and protein storage vacuole (Bethke et al. 1996). A lytic vacuole maintained at an acidic pH and contains degradative enzymes, including a wide variety of acid hydrolases. A protein storage vacuole functions primarily in the storage of materials, including nutrients, pigments, waste products, and small molecules.

Vacuolar proteins are associated with various functions,

such as germination, senescence and hypersensitive response (HR). Precursors of most vacuole-related proteins are synthesized in the rough endoplasmic reticulum (rough ER) in higher plants. They are transported to the vacuoles, and converted into the respective mature forms (Hara-Nishimura et al. 1985; Hara-Nishimura and Nishimura 1987). Vacuolar processing enzyme (VPE, also called vacuolar protease or C13 peptidase) is isolated in maturing castor bean seed and is characterized that it is associated with maturation and activation of seed storage proteins (Hara-Nishimura et al. 1991). After that, many intensive studies revealed that VPE is widely existed in plant, such as soybean (Shimada et al. 1994), vetch (Becker et al. 1995), citrus (Alonso and Granell 1995), tobacco (Zakharov and Muntz 2004) and *Arabidopsis* (Kinoshita et al. 1995a; Kinoshita et al. 1995b). Research of cereal crop VPE displayed that VPEs were detected in rice (Wang et al.

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2009) and barley (Linnestad et al. 1998). These VPEs belong to a novel family of cysteine protease. Legumains are a new family of cysteine protease in tobacco. Due to their vacuolar localization and processing function, legumains have been also named VPE (Zakharov and Muntz 2004). VPEs are synthesized as precursors that are self-catalytically converted into the mature forms. The self-catalytic activation was found with *Arabidopsis* VPE. It was expressed in the insect cell and occurred under acidic condition (Kuroyanagi et al. 2002).

Seed storage proteins are accumulated in protein storage vacuoles. It provides a nitrogen source for seedling growth during early stage of germination (Müntz 1998). VPEs perform unique functions in seed storage protein processing. Intensive studies revealed that a mechanism for VPE-mediated maturation of seed storage proteins was proved in *Arabidopsis* knock-out mutant series (Gruis et al. 2004). In the analysis of VPEs activity in dry seed of *Arabidopsis*, β VPE knockout mutants did not have matured seed storage proteins but precursors of seed storage proteins. Programmed cell death (PCD) is a basic physiological process under various stressed conditions and development of higher organisms including plant (Vaux and Korsmeyer 1999). Apoptosis, a representative type of PCD, occurs in the immune system under various stressed condition and development stage of organism. However, plant defense mechanism is so different from animal defense mechanism because plants have no immune system unlike animals. Many intensive studies carried out to explain ambiguous mechanism and characterization of plant PCD mechanism and it is revealed that PCD in plant and animals shares components that include caspase-like activity (Cohen 1997; Woltering et al. 2002; Bonneau et al. 2008). Recently, VPE has been displayed to be a protease that exhibits caspase-1-like activity. For example, VPE is responsible for tobacco mosaic virus (TMV) induced hypersensitive cell death in tobacco leaves (Hatsugai et al. 2004). In case of *Arabidopsis*, δ VPE is highly expressed in two cell layers of the inner integument of the seed coat during early stage in embryogenesis. δ VPE has shown that PCD occurs not only under disease or stressed conditions but also during plant development including seed coat formation (Nakaune et al. 2005). As undergoing senescence or stress, the

excreted endogenous hormone is changed and many genes are ready to activate. *Arabidopsis* VPE is activated by senescence or stressed condition (Kinoshita et al. 1999). Wheat vacuolar protease was up-regulated in senescing leaf induced by different factors (Martinez et al. 2007).

With the aid of completed *Arabidopsis* genome sequencing, four subfamilies of VPEs in *Arabidopsis* (α , β , γ , and δ VPE), were isolated and well characterized (Kinoshita et al. 1995a; Kinoshita et al. 1995b; Nakaune et al. 2005). Many roles of VPE, such as seed storage protein maturation, apoptosis mediation and developmental cell death mediation have been examined by *Arabidopsis* (Hara-Nishimura et al. 2005; Yamada et al. 2005).

Despite of functional importance of VPE in plants, wheat cysteine proteases including VPE have not been intensively studied at gene level (Kiyosaki et al. 2009). Recently, using a map-based cloning strategy, we isolated three VPE genes of wheat (*TaVPEs*) from cDNA library of wheat spikelet at DAF16 and DAF18. The isolated three putative VPE wheat subfamily genes, *TaVPE2*, 3 and 4 are homologs of wheat VPE1 that is deposited in the GenBank database. Here, we report temporal and spatial expression patterns of *TaVPEs* in tissues of wheat such as spikelet and seedling. We also discuss promoter region analysis and expression of the VPE genes under various stress conditions and exogenous phytohormone treatments.

MATERIALS AND METHODS

Isolation of *TaVPE* subfamilies of wheat

We constructed cDNA library to isolate genes expressed during seed development stage. Total RNA of infested seedling was extracted from spikes at 16 and 18 DAF (days after fertilization) using Trizol according to commercial protocols (Gibco/BRL, USA.). Poly(A)⁺ RNA was isolated with the PolyATract mRNA isolation system (Promega, USA). A cDNA library was constructed using Uni-ZAP XR vector (Stratagene, USA.). The phage library was excised to pBluescript plasmids as described by the manufacturer (Stratagene). The obtained phages of the library were transformed into *Escherichia coli* strain SOLR. Plasmid DNAs were isolated from the randomly selected

clones, and 74 clones were successfully sequenced. The K59 clone that showed highly homology to sequence of *TaVPE1* (*Triticum aestivum* vacuolar processing enzyme 1, access no. EF682132) was used in this study. Additionally,

we collected 74 expressed sequence tag (EST) sequences to isolate *TaVPE* family genes using the term “vacuolar processing enzyme” in NCBI. ClustalW at the European Bioinformatics Institute (EBI) was used (<http://www.ebi>.

Table 1. List of primer sequences and their purposes.

Name	Forward (5' to 3')	Reverse (3' to 5')	Purpose
<i>TaVPE1</i>	GATGAAC TCGAGGGTGGCGATGGC	TCAGGCGCTG TAGCCTCGAA CCAGC	Isolation of <i>TaVPE1</i> cDNA
<i>TaVPE1</i> RT	TTTTACCTGTTTAGGTGACTT	TCGGAGACGCCATTGTTGCAC	Analysis of <i>TaVPE1</i> expression
<i>TaVPE1</i> UP1		CCGTTGGGCATACCAAGTGAACCAGGAGA	Isolation of the <i>TaVPE1</i> promoter
<i>TaVPE1</i> UP2		AGCGGGAGGAGTCCACAAACCCACCACG	Isolation of the <i>TaVPE1</i> promoter
<i>TaVPE2</i>	GGCTCTAGCACACGCACATGAAC	GAGCATTTGGTACTGCTCCCATGTA	Isolation of <i>TaVPE2</i> cDNA
<i>TaVPE2</i> RT	TGCCGTTTGTTTATGCTGGCGAC	AAACGCTGCCTGAAATAACTGGA	Analysis of <i>TaVPE2</i> expression
<i>TaVPE2</i> UP1		CACCGCCAGCAGCAGAAGCGGGAGGAGT	Isolation of the <i>TaVPE2</i> promoter
<i>TaVPE2</i> UP2		AGCGGGAGGAGTCCACAAACCCACCACG	Isolation of the <i>TaVPE2</i> promoter
<i>TaVPE2</i> UP3		TATGCGCTTCTGCGCTGCAGCTGAAAA	Isolation of the <i>TaVPE2</i> promoter
<i>TaVPE</i> UP4		GAAGGACTCAGCTTAGTCCAGATCCTT	Isolation of the <i>TaVPE2</i> promoter
<i>TaVPE3</i> IPCR1	TTGCAAACATGTGCAACAATGGCATC	CAATGTTCTCCTCCTTTAGGCCCCC	Isolation of <i>TaVPE3</i> cDNA
<i>TaVPE3</i> IPCR2	CGAAATGAGGGAAGCAAGCATCAG	CTTCCTCAGGATCTGGTAGGCGTG	Isolation of <i>TaVPE3</i> cDNA
<i>TaVPE3</i> RT	TGCCGTTTGTTTATGCTGGCGAC	AACCTATACTGGACTCATTTT	Analysis of <i>TaVPE3</i> expression
<i>TaVPE3</i> UP1		TTTTTCAGGATCTGGTACGCGTGGCACACA	Isolation of the <i>TaVPE2</i> promoter
<i>TaVPE3</i> UP2		AGCGGGAGGAGTCCACAAACCCACCACG	Isolation of the <i>TaVPE2</i> promoter
<i>TaVPE4</i> IPCR1	CATGAGGGCGTTCGCCAACATATGCAAC	CAGCAGAAGCGGGAGGAGTCCACAAAC	Isolation of <i>TaVPE4</i> cDNA
<i>TaVPE4</i> IPCR2	AAGCATCAGGGCCTGCGACGATTAC	AGAAGCGGGAGGAGTCCACAAACCCAG	Isolation of <i>TaVPE4</i> cDNA
<i>TaVPE4</i> RT	TGATATCGCCAAAAACGACCTC	GAACCCCTATCGAATTCCTTTGA	Analysis of <i>TaVPE4</i> expression
<i>TaVPE4</i> UP1		TCCAGTAACCGCGGTTTTGTCCCAAT	Isolation of the <i>TaVPE4</i> promoter
<i>TaVPE4</i> UP2		CCGCCAGGAACGGGAGGAATCCACAA	Isolation of the <i>TaVPE4</i> promoter
<i>Ta Actin</i>	GCCACACTGTTCCAATCTATGA	TGAAATTGTATGTCGCTTC	Analysis of <i>TaVPEs</i> expression

ac.uk/clustalw/) to perform multiple alignments of the EST sequences. Two pairs of forward and reverse primers of *TaVPE 2* gene were designed from the sequences of the putative 5' and 3' untranslated regions (UTRs) by comparison with the EST sequences. PCR was performed using specific primers for *TaVPE1* and *TaVPE2*. The PCR cycling conditions consisted of 31 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s. In addition, Inverse polymerase chain reaction (IPCR) was conducted to isolate *TaVPE3* and *TaVPE4* based on the EST sequence from NCBI according to protocol of Sambrook and Russell (2006). Briefly, genomic DNA (1.5 µg) was digested with 15U of *EcoRI* and *HindIII* for 2h. The digested DNA was dissolved in 0.1–0.2 µg/ml distilled water followed by phenol/chloroform extraction and ethanol precipitation. The fragments were self-ligated into circular DNA in a mixture containing 10 µl of isolated fragments, 3 µl of 10× ligation buffer (TaKaRa), 3 U (1 µl) of T4 DNA ligase, and 16 µl of distilled water at 16°C for 16 h. The IPCR cycling conditions for *TaVPE3* and *TaVPE4* consisted of 35 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s, with a final cycle of 10 min at 72°C. The sequence of all primers used in this study is shown in Table 1. Amplification products were cloned into the T&A cloning vector and sequenced.

Plant materials

Seeds of Korean common wheat (*Triticum aestivum* L. cv. Geumgangmill) were germinated on moistened filter paper as described by Lee et al (2007). For a synchronized germination pattern, seeds with moistened filter paper were stored in a refrigerator at 4°C. After 36 hours, 16 imbibed seeds were transferred to a container (6.5 x 6.5 x 20 cm) containing a polypro-mesh and supported by acryl struts. Seedlings were positioned on the polypro-mesh into a solution of 200 ml of distilled water. Seedlings were grown in a growth chamber (Vision scientific, Korea) set at a photo-cycle of 13/11 h (day/night) 22/18°C (day/night) and exposed to light (10,000 lux) during day portion of the cycle. The water in the container was replaced with distilled water once a day. After 5 days of imbibitions, plants were transferred to 200 ml in half-strength of Hoagland's nutrient solution, until further treatments. Control plants were

prepared and grown under same environmental conditions.

For the study at seed development stage, seeds of wheat (*Triticum aestivum* L.) cv. Geumgangmill were planted in the field (Deokso, Namyangju, republic of Korea). The field was managed under standard agricultural practices, and received non-limiting rainfall during the growing season. Individual spike was tagged 1 DAF, as judged by the 3/4-projection of the awns from the leaf sheath. These spikes were tagged at 1, 5, 10, 15, 22, 30, 40 DAF and selected in main stem. Caryopsis and pericarp were embedded in tube with RNAlater solution (Ambion, USA). Leaf and stem were frozen immediately in liquid nitrogen. Then samples pre-mixed with RNAlater solution were plunged in liquid nitrogen and stored at -80°C.

Abiotic stress treatment, phytohormone application and tissue sampling

The seedlings at 13 days after germination (secondary leaf was fully-expanded) were transferred to 200 ml of half-strength of Hoagland's nutrient solution, which contains 5 mM of salicylic acid (SA), 100 µM of gibberellic acid (GA3) and 100 µM of abscisic acid (ABA). In order to examine the responses of the *TaVPE* genes to abiotic stress, seedlings were treated via dissolving root parts in 200 ml of half-strength of Hoagland's nutrient solution dissolved with each of polyethylene glycol (PEG, 25%, MW 10,000), NaCl (250 mM) and H₂O₂ (100 mM). The solution in the each container was replaced with fresh solution once a day. Leaf, stem and root tissues of samples were collected at 0, 6, 12, 24, 48 hours after each treatment. Samples were immediately frozen in liquid nitrogen and stored at -80°C in deep freezer until further usage. Plant tissues including caryopsis, pericarps, stems and leaves were collected in the field at scheduled days (DAF 1, 3, 5, 8, 10, 12, 15, 17, 19, 22, 25, 30, 35 and 40). All samples were frozen immediately in liquid nitrogen, and then stored at -80°C.

RNA extraction and RT-PCR analysis

Total RNA was extracted from individual tissues with TRIzol reagent (Molecular Research Center, Inc). RNA extraction was conducted according to the manufacturer's instructions. The extracted RNA from each tissue was utilized as a template for first-strand cDNA synthesis using

a first-strand cDNA synthesis Kit (iNtRON Biotechnology, Korea). First-strand cDNA synthesis was performed at 42 °C for 60 min and terminated by enzyme inactivating reaction at 70 °C for 5 min. For Semi-quantitative RT-PCR analysis, gene-specific primers were designed by cDNA sequence alignment of *TaVPE* genes (Table. 1). The RT-PCR products were cloned and sequenced to confirm the target gene specificity of each gene-specific primer. *TaVPE2* and *TaVPE3* share 5' gene specific primer because of high homology between *TaVPE2* and *TaVPE3*. The actin primers were designed from *Taactin* sequence at the GenBank and served as an internal control. RT-PCR experiments were processed in a thermo-cycler (MJ research, USA) and PCR conditions of the RT-PCR are listed in Table 1. The RT-PCR products were analyzed by 1% agarose gel electrophoresis in 1X TAE buffer and stained with ethidium bromide (EtBr).

Promoter analysis of *TaVPEs*

Genomic DNA was isolated from the leaf tissues of common wheat cv. "Geumgangmill". In order to isolate promoter region of the *TaVPE* genes, we used the universal GenomeWalker kit (Clontech). The genomic DNA was digested with five blunt end-forming restriction enzyme (*PvuII*, *EcoRV*, *StuI*, *SmaI*, *DraI*). After digestion, GenomeWalker adaptor was ligated with digested genomic DNA. After adapter primer annealed digested genomic DNA with gene-specific primer, PCR was conducted. The gene-specific primers used are shown in Table 1. The PCR product was cloned into RBC vector and sequenced. Because of high similarity among *TaVPEs*, one gene-specific primer was applied to PCR *VPE1*, 2 and 3. After sequence conformation, secondary PCR was conducted because primary PCR products have insufficient nucleotide

length for promoter analysis. Gene specific primer of secondary PCR was made from promoter region of *TaVPE*. PCR was processed according to the manufacturer's instructions (The Universal Genome Walker Kit, Clontech). The search for *cis*-acting elements on the promoter region of *TaVPE* genes was performed using plantCARE (Lescot et al. 2002) and PLACE Web Signal Scan (Higo et al. 1999). The correlation between the *cis*-acting elements and expression pattern was analyzed for each *TaVPE* gene.

RESULTS

Sequence comparison of the *VPE* genes of wheat and other plants

We constructed cDNA library to isolate genes expressed during seed development stage. About 69, out of 180 clones, showed a significant degree of homology to sequence registered in the National Center for Biotechnological Information (NCBI) (Appendix Table 1). The K59 clone that showed high homology to partial sequence of *TaVPE1* (*Triticum aestivum* vacuolar processing enzyme 1, access no. EF682132) was used in this study. The isolated cDNAs encoding *TaVPE1*, 2, 3 and 4 contained 1,482, 1,482, 1,482 and 1,467 bp open reading frames and encoded 493, 493, 493 and 488 amino acids, respectively. Nucleotide sequence similarities among *TaVPEs* ranged from 84% to 93%. The N-terminal of all deduced *TaVPE* sequences was analyzed using the SignalIP program (<http://www.cbs.dtu.dk/services/SignalIP>). All deduced *TaVPEs* did not contain any potential cleavage site. Predicted isoelectric points of *TaVPEs* ranged from 6.80 to 6.94, and molecular weights were from 54.07 to 54.5 kDa. Characteristics of cDNAs, predicted proteins and similarities

Table 2. Characteristics of *TaVPEs* cDNAs and proteins from *Triticum aestivum* cv. Geumgangmill.

Gene	cDNA				Mature Protein					
	Length	Identity (%)			Length	kDa	pI	Identity (%)		
		<i>TaVPE1</i>	<i>TaVPE2</i>	<i>TaVPE3</i>				<i>TaVPE1</i>	<i>TaVPE2</i>	<i>TaVPE3</i>
<i>TaVPE1</i>	1482				493	54.4	6.8			
<i>TaVPE2</i>	1482	89			493	54.35	6.84	86		
<i>TaVPE3</i>	1482	92	93		493	54.5	6.8	90	92	
<i>TaVPE4</i>	1467	85	85	85	488	54.07	6.94	79	79	81

were summarized in Table 2.

The four *TaVPE* genes were closely related from the peptide comparisons (Fig. 1). Amino acid sequence homologies among *TaVPEs* ranged from 79% to 92%. *TaVPE2* and *TaVPE3* showed most close relationship with 92% sequence similarity, while relatively lower similarities were observed between *TaVPE4* and other *TaVPE* genes.

To find a significant consensus pattern, we analyzed the *TaVPE1*, 2, 3 and 4 proteins with ExPASy Proteomic Server System (www.expasy.org/prosite). We also added the amino acid sequence of Rice NP1 (AAL40390) and barley NP1 (AAD04882). Rice NP1 and barley NP1 showed higher homologies to wheat than amino acid sequences of other plants VPE. Additionally, these two VPE genes were

TaVPE2	MNSRVAMAANWVCGLLPLLLAVA-----ADGDPLIRLPTENGHAPAPAGPAAPEE 54
TaVPE3	MNSRVAMAANWVCGLLPLLADAAK-----GNSEPLIRLPTENGHAPAPAGPAAPEE 54
TaVPE1	MNSRVAMAANWVCGLLPLLADAAK-----GNSEPLIRLPTENGHAPAPAGPAAPEE 54
TaVPE4	-----MRAWVCGFLPFLAAAAAADPAKGNWEPLIRLPTTEKGDAPAPA----ASAAED 50
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	GVTKWAVLVADSSGYENYRHQADVCHAYQILKKGGLKDENIVVFMYYDDIANSPENPRRGV 114
TaVPE3	EVTKWAVLVAGSSGYENYRHQADVCHAYQILKKGGLKDENIVVLMYYDDIANSPENPRRGV 114
TaVPE1	EVTKWAVLVAGSSGYENYRHQADVCHAYQILKKGGLKDENIVVFMYYDDIANSPDNPRRGV 114
TaVPE4	GVTKWAVFVAGSSGYENYRHQADVCHAYQILKKGGLKEENIVVFMYYDDIANLNPARGV 110
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	VINHFKGKDVYHGVPKDYTGKHVTAKNLYAVLLGNKTAVTGGSRKVINSKPNDFHIFYT 174
TaVPE3	VINHFKGKDVYHGVPKDYTGQVTAKNLYAVLLGNKTAVTGGSRKVINSKPNDFHIFYT 174
TaVPE1	VINHFKGKDVYHGVPKDYTGQVTAKNLYAVLLGNKTAVTGGSRKVINSKPNDFHIFYT 174
TaVPE4	IINHFKGEDVYAGVPKDYTGKQVTAKNFFAVLLGNKTAVTGGSRKVINSKPNDFHIFYA 170
	: * * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	DHGGAGSLGMPNVFVYAGDFIKVLRQKHASKSYSKMIIYVEACESGSIFEGLMFPQDHNI 234
TaVPE3	DHGGAGALGMPNVFVYAGDFIKVLRQKHASKSYSKMIIYVEACESGSIFEGLMFPQDHNI 234
TaVPE1	DHGGAGSLGMPNVFVYAGDFIKVLRQKHASKSYSKMIIYVEACESGSIFEGLMFPQDNI 234
TaVPE4	DHGGAGVLGMPNRPYIYAGDFIKVLRQKHASKSYSKMIIYVEACESGSIFEGLLPEDLNI 230
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	YVTTAANAESSWAAYCPGMEVPPSEYKTCGLDAYSVSWMEDSETHNLKESIKQQYEV 294
TaVPE3	YVTTAANAESSWAAYCPGMEVPPSEYKTCGLDAYSVSWMEDSETHNLKESIKQQYEV 294
TaVPE1	YVTTAANAESSWAAYCPDGTGPPPEYFTCLGDLYSVSWMEDSETQNLKNETIKQQYEV 294
TaVPE4	YVTTASNAVENSWGAYCPGKSSPTEYDTCGLDIYSVSWMEDSETHNLKESIKQQYEV 290
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	VKARTAPPNESSIGSHVMEYGDKTFKGDMLFLYQGFDPKSSYSSYFRQLPSLKGAINQR 354
TaVPE3	VKARTAPPNESSIGSHVMEYGDRTFKDEMLFLYQGFDPKSSITNRPRLRSLKGAINQR 354
TaVPE1	VKARTAPPNESSIGSHVMEYGDKTFKEDMLFLYQGFDPKSSIRNRPPLPMPSLKGAIKQR 354
TaVPE4	VKTRTSKSKEFDKSHVMEYGDKTFKDEKLFLYQGFDPANSNVAN-RLLLPDLEGAINQR 349
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	DADILFMWKYEQNLGGSEEEKQALREIKETVLHRKHLDSIDFIGKLTVFGFEKGPSVLD 414
TaVPE3	DADILFMWKYEQNLGGSEEEKQALREIKETVLHRKHLDSIDFIGKLTVFGFEKGPSVLD 414
TaVPE1	DADILFMWKYEQNLGGSEEEKQALREVKETVLHRKHLDSIDFIGKLTVFGFDKGPLVLE 414
TaVPE4	DADILFMWKYEQNLGGSEEEKQALREVKETVVRKHLDSIDFIGKLTVFGFENGPSMIE 409
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	AARGSGQPLVDDWDLCKTMVRVFSQCGSLTQYGMKHMRAFANICNNGVSGAEMKEASIS 474
TaVPE3	ASRGSGQPLVDDWDLCKTMVRVFSQCGSLTQYGMKHMRAFANICNNGISEAEMKEASIS 474
TaVPE1	AARGSGQPLVDDWDLCKTMVRVFSQCGSLTQYGMKHMRAFANICNNGASEAEMKEASIS 474
TaVPE4	AARSSGQPLVDDWDLCKTRVRFVFSQCGSLTQYGMKHMRAFANICNNGISEAEMREASIS 469
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *
TaVPE2	ACDDYDMGKWSPLVRGHS 493
TaVPE3	ACDDYDMGKWSPLVRGHS 493
TaVPE1	ACDGYDMGKWSPLVRGYS 493
TaVPE4	ACGGYDSAKWSPLAVGHS 488
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *

Fig. 1. Comparison chart of amino acid sequence of the *TaVPE* genes. The numbers at the end of right side in each line were the cumulative total number of amino acids sequence in each line. “*” indicated the same 4 amino acid sequence at that position among all amino acid sequence. “:” indicated the same 3 amino acid sequence and “.” the same 2 amino acid sequence.

Fig. 2. Phylogram of the relationship between *TaVPE* family and other plant VPE proteins. VPE can be separated into three types that are seed (A), novel (B, also called uncharacterized) and vegetative types (C).

isolated from major cereal crops (Linnestad et al. 1998).

VPE genes had 4 conserved domain, casein kinase II phosphorylation site, N-myristoylation site, N-glycosylation site and protein kinase C phosphorylation site. In barley NP1, we observed absence of Cyclic adenosine 5'-monophosphate (cAMP)- and cyclic guanosine 5'-monophosphate (cGMP)- dependent protein kinase phosphorylation site whereas other VPEs possessed this domain. Otherwise, rice NP1 contained additional domain, Tyrosine kinase phosphorylation site. *TaVPE1*, 2, 3 and 4 contains a conserved vacuolar processing enzyme domain (<http://www.ebi.ac.uk/Tools/InterProScan>).

A phylogram for the phylogenetic relationship of the *TaVPEs* and other plant *TaVPEs* were constructed (Fig. 2). The 24 protein sequences except for *TaVPE* were obtained in NCBI.

Developmental and tissue-specific expression of *TaVPE* genes

To investigate the seed developmental expression of *TaVPE* genes, RT-PCR was carried out with RNAs isolated from leaf, stem, pericarp and caryopsis at different development stages. The amplified RT-PCR products were 610 bp (*TaVPE1*), 472 bp (*TaVPE2*), 365 bp (*TaVPE3*) and 678 bp (*TaVPE4*) in length. Each *TaVPE* gene exhibited different expression pattern (Fig. 3). Expression of *TaVPE1* increased gradually until 40 DAF in caryopsis. No signal of *TaVPE1* was detected in stem and pericarp. Otherwise, transcripts of *TaVPE1* were detected at 35 DAF whereas *TaVPE1* was not observed until 25 DAF in leaf. *TaVPE2* was expressed during seed development in caryopsis. However, accumulation of *TaVPE2* transcripts was not detected in vegetative tissues. In caryopsis, *TaVPE3* expression was found at 5 DAF, reached a peak at 10 DAF

Fig. 3. Transcripts accumulation of the *TaVPEs* in different tissue during seed development stage. (A) caryopsis, (B) pericarp, (C) leaf, and (D) stem. DAF: days after fertilization.

and decreased until 22 DAF. After 30 DAF, transcripts of *TaVPE3* were not detected. *TaVPE3* was not expressed in vegetative tissues, but its transcript was detected in senescence leaf. *TaVPE4* was expressed in all tissues during seed development stage. However, expression pattern of *TaVPE4* is different in each tissue. Signal strength of *TaVPE4* was similar during DAF in leaf. However, *TaVPE4* expression was increased gradually in other tissues. Transcription level of *TaVPE4* was especially high compared with other expression pattern.

Expression of *TaVPE* genes in response to exogenous hormones and abiotic stress treatments

Semi-quantitative RT-PCR was used to investigate the accumulation of *TaVPE* transcripts in response to exogenous hormones and abiotic stress treatments. Expression analysis of *TaVPE* genes displayed various transcripts accumulation pattern, compared to the non- treated samples (Fig. 4, Fig. 5). *TaVPE1* was not expressed in PEG, ABA and GA₃ treatment. In leaf tissue, accumulation of *TaVPE1* was slightly increased under NaCl treated condition. On the other hand, *TaVPE1* was expressed in root under both of NaCl and H₂O₂ treated condition and gradually decreased

after treatment. *TaVPE1* was more significantly expressed in root than in leaf. Unlike, *TaVPE1*, transcripts of *TaVPE2* were not present under most treated conditions. Only *TaVPE2* expression was slightly increased after PEG treatment and was detected slightly in root during H₂O₂ treatment. Expression of *TaVPE2* was not observed in stem. A weak expression of *TaVPE3* is detected in the roots. In case of stem, *TaVPE3* was weakly expressed at 12 h after NaCl treatment. In SA treatment, *TaVPE3* was expressed within a short time. Expression level of *TaVPE3* was very low in tissues comparing with other VPEs. *TaVPE4* transcripts accumulation was kept in steady state than other *TaVPEs*. Signal strength of *TaVPE4* was higher than other *TaVPEs*. However, *TaVPE4* transcripts showed most variable expression patterns. In many cases, *TaVPE4* was down-regulated under phytohormone-and stress-treated conditions. Increased transcripts accumulation of *TaVPE4* was observed in each tissue during NaCl treatment. However, directly after NaCl treatment, *TaVPE4* expression level was rapidly decreased in stem. After rapidly decrease, transcription level of *TaVPE4* was gradually increased. *TaVPE4* expression pattern in stem after NaCl treatment can be observed in leaf during H₂O₂ treatment.

Fig. 4. Expression profiles of *TaVPE* genes. *TaVPEs* were differently expressed in each tissue under NaCl (A) and H₂O₂ (B) treatment. All samples were treated at secondary leaf fully expanded stage. (NaCl : 250 mM, H₂O₂ : 100 mM).

Fig. 5. Transcripts accumulation profiles of *TaVPEs* in wheat leaf expressed in responsive to (A) 5mM SA, (B) 25% PEG, (C) 100 μM GA₃, and (D) 100μM ABA using RT-PCR analysis. All samples were treated at secondary leaf stage.

Promoter Analysis

To understand the mechanism associated with the regulation of *TaVPE* gene expression, the GenomeWalker strategy was used to isolate the *TaVPE* gene promoter region. The length of isolated promoter region was 846 bp for *TaVPE1*, 2,048bp for *TaVPE2*, 843 bp for *TaVPE3* and

537 bp for *TaVPE4*.

Searches for putative *cis*-elements in promoter regions of the *TaVPE* genes were performed using plant promoter databases, PLACE Web Signal Scan (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) and plantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>).

cis-Elements related to transcriptional regulation by hormones and stresses were selected from a number of putative *cis*-elements and analyzed. Schematic diagrams of *cis*-elements of the *TaVPEs* promoter region are shown in Fig. 6.

The putative promoter regions of *TaVPE1*, 2 and 3 contained several common *cis*-elements such as CAAT box, CGTCA motif, G box, Skn-1, MBSI and LTR. *TaVPE1*, 2 and 3 promoter regions contained the *cis*-acting elements, Skn-1, that is required for endosperm expression. In the presence of Skn-1, transcripts accumulation of *TaVPE1*, 2 and 3 were detected in caryopsis during seed development stage. Instead of Skn-1, promoter region of *TaVPE4* possessed GCN4 motif that involved in endosperm expression. In spite of many light- and Methyl Jasmonate (MeJA)- related elements, we could not find relationship between these *cis*-element and VPE. Wheat seedlings were not treated with dark condition and MeJA application. In case of *TaVPE2*, correlations were detected between the *cis*-elements found by plantCARE and PLACE and expression pattern of VPE2. The presence of the TC-rich motif corresponded to transcripts accumulation of *TaVPE2* in leaf and root under NaCl and H₂O₂ treated condition.

PEG treatment induced at least 2.0-fold *TaVPE2* transcript accumulation as indicated by results of RT-PCR. Many other *cis*-elements were found in the *TaVPE2* promoter region, but few responses to the corresponding stress treatments were observed.

DISCUSSION

The wheat cv. Geumgangmill has good characteristics such as early maturity and protein quality, providing a good source for wheat flour in South Korea (<http://oneclick.rda.go.kr/htmlfile/A0108050302/A010805030215.html>; Rural Development Administration, Suwon, Korea). We isolated the 3 novel genes from wheat Geumgangmill that had high sequence homologies and determined as *TaVPE* genes. A number of VPEs have subfamily genes in a single plant species (Gruis et al. 2004; Zakharov et al. 2004). In case of wheat, isolation of *TaVPE1*, registered in the NCBI, and 3 novel wheat VPEs, designated *TaVPE2*, 3 and 4, were isolated in this study.

In silico analysis of the deduced amino acids sequences of *TaVPE* genes and VPEs of other plants showed that they

Fig. 6. Schematic comparisons of the *cis*-elements of the *TaVPEs* promoter regions.

possessed five highly conserved domains, Casein kinase II phosphorylation site, N-myristoylation site, N-glycosylation site, cAMP- and cGMP- dependent protein kinase phosphorylation site and protein kinase C phosphorylation site. These conserved domains are present in other plant VPE such as *Arabidopsis*. The absence of cAMP- and cGAMP- dependent protein kinase phosphorylation site in barley NP1, resulted to an incomplete sequence (Linnestad et al. 1998). Casein kinase II, protein kinase C, cAMP- and cGMP- dependent protein kinase play a key role in the control of phosphorylation in plant (Vandromme et al. 1996). Many enzymes are switched “on” or “off” by phosphorylation and dephosphorylation (Barford et al. 1998). It seems that these domains contributed to the activation of VPE. It was reported that inactivated VPE by caspase-1 inhibitor was associate with inhibition of HR (Hatsugai et al. 2004). Activated VPE exhibited typical HR as a response to pathogen (Hatsugai et al. 2004).

Kernel generally can be divided into two types of tissues, caryopsis and pericarp. The results of semi-quantitative RT-PCR suggested that expression of *TaVPEs* was observed in caryopsis, whereas only *TaVPE4* gene was expressed in pericarp during seed development (Fig. 3). Wheat grain filling lasts for about twenty days after fertilization. Expression of *TaVPE3* gene was detected in seed development stage and peaked at 10 DAF. Contrary to other *TaVPEs*, *TaVPE3* gene was not expressed at 22 DAF. This expression pattern of *TaVPE3* is similar to transcripts accumulation of barley NP1 (Linnestad et al. 1998). *TaVPE1* and *TaVPE3* exhibited a slightly increased expression pattern in caryopsis during seed development stage whereas expression levels of *TaVPE4* were kept at steady-state. Furthermore, *TaVPE1* and *TaVPE3* were expressed in leaf at later stage of seed development.

Satoru Nakaune et al (2005) has suggested that plant VPE can be divide into three types, vegetative type, seed type and uncharacterized (novel) type by their functional characteristics, expression pattern and sequence homology (Fig. 2). *TaVPE1*, 2, 3 and 4 belong to the seed type VPE group in phylogenetic grouping (A in Fig. 2). Seed type VPEs were reported that they are responsible for seed storage protein maturation. Studies using *Arabidopsis* mutant showed that VPE mutant plant could not produce

matured seed storage protein and have only protein precursors (Gruis et al. 2004). Expression pattern of seed type VPE was mainly concentrated in seed development stage and early germination stage (Kinoshita et al. 1999). *TaVPEs* were also mainly expressed in seed development stage.

Many plant genes are up- or down-regulated by a various growth hormones during plant development (Kende and Zeevaart 1997). ABA plays regulatory roles in the plants seed and bud dormancy and responses to various stress conditions (Kende and Zeevaart 1997). GAs are plant hormones that regulate growth and influence various development processes, including stem elongation, germination, dormancy, flowering, enzyme induction and senescence (Kende and Zeevaart 1997). In ABA and GA₃ treatment, only *TaVPE4* exhibited a slightly decreased expression pattern. Other VPEs were not expressed in these phytohormones. SA is found in plants with roles in plant growth and development, photosynthesis, and ion uptakes (Van Huijsduijnen et al. 1986). It also plays a role in the resistance to pathogens by inducing the production of pathogenesis-related proteins (Van Huijsduijnen et al. 1986). It was reported that vegetative type VPE of *Arabidopsis*, α VPE and γ VPE were up-regulated under SA treated condition (Kinoshita et al. 1999). Contrary to previous phytohormone treatments, *TaVPE1* and *TaVPE3* were expressed at 6h after SA treatments. However, their transcripts accumulations vanished at 12 h after treatment.

Under stressed conditions, pH levels could be altered to acidic very easily (Yamada et al. 2005). These acidic conditions result in the reverse effects on viability of plant roots. The self-catalytic activation and maturation of VPEs occurs under the acidic condition (kuroyanagi et al. 2002). After NaCl and H₂O₂ treatment, *TaVPEs* were up-regulated in root. Especially, *TaVPE4* is extensively expressed during these treatments. Transcription level of *TaVPE4* was remarkably high during 24 h after NaCl treatment then its expression was decreased. Under H₂O₂ treatment condition, expression of *TaVPE4* was significantly high in root which did not change across observation time. NaCl treatment showed a slight induction of *TaVPE1* and *TaVPE2* as shown in Fig. 4. At 48h after treatment, significant visible transcripts of *TaVPE1* and *TaVPE2* were

detected in leaf. It seems likely that VPE gene is very sensitive to salinity. Interestingly, all *TaVPEs* were significantly expressed in root after H₂O₂ treatment. It is thought that VPE genes of root are very sensitive to stress treatment because stress factors are directly absorbed through root.

Using the PlantCARE and the PLACE, it was revealed that promoter region of *TaVPE1* and 2 contained a number of *cis*-acting elements involved in stress, phytohormone and light-responsiveness. A number of light responsive elements such as G box, GAG motif, SP1 and AS2 box were involved in their promoter sequence. The comparison of putative *cis*-elements taken from the promoter database in each *TaVPE* promoter region suggests that some identified sets of putative regulatory correlated with the responses of the *TaVPE* genes to the hormones and abiotic stresses. Promoter sequence of *TaVPE2* contained two putative *cis*-elements, MBSI and ARE. Flavonoids are known to function in resistance to drought stress. They may protect the membrane against desiccation by interacting with lipid bilayer and function as protectants by scavenging ROS (Perez et al. 2002; Moore et al. 2005). MBSI is a *cis*-elements myb binding site involved in flavonoid biosynthetic genes regulation and ARE is known as a regulatory element essential for the anaerobic induction. The *TaVPE2* and *TaVPE4* expressed in the PEG treatments. However, some contradictions between the *cis*-elements and expression of *TaVPEs* imply the presence of unidentified *cis*-elements of promoter region.

TaVPE4 showed unique expression pattern compared with other *TaVPEs*. *TaVPE4* belongs to seed-type VPE, but expression patterns in tissue-types and treatment conditions are different from seed-type VPE. *TaVPE4* displayed tissue non-specific expression. It seems that promoter region of *TaVPE4* would be different from those of other *TaVPEs*. VPEs have not been extensively studied under conditions with phytohormone and stresses. Thus, further expressional analysis of *TaVPE* genes and search of promoter region of *TaVPE3* and 4 should be conducted to clarify the regulatory mechanism controlling the expression of these genes. Additional expression analysis under wound-, dark-, and MeJA-treated condition would provide the examination of relationship between *cis*-elements and

transcripts accumulation of *TaVPEs*.

As undergoing senescence and stress, the secreted enzyme such as cysteine protease is changed and cells in specific tissues are ready to die (Kinoshita et al. 1999). VPEs are closely related to senescence and stressed-condition including disease-induced PCD in plants (Kinoshita et al. 1999). *TaVPE1*, *TaVPE2* and *TaVPE4* are strongly expressed in vegetative tissues such as senescing leaves. Especially, *TaVPE4* showed the ordinary expression pattern of vegetative-type VPEs. Vegetative-type VPEs are expressed in vegetative tissue, such as stem and leaf. (Kinoshita et al. 1995a; Zakharov and Muntz 2004). However, *TaVPE4* is also expressed in caryopsis and is belong to the seed-type VPE group on phylogenetic grouping (Fig. 2). We speculate that the classification into vegetative- and seed- specific VPE according to gene expression patterns and sequence homologies could not be applied to *TaVPEs*.

In this study, we isolated three novel VPEs, *TaVPE2* and 3 and 4. *TaVPEs* including *TaVPE1* differently expressed in separate tissues during seed development stage, stress- or phytohormone- treatment. These results suggest that the *TaVPEs* have different roles during seed development and under stress- or phytohormone- treated condition. Expression analysis of tobacco legumain *NtPB-1* and *Arabidopsis* δ VPE revealed that these genes displayed highly tissue-specific expression patterns (Zakharov and Muntz 2004; Nakaune et al. 2005).

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REFERENCES

- Alonso JM and Granell A. 1995. A putative vacuolar processing protease is regulated by ethylene and also during fruit ripening in citrus fruit. *Plant Physiol.* 109:541-547.

- Barford D, Das AK and Egloff M-P. 1998. The structure and mechanism of protein phosphatases: insights onto catalysis and regulation. *Annu. Rev. Bioph. Biom.* 27:133-164.
- Becker C, Shutov AD, Nong VH, Senyuk VI, Jung R, Horstmann C, Fischer J, Nielsen NC and Klaus M. 1995. Purification, cDNA cloning and characterization of proteinase B, an asparagine-specific endopeptidase from germinating vetch (*Vicia sativa* L.) seeds. *Eur. J. Biochem.* 228:456-462.
- Bethke PC, Hillmer S and Jones RL. 1996. Isolation of intact protein storage vacuoles from barley aleurone (identification of aspartic and cysteine proteases). *Plant Physiol.* 110: 521-529.
- Bonneau L, Ge Y, Drury GE and Gallois P. 2008. What happened to plant caspases? *J. Exp. Bot.* 59:491-499.
- Cohen GM. 1997. Caspases: the executioners of apoptosis. *Biochem. J.* 326:1-16.
- Fischer J, Becker C, Hillmer S, Horstmann C, Neubohn B, Schlereth A, Senyuk V, Shutov A and Müntz K. 2000. The families of papain- and legumain-like cysteine proteinases from embryonic axes and cotyledons of *Vicia* seeds: development patterns, intracellular localization and functions in globulin proteolysis. *Plant Mol. Biol.* 43:83-101.
- Gruis D, Schulze J and Jung R. 2004. Storage protein accumulation in the absence of the vacuolar processing enzyme family of cysteine proteases. *Plant Cell* 16: 270-290.
- Hara-Nishimura I, Hatsugai N, Nakaune S, Kuroyanagi M and Nishimura M. 2005. Vacuolar processing enzyme: an executor of plant cell death. *Curr. Opin. Plant Biol.* 8: 404-408.
- Hara-Nishimura I, Inoue K and Nishimura M. 1991. A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. *FEBS Lett.* 294:89-93.
- Hara-Nishimura I and Nishimura M. 1987. Proglobulin processing enzyme in vacuoles isolated from developing pumpkin cotyledons. *Plant Physiol.* 85:440-445.
- Hara-Nishimura I, Nishimura M and Akazawa T. 1985. Biosynthesis and intracellular transport of 11S globulin in developing pumpkin cotyledons. *Plant Physiol.* 77: 747-752.
- Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M and Hara-Nishimura I. 2004. A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* 305:855-858.
- Higo K, Ugawa Y, Iwamoto M and Korenaga T. 1999. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucl. Acids. Res.* 27:297-300.
- Kende H and Zeevart J. 1997. The five "Classical" plant hormones. *Plant cell* 22:1197-1210.
- Kinoshita T, Yamada K, Hiraiwa N, Kondo M, Nishimura M and Hara-Nishimura I. 1999. Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions. *Plant J.* 19:43-53.
- Kinoshita T, Nishimura M and Hara-Nishimura I. 1995a. Homologues of a vacuolar processing enzyme that are expressed in different organs in *Arabidopsis thaliana*. *Plant Mol. Biol.* 29:81-89.
- Kinoshita T, Nishimura M and Hara-Nishimura I. 1995b. The sequence and expression of the γ VPE gene, one member of a family of three genes for vacuolar processing enzymes in *Arabidopsis thaliana*. *Plant Cell Physiol.* 36: 1555-1562.
- Kiyosaki T, Asakura T, Matsumoto I, Tamura T, Terauchi K, Funaki J, Kuroda M, Misaka T and Abe K. 2009. Wheat cysteine proteases triticain α , β and γ exhibit mutually distinct responses to gibberellin in germinating seeds. *J. Plant Physiol.* 166:101-106.
- Kuroyanagi M, Nishimura M and Hara-Nishimura I. 2002. Activation of arabidopsis vacuolar processing enzyme by self-catalytic removal of an auto-inhibitory domain of the C-terminal propeptide. *Plant Cell Physiol.* 43:143-151.
- Lee TG, Jang CS, Kim JY, Kim DS, Park JH, Kim DY and Seo YW. 2007. A Myb transcription factor (*TaMyb1*) from wheat roots is expressed during hypoxia: roles in response to the oxygen concentration in root environment and abiotic stresses. *Physiol. Plantarum* 129:375-385.
- Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P and Rombauts S. 2002. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucl. Acids. Res.* 30:325-327.
- Linnestad C, Doan DNP, Brown RC, Lemmon BE, Meyer DJ, Jung R and Olsen O-A. 1998. Nucellain, a barley homolog of the dicot vacuolar-processing protease, is localized in nucellar cell walls. *Plant Physiol.* 118:1169-1180.
- Martinez DE, Bartoli CG, Grbic V and Guamet JJ. 2007. Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. *J. Exp. Bot.* 58:1099-1107.
- Moore JP, Westall KL, Ravenscroft N, Farrant JM, Lindsey GG and Brandt WF. 2005. The predominant polyphenol in the leaves of the resurrection plant *Myrothamnus flabellifolius*, 3,4,5 tri-O-galloylquinic acid, protects membranes against desiccation and free radical-induced oxidation. *Biochem. J.* 385:301-308.
- Müntz K. 1998. Deposition of storage proteins. *Plant Mol. Biol.* 38:77-99.
- Nakaune S, Yamada K, Kondo M, Kato T, Tabata S, Nishimura M and Hara-Nishimura I. 2005. A vacuolar processing enzyme, δ VPE, is involved in seed coat

- formation at the early stage of seed development. *Plant Cell* 17:876-887.
- Perez FJ, Villegas D and Mejia N. 2002. Ascorbic acid and flavonoid-peroxidase reaction as a detoxifying system of H₂O₂ in grapevine leaves. *Phytochemistry* 60:573-580.
- Shimada T, Hiraiwa N, Nishimura M and Hara-Nishimura I. 1994. Vacuolar processing enzyme of soybean that converts proproteins to the corresponding mature forms. *Plant Cell Physiol.* 35:713-718.
- Van Huijsduijnen RAMH, Alblas SW, De Rijk RH and Bol JF. 1986. Induction by salicylic acid of pathogenesis-related proteins and resistance to alfalfa mosaic virus infection in various plant species. *J. Gen. Virol.* 67: 2135-2143.
- Vandromme M, Gauthier-Rouviere C, Lamb N and Fernandez A. 1996. Regulation of transcription factor localization: fine-tuning of gene expression. *Trends Biochem. Sci.* 21: 59-64.
- Vaux DL and Korsmeyer SJ. 1999. Cell death in development. *Cell* 96: 245-254.
- Wang Y, Zhu S, Liu S, Jiang L, Chen L, Ren Y, Han X, Liu F, Ji S, Liu X and Wan J. 2009. The vacuolar processing enzyme *OsVPE1* is required for efficient glutelin processing in rice. *Plant J.* 58:606-617.
- Woltering EJ, van der Bent A and Hoeberichts FA. 2002. Do plant caspases exist? *Plant Physiol.* 130:1764-1769.
- Yamada K, Shimada T, Nishimura M and Hara-Nishimura I. 2005. A VPE family supporting various vacuolar functions in plants. *Physiol. Plantarum* 123:369-375.
- Zakharov A and Muntz K. 2004. Seed legumains are expressed in stamens and vegetative legumains in seeds of *Nicotiana tabacum* L. *J. Exp. Bot.* 55:1593-1595.

Appendix Table 1. The nucleotide-homology and expression patterns of ESTs from cDNA library expressed during 16 and 18 day after fertilization in wheat.

No.	accession No.	function	E-value	Organism
1	XM_003572974.1	unknown	0	<i>Triticum aestivum</i>
3	XM_003572368	fatty acid beta-oxidation multifunctional protein-like	0	<i>Brachypodium distachyon</i>
4	AK335059.1	unknown	0	<i>Triticum aestivum</i>
6	AJ438611.1	nucleosome assembly protein 1	0	<i>Oryza sativa</i>
7	XM_003575523.1	ethylene-insensitive protein 2-like	0	<i>Brachypodium distachyon</i>
8	XM_003560684	LRR receptor-like serine/threonine-protein kinase	0	<i>Brachypodium distachyon</i>
13	XM_003577452.1	Xaa-Pro aminopeptidase P-like	0	<i>Brachypodium distachyon</i>
16	XM_003557695.1	nucleoredoxin 1-2-like	0	<i>Brachypodium distachyon</i>
17	GAJL01224176.1	unknown	0	<i>Triticum aestivum</i>
23	XM_003567746.1	receptor-like protein kinase	0	<i>Brachypodium distachyon</i>
24	AB029888.1	sucrose 1-fructosyltransferase	0	<i>Triticum aestivum</i>
27	GAJL01276045	unknown	0	<i>Triticum aestivum</i>
33	GAJL01254795.1	unknown	0	<i>Triticum aestivum</i>
37	M90077.1	elongation factor 1 alpha-subunit (TEF1)	0	<i>Triticum aestivum</i>
39	GAJL01262300.1	unknown	0	<i>Triticum aestivum</i>
40	AF327046.1	1-Cys peroxiredoxin (PER1)	0	<i>Triticum aestivum</i>
43	XM_003559238.1	DEAD-box ATP-dependent RNA helicase like	1.00E-158	<i>Brachypodium distachyon</i>
51	XM_003579618.1	acetyl-coenzyme A synthetase-like	0	<i>Brachypodium distachyon</i>
52	XM_003563516.1	indole-3-acetic acid-induced protein	4.00E-73	<i>Brachypodium distachyon</i>
53	GAJL01272262	unknown	0	<i>Triticum aestivum</i>
55	AF543198.1	phosphate transporter 6	0.00E+00	<i>Hordeum vulgare</i>
57	M98254.1	PSI-D subunit of photosystem I	0	<i>Hordeum vulgare</i>
58	FN429985.1	glyceraldehyde-3-phosphate dehydrogenase	0.00E+00	<i>Triticum aestivum</i>
59	EF682132.1	vacuolar processing enzyme 1 (VPE 1)	0	<i>Triticum aestivum</i>
60	XM_003562216.1	2-oxoglutarate dehydrogenase complex 1		<i>Brachypodium distachyon</i>
62	X94352.1	catalase	0	<i>Triticum aestivum</i>
63	GAJL01046966.1	unknown	1.00E-118	<i>Triticum aestivum</i>
64	XM_003560068	oxygen-evolving enhancer protein 3-1, chloroplastic-like	0	<i>Brachypodium distachyon</i>
65	XM_003558796.1	cytochrome P450 86B1-like	0	<i>Brachypodium distachyon</i>
67	NM_001155699.1	BAX inhibitor motif-containing protein 4	0	<i>Zea mays</i>
68	XM_003559284.1		0	<i>Brachypodium distachyon</i>
70	XM_003581500.1	cytochrome P450 704C1-like	0	<i>Brachypodium distachyon</i>
71	XM_003573279.1	lysine histidine transporter 1-like	0	<i>Brachypodium distachyon</i>
72	M96940.1	endo-1,3-beta-glucosidase (isoenzyme IV)	0	<i>Hordeum vulgare</i>
73	XM_003564446.1	serine/threonine-protein kinase like	0	<i>Brachypodium distachyon</i>
74	GAJL01232596.1	unknown	0	<i>Triticum aestivum</i>
79	XM_003563826	formate dehydrogenase 1	2.00E-160	<i>Brachypodium distachyon</i>
91	GAJL01259873.1		0	<i>Triticum aestivum</i>
96	AY057118.1	WCOR413-like protein beta form	0	<i>Triticum aestivum</i>
95	XM_003573173.1	ABC transporter F family member 1-like	0.00E+00	<i>Brachypodium distachyon</i>
97	XM_003558923.1	COP9 signalosome complex subunit	2.00E-93	<i>Brachypodium distachyon</i>

Appendix Table 1. Continued.

No.	accession No.	function	E-value	Organism
98	AK330527.1	unknown	0	<i>Triticum aestivum</i>
99	AK333039.1	unknown	0	<i>Triticum aestivum</i>
101	GAJL01271751.1	unknown	0	<i>Triticum aestivum</i>
107	XM_003580747.1	polyamine oxidase 4-like	0	<i>Brachypodium distachyon</i>
110	AK373155.1	unknown	0	<i>Hordeum vulgare</i>
114	XM_003567348.1	THO complex subunit 2-like	0	<i>Brachypodium distachyon</i>
115	GAJL01227155.1	unknown	0	<i>Triticum aestivum</i>
117	GAJL01275155.1	unknown	0	<i>Triticum aestivum</i>
121	M90077.1	translation elongation factor 1 alpha-subunit (TEF1)	0	<i>Triticum aestivum</i>
124	XM_003568881.1	serine/threonine-protein phosphatase 2A regulatory subunit B	0	<i>Brachypodium distachyon</i>
130	XM_003572633.1	cytochrome P450 86B1-like	0	<i>Brachypodium distachyon</i>
132	GAJL01187384.1	unknown	0	<i>Triticum aestivum</i>
133	XM_003576973.1	40S ribosomal protein S19-like	6.00E-151	<i>Brachypodium distachyon</i>
134	AK330414.1	unknown	0	<i>Triticum aestivum</i>
142	AK359789.1	unknown	0	<i>Hordeum vulgare</i>
143	XM_003562590.1	GTP-binding protein 1	0	<i>Brachypodium distachyon</i>
144	XM_003563994.1	exosome complex component rrp4-lik	0	<i>Brachypodium distachyon</i>
147	XM_003569220.1	myosin-Va-like	0	<i>Brachypodium distachyon</i>
150	XM_003574440.1	gibberellin 3-beta-dioxygenase 1-like	0	<i>Brachypodium distachyon</i>
155	XM_003573973.1	60S ribosomal protein L21-2-like	0	<i>Brachypodium distachyon</i>
158	XM_003576493.1	disease resistance protein RGA2-like	7.00E-171	<i>Brachypodium distachyon</i>
160	XM_003560701.1	alpha-glucan water dikinase	0	<i>Brachypodium distachyon</i>
161	GAJL01275155.1	unknown	0	<i>Triticum aestivum</i>
162	GAJL01261874.1	unknown	0	<i>Triticum aestivum</i>
164	GAJL01263710.1	unknown	0	<i>Triticum aestivum</i>
165	XM_003570714.1	chaperone protein ClpB3	0	<i>Brachypodium distachyon</i>
170	XM_003569551.1	phytochrome-associated serine/threonine protein phosphatase 1-like	0	<i>Brachypodium distachyon</i>
172	GAJL01239546.1	unknown	0	<i>Triticum aestivum</i>
173	XM_003578624.1	monodehydroascorbate reductase-like	0	<i>Brachypodium distachyon</i>
174	GAJL01174843.1	unknown	1.00E-173	<i>Triticum aestivum</i>
177	XM_003563760.1	T-complex protein 1 subunit gamma-like	0	<i>Brachypodium distachyon</i>
178	GAJL01183894.1	unknown	0	<i>Triticum aestivum</i>
180	GAJL01226876.1	unknown	0	<i>Triticum aestivum</i>