Identification and Functional Analysis of S-AdenosylMethionine Synthetase (*HvSAMS*) genes in Early Maturing Barley (*Hordeum vulgare subsp. vulgare*)

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ABSTRACT Grain development has been shown to involve a complex series of physiological and molecular events. In order to provide information on molecular events during grain development, we isolated four noble genes, *HvSAMS1*, 2, 3 and 4 (*Hordeum vulgare* *S-AdenosylMethionine Synthetase1*, 2, 3 and 4). The four *HvSAMS* genes were closely related based on peptide comparisons and sequence homologies that ranged from 89% to 99%. To determine the developmental expression of *HvSAMS* genes, Northern blot analysis and RT-PCR were performed with the kernels of -3 to 30 days after fertilization (DAF). The transcripts of *HvSAMS1* and 3 genes peaked at -3 DAF that lasted until 7 DAF, and started to reduce at 10 DAF. A strong expression signal of *HvSAMS2* was detected from -3 DAF and reached the maximum level at 3 DAF, then decreased until 30 DAF. The expression of *HvSAMS4* initially elevated from -3 DAF, reaching the peak at 10 DAF, and decreased gradually until 30 DAF. The *HvSAMS* gene transcripts were accumulated abundantly in grains, stems and leaves. To evaluate subcellular localization molecular functions of *HvSAMS1* gene, we transformed the *HvSAMS1* gene into onion epidermal cell and *Arabidopsis*. Expression of *HvSAMS1* recognized by 35S::*HvSAMS1*::*GFP* was detected in the nucleus and slightly in the cytosol, whereas 35S::*GFP* expressed throughout the cell. The transgenic lines showed slightly early germination on MS-medium containing 1 μ M GA₃ coupled with accelerated extension of bolts. The *HvSAMS* genes were dominantly expressed in grains during grain development (3 DAF). The *HvSAMS* genes showed various transcript accumulations in response to the abiotic stresses and exogenous application of phytohormones. Especially, *HvSAMS* genes were regulated by exogenous GA₃. The subcellular localization of *HvSAMS1* and histochemical localization of *HvSAMS2* promoter provided opportunities to elucidate their possible cellular functions. The phenotypic attributes displayed by *HvSAMS1* overexpressing transgenic plants suggested the role of *HvSAMS1* in the germination and GA₃ response mechanism.

Keywords Barley, Eam10, Differential expression, S-adenosylmethionine synthetase, *HvSAMS*

INTRODUCTION

Developing cereal grain which consists of several organs such as embryo, endosperm, and pericarp undergoes very important processes that determine its quality and quantity. After fertilization, embryo and endosperm were developed via cell division followed by grain filling. During the grain filling process, cellularization, differentiation and deposition of protein and starch were accelerated. After completion of accumulation of storage materials followed by dehydration, the grain entered seed dormancy (Schütz 1997). Many genes involved during dormancy cycling were profiled

(Han et al. 1999; Cadman et al. 2006). Colucci et al (2002) reported that overexpression of *GCR1*, the G protein – coupled receptor gene, could abolish the seed dormancy.

Plants are sources of secondary metabolites that often have high value (Belbahri. et al. 2000). Plant secondary metabolites are defined as compounds that have not exactly recognized roles in the maintenance of fundamental life processes in the plants, but they do have important roles in the interaction of the plant with its environments grown (Oksman-Caldentey and Inzé 2004). The plant secondary metabolites are reported with poor productivity because of their extremely low level of accumulation in plants species

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(Goossens et al. 2003). Plenty of secondary metabolites possess a methyl group involving S-adenosylmethionine (SAM) in the biosynthetic pathway (Kutchan 1995; Belbahri et al. 2000).

S-adenosylmethionine synthetase (SAMS) catalyzes the biosynthesis of S-adenosylmethionine (SAM) from ATP and L-methionine (Cantoni 1953). As SAMS is an important methyl donor (Schlenk and DePalma 1957), it plays a role in transmethylation of proteins, nucleic acids, polysaccharides and fatty acids. Decarboxylated SAMS serves as an aminopropyl donor in polyamine synthesis (Kumar et al. 1997) and SAM is a precursor in the synthesis of ethylene (Yang and Hoffman 1984). SAM was produced in the cycle of active methyl groups via S-adenosylhomocysteine hydrolase (SAHH) (Schröder et al. 1997). SAHH hydrolyzed homocysteine and adenine from S-adenosylhomocysteine (SAH). Homocysteine was methylated by methionine synthase (MS) to yield methionine and finally regenerated SAM using SAMS (Heim et al. 2006).

The cloning and sequence analysis of genes for *SAMS* have been reported in many plants. Two *Arabidopsis SAMS* genes were isolated by Peleman et al (1989a). Three different *SAMS* genes were reported from tomato (Espartero et al. 1994), three from periwinkle (Schröder et al. 1997), two from pea (Gómez-Gómez and Carrasco 1998), one from *Lycoris radiata* (Li et al. 2013). Because SAM is an important factor in primary and secondary metabolism, various effects may be explained via plant transformation with a *SAMS* gene (Belny et al. 1997). *SAMS* genes under the control of the CaMV 35S constitutive promoter were introduced in *Arabidopsis thaliana* (Peleman et al. 1989a, b), *Papaver somniferum* (Belny et al. 1997), and *Nicotiana tabacum* (Boerjan et al. 1994; Belbahri et al. 2000; Qi et al. 2010).

Usually, SAMS is ubiquitous enzyme and has been found in different tissue parts, such as vascular tissues and sclerenchyma of *Arabidopsis* (Peleman et al. 1989), elongated stems of petunia (Izahaki et al. 1996), leaves of rice (Dekeyser et al. 1990), and developing ovary of pea (Gómez-Gómez and Carrasco 1998). However, the relationship of SAMS between its expression and biosynthesis is still unknown (Qi et al. 2010).

In this paper, we isolated a gene family encoding SAMS

in the barley (GSHO 2504) which has a characteristic of early maturity; and their expression modes in the early stage of kernel development were evaluated. Furthermore, transcripts accumulations of the *HvSAMS* genes in response to the exogenous elicitors were investigated.

MATERIALS AND METHODS

Plant materials and elicitor treatment

Early maturing barley germplasm “GSHO 2504” provided by USDA-ARS, National Small Grains Research Facility, Barley Genetic Stocks Collection, was grown at the Korea University research field. Procedures for the determination of days after fertilization (DAF) and sampling of grain materials were described by Jang et al (2003). The grain tissues used in the experiments were collected at -3, 1, 3, 7, 10, 20 and 30 DAF. Different tissue parts including grain, stem, leaf and root parts were harvested from plants at 10 DAF.

Transcriptional analyses were performed on the leaves of 4-week-old plants after spraying each with 100 μ M of ABA, GA₃, and spermidine, as well as ABA in combination with GA₃. Each spray contained 0.05% Tween 20. For the NaCl treatment, the leaves of 4-week-old plants were sprayed with 100 mM NaCl solution with 0.05% Tween 20. For the ethephon treatment, ethephon gas was inserted into a transparent plastic bag, which was used to cover 4-week-old plants. Because ethephon releases ethylene with HCl and H₃PO₄, separate 4-week-old plants were treated with 10 mM HCl and 10 mM H₃PO₄. For the mock treatment, non-treated plants were covered with a transparent plastic bag for 24 h. In the case of mechanical wounding treatment, the leaves were pierced using a surface-sterilized needle. Treated leaves were subsequently harvested at 1, 6, 12, 24 and 48 h after each treatment except for the wounded-plant treatment which was sampled after 0.5, 1, 6, 12 and 24 h.

Isolation of *HvSAMS* gene family

A cDNA library was constructed from barley grains of 3 DAF and 7 DAF using Uni-ZAP XR vector (Stratagene, Cedar Creek, TX, USA) described by Jang et al (2003). The plasmid DNAs were prepared from 800 randomly selected

colonies using the QIAprep Spin Miniprep kit (Qiagen, Germantown, MD, USA). About 300 ng of individual plasmid DNA was blotted onto a positive nylon membrane (GE Osmonics, Minnetonka, MN, USA) using a 96-well vacuum dot blot apparatus (Invitrogen, Garlsbad, CA, USA). After aspiration, the membrane was washed with 2X SSC buffer for 2 min, air-dried and then fixed with UV

cross-linking. In order to isolate *HvSAMS* genes, total RNA extracted from 3 DAF grains was reverse-transcribed into cDNA using the First Strand cDNA Synthesis kit (Roche, Florence, SC, USA). Among the four *HvSAMS* clones, one was obtained by DH analysis as described by Lee et al (2006), while the other three were isolated using PCR amplification with gene specific primers (cDNA isolation

Table 1. List of primers used for gene cloning from cDNA and genomic DNA, promoter isolation, and RT-PCR.

Gene	Sequence	Direction	Purpose
<i>HvSAMS1</i>	5'-CGGCCTCCTGAACAATAGCATCAGCAC-3'	sense	C
	5'-GGATTGTTGAACTAGAGTGGCAGCAGAC-3'	antisense	
	5'-GATTCGACCTCTTTCGGTTTCG-3'	sense	R
	5'-CAGCACACTCAAAAAGAAAATGAA-3'	antisense	
	5'-CCACGAAAGAAATGGCGG-3'	sense	G
	5'-CCAATTGCACAAACCATGAT-3'	antisense	
	5'-AAATTGCAACGTTTCATGGGCGGCATGGG-3'	antisense	P
	5'-TAGTCGCTTGATGCGTGTCTTACGG-3'	antisense	
<i>HvSAMS2</i>	5'-GAGAGCATCTCTACCACCAAAG-3'	sense	C
	5'-CCTTGCCGAACCTTGAGAGGCAT-3'	antisense	
	5'-CGACCTCTTCCTTTCGGTTTCTC-3'	sense	R
	5'-GAGGAGCAAAAGGATCCCGCCT-3'	antisense	
	5'-AAAGAAATGGCGGCCGAGACGTTTC-3'	sense	G
	5'-TCTTGTCTTAAGCAGATGCCTTG-3'	antisense	
	5'-CCTCTCAACTGCTTCATGGCCGACGAAG-3'	antisense	P
	5'-GTAATTTTGCTACATTCTTCAACCGGCATC-3'	antisense	
<i>HvSAMS3</i>	5'-GGAGCAATAGAAGCGGCACAAG-3'	sense	C
	5'-CCGCCTTGCCGAACCTGAGA-3'	antisense	
	5'-CGGCACGAGGTCGCCTCTTC-3'	sense	R
	5'-CAGTGTAGCACGTCGCAGCG-3'	antisense	
	5'-GAAAGAAATGGCGGCCGAGACA-3'	sense	G
	5'-TCTTGTCTTAGGCAGATGCCTTG-3'	antisense	
	5'-GTCGACGAAAACGTCTCTTTCCACTGAAAGCACA-3'	antisense	P
	5'-CTGAGACTTAACTCTGGTGGGCAGTGGA-3'	antisense	
<i>HvSAMS4</i>	5'-ATCGTGCGGTCCGATCTGGC-3'	sense	C
	5'-TAAACTAATTTTATTCAGAGGCTTC-3'	antisense	
	5'-CGAGGAGGCGGCTTCATTTTGC-3'	sense	R
	5'-CAGCACTGGAGCAATGAATAGAG-3'	antisense	
	5'-GGCCAAAGAAGATGGCTGAAGTTG-3'	sense	G
	5'-GTGTTTGCTCAGGCAGAAGGCTTC-3'	antisense	
	5'-GGGAGTTGAGGAACTAATGGCTCCA-3'	antisense	P
	5'-GGCCGAATTAGTTGACTCAGATTTGTCTAG-3'	antisense	

C indicates cDNA isolation, R indicates RT-PCR, G indicates genomic DNA isolation, and P indicates promoter isolation

in Table 1). The amplified fragments were cloned into pGEM easy vector (Promega, Madison, WI, USA), and DNA sequencing analysis was performed using an ABI PRISM 310 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Amino acid homology between isolated *HvSAMS* clones was analyzed using BLASTX alignment (Altschul et al. 1997) and CLUSTAL W (Thompson et al. 1994).

Southern blot analysis

Genomic DNA was extracted from 1 g of fresh leaf tissue of barley germplasm “GSHO 2504” using a modified CTAB protocol described by James et al (2008). A total of 10 µg of genomic DNA was digested using restriction enzymes *EcoRI*, or *XbaI*, which do not cut the coding sequence of *HvSAMS1*, 2, 3, and 4. Following digestion, genomic DNA fragments were separated by gel electrophoresis on a 0.8% (w/v) agarose gel and blotted onto a Hybond-N membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The full-length of *HvSAMS1* coding sequence was amplified by PCR and purified with the QIAquick PCR purification kit (Qiagen, Germantown, MD, USA). Following amplification, the coding sequence was used as a probe for Southern blot analysis. Probe labeling, hybridization, and signal detection were performed as described by Kim et al (2011).

Northern blot analysis

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). A total of 10 µg total RNA from each plant sample was separated on 1% (w/v) formaldehyde agarose gel, and then transferred onto a Magna charge nylon membrane (GE Osmonics, Minnetonka, MN, USA). Using the Biotin-dCTP and PCR method with each gene specific primer, the full-length cDNA, which contained 5′ and 3′ untranslated regions were labeled and used as a probe. After membrane transfer, the membrane was equilibrated in 250 mM sodium phosphate for 5 min, prehybridized with hybridization buffer [0.1 mM EDTA, 7% SDS, 250 mM sodium phosphate (pH 7.2), and 5% dextran sulfate] for 1 h at 65°C. The hybridization procedure was performed with membrane using a Biotin-labeled

probe in fresh hybridization buffer for 16 h at 65°C. The hybridized membrane was washed twice with 2X SSC and 1% SDS for 5 min at room temperature, followed by washing twice with 0.1X SSC and 1% SDS for 15 min at 65°C. An additional washing procedure was carried out with 1X SSC for 5 min at room temperature. The detection procedure was performed with Southern-Light and Southern-stat system (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instruction. Analysis was done in triplicate.

RT-PCR

For RT-PCR, 5 µg of total RNA was reverse-transcribed into cDNA using the First Strand cDNA Synthesis kit (Roche, Florence, SC, USA) following the manufacturer’s instructions. The transcript of barley *Actin* gene served as an endogenous expression control. The gene-specific primers for RT-PCR were designed from 5′ and 3′ UTR region of cDNA sequence of *HvSAMS1*, 2, 3, and 4 (Table 1) based on comparison of UTR sequences. The first strand cDNA product was diluted five times for use as a template for the synthesis of second strand cDNAs with gene-specific primers or *Actin* primer (sense; 5′-AGACCTTC AACACCCCTGCTATGT-3′, antisense; 5′-CCAATCCA GACACTGTACTTCCTT-3′). The PCR reactions (25 µl) were denatured at 94°C for 1 min, followed by 27 cycles for *HvSAMS1*, 2, 3, and 4 or 24 cycles for *Actin* at 94°C for 1 min, 60°C for 45 sec, 72°C for 1 min 30 sec, and a final extension cycle of 72°C for 4 min. The PCR products were analyzed by electrophoresis on 1% agarose gel (w/v) with 1X TAE buffer and visualized by ethidium bromide staining. The set up was done in triplicate.

Isolation of *HvSAMS1*, 2, 3, and 4 promoters

Genomic DNA was extracted from barley germplasm “GSHO 2504” and genome walker construction was performed as described by Kim et al (2007) using the Universal GenomeWalker kit (Clontech, Mountain View, CA, USA). Genome walker construction was used to isolate the promoter regions of *HvSAMS1*, 2, 3, and 4 after digestion with seven restriction enzymes forming blunt end (*DraI*, *EcoRV*, *PvuII*, *StuI*, *BbrPI*, *SmaI*, *SnaBI*). The

gene-specific primers (Table 1) were designed based on the alignment of nucleotide homology between *HvSAMS1*, *HvSAMS2*, *HvSAMS3*, and *HvSAMS4* genomic DNA sequences. PCR products amplified from Genome walker library were cloned into the pGEM easy vector (Promega, Madison, WI, USA). After sequence analysis, the *cis*-elements in each UTR region of *HvSAMS1*, 2, 3, and 4 were analyzed with the BLAST alignment (Altschul et al. 1997), PLACE Web Signal Scan (Higo et al. 1999) and PlantCARE (Lescot et al. 2002).

Vector Constructions

Transient GUS plasmid, pBI101::HvSAMS(-1459), pBI101::HvSAMS(-301), and pBI101::HvSAMS(-211), containing GUS reporter gene under control of the 1,459 bp, 301 bp, and 211 bp of upstream regions of *HvSAMS2* gene (Kim et al. 2007) were introduced into the *Agrobacterium tumefaciens* strain (GV3101) via freeze-thaw method as described by Chen et al (1994).

For GFP expression vector, *HvSAMS1* cDNA that codes for the full-length HvSAMS1 protein without stop codon was amplified with 5'-TCTAGAAATGGCGGCCGAGAC GTTCCT-3' containing *Xba*I (forward primer) and 5'-GGATCCGGCAGATGCCTTGTCGAACTTG-3' containing *Bam*HI (reverse primer). The 35S::smGFP::HvSAMS1 plasmid was constructed by subcloning into the 326 GFP vector (Lee et al. 2001) after *Xba*I and *Bam*HI digestion.

For transformation binary vector, the full-length cDNA *HvSAMS1* gene under the control of maize ubiquitin promoter and first intron were subcloned into the pCAMBIA 3301 binary vector after digestion with *Eco*RI and *Hind*III.

Histochemical staining

In order to identify the localization of *HvSAMS2* expression, histochemical staining was conducted on pBI101::HvSAMS(-1459), pBI101::HvSAMS(-301), and pBI101::HvSAMS(-211). The *Arabidopsis* (Col-0) transformation was performed with *A. tumefaciens* (GV3101) as described by Clough and Bent (1998). To select transformed plants, sterilized seeds were sown on kanamycin (50 mg/l) selection plates, and then allowed to grow for 1-2 weeks in a controlled

environment at 22°C under 16 h light photoperiod. Some transformants were tested in a X-gluc solution {1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide), 100 mM phosphate buffer, pH 7.0, 5 mM K₃[Fe(CN)₆], and 10 mM EDTA} for 16 h at 37°C. After staining, the tissues were fixed in a solution consisting of 5% formaldehyde, 5% acetic acid and 50% ethanol for 4 h at room temperature, and then cleared by sequential incubation in 60, 80, and 100% ethanol for 1 h at room temperature. Analysis was done twice.

Subcellular localization of HvSAMS1

To identify the subcellular localization of HvSAMS1, onion epidermal cells were sterilized and incubated on MS medium (Murashige and Skoog 1962) for 1 day at 25°C without light. After incubation, 35S::smGFP::HvSAMS1 or 35S::smGFP were precipitated on 1.0 μ m gold particle and bombarded by a biolistic particle delivery system, PDS-1100 (Bio-Rad, Hercules, CA, USA). After incubation of transformed onion epidermis cells for 16 h at 22°C, subcellular localization of the 35S::smGFP::HvSAMS1 or 35S::smGFP fusion constructs was monitored using a Bio-Rad MRC-1024 confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA). All photographs were taken at the same magnification. Analysis was done in duplicate.

Plant transformation

The *A. thaliana* plant used in this study is descendant of Columbia (Col-0) ecotype. Plants were grown in a soil mixture (loam soil/perlite/vermiculite, 3/1/1) or on MS salt medium (Murashige and Skoog, 1962) at 22°C, 16 h photoperiod. The recombinant plasmid was introduced into an *A. tumefaciens* strain GV3101 via the freeze-thaw method (Chen et al. 1994) which was then transformed into *A. thaliana* (Col-0) following the protocol described by Clough and Bent (1998).

Seeds collected from the transformed plants were sterilized with Clorox bleach for 5 min, and washed eight times with sterile distilled water. After surface sterilization, T₁ seeds were plated on MS medium (Murashige and Skoog 1962) with phosphinothricin (15 μ g/L) and stored

at 4°C for 2 days for vernalization. The vernalized seeds were transferred to a growth chamber under a controlled environment at 22°C, 16 h photoperiod. Eight individual phosphinothricin resistant plants (T_1) were transplanted to a soil mixture (loam soil/perlite/vermiculite, 3/1/1), and T_2 seeds were harvested. The T_2 seeds were further advanced to T_3 generation.

RESULTS

Isolation and identification of *HvSAMS1*, 2, 3, and 4

The kernel-specific expressed genes during early stage of grain development were isolated via DH from randomly selected 800 cDNA clones. A total of 61 individual cDNA clones showed significant degree of homology to sequences registered in NCBI (Appendix Table 1). One clone whose transcripts were highly expressed at 3 DAF kernel and showed high homology to S-adenosylmethionine synthetase was identified and designated as *HvSAMS1* (*Hordeum vulgare* *S-AdenosylMethionine Synthetase1*). Another 3 *SAMS* genes were isolated from cDNA of 3 DAF kernels and designated as *HvSAMS2*, 3 and 4. The cDNAs encoding *HvSAMS1*, 2, 3 and 4 contained 1,185 bp, 1,185 bp, 1,185 bp and 1,191 bp open reading frames that code 394, 394, 394 and 396 amino acids, respectively (Table 2). All *HvSAMS1*, 2, 3 and 4 did not contain intron region in protein coding region. The N-terminal of all deduced *HvSAMS* family sequences did not contain any potential cleavage site and their predicted isoelectric points ranged from 5.40 to 5.58, and molecular weights were from 42.7 to 43.2 kDa. Amino acid sequence homologies among *HvSAMS1*, 2, 3, and 4 ranged from 89% to 99% (Table 2).

HvSAMS1 and *HvSAMS2* showed the closest relationship with 99% sequence similarity with only one amino acid difference (K to N) at the 361st amino acid position. The four deduced amino acids sequences contained highly conserved two domains. One was the ATP-binding site hexapeptide, GAGDQG, and the other was phosphate-binding P-loop, glycine-rich nonapeptide, GGGAFSGKD (Fig. 1A). A phylogram for the phylogenetic relationship of the *HvSAMS* and other plant *SAMS* were constructed (Fig. 1C). It could be observed that 23 *SAMS* proteins were clustered in two groups. *HvSAMS1*, 2, 3 and 4 belonged to the same group (type I).

Genomic Southern blots were performed by hybridization of *EcoRI* or *XbaI* digested barley genomic DNA using a ³²P labeled full-length *HvSAMS1* gene as a probe. Three bands were detected with *EcoRI* and *XbaI* digestion (Fig. 1B). The sizes of hybridization signals were approximately 20 kb and 10 Kb for *EcoRI* digestion and approximately 14 kb, 13.5 kb and 4.5 kb for *XbaI* digestion. Two hybridization signals of approximately 20 kb in size were detected in *EcoRI* digestion. However, they were detected as two hybridization signals on short expose period x-ray film (data not shown).

Developmental and tissue-specific expression of *HvSAMS* gene

Northern blot analysis and RT-PCR were performed using kernels at -3, 0, 3, 7, 10, 20 and 30 DAF to determine the developmental expression of *HvSAMS* genes. The transcripts of *HvSAMS1* and *HvSAMS3* reached highest levels at -3 DAF which continued until 7 DAF, and then started to decrease at 10 DAF (Fig. 2A). A strong expression of *HvSAMS2* was detected from -3 DAF which

Table 2. Characteristics of *HvSAMS* cDNAs and proteins from *Hordeum vulgare* subsp. *vulgare*.

Gene	cDNA				Mature Protein					
	Length (bp)	Identity (%)			Length (aa)	kDa	pI	Identity (%)		
		<i>HvSAMS1</i>	<i>HvSAMS2</i>	<i>HvSAMS3</i>				<i>HvSAMS1</i>	<i>HvSAMS2</i>	<i>HvSAMS3</i>
<i>HvSAMS1</i>	1185				394	42.8	5.58			
<i>HvSAMS2</i>	1185	95			394	42.8	5.49	99		
<i>HvSAMS3</i>	1185	92	93		394	42.7	5.52	96	97	
<i>HvSAMS4</i>	1191	79	80	80	396	43.2	5.40	90	90	89

further intensified at 3 DAF, and subsequently decreased until 30 DAF. *HvSAMS4* expression was notably high from -3 DAF and reached the peak at 10 DAF, and decreased gradually until 30 DAF. Additional transcript signals were detected in northern blots for *HvSAMS1*, 2 and 3 (Fig. 2A). Except for *HvSAMS4*, additional transcript signals of higher molecular size than the major transcripts of *HvSAMS1*,

2 and 3 were observed. These additional detected transcripts exhibited similar expression pattern with those of *HvSAMS*.

The RT-PCR was also carried out with RNA isolated from kernels at different developmental stages to confirm *HvSAMS* genes expression. Because *HvSAMS* genes had very high sequence homology in their open reading frame, the gene-specific primers were designed from 5'UTR and

Fig. 1. (A) Comparison of amino acid sequence of the *HvSAMS*. The numbers at the end of right side in each line are the cumulative total number of amino acids sequence in each line. The conserved domains were indicated by asterisks. Black shading indicated the same amino acid at that position among all amino acids. Gray shading indicated the conserved amino acid residues. Dashes showed gaps in the amino acid sequences that were presented to optimize alignment. (B) Southern blot detection of *HvSAMS* genes in barley. Each lane contained 40 μ g of genomic DNA digested with *Eco*RI or *Xba*I. The digested DNA fragments were separated by 0.8% agarose gel, electrophoresis, transferred onto nylon membrane and hybridized with 32 P labeled full-length *HvSAMS1* probe. Arrows indicate hybridization signal, M, molecular size marker; E, *Eco*RI; X, *Xba*I. (C) Phylogram of the relationship between *HvSAMS* proteins and various plant SAMS proteins. The scale bar represented 0.1 unit.

3'UTR region (Table 1 and Table 2). The amplified RT-PCR products were detected at molecular sizes of 1,704 bp (*HvSAMS1*), 1,328 bp (*HvSAMS2*), 1,449 bp (*HvSAMS3*) and 1,389 bp (*HvSAMS4*). *HvSAMS* transcripts predominantly accumulated at early and intermediate time points (from -3 DAF to 10 DAF) with highest accumulation frequency observed at 3 DAF. However, the additional transcript accumulations were not detected in RT-PCR analysis (Fig. 2B).

Tissue-specific expression of *HvSAMS* genes were also analyzed using tissues of grains, roots, leaves and stems at 10 DAF. The results of RT-PCR showed that the transcripts of all *HvSAMS* genes accumulated abundantly in grains, stems, and leaves. The lowest transcript level was observed in the roots for all *HvSAMS* genes. Expression of *HvSAMS2*, 3 and 4 were similar in grains, stems and leaves, but at level relatively lower than that of *HvSAMS1* (Fig. 2C).

Fig. 2. Transcript profiling of *HvSAMS* genes during grain development. (A) Northern blot hybridization of the *HvSAMS* genes during the grain development in barley. Total RNAs of different stage grains were fractionated on a 1% denaturing agarose gel. The grain materials were harvested at -3, 0, 3, 7, 10, 20 and 30 DAF. 1, -3 DAF; 2, 0 DAF; 3, 3 DAF; 4, 7 DAF; 5, 10 DAF; 6, 20 DAF; 7, 30 DAF. (B) RT-PCR analysis of the barley grain with same RNA in Fig. 2A. (C) RT-PCR analysis of the *HvSAMS* genes in different tissues. Total RNAs of four tissues from the barley (cv. K800) at 10 DAF were fractionated on a 1% denaturing agarose gel. G, grain; R, root; S, stem; L, leaf.

Expression of *HvSAMS* genes in response to abiotic stress and phytohormone treatments

RT-PCR was used to investigate the accumulation of *HvSAMS* transcripts in response to the abiotic stress and exogenous application of phytohormones. Various expression patterns of *HvSAMS* genes showing up- and down-regulation by each treatment were noted (Fig. 3). Transcripts of *HvSAMS1* were present at all-time points in wounding, NaCl, ABA and spermidine treatments. *HvSAMS1* transcripts

accumulations were elevated from 24 h in GA₃ and GA₃ together with ABA treatment. Accumulation of *HvSAMS1* transcripts were also increased from 24 h in ethylene treatment, but subsequently decreased at 48 h.

HvSAMS2 transcripts were present at all-time points in NaCl treatment and were highly accumulated in GA₃ and GA₃ - ABA treatment. Similar to *HvSAMS1*, mRNA levels of *HvSAMS2* were at highest level at 24 h. For spermidine treatment, the strongest signal of *HvSAMS2* was detected at

Fig. 3. Transcript accumulation profiles of *HvSAMS* genes expressed in response to exogenous phytohormones and abiotic stress treatment using RT-PCR analysis. Plant material was 4-week-old leaves of barley treated with a solution of 100 μ M each of ABA, GA₃, spermidine, ethephon and 100 mM NaCl; C, non-treated; M, mock treatment (24 hrs).

Fig. 4. Histochemical localization of GUS activity in transgenic *Arabidopsis* seedlings. (A) Schematic diagram of different *HvSAMS* 2 promoter::GUS fusions. The different length promoter fragments were subcloned into pBI 101. The putative *cis*-acting elements were represented by symbols. (B) *Arabidopsis* Columbia was transformed with transformation vectors. i, iv, vii, and x were introduced with *pBI101::HvSAMS* (-1459); ii, v, viii were introduced *pBI101::HvSAMS* (-301); iii, vi, ix were introduced *pBI101::HvSAMS* (-211) GUS fusion vector. I, ii, iii were 1-week-old transgenic plants (T_2); iv, v, vi were 2-week-old transgenic plants (T_2); vii, viii, ix, x were 3-week-old transgenic plants (T_2); x magnified to 3 times vii.

48 h. However, *HvSAMS2* transcript level was particularly low in ABA, and wounding treatments, as well as in ethylene treatment (except the transcript profile at 24 h).

The transcripts of *HvSAMS3* were present at all-time points in spermidine treatment. *HvSAMS3* gene transcript was less abundant in response to wounding, NaCl, GA₃ alone and GA₃ - ABA. A low transcript accumulation during ethylene treatment was observed except at 24 h after treatment. Accumulation of *HvSAMS3* mRNA transcripts continuously dropped until 12 h showing similar level as 0 h in ABA treatment.

HvSAMS4 transcripts accumulation was at steady state and was present at all-time points in spermidine, NaCl, GA₃ and ethylene treatment. *HvSAMS4* showed that its transcripts were slightly increased by wounding and ABA treatment. In case of GA₃ - ABA treatment, the transcripts were negligible at 0 h treatment and increased after 1 h.

Isolation of the *HvSAMS* genes promoter and expression

The genome walker strategy was used to isolate the *HvSAMS* gene promoter region from the genomic DNA of

barley. Because *HvSAMS* genes contained no intron region in their ORF, a couple of nested gene-specific primers were designed using the *HvSAMS* gene cDNA sequence (Table 1). The amplified promoter region was 1,788 bp for *HvSAMS1*, 1,698 bp for *HvSAMS2*, 1,916 bp for *HvSAMS3*, and 1,537 bp for *HvSAMS4*. After sequence confirmation, the putative *cis*-acting elements in each promoter region were analyzed using PlantCARE (Appendix Table 2).

In order to determine the spatial regulation of *HvSAMS2* gene expression, the transgenic plants harboring *pBI101::HvSAMS(-1459)*, *pBI101::HvSAMS(-301)*, and *pBI101::HvSAMS(-211)* constructs were investigated using histochemical GUS assay (Fig. 4A). Fig. 4B showed histochemical staining analysis for the GUS activity in 1-, 2-, and 3-week-old T₂ transgenic *Arabidopsis* plants. The GUS activity was only detected in 2-week-old plants, particularly in *pBI101::HvSAMS* - and *pBI101::HvSAMS* - containing transgenic plants which showed expression in the stem. The GUS expression in *pBI101::HvSAMS* transgenic plants lasted until 3 weeks (Fig. 4B).

Fig. 5. Subcellular localization of the *HvSAMS1* protein in onion cells. Onion epidermal cells expressing cytosolic GFP marker and *HvSAMS1* :: GFP fusion protein were examined at 24 h after transformation under a confocal laser scanning microscope (Bio-Rad MRC-1024) fitted with filters (excitation filter, 450-490 nm; emission filter, 520 nm).

Subcellular localization of HvSAMS1

The localization of HvSAMS1 was examined in onion epidermal cells using the HvSAMS 1::GFP fusion protein. Onion cells transformed with a plasmid containing GFP alone showed fluorescence dispersed throughout the cell. In contrast, the fluorescence was localized in the nucleus of the cells and slightly in the cytosol transformed with the plasmid expressing HvSAMS 1::GFP fusion protein (Fig. 5).

Transgenic *Arabidopsis* with *HvSAMS1* overexpression

In order to determine the *HvSAMS1* gene function, we generated the *Arabidopsis* transgenic plant overexpressing the coding sequence for the entire *HvSAMS1* under the control of an ubiquitin promoter. A total of 24 putative T₁ plants were produced via *Agrobacterium*-mediated transformation,

19 of which were selected through PCR screening (Appendix Fig. 2). Subsequent selections were carried out for T₂ and T₃ plants. In the T₃ generation, three homozygous lines for *HvSAMS1* were obtained.

The T₃ homozygous lines did not exhibit distinct phenotypic differences with wild-type for the color, leaf shape and plant height. Germination rate was significantly different between the *HvSAMS1* transgenic lines and wild-type plants (Fig. 6A). Transgenic plants showed faster growth for an initial two weeks, after which no difference in growth rate between transgenic lines and wild-type plants was observed (Fig. 6B). The transgenic lines exhibited slightly faster bolting than the wild-type plants (Fig. 6C). However, there was no significant difference in maturity between transgenic lines and wild-type. The transgenic lines vigorously grew on MS medium containing 1 μ M GA₃ (Fig. 6D).

Fig. 6. Growth characteristics of *Arabidopsis* transgenic plants overexpressing *HvSAMS1*. (A) comparison of the phenotypes of 1-week-old transgenic line (T₃) and wild-type. (B) comparison of the phenotypes of 2-week-old transgenic line (T₃) and wild-type. (C) bolt appearance and extension of 4-week-old transgenic line (T₃) and wild-type. (D) comparison of seed germination phenotypes of 1-week-old transgenic line (T₃) and wild-type on MS medium which contained 1 μ M of GA₃.

DISCUSSION

The barley germplasm “GSHO 2504” known as an early maturity 10, has an *eam10* gene and provided a good source for the barley breeding (Börner et al. 2002). The Barley lines possessing such early maturity related major genes (*eam8*, *eam9* and *eam10*) also provide sources for genetic mapping using RFLP (Restriction Fragment Length Polymorphism) and SSR (Single Sequence Repeat) (Gallagher et al. 2001; Börner et al. 2002). In our previous paper, the DH method was used for the isolation of grain-specific genes during the middle phase of grain development (Lee et al. 2006).

We isolated the four cDNAs that had high sequence homologies and designated as *HvSAMS* genes. Southern blot analysis of genomic DNA revealed the presence of at least 3 copies of *SAMS* genes in barley (Fig. 1B). Because *HvSAMS1* has only 79% sequence homology with *HvSAMS4*, the probe could not be hybridized with *HvSAMS4* gene. Nevertheless, this study demonstrates that barley has four copies of *HvSAMS* genes. This has been demonstrated in *Arabidopsis* (Peleman et al. 1989), periwinkle (Schröder et al. 1997), pea (Gómez-Gómez and Carrasco 1998) and tomato (Espartero et al. 1994). But this does not apply to all plant species. Only one *SAMS* gene was identified in petunia (Gómez-Gómez and Carrasco 1998) and soybean (Kim et al. 1995). To date, four *SAMS* genes were identified in barley endosperm (Radchuk et al. 2005).

HvSAMS gene family shared highly conserved two domains. One was ATP-binding domain, GAGDQG, and the other was P-loop-like domain, GGGAFSGKD. The traditional *SAMS* domain, ATP-binding domain, was involved with *SAMS* activity, SAM concentration and free methionine concentration (Shen et al. 2002). A P-loop-like sequence has been found in the *HvSAMS* genes sequence around the ATP-binding site (Fig. 1A) (Chiang et al. 1999). The typical P-loops were composed of the serine/threonine [S/T] residue, but *HvSAMS* genes were replaced by an aspartate [D]. This replacement was not an unusual phenomenon. There are two consecutive β -turns in the P-loop-like sequence in *SAMS*, instead of only a single turn in the typical P-loop. The first β

-turns consist of four residues, F-S-G-K, and the second β -turns consist of another four residues, D-P-S-K (Takusagawa et al. 1996). The serine [S] in the second β -turns was replaced by threonine [T] in *HvSAMS* genes. Because serine and threonine are classified as polar amino acids with uncharged residue groups, the P-loop-like region of *HvSAMS* genes might consist of two consecutive β -turns.

Schröder et al (1997) reported that plant *SAMS*s were fallen into two types. Although *HvSAMS* genes belong to the same group (type I), differential gene expression for *HvSAMS1*, 2, 3 and 4 were observed (Fig. 3). This result indicated that different regulatory regions might be responsible for different responses. The *HvSAMS* genes exhibited the highest expression pattern during early grain development (i.e., -3, 0 and 3 DAF) followed by a subsequent reduction as kernel development elapsed (Fig. 2A and B). It has been reported that *SAMS* genes were usually expressed in stem and root tissue (Peleman et al. 1989; Espartero et al. 1994). In only a few plants, *SAMS* genes were predominantly expressed in reproductive tissue such as grain (Radchuk et al. 2005) and ovary (Gómez-Gómez and Carrasco 1998). Radchuk et al (2005) profiled methylation cycle involved in enzymes, including *SAMS* genes, in different developmental stage of barley endosperm. *SAMS* genes from barley (*HvSAMS1*, 2, 3 and 4) were highly expressed during pre-storage and intermediate phase, considerably low near 12 DAF, and eventually high at 20 DAF. Although the sequence of *HvSAMS* genes and *HvAMS* genes showed high similarity, the transcriptional modes of *HvSAMS* genes and *HvAMS* genes observed by Northern blotting and RT-PCR were different (Fig. 2A and B).

With high sequence homology among *HvSAMS1*, 2 and 3, we speculated that the different expression pattern of these genes were unlikely the result of sequence differences. Radchuk et al (2005) reported expression profiling of *HvAMS* gene family using *in silico* analysis and microarray. The hybridized probe used was prepared using poly(A)⁺ RNA of cDNA sequence. Non-specific cross-hybridization could occur by cross-hybridization resulting in sequence similarity of probes because *HvSAMS1* and 2 sequences differed by only one amino acid position.

HvAMS3 had also 12 amino acids that were different from *HvAMS1*. When homology of gene sequences were very high, designing gene-specific (e.g. Northern hybridization) primers would be difficult. Alternatively, Ciaffi et al (2006) used RT-PCR to avoid non-specific hybridization. Gene-specific primers for each *HvSAMS* gene cloning and RT-PCR (Table 1) showed low sequence homology (5.1% ~ 50%) to each other. RT-PCR suggested that *HvSAMS1*, 2 and 3 transcripts were abundant at early stage (from -3 DAF to 3 DAF) and *HvSAMS4* expression remained at steady state throughout the observation time used in this study.

Plant genes are up- and down-regulated by a variety of growth regulatory hormones during plant development (Kende and Zeveaart 1997). The *SAMS* gene has been regarded as housekeeping gene involving methyl donor in numerous methylation systems. But abundant plant *SAMS* genes were significantly stimulated by an elicitor treatment (Gowri et al. 1991). In petunia, *SAMS* gene was examined by means of the temporal-, special-regulation against exogenous phytohormones (Izhaki et al. 1996). Espartero et al (1994) investigated differential transcripts accumulation of three *SAMS* genes in response to salt, ABA and wounding treatment in tomato. In our study, a low transcripts accumulation for *HvSAMS2* and 3, and no significant change for *HvSAMS1* and 4 were observed. This finding was in accordance with the previous work with tomato leaves where the *SAMS* genes expression was low under wounding stress (Esparteto et al. 1994). Transcript accumulations of *SAMS* genes in periwinkle were increased in response to salt stress at seedling stage (Schröder et al. 1997). However, the expression was merely detected in 5- or 6- leaf stage of tomato plants under the salt stress (Espartero et al. 1994). *HvSAMS* family genes might follow similar regulation process as seen in tomato leaves.

Exogenous application of spermidine induced up-regulated *ADC* transcripts in mustard leaves (Mo and Pua 2002). However, *HvSAMS* genes were not significantly stimulated by exogenous application of spermidine. In kiwifruit, the *SAMS* genes and ACC oxidase gene family were induced by the application of exogenous ethylene, but ACC synthetase gene was not affected by ethylene treatment (Whittaker et al. 1997). They also suggested that

the regulation of *SAMS* transcripts by ethylene could occur as the methionine salvage pathway. As shown in Fig. 3, *HvSAMS2* and 3 genes showed minimum level of transcripts, whereas *HvSAMS1* and 4 genes displayed stable expressions. It can be deduced that *HvSAMS* genes might be involved in some parts of the methionine salvage pathway at 4-week-old barley leaves.

SAMS isozymes from dwarf pea was stimulated in GA₃ treatment, and stimulatory response of GA₃ was nullified by ABA treatment (Mathur and Sachar 1991). GA₃ might control the induction of isozymes of *SAMS* in wheat aleurones (Mathur et al. 1992). *HvSAMS* transcripts were slightly elevated at the late observation time (24 - 48 h). Although ABA is a known antagonistic regulator of GA₃, we did not observe any inverse expression of *HvSAMS* genes. Interestingly, the *HvSAMS1*, 2 and 3 genes showed similar expression pattern in response to GA₃ and GA₃ - ABA treatment, but not in ABA.

Robertson (2003) reported that *HvSPY* was up-regulated in ABA treatment whereas the *HvSPY* expression was not affected by GA₃ when ABA was also present. He explained that *HvSPY* expression was influenced by *cis*-element (G box, GC rich element). It was observed that expression of *HvSAMS1*, 2 and 3 can be triggered in both GA₃ and ABA treatments through enhanced GA₃ signal. Hence we suggested that *HvSAMS1*, 2 and 3 were regulated by the GA₃-dependent signal pathway, although they might as well carry different regulatory elements as their responses to GA₃ treatment varied. *HvSAMS4* transcripts on the other hand were not regulated by GA₃ and ABA. This finding suggested that the *HvSAMS4* gene might be driven by another regulative pathway that is unique from the other *HvSAMS* genes.

HvSAMS family shared sequence identity that ranged from 89% to 99%. Expressions were tissue-specific, and were found similar throughout the grain development stage. However, the transcripts accumulations of *HvSAMS* gene family showed significant differences to each treatment. One possible explanation for the difference expression is that *HvSAMS* family can be regulated by other elements. We speculate that 5' untranslated regions (5'UTRs) of *HvSAMS* genes play key roles in the transcription regulation. *SAM decarboxylase* of plants was strongly suggested that

uORF regulated the translation mechanism (Franceschetti et al. 2001). Chung et al (2006) reported that an intron in the 5'UTR significantly enhanced the gene expression, and the length of intron also influenced the gene expression. Northern blot analysis of *HvSAMS1*, 2 and 3 showed two signals, including a major signal and an additional weak signal (Fig. 2A). The minor signal corresponded to the expression pattern of major signal, but this signal was not detected in *HvSAMS4*. *Waxy* gene led to an aberrant splicing in intron of 5'UTR from rice (Cai et al. 1998). Fujikake et al (2005) identified new alternative splicing transcription factors and detected their isoforms using RT-PCR. Possible hybridization of gene-specific probe with alternatively spliced transcripts in Northern blotting resulted in the presence of an additional band which contained 5'UTR intron as well as *HvSAMS* transcript. However, RT-PCR using 5' gene specific primer that was designed in compliance with upstream region of 5' UTR showed expected mature *HvSAMS* transcript.

In previous study, we reported *HvSAMS1* promoter expression (Kim et al. 2007). But according to present study, the reported promoter was not that of *HvSAMS1* upstream region but that of *HvSAMS2* upstream region. The promoters of *HvSAMS1*, 2, 3, and 4 contains two introns (Appendix Fig. 1). The first exon of *HvSAMS1*, 2, 3 were located immediately before the start codon which was exactly 23 bp, while that of *HvSAMS4* was 12 bp away from the start codon. Further study on the molecular characterization using promoters of *HvSAMS* genes is highly recommended for better understanding of the *HvSAMS* gene expression mechanisms.

As shown in Fig. 5, the *HvSAMS1* was expressed in nucleus and slightly in cytosol. Ravel et al (1998) suggested three possibilities in the integration of the methionine and SAM biosynthetic/recycling pathways in the plant cell. The first and second models proposed that SAMS or SAM synthesis occurred in the cytosol. Whereas, the third model proposed that methionine and SAM biosynthesis reacted autonomously, with methionine recycling and SAM metabolism occurred both in the cytosol. Results in this study might be in accordance with the third model. The *HvSAMS1* transgenic *Arabidopsis* plants initially exhibited faster growth than the wild-type plants. In

addition, the *HvSAMS1* transgenic *Arabidopsis* plants responded vigorously to high GA₃ concentration. These results suggested that overexpression of *HvSAMS1* might play roles in the germination and in GA₃ response mechanism.

In order to elucidate the molecular mechanisms inherent to grain development in the extra early maturing barley, we identified early maturity grain enriched genes, *HvSAMS* family, and characterized their extent of regulation by several phytohormones and stresses. However, further studies on the molecular characterization of *HvSAMS* genes together with other plant *SAMS* genes are still needed to better understand their roles during grain development.

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Appendix Table 1. Differentially expressed clones in grain of K800 (GSHO 2504, *eam10*)

Clone	Putative Identification	origin	E-value	Length (A.A)
EA3	Methionine synthase enzyme 1	<i>Hordeum vulgare subsp. Vulgare</i>	1e-65	765
EA4	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-82	679
EA5	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	4e-67	392
EA6	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	7e-43	389
EA13	S-adenosylmethionine synthetase	<i>Populus nigra</i>	4e-43	95
EA16	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-66	525
EA18	H2A2_Wheat	<i>Triticum aestivum</i>	3e-22	151
EA19	Vacuolar proton-inorganic pyrophosphatase	<i>Hordeum vulgare subsp. Vulgare</i>	4e-65	771
EA24	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	8e-74	171
EA30	Putative syntaxin-related protein	<i>Triticum aestivum</i>	5e-83	302
EA31	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-46	422
EA32	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-54	306
EA33	Cytosolic Cu/Zn superoxide dismutase	<i>Triticum aestivum</i>	9e-49	152
EA34	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	6e-07	182
EA35	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-85	324
EA36	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-94	948
EA41	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	9e-98	507
EA42	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-96	270
EA43	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-80	333
EA44	S-adenosylmethionine decarboxylase	<i>Triticum monococcum</i>	0.034	388
EA45	70 kDa heat shock protein	<i>Sandersonia aurantiaca</i>	2e-67	336
EA46	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-90	507
EA47	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	4e-82	128
EB5	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-101	509
EB7	Hypothetical protein ZAEMMB73_626728	<i>Zea mays</i>	1e-07	280
EB25	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	4e-04	963
EF49	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-111	581
EB97	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-20	257
EF51	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	1e-94	272
EB100	OSIGBa0096P03.8	<i>Oryza sativa indica Group</i>	2e-30	381
EF52	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	7e-112	563
EC13	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-54	293
EF54	Os02g0615800	<i>Oryza sativa Japonica Group</i>	1e-71	1001
EB43	Non-specific lipid-transfer protein 3	<i>Hordeum vulgare</i>	2e-06	118
ED4	RuBisCO large subunit-binding protein beta	<i>Secale cereal</i>	6e-50	499

Appendix Table 1. Continued.

Clone	Putative Identification	origin	E-value	Length (A.A)
ED14	Vacuolar processing enzyme 2d	<i>Hordeum vulgare subsp. Vulgare</i>	4e-91	493
EB58	Hypothetical protein Osl_10238	<i>Oryza sativa indica Group</i>	2e-14	156
EE65	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	1e-57	129
EB61	Elongation factor 1-alpha	<i>Triticum aestivum</i>	5e-36	447
EE99	Lipoxygenase 2	<i>Hordeum vulgare subsp. Vulgare</i>	4e-58	864
EF67	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	1e-88	436
EE100	Chain K	<i>Triticum aestivum</i>	3e-24	206
EF68	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	5e-67	694
EF69	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-54	226
EB76	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	4e-31	447
EF3	Probable galaturonosyltransferase-like 7-like	<i>Brachypodium distachyon</i>	3e-79	367
EF72	Hypothetical protein OsJ_18634	<i>Oryza sativa Japonica Group</i>	1e-82	586
EB77	QM	<i>Triticum aestivum</i>	2e-08	178
EF82	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	1e-146	388
EF29	Gene X-like protein	<i>Brachypodium distachyon</i>	2e-26	813
EF84	Putative carbonic anhydrase	<i>Secale cereale x Triticum durum</i>	2e-117	259
EB82	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-11	487
EF33	Papin-like cysteine proteinase	<i>Hordeum vulgare subsp. Vulgare</i>	8e-82	381
EF88	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	1e-86	219
EB92	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	2e-48	538
EF34	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	1e-98	401
EF89	Root abundant factor	<i>Hordeum vulgare</i>	7e-50	328
EF43	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-135	376
EF98	Uncharacterized protein LOC100836267	<i>Brachypodium distachyon</i>	6e-84	1305
EF44	Predicted protein	<i>Hordeum vulgare subsp. Vulgare</i>	3e-64	253
EF99	Uncharacterized membrane protein At1g16860-like	<i>Brachypodium distachyon</i>	4e-84	423

Appendix Table 2. Analysis of 5' untranslated regions of *HvSAMS1*, 2, 3, and 4. The PlantCare was used for each *cis*-elements

Gene	cDNA		genomic DNA		conserved sequence	function
	<i>cis</i> -element	location	<i>cis</i> -element	location		
<i>HvSAMS1</i>	AAGAA-motif	(+)78	AAGAA-motif	(-)601 (+)1825	GAAAGAA	
	CAAT-box	(+)37	CAAT-box	(-)1553 (-)920 (-)1718 (+)1176 (-)1562	CAAT	common <i>cis</i> -acting element in promoter and enhancer regions
	EIRE	(+)5	EIRE	(+)959	TTCGACC	elicitor-responsive element
	LTR	(-)14	LTR	(-)968	CCGAAA	<i>cis</i> -acting element involved in low-temperature responsiveness
	MBS	(+)51	MBS	(-)480 (+)1005	CAACTG	MYB binding site involved in drought-inducibility
	SARE	(+)5	SARE	(+)959	TTCGACCTCCTT	<i>cis</i> -acting element involved in salicylic acid responsiveness
	circadian	(+)37			CAANNNNATC	<i>cis</i> -acting regulatory element involved in circadian control
			A-box	(-)515	CCGTCC	<i>cis</i> -acting regulatory element
			C-box	(+)1367	CTGACGTCAG	<i>cis</i> -acting regulatory element involved in light responsiveness
			G-box	(+)570 (+)891 (+)675 (+)1756	CACGTT	<i>cis</i> -acting regulatory element involved in light responsiveness
			TCA-element	(+)453	CCATCTTTTT	<i>cis</i> -acting element involved in salicylic acid responsiveness
			TGACG-motif	(+)898 (+)1519 (+)1368	TGACG	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
			CGTCA-motif	(+)1179 (+)1472	CGTCA	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
<i>HvSAMS2</i>			G-Box	(-)928	CACGTA	<i>cis</i> -acting regulatory element involved in light responsiveness
	CAAT-box	(+)9	CAAT-box	(+)831 (-)1585 (+)469 (+)1064 (-)589 (+)1494 (-)758 (-)289	CAAT	common <i>cis</i> -acting element in promoter and enhancer regions

Appendix Table 2. Continued.

Gene	cDNA		genomic DNA		conserved sequence	function
	<i>cis</i> -element	location	<i>cis</i> -element	location		
<i>HvSAMS2</i>	GAG-motif	(-)37			AGAGATG	light responsive element
	MBS	(+)23	MBS	(+)845	CAACTG	MYB binding site involved in drought-inducibility
	circadian	(+)9	circadian	(-)583 (+)831	CAANNNNATC	<i>cis</i> -acting regulatory element involved in circadian control
			A-box	(-)1311 (-)1626 (-)1562	CCGTCC	<i>cis</i> -acting regulatory element
			ABRE	(+)928	TACGTG	<i>cis</i> -acting element involved in the abscisic acid responsiveness
			GCN4_motif	(-)628	TGAGTCA	<i>cis</i> -regulatory element involved in endosperm expression
			LTR	(-)808	CCGAAA	<i>cis</i> -acting element involved in low-temperature responsiveness
			Skn-1_motif	(+)448 (-)461	GTCAT	<i>cis</i> -acting regulatory element required for endosperm expression
			TGA-element	(-)1300	AACGAC	auxin-responsive element
			TGACG-motif	(-)1179 (-)1472	TGACG	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
<i>HvSAMS3</i>	CAAT-box	(+)29	CAAT-box	(+)1594 (-)1894 (+)781 (+)1147 (-)453	CAAT	common <i>cis</i> -acting element in promoter and enhancer regions
	circadian	(+)43			CAANNNNATC	<i>cis</i> -acting regulatory element involved in circadian control
			ABRE	(+)876 (-)1139	TACGTG	<i>cis</i> -acting element involved in the abscisic acid responsiveness
			AuxRR-core	(+)1802	GGTCCAT	<i>cis</i> -acting regulatory element involved in auxin responsiveness
			CGTCA-motif	(+)854	CGTCA	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
			G-box	(+)678 (-)1430 (+)1141 (+)1868 (+)876 (-)1491	CACGTA	<i>cis</i> -acting regulatory element involved in light responsiveness
			MBS	(-)982	CGGTCA	MYB Binding Site

Appendix Table 2. Continued.

Gene	cDNA		genomic DNA		conserved sequence	function
	<i>cis</i> -element	location	<i>cis</i> -element	location		
<i>HvSAMS3</i>			Skn-1_motif	(+)855 (+)1857	GTCAT	<i>cis</i> -acting regulatory element required for endosperm expression
			TCA-element	(+)549	GAGAAGAATA	<i>cis</i> -acting element involved in salicylic acid responsiveness
			TGACG-motif	(-)854	TGACG	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
<i>HvSAMS4</i>	CGTCA-motif	(+)23	CGTCA-motif	(-)316	CGTCA	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
	Skn-1-motif	(+)24	Skn-1-motif	(-)1364	GTCAT	<i>cis</i> -acting regulatory element required for endosperm expression
	circadian	(+)20	circadian	(-)1054	CAANNNNATC	<i>cis</i> -acting regulatory element involved in circadian control
			ABRE	(-)220 (-)305	TACGTG	<i>cis</i> -acting element involved in the abscisic acid responsiveness
			AuxRR-core	(+)951	GGTCCAT	<i>cis</i> -acting regulatory element involved in auxin responsiveness
			CAAT-box	(-)216 (+)981 (-)987 (-)1288 (+)1350	CAAT	common <i>cis</i> -acting element in promoter and enhancer regions
			ERE	(+)1465	ATTTCAAA	ethylene-responsive element
			G-Box	(+)220 (+)1538 (-)270	CACGTA	<i>cis</i> -acting regulatory element involved in light responsiveness
			GCN4_motif	(+)96	TGAGTCA	<i>cis</i> -regulatory element involved in endosperm expression
			LTR	(+)157	CCGAAA	<i>cis</i> -acting element involved in low-temperature responsiveness
			MBS	(+)1384	CAACTG	MYB binding site involved in drought-inducibility
			TCA-element	(+)1162 (-)1500	GAGAAGAATA	<i>cis</i> -acting element involved in salicylic acid responsiveness
			TGA-element	(+)297	AACGAC	auxin-responsive element
			TGACG-motif	(+)316	TGACG	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness