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 μM 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 GAs. 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 HvSAMS 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
Cloning and Expression Analysis of HvSAMS genes in Barley (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 aminepropyl 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 μM 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 |
|--------|-----------------------------------------------|-----------|---------|
| *HvSAMS1* | 5’-CGGCCCTCCTGAAACAATAGCAGTCAGCAG-3’          | sense     | C       |
|        | 5’-GGATTTGTTGAAGAGTAGGTGGCAGCAGAC-3’          | antisense |         |
|        | 5’-GATTCCCGCTTCTTTCCGGTTG-3’ C               | sense     | R       |
|        | 5’-CAGCACAATCCAAAAAGAAAAATGAA-3’             | antisense |         |
|        | 5’-CCACGAAAGAAATGGCAGG-3’ G                 | sense     | G       |
|        | 5’-CTTTCTCTTCAATGCTGCTGTCG-3’                | antisense | P       |
|        | 5’-TAGTGCCTTTGGATGCTGTCCTACG-3’             | antisense |         |
| *HvSAMS2* | 5’-GAGAGCATCCTCTAACCACCAAAG-3’               | sense     | C       |
|        | 5’-CCCTGCGCAACTTGAGGCACT-3’ C               | antisense |         |
|        | 5’-CGACCTCTTCTCTTTCCGGTTTTC-3’              | sense     | R       |
|        | 5’-GAGGAGCAAAAGGATCCCGCCT-3’                | antisense |         |
|        | 5’-AAGAGAACGTGGCGGGACAGCCT-3’ C             | sense     | G       |
|        | 5’-CTTTGTTCTTAAGCAGATGCGCTTG-3’             | antisense | P       |
|        | 5’-CTCTTTCAACTGCTTCAATGGCAGCAGAAG-3’        | antisense |         |
|        | 5’-GTTTTGCTACTATCTCTTCAACCGGGCCT-3’         | antisense |         |
| *HvSAMS3* | 5’-GGAGCAATAGAAGCCGAGCACAAG-3’               | sense     | C       |
|        | 5’-CCGCCTCCTGAGAAGCTCTGGA-3’                | antisense |         |
|        | 5’-CGGCAGGGTCGCTCCTCTC-3’ C                | sense     | R       |
|        | 5’-CAGTGTAGCAGTGTCAGCGCAGG-3’               | antisense |         |
|        | 5’-GAAGAATGCGGGGCAGAAGGAC-3’ C             | sense     | G       |
|        | 5’-CTTTGTTCTTAAGCAGATGCGCTTG-3’             | antisense |         |
|        | 5’-GTCGAGCGAAAGACTCTCTACTCCCTCACTGAAAGCACA-3’ | antisense |         |
|        | 5’-CTGAGACTTAAACTCTGGTGGGCGAGTGA-3’         | antisense |         |
| *HvSAMS4* | 5’-ATCGTGCGGTCCGTCCGATCGGCC-3’               | sense     | C       |
|        | 5’-TAACACTATTTATTCAAGACCGTCT-3’             | antisense |         |
|        | 5’-CGAGGAGCGGCGGCTTTGATGGG-3’               | sense     | R       |
|        | 5’-CAAGCAGCGAATGCGTAAGAG-3’ C              | sense     | G       |
|        | 5’-GGCCAAAGAGATGGGCTGAGTTG-3’              | antisense |         |
|        | 5’-GTGTTTGGTCTAGCAGGCAAGGCTCTC-3’          | antisense |         |
|        | 5’-GGGAGTGGTGAATACTTAGGCTCAGT-3’ GC        | antisense |         |
|        | 5’-GGCCGAATTAGGATGACTCAGATTTTGCTAG-3’       | antisense |         |

C indicates cDNA isolation, R indicates RT-PCR, G indicates genomic DNA isolation, and P indicates promoter isolation.
Cloning and Expression Analysis of HvSAMS genes in Barley

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 HvSAMS 1 coding sequence was amplified by PCR and purified with the QIAquik 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 HvSAMS 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 GACACTGTACTTCTT-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 HvSAMS I, 2, 3, and 4 after digestion with seven restriction enzymes forming blunt end (DraI, EcoRV, PvuII, StuI, BbrPI, Smal, 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’-TCTAGAATGGCGGCCGAGAC GTTCCT-3’ containing XbaI (forward primer) and 5’-GGATCCGGCAGATGCCTTGTCGAACTTG-3’ containing BamHI (reverse primer). The 35S::smGFP::HvSAMS1 plasmid was constructed by subcloning into the 326 GFP vector (Lee et al. 2001) after XbaI and BamHI 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 EcoRI and HindIII.

**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 μm 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 μg/ 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 (T₁) were transplanted to a soil mixture (loam soil/perlite/vermiculite, 3/1/1), and T₂ seeds were harvested. The T₂ seeds were further advanced to T₃ 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 361̅th 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

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

| Gene     | Length (bp) | cDNA Identity (%) | Mature Protein |
|----------|-------------|-------------------|----------------|
|          |             | *HvSAMS₁* *HvSAMS₂* *HvSAMS₃* | *Identity (%)* |
| HvSAMS₁  | 1185        | 95                | 394            |
| HvSAMS₂  | 1185        | 92                | 394            |
| HvSAMS₃  | 1185        | 80                | 394            |
| HvSAMS₄  | 1191        | 80                | 396            |
further intensified at 3 DAF, and subsequently decreased until 30 DAF. *HvSAMS* 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 μg 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 32P 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 µM each of ABA, GA3, spermidine, ethephon and 100 mM NaCl; C, non-treated; M, mock treatment (24 hrs).](image-url)
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 (T2); iv, v, vi were 2-week-old transgenic plants (T2); vii, viii, ix were 3-week-old transgenic plants (T2); 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 *pBl101::HvSAMS(-1459), pBl101::HvSAMS(-301), and pBl101::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 *pBl101::HvSAMS* - and *pBl101::HvSAMS* - containing transgenic plants which showed expression in the stem. The GUS expression in *pBl101::HvSAMS* transgenic plants lasted until 3 weeks (Fig. 4B).

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**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 μM 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 1μM 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 Polymorphysm) 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 SAMSs 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 (HvAMS1, 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 HvAMS1 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 Zeevaart 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 (Espinat 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 (Espinat 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. Ravanel 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.

ACKNOWLEDGEMENT

This work was supported by a grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center No. PJ008031012013), Rural Development Administration, Republic of Korea. Jae Yoon Kim was supported by a Korea University Grant.

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

| Clone | Putative Identification | origin | E-value | Length (A.A) |
|-------|-------------------------|--------|---------|--------------|
| ED14  | Vacuolar processing enzyme 2d | *Hordeum vulare subsp. Vulgare* | 4e-91  | 493          |
| EB58  | Hypothetical protein OsL_10238 | *Oryza sativa indica Group* | 2e-14  | 156          |
| EE65  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 1e-57  | 129          |
| EB61  | Elongation factor 1-alpha | *Triticum aestivum* | 5e-36  | 447          |
| EE99  | Lipoxygenase 2            | *Hordeum vulare subsp. Vulgare* | 4e-58  | 864          |
| EF67  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 1e-88  | 436          |
| EE100 | Chain K                  | *Triticum aestivum* | 3e-24  | 206          |
| EF68  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 5e-67  | 694          |
| EF69  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 3e-54  | 226          |
| EB76  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 4e-31  | 447          |
| EF3   | Probable galacturonosyltransferase-like 7-like | *Brachypodium distachyon* | 3e-79  | 367          |
| EF72  | Hypothetical protein OsJ_18634 | *Oryza sativa Japonica Group* | 1e-82  | 586          |
| EB77  | QM                       | *Triticum aestivum* | 2e-08  | 178          |
| EF82  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 1e-146 | 388          |
| EF29  | Gene X-like protein       | *Brachypodium distachyon* | 2e-26  | 813          |
| EF84  | Putative carbonic anhydrase | *Secale cereale x Triticum durum* | 2e-117 | 259          |
| EB82  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 2e-11  | 487          |
| EF33  | Papin-like cysteine proteinase | *Hordeum vulare subsp. Vulgare* | 8e-82  | 381          |
| EF88  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 1e-86  | 219          |
| EB92  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 2e-48  | 538          |
| EF34  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 1e-98  | 401          |
| EF89  | Root abundant factor      | *Hordeum vulgare* | 7e-50  | 328          |
| EF43  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 3e-135 | 376          |
| EF98  | Uncharacterized protein LOC100836267 | *Brachypodium distachyon* | 6e-84  | 1305         |
| EF44  | Predicted protein         | *Hordeum vulare subsp. Vulgare* | 3e-64  | 253          |
| EF99  | Uncharacterized membrane protein At1g16860-like | *Brachypodium distachyon* | 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 cis-element location | genomic DNA cis-element location | conserved sequence | function                                                                 |
|--------|---------------------------|---------------------------------|-------------------|--------------------------------------------------------------------------|
| **HvSAMS1** |
| AAGAA-motif | (+)78  | AAGAA-motif (-)601 (+)1825  | GAAAGAA  | common cis-acting element in promoter and enhancer regions |
| CAAT-box | (+)37  | CAAT-box (-)1553 (-)920 (-)1718 (+)1176 (-)1562  | CAAT  | |
| EIRE  | (+)5  | EIRE (+)959  | TTCGACC  | elicitor-responsive element |
| LTR   | (-)14  | LTR (-)968  | CCGAAA  | cis-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  | cis-acting element involved in salicylic acid responsiveness |
| circadian | (+)37  | CAANNNNATC  | |
| A-box | (-)515 | CCGTCC  | cis-acting regulatory element involved in circadian control |
| C-box | (+)1367  | CTGACGTCAG  | |
| G-box | (+)570 (+)675 (+)1756  | CACGTT  | cis-acting regulatory element involved in light responsiveness |
| TCA-element | (+)453  | CCATCTTTTT  | cis-acting element involved in salicylic acid responsiveness |
| TGACG-motif | (+)898 (+)1519 (+)1368  | TGACG  | cis-acting regulatory element involved in the MeJA-responsiveness |
| CGTCA-motif | (+)1179 (+)1472  | CGTCA  | cis-acting regulatory element involved in the MeJA-responsiveness |
| G-Box | (-)928  | CACGTA  | cis-acting regulatory element involved in light responsiveness |
| **HvSAMS2** |
| CAAT-box | (+)9  | CAAT-box (+)831 (-)1585 (+)469 (+)1064 (-)589 (+)1494 (-)758 (-)289  | CAAT  | common cis-acting element in promoter and enhancer regions |
| Gene     | cDNA conserved sequence | genomic DNA conserved sequence | cis-element function |
|----------|-------------------------|-------------------------------|----------------------|
| GAG-motif | AGAGATG                | light responsive element      |
| MBS      | (+)845                 | CAACTG                       | MYB binding site involved in drought-inducibility |
| circadian | (-)583 (+)831         | CAAANNNATC                   | cis-acting regulatory element involved in circadian control |
| A-box    | (-)1311 (-)1626 (-)1562 | CCGTCC                       | cis-acting regulatory element |
| ABRE     | (+)928                 | TACGTG                       | cis-acting element involved in the abscisic acid responsiveness |
| GCN4_motif | (-)628               | TGAGTCA                      | cis-regulatory element involved in endosperm expression |
| LTR      | (-)808                 | CCGAAA                       | cis-acting element involved in low-temperature responsiveness |
| Skn-1_motif | (+)448 (-)461      | GTCAT                        | cis-acting regulatory element required for endosperm expression |
| TGA-element | (-)1300         | ACGAC                         | auxin-responsive element |
| TGACG-motif | (-)1179 (+)1472   | TGACG                         | cis-acting regulatory element involved in the MeJA-responsiveness |
| CAAT-box (+)29 | CAAT               | common cis-acting element in promoter and enhancer regions |
| circadian (+)43 | CAANNNNATC | cis-acting regulatory element involved in circadian control |
| ABRE     | (+)876 (-)1139        | TACGTG                       | cis-acting element involved in the abscisic acid responsiveness |
| AuxRR-core | (+)1802             | GGTCCAT                      | cis-acting regulatory element involved in auxin responsiveness |
| CGTCA-motif | (+)854              | CGTCA                         | cis-acting regulatory element involved in the MeJA-responsiveness |
| G-box    | (+)678 (+)1141 (+)1868 (+)876 (+)1491 | CACGTA                       | cis-acting regulatory element involved in light responsiveness |
| MBS      | (-)982                | CGGTCA                       | MYB Binding Site     |
| Gene     | cDNA    | genomic DNA | conserved sequence | function                                           |
|----------|---------|-------------|--------------------|----------------------------------------------------|
|          | cis-element | cis-element | location           | function                                           |
|          | location | location    |                    |                                                    |
| HV4     | Skn-1_motif | (+)855 | GTCAT | cis-acting regulatory element required for endosperm expression |
|         | (+)1857 |             |                    |                                                    |
| HV4     | TCA-element | (+)549 | GAGAGAATA | cis-acting element involved in salicylic acid responsiveness |
|         |             | (-)854 | TGACG | cis-acting regulatory element involved in the MeJA-responsiveness |
| HV4     | CGTCA-motif | (+)23 | CGTCA | cis-acting regulatory element involved in the MeJA-responsiveness |
|         | (-)316 |             |                    |                                                    |
| HV4     | Skn-1-motif | (+)24 | GTCAT | cis-acting regulatory element required for endosperm expression |
|         | (-)1364 |             |                    |                                                    |
|         | circadian | (+)20 | CAANNNNATC | cis-acting regulatory element involved in circadian control |
|         | (-)1054 |             |                    |                                                    |
|         | ABRE | (-)220 | TACGTG | cis-acting element involved in the abscisic acid responsiveness |
|         | (-)305 |             |                    |                                                    |
|         | AuxRR-core | (+)951 | GGTCCAT | cis-acting regulatory element involved in auxin responsiveness |
|         | CAAT-box | (-)216 | CAAT | common cis-acting element in promoter and enhancer regions |
|         | (-)987 |             |                    |                                                    |
|         |         | (-)1288 |             |                                                    |
|         |         | (+)1350 |             |                                                    |
| HV4     | ERE | (+)1465 | ATTTCAA | ethylene-responsive element |
|         | G-Box | (+)220 | CACGTA | cis-acting regulatory element involved in light responsiveness |
|         | (+)1538 |             |                    |                                                    |
|         |         | (-)270 |             |                                                    |
|         | GCN4_motif | (+)96 | TGAGTCA | cis-regulatory element involved in endosperm expression |
|         | LTR | (+)157 | CCGAAA | cis-acting element involved in low-temperature responsiveness |
|         | MBS | (+)1384 | CCACTG | MYB binding site involved in drought-inducibility |
|         | TCA-element | (+)1162 | GAGAGAATA | cis-acting element involved in salicylic acid responsiveness |
|         | (+)1500 |             |                    |                                                    |
|         | TGA-element | (+)297 | AACGAC | auxin-responsive element |
|         | TGACG-motif | (+)316 | TGACG | cis-acting regulatory element involved in the MeJA-responsiveness |
|         |             | (-)854 | TGACG | cis-acting regulatory element involved in the MeJA-responsiveness |
Appendix Fig. 1. Schematic representation of the 5’ UTRs of the HvSAMS1, 2, 3, and 4. Solid bars represent exons (E1 and E2), whereas introns were indicated by the straight/“Λ” lines. The nucleotide of the ATG translation initiation codon was assigned as position 1 in the nucleotide sequence, and the nucleotide positions upstream of position 1 were presented as negative numbers.

Appendix Fig. 2. PCR screening of transgenic HvSAMS lines. Lane 1-24 represents putative transgenic plants that survived on selection medium. M, molecular size marker; NC, negative control (wild-type); PC, HvSAMS1 overexpression plasmid DNA.