Analysis of a Sugar Response Mutant of Arabidopsis Identified a Novel B3 Domain Protein That Functions as an Active Transcriptional Repressor[1]

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A recessive mutation *hsi2* of Arabidopsis (*Arabidopsis thaliana*) expressing luciferase (LUC) under control of a short promoter derived from a sweet potato (*Ipomoea batatas*) sporamin gene (*Spo^{min}::LUC*) caused enhanced LUC expression under both low- and high-sugar conditions, which was not due to increased level of abscisic acid. The *hsi2* mutant contained a nonsense mutation in a gene encoding a protein with B3 DNA-binding domain. HSI2 and two other Arabidopsis proteins appear to constitute a novel subfamily of B3 domain proteins distinct from ABI3, FUS3, and LEC2, which are transcription activators involved in seed development. The C-terminal part of HSI2 subfamily proteins contained a sequence similar to the ERF-associated amphiphilic repression (EAR) motif. Deletion of the C-terminal portion of HSI2 lost in the *hsi2* mutant caused reduced nuclear targeting of HSI2. Null allele of *HSI2* showed even higher *Spo^{min}::LUC* expression than the *hsi2* mutant, whereas overexpression of HSI2 reduced the LUC expression. Transient coexpression of *35S::HSI2* with *Spo^{min}::LUC* in protoplasts repressed the expression of LUC activity, and deletion or mutation of the EAR motif significantly reduced the repression activity of HSI2. These results indicate that HSI2 and related proteins are B3 domain-EAR motif active transcription repressors.

In addition to transcriptional activators, transcriptional repressors play important roles in the regulation of transcription. Transcriptional repressors are basically classified into passive repressors and active repressors (Hanna-Rose and Hansen, 1996; Thiel et al., 2004). Passive repressors do not have intrinsic repressing activity and inhibit the activation of transcription by inhibiting the function of transcriptional activators through a competition with activators for binding with DNA or a formation of inactive heterodimers with activators. On the other hand, active repressors inhibit transcription in an activator-independent manner by binding with basic transcription factors or corepressors.

The expression of a number of plant genes is regulated by changes in sugar status via multiple signal transduction pathways (for review, see Koch, 1996; Smeekens, 2000; Rolland et al., 2002). Genetic analyses of sugar signaling mutants of Arabidopsis (*Arabidopsis thaliana*), obtained by genetic screen based on inhibition of germination and early seedling development by sugars, revealed a close link between sugar signaling and the production of ethylene and abscisic acid (ABA; for review, see Gazzarrini and McCourt, 2001; Rook and Bevan, 2003). In addition, direct screening based on sugar-responsive expression of endogenous genes or transgenes with reporters also resulted in identification of mutants with altered sugar-responsive gene expression (Dijkwel et al., 1997; Martin et al., 1997; Mita et al., 1997a, 1997b; Rook et al., 2001; Baier et al., 2004). In general, these mutants affected the expression of a subset of sugar-regulated genes, and mutation either diminished or enhanced the sugar-responsive expression of the target gene.

The recessive *hba1* mutation of Arabidopsis enhances the expression of a gene for the major β-amylase under conditions of low sugar (Mita et al., 1997a). Mutations in PRL1, a regulatory WD protein that interacts with Snf1-related kinase, cause derepression of Glc-responsive genes (Németh et al., 1998; Bhalerao et al., 1999). The *hsr* mutants exhibit elevated expression of several sugar-inducible genes under high-sugar conditions while keeping the expression low under noninducing low-sugar conditions (Baier et al., 2004). These results suggest that negative factors are involved in the sugar-regulated gene expression in plants. In yeast (*Saccharomyces cerevisiae*), a complex network of positive and negative regulators operates in the Glc-responsive regulation of gene expression,

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and transcriptional repressors such as Mig1 (Ostling et al., 1996) and Rgt1 (Ozcan and Johnston, 1995) play important roles in the regulation of expression of variety of genes in response to low or high levels of Glc (for review, see Ozcan and Johnston, 1999). However, transcriptional repressors involved in the expression of sugar-regulated genes in plants are not known at present.

Expression of a family of sporamin genes of sweet potato (*Ipomoea batatas*) is inducible by Suc, Glc, or other metabolizable sugars (Hattori et al., 1990; Nakamura et al., 1991). Dissection of the promoter region of *gSpoA1* using a β-glucuronidase (GUS) reporter in tobacco (*Nicotiana tabacum*) yielded a 210-bp sugar-inducible "minimal" promoter (Spo^{min}; Morikami et al., 2005). Expression of sporamin genes is also inducible by ABA (Ohto et al., 1992), and Spo^{min} directed sugar- and ABA-inducible expression of GUS and luciferase (LUC) reporters in Arabidopsis. A transgenic Arabidopsis line harboring the Spo^{min}::*GUS*-Spo^{min}::*LUC* dual reporter genes was used to screen mutants displaying altered patterns of Suc-inducible LUC and GUS expression, and a number of mutants that show higher levels of LUC and GUS expression under low-sugar conditions or lower levels of expression under high-sugar conditions compared to the wild type were isolated (A. Morikami, T. Saijo, M. Yamada, H. Tsukagoshi, T. Hattori, and K. Nakamura, unpublished data). The *hsi2* mutant is one of the mutants showing expression of LUC and GUS that are significantly higher than the wild-type plants under both low- and high-sugar conditions. In this

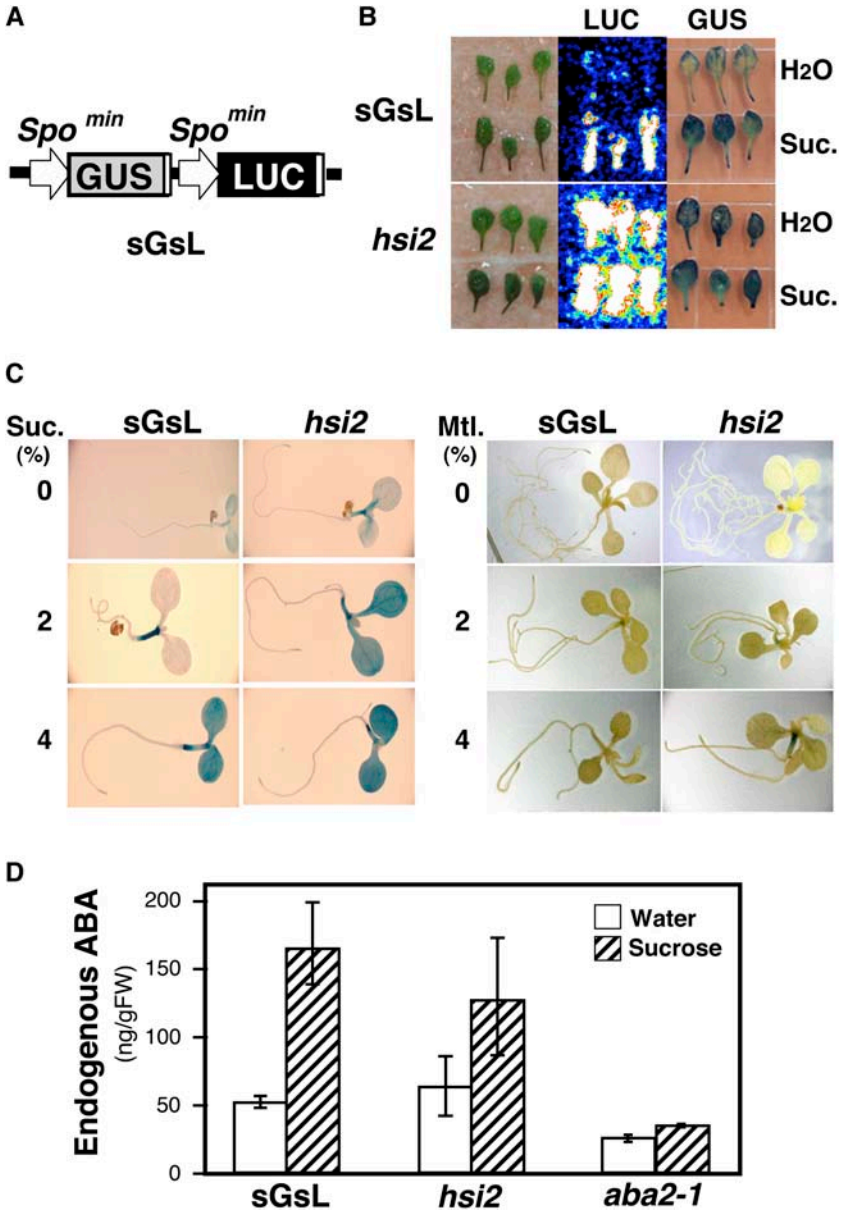

Figure 1. Arabidopsis *hsi2* mutant with high-level expression of Spo^{min}::*LUC*-Spo^{min}::*GUS* reporters. A, Spo^{min}::*LUC*-Spo^{min}::*GUS* dual reporter genes in the sGsL line. B, Luminescence image and GUS staining of leaves of the wild-type sGsL plants (top) and *hsi2* mutants (bottom) that were excised from 3-week-old plants and treated with water or 5% Suc for 48 h. C, Histochemical GUS staining of the sGsL and *hsi2* seedlings. Seedlings were grown for 7 and 10 d on Murashige and Skoog agar plates that contained 0%, 2%, or 4% Suc (Suc., left) and mannitol (Mtl., right), respectively, and stained for GUS activity with X-glucuronide. D, Endogenous level of ABA in leaves of the sGsL, *hsi2*, and *aba2-1* plants after treatment with water (white bars) or 6% Suc (hatched bars) for 48 h. Results shown are averages of two experiments, and the error bars represent ±SD. FW, Fresh weight.

article, we present data showing that HSI2 is a member of a novel family of B3 domain proteins with the sequence similar to ERF-associated amphiphilic repression (EAR) motif (Ohta et al., 2001) and that it functions as an active repressor of the *Spo*^{min} promoter through the EAR motif.

RESULTS

hsi2 Mutant Exhibits High-Level Expression of the *Spo*^{min} Promoter

A 210-bp "minimal" promoter derived from a gene for sweet potato sporamin A1 (*Spo*^{min}; Morikami et al., 2005) directed sugar- and ABA-inducible expression of the LUC and GUS reporter gene in leaves of Arabidopsis. Mutants displaying altered patterns of Suc-inducible expression of LUC were isolated from the transgenic line sGsL, which carries a single copy of T-DNA containing the *Spo*^{min}::*GUS-Spo*^{min}::*LUC* dual reporter genes (Fig. 1A) in the upper arm of chromosome 5. Among 29 *hsi*-type mutants showing enhanced expression of LUC under low-sugar conditions, the *hsi2* mutant displayed highest levels of LUC and GUS activities under both low- and high-sugar conditions. The levels of LUC and GUS activities in the leaves of *hsi2* plants grown for 3 weeks were higher than those in the wild-type sGsL plants. The treatment of excised leaves with 6% Suc caused an increase in LUC and GUS activities in both sGsL and *hsi2* plants (Fig. 1B). The LUC and GUS activities in *hsi2* plants were always higher than those in the sGsL line at various time points after the treatment and at various concentrations of Suc or ABA (data not shown).

In sGsL seedlings grown on medium containing 2% Suc, we observed strong GUS expression in the hypocotyls, which was not observed in seedlings grown without Suc (Fig. 1C). On medium containing 4% Suc, strong GUS staining extended to the cotyledons of the sGsL seedlings. By contrast, the *hsi2* seedlings grown without Suc already showed GUS expression in hypocotyls, and strong GUS expression extended to the cotyledons in the seedlings grown with 2% Suc. Unlike Suc or Glc (data not shown), mannitol did not induce expression of LUC and GUS activities in either sGsL or *hsi2* plants, except for weak expression in the hypocotyls of *hsi2* plants grown on 4% mannitol (Fig. 1C). These results suggest that the *hsi2* mutation caused an enhancement of the expression of the *Spo*^{min} promoter in response to Suc without affecting the spatial pattern of expression or the mode of regulation.

Given that the *Spo*^{min} promoter is induced by both sugars and ABA and that sugar enhances the endogenous level of ABA (Arenas-Huertero et al., 2000; Rook et al., 2001), the high-level expression of *Spo*^{min} in the *hsi2* mutant could be due to a high endogenous level of ABA or to altered starch metabolism. However, the level of ABA in leaves of the *hsi2* plants and its increase after treatment with Suc were similar to those in leaves of the sGsL plants (Fig. 1D). The levels of starch in leaves of the mutant plants were also similar to those in the sGsL plants (data not shown).

Genetic Analysis and Positional Cloning of the *HSI2* Gene

When the *hsi2* plants (Columbia [Col]-0 ecotype) were crossed with the wild-type Wassilewskija (Ws) ecotype, all of the F_1 plants showed LUC activities similar to the sGsL line. In the F_2 generations, 502 out of 2,074 kanamycin-resistant plants displayed high LUC activities in the leaves (χ^2 for a ratio of

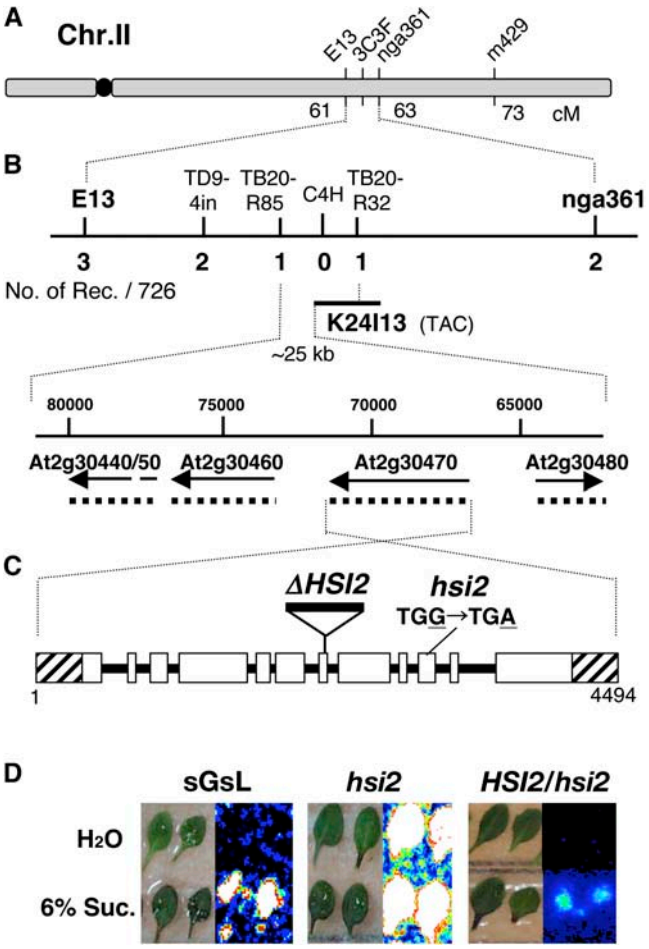

Figure 2. Positional cloning of the *HSI2* gene. A, Rough mapping of *hsi2* after crossing with the wild-type Ws ecotype. E13, 3C3F, nga361, and m429 are SSLP markers. B, Mapping of *hsi2* after crossing with the wild-type L*er* ecotype. The numbers of recombinants per 726 chromosomes at each CAPS or SSLP marker are indicated. Transformation of the *hsi2* mutant with the genomic fragment contained in a TAC clone K24I13 did not complement the mutation, which narrowed the location of *hsi2* mutation within a 25-kb region between the CAPS markers TB20-R85 and C4H. C, A genomic structure of At2g30470 and positions of the *hsi2* mutation and T-DNA insertion in the ΔHSI2 mutant. Hatched and white boxes indicate exons covering the untranslated and translated regions, respectively. D, Complementation of *hsi2* mutation by the At2g30470 gene. Luminescence images of leaves of the sGsL plants, *hsi2* mutant, and *hsi2* mutant transformed with the At2g30470 gene (*HSI2*) after treatment with water or 6% Suc.

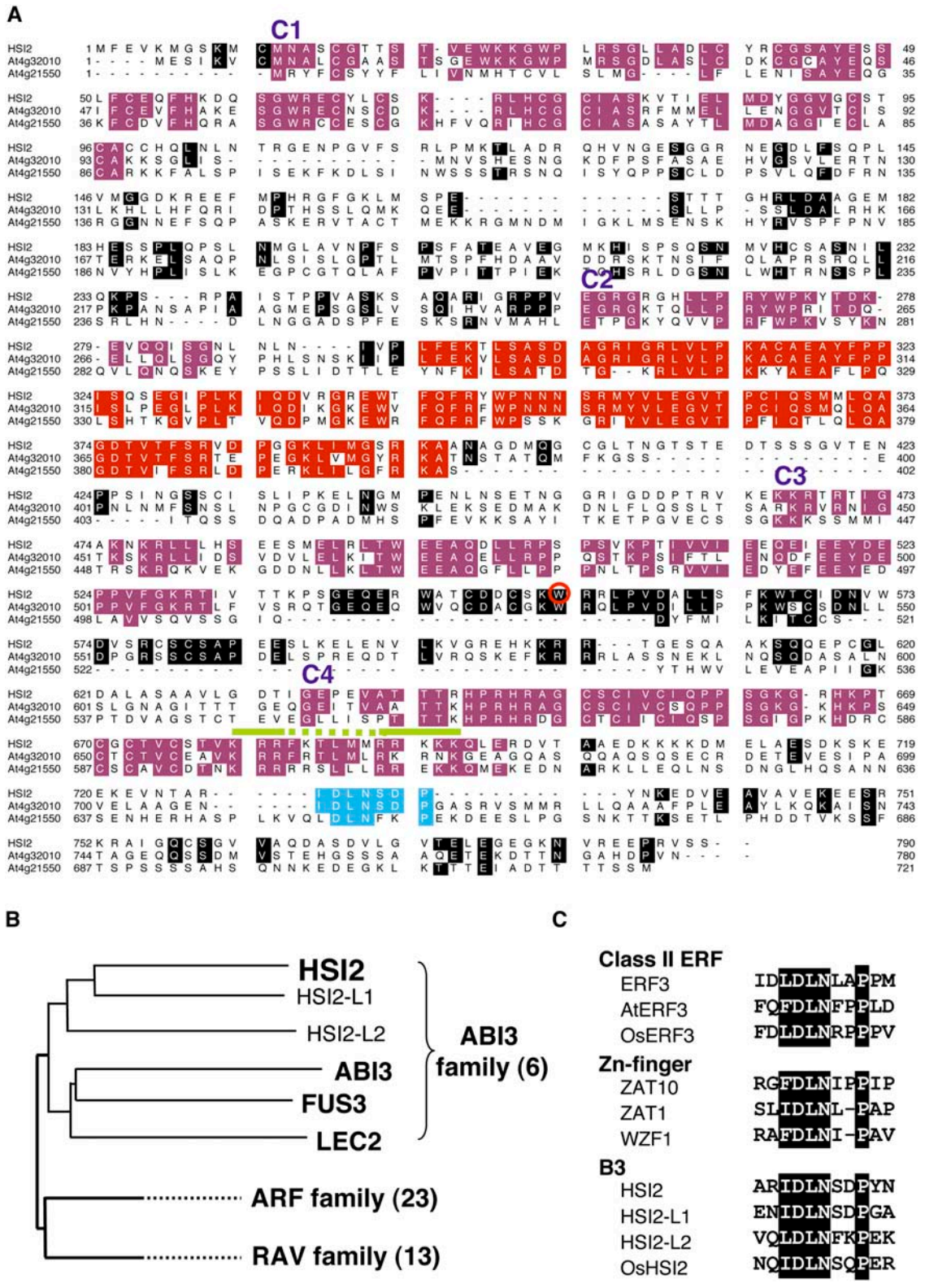

Figure 3. Amino acid sequence of HSI2 and related B3 domain-EAR motif proteins of Arabidopsis. A, Amino acid sequence of HSI2 and two other B3 domain-EAR motif proteins encoded by At4g32010 and At4g21550 were aligned using the Clustal alignment program. The B3 DNA-binding domain is indicated by red boxes, and four conserved regions (C1–C4) are indicated by pink boxes. The blue boxes indicate the EAR motif. A green line above the C terminus of the C4 region indicates the NLS-like sequence. The Trp-553 changed to a nonsense mutation in the *hsi2* mutant is indicated by a red circle. B, A phylogenetic tree of the 42 B3 domain proteins in Arabidopsis. They are classified into three families, represented by ABI3, ARF1, and RAV1. Within

3:1 = 0.639; $P > 0.05$), suggesting that *hsi2* is a single recessive mutation. Using simple sequence length polymorphism (SSLP) markers, *hsi2* was located near the *nga361* marker (63 cM) on the lower arm of chromosome 2, and further analysis with new cleaved-amplified polymorphic sequence (CAPS) markers located *hsi2* within 1 cM of the 3C3F marker (61.8 cM; Fig. 2A).

For further mapping, *hsi2* was crossed with the wild-type Landsberg erecta (L*er*), and DNAs were isolated from 363 kanamycin-resistant F_2 individuals showing high LUC activity in water-treated leaves. Mapping with CAPS and SSLP markers indicated that *hsi2* was located near the C4H marker and between the TB20-R85 and TB20-R32 markers (Fig. 2B). A genomic DNA carried on a transformation-competent bacterial artificial chromosome (TAC) clone, K24I13, covering the C4H and TB20-R32 loci was introduced into the *hsi2* mutant. However, transformed plants showed high LUC activities similar to the *hsi2* plants (data not shown). These results indicated that the *hsi2* mutation is located within a 25-kb region between the TB20-R85 and C4H markers.

According to the public database, the 25-kb region contained four putative protein-coding genes (Fig. 2B). Sequencing of genomic DNAs covering these putative genes from both the sGsL and *hsi2* plants identified one base substitution in the At2g30470 gene. This G-to-A substitution was present in the 10th exon, and it could result in nonsense mutation from the Trp codon (TGG) in sGsL to TGA in *hsi2* (Fig. 2C). When a genomic fragment of sGsL covering the At2g30470 gene was introduced into the *hsi2* plant, transformed plants showed low LUC activities in leaves treated with water or Suc (Fig. 2D). Based on these results, we concluded that the At2g30470 gene is the *HSI2* gene. Determination of the nucleotide sequence of a full-length cDNA clone for HSI2 (RAFL07-13-K13; Seki et al., 2002) verified the genomic structure of the *HSI2* gene (Fig. 2C).

HSI2 Gene Codes for a Novel B3 Domain Protein

The coding sequence of the *HSI2* gene was composed of 2,370 bp, and it could encode a polypeptide of 790 amino acids (Fig. 3A). The HSI2 protein contained a plant-specific B3 DNA-binding domain. The Arabidopsis genome contains 42 genes that could code for proteins with B3 DNA-binding domain. Based on the amino acid sequence of the encoded proteins, these genes could be classified into three families that are represented by *ABI3* (*abscisic acid-insensitive 3*; Giraudat et al., 1992), *ARF1* (*auxin responsible factor 1*; Ulmasov et al., 1997) and *RAV1* (*related to ABI3/VP1*; Kagaya et al., 1999). HSI2 belongs to the ABI3 family (Fig. 3B). Among the six genes in the ABI3 family, *HSI2* and two other predicted genes, At4g320110 and At4g215510, form a subfamily that is distinct from the other three members (ABI3, FUS3, and LEC2), which are transcriptional activators involved in embryogenesis and seed development (Baumlein et al., 1994; Luerßen et al., 1998; Nambara et al., 2000; Raz et al., 2001).

Other than the B3 domain, the HSI2 subfamily proteins do not share sequence similarities with ABA3, FUS3, and LEC2. However, in addition to the B3 domain, the three HSI2 subfamily proteins shared conserved sequences from C1 to C4 (Fig. 3A), which may have some functional significance. The C-terminal part of the C4 region contains a sequence similar to a bipartite nuclear localization signal (NLS; Liu et al., 1999). In addition to these, the C-terminal region of the three HSI2 subfamily proteins contains sequences similar to the EAR motif, which is involved in active repression of class II ERF transcriptional repressors and TFIIIA-type zinc-finger proteins ([L/F]DLN[L/F]xP; Ohta et al., 2001). The Trp-553 that was mutated in the *hsi2* mutant is located between the C3 and C4 regions.

Expression of HSI2 and Its Nuclear Localization

We examined the expression of mRNAs for HSI2 and two other related genes in various organs of Arabidopsis by semiquantitative reverse transcription (RT)-PCR. Actin 2 (ACT2) mRNA served as a control. The HSI2 mRNA was detected in roots, flowers, stems, and leaves, and the highest levels appeared to be in the flowers (Fig. 4A). The mRNA for HSI2-L1 (At4g32010) was also detected in all of these organs at similar levels. The level of mRNA for HSI2-L2 (At4g21550), on the other hand, was very low in organs other than the flowers.

The level of HSI2 mRNA in leaves showed a slight increase following a 24-h treatment with 6% Suc, whereas treatment with 50 μM ABA did not affect the level of HSI2 mRNA (Fig. 4B). The levels of mRNAs for HSI2-L1 and HSI2-L2 were not affected by treatment of plants with Suc or ABA (data not shown). The *hsi2* plants contained the mutant HSI2 mRNA at levels similar to the HSI2 mRNA in the sGsL plants.

To examine the cellular localization of HSI2, genes for translational fusions of synthetic green fluorescent protein (sGFP) at either the C (HSI2-GFP) or N terminus (GFP-HSI2) of HSI2 were constructed

Figure 3. (*Continued.*)
the ABI3 family, HSI2 and two other proteins form a subfamily distinct from the ABI3, FUS3, and LEC2 subfamilies. C, Comparison of the EAR motifs in class II ERFs, TFIIIA-type zinc-finger proteins, and HSI2-type B3 domain proteins. Sequences of ERF3 (T02433), AtERF3 (At1g50640), OsERF3 (AB036883), ZAT10 (At1g27730), ZAT1 (At1g02030), and WZF1 (S39045) are shown.

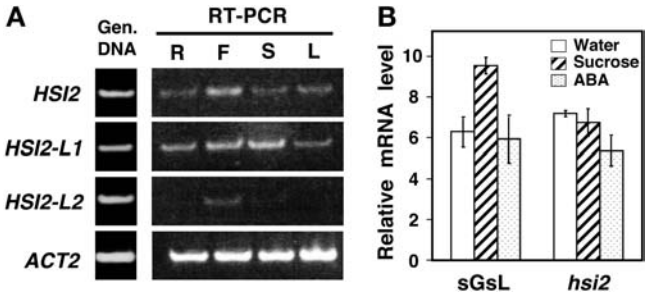

Figure 4. Expression of the *HSI2* gene. A, Detection of transcripts of *HSI2*, HSI2-L1 (At4g32010), and HSI2-L2 (At4g21550) in various organs. Semiquantitative RT-PCR was performed using RNA isolated from roots (R), flowers (F), stems (S), and leaves (L) from 35-d-old Col-0 plants. ACT2 served as a control. No bands were detected without the RT reaction. PCR using genomic DNA and the same primers used for RT-PCR was carried out to compare the efficiency of PCR. B, The levels of *HSI2* mRNA in seedlings treated with water, Suc, or ABA. Real-time RT-PCR was performed using RNA isolated from the 21-d-old seedlings of the sGsL line (left) or the *hsi2* mutant (right) after treatment with water, 6% Suc, or 50 μM ABA for 24 h. Data represents the mean value, and the error bars represent ±SD of *HSI2* mRNA levels relative to ACT2 mRNA.

downstream of the cauliflower mosaic virus (CaMV) 35S promoter. These fusion gene constructs were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment, and GFP expression was monitored by fluorescence microscopy. In contrast to cells expressing the control GFP, in which both cytoplasm and nucleus showed fluorescence, strong fluorescent signals were detected only in the nucleus of cells expressing HSI2-GFP and GFP-HSI2 (Fig. 5).

Because a nonsense *hsi2* mutation was located between the B3 DNA-binding domain and the C4 region containing the putative NLS (Fig. 3A) and because a mutant transcript of HSI2 was detected in the *hsi2* plants (Fig. 4B), it seemed likely that the *hsi2* plants produce a truncated HSI2 protein with a B3 domain that is not efficiently targeted to the nucleus. To examine this possibility, sGFP was fused to the C or N terminus of a truncated *hsi2* mutant form of HSI2 (HSI2[Δ553-790]). Unlike GFP fusions with the full length of HSI2, fluorescence from these fusion proteins was observed both in nucleus and cytoplasm (Fig. 5). The residual nuclear localization of GFP-HSI2[Δ553-790] could be due to remaining weak NLS or interaction of HSI2[Δ553-790] with other nuclear protein.

The HSI2 Null Mutant Exhibits a Higher Level of *Spo^{min}*::*LUC* Expression than the *hsi2* Mutant, and Overexpression of HSI2 Reduces *Spo^{min}*::*LUC* Expression

A T-DNA insertion line, K_24I13, from the Salk Institute (La Jolla, CA) contained a T-DNA inserted in the seventh exon of the HSI2 gene (Fig. 2C). This T-DNA insertion line was crossed with the sGsL line, and F_2 plants harboring the sGsL dual reporters and homozygous for disruption of the HSI2 gene were selected. Unlike the *hsi2* mutant, the HSI2 mRNA was not detected in the ΔHSI2 plants (Fig. 6A). The levels of LUC activities in the leaves of the ΔHSI2 null mutant were higher than those in the *hsi2* plants (Fig. 6B). These results suggest that *hsi2* is a leaky mutant of the negative regulator of *Spo^{min}*.

To determine whether overexpression of HSI2 causes negative effects on the expression of *Spo^{min}*, full-length HSI2 cDNA was placed downstream of the CaMV 35S promoter and used to transform the sGsL plants. The T_2 generation of the transformed plants expressed higher levels of HSI2 mRNA than the

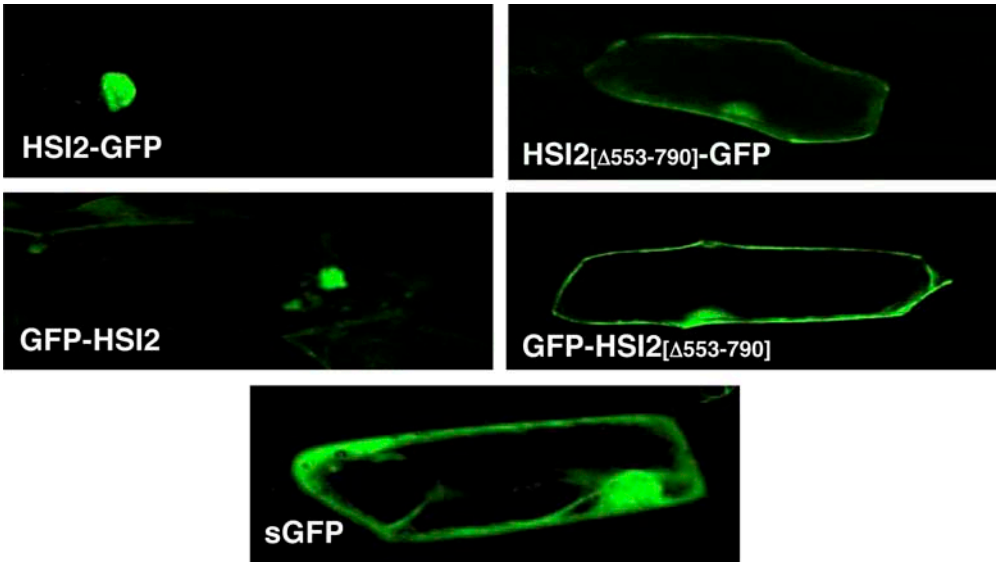

Figure 5. Nuclear localization of HSI2-GFP fusion proteins. The N- and C-terminal translational fusions of sGFP and HSI2 or the *hsi2*-type truncated form of HSI2 (HSI2[Δ553-790]) under the CaMV 35S promoter were introduced into onion epidermal cells, and expression of GFP fluorescence was observed by confocal microscopy. The expression of sGFP alone served as a control.

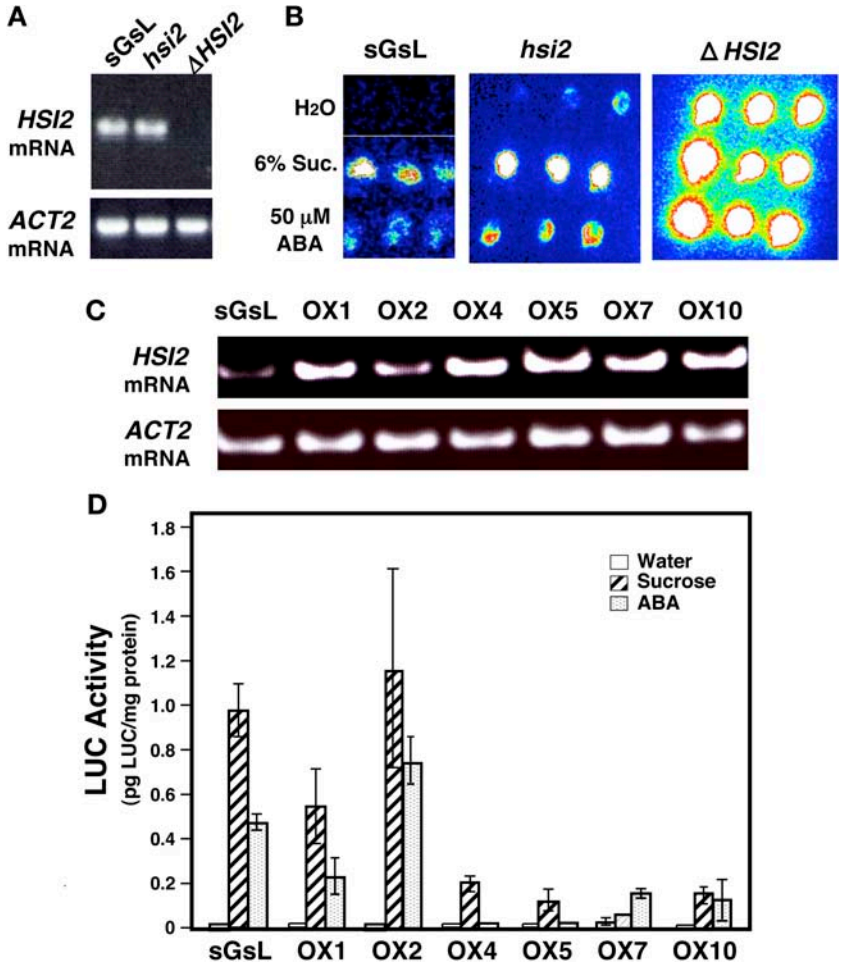

Figure 6. Expression of *Spo*^{min}::*LUC* in the *HSI2* null mutant (*ΔHSI2*) and in the overexpressor of HSI2. A, The levels of *HSI2* mRNA in the wild-type sGsL line, *hsi2* mutant, and *ΔHSI2* mutant detected by RT-PCR. B, Luminescence images of leaves of the sGsL line, *hsi2* mutant, and *ΔHSI2* mutant after treatment with water, 6% Suc, and 50 μM ABA. C, RT-PCR determination of the levels of *HSI2* mRNA in the wild-type sGsL line and six independent lines of sGsL transformed with *35S*::*HSI2*. D, LUC activities in leaves of the sGsL line and six independent lines of HSI2 overexpressors after treatment with water, 6% Suc, or 50 μM ABA. Results represent the means, and error bars represent the ±SD.

sGsL plant (Fig. 6C). In the T_2 generation, 14 out of 20 independent transformants (e.g. OX4, OX5, OX7, and OX10) had LUC activities in the leaves that were less than 20% of the levels in the sGsL plants (Fig. 6D). Some of the transformants (e.g. OX1 and OX2) had LUC activities that were similar to those in the sGsL plants, despite an increased level of HSI2 mRNA.

Transient Expression of *Spo*^{min}::*LUC* in Protoplasts Is Repressed by Coexpression of HSI2

To further characterize HSI2 as a negative regulator of *Spo*^{min}, we conducted transient coexpression of the *Spo*^{min}::*LUC* reporter and the *35S*::*HSI2* effector in protoplasts derived from suspension-cultured Arabidopsis cells. The *35S*::*GUS* plasmid DNA was also added to each assay, and LUC activity was normalized according to the GUS activity. The expression of LUC activity from the *Spo*^{min}::*LUC* reporter was strongly repressed when the HSI2 effector was coexpressed, and the activity was reduced to less than 20% of the level with the empty vector (Fig. 7B). In these assays, the level of GUS activity did not vary significantly, and the expression of the *35S*::*LUC* reporter was not affected by coexpression of *35S*::*HSI2* (Fig. 7B). These results suggest that repression of LUC reporter expression by coexpression of HSI2 is promoter dependent.

Compared to the 80% reduction of the level of expression of *Spo*^{min}::*LUC* by HSI2, only about 25% reduction of the expression of *Spo*^{min}::*LUC* was observed when C-terminally truncated *hsi2* effector was coexpressed instead of HSI2 (Fig. 7, A and B). Coexpression of the ΔEAR or mEAR effector, in which the EAR motif-like sequence (IDLNSDP) in the C-terminal region of HSI2 was either deleted or mutated to IAANADP (Fig. 7A), respectively, also only weakly repressed the LUC activity (Fig. 7B). These results indicate that the EAR motif is important for the transrepression activity of HSI2. When the activation domain of VP16 was fused to the N terminus of ΔEAR and mEAR effectors, these VP16-ΔEAR and VP16-mEAR caused more than 2-fold transactivation of *Spo*^{min}::*LUC* (Fig. 7B). These results suggest that loss of transrepression activity in the ΔEAR and mEAR effectors is not due to loss of DNA-binding activity or rapid degradation of these modified effectors. In contrast to ΔEAR and mEAR effectors, HSI2 showed strong transrepression activity even when the activation domain of VP16 was fused to its N terminus (Fig. 7B), indicating that the EAR motif of HSI2 is an

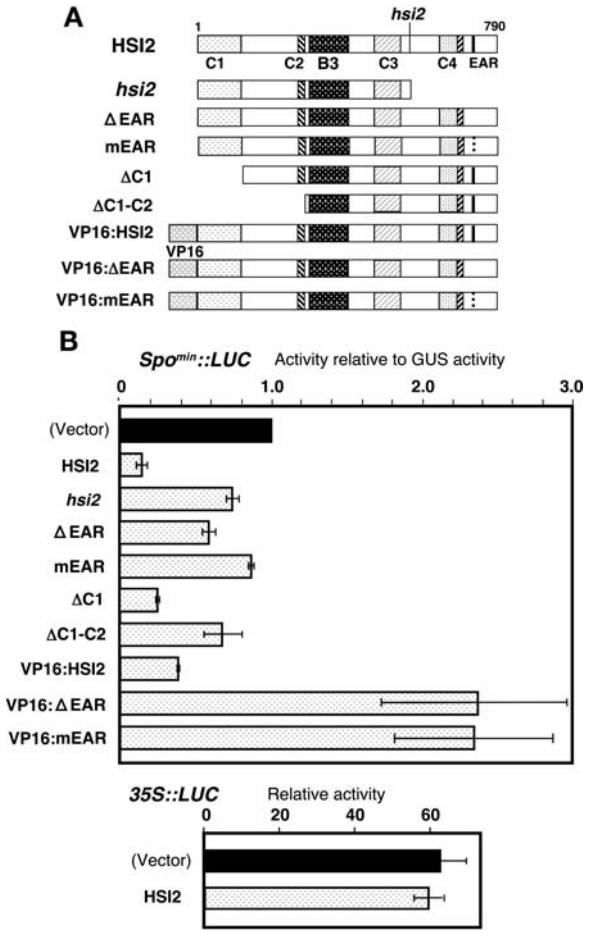

Figure 7. Repression of *Spo*^{min}::*LUC* expression by coexpression of HSI2 in Arabidopsis protoplasts. A, Schematic representations of the structure of HSI2 and its deletion derivatives. B, Effects of coexpression of HSI2 on transient expression of *Spo*^{min}::*LUC* and *35S*::*LUC*. B, Top, Protoplasts were cotransfected with the *Spo*^{min}::*LUC* reporter plasmid, various effector plasmids as shown in A, and the *35S*::*GUS* internal control plasmid. The empty effector plasmid served as a control. The LUC activity in each assay was normalized according to the GUS activity. The normalized LUC activities are expressed relative to values obtained with the empty effector plasmid. Results represent the means of three experiments, and the error bars represent ±SD. B, Bottom, Protoplasts were cotransfected with the *35S*::*LUC* reporter plasmid and *35S*::*HSI2* or empty effector plasmid. Results represent the mean LUC activities from three experiments, and the error bars represent ±SD.

active repression domain that can negate the function of VP16 activation domain.

Although deletion of the N-terminal C1 region did not affect the repressor activity of HSI2, deletion of both the C1 and C2 regions resulted in significant reduction of the repressor activity. These N-terminal regions could be required for correct DNA-binding activity or repression activity of HSI2.

DISCUSSION

The *hsi2* mutant exhibiting highly enhanced expression of *Spo*^{min}::*LUC* was due to a single recessive mutation. In the *hsi2* mutant, the levels of expression of *Spo*^{min}::*LUC* were always higher than the sGsL line not only under noninductive condition but also after treatment of plants with Suc or ABA. In addition, the spatial pattern of expression of *Spo*^{min}::*GUS* in the *hsi2* mutant was not changed compared to the sGsL line, and the *hsi2* mutant did not exhibit increased levels of ABA or defective starch synthesis. These results suggest that HSI2 has negative effects on the basal transcription level from the *Spo*^{min} promoter.

The *hsi2* mutation was a nonsense mutation in a gene encoding a protein with B3 DNA-binding domain. The HSI2-GFP fusion protein was localized in the nucleus, while truncation of HSI2 at the *hsi2* mutation (GFP-HSI2[Δ553-790]) reduced the nuclear localization. Since the *hsi2* mutant plants contained the mutant HSI2 mRNA at levels similar to the HSI2 mRNA in the sGsL plants, the *hsi2* mutant might produce a truncated HSI2 that is not efficiently targeted to the nucleus. The null mutation of the *HSI2* gene due to T-DNA insertion resulted in a level of *Spo*^{min}::*LUC* expression even higher than that observed in the *hsi2* mutant. These results indicate that HSI2 functions as a negative regulator of *Spo*^{min}::*LUC* expression and that the function of HSI2 is not completely abolished by the *hsi2* mutation. That HSI2 negatively regulates *Spo*^{min}::*LUC* expression is further supported by the results showing that most of the transgenic lines with *35S*::*HSI2* exhibited less than 20% of the levels of expression of *Spo*^{min}::*LUC* compared to the sGsL plants.

In addition to *HSI2*, the Arabidopsis genome contained two other expressed genes, *HSI2-L1* (At4g320110) and *HSI2-L2* (At4g215510), which code for proteins with high similarities to HSI2. Despite the similarity of the B3 domain, the HSI2 subfamily proteins do not show structural similarities other than the B3 domain with ABI3, FUS3, and LEC2. Three HSI2 subfamily proteins share conserved C1 to C4 regions in addition to the B3 domain. They also contain sequences similar to the EAR motif in the C-terminal region. The rice (*Oryza sativa*) genome contains one gene that could code for a protein with overall similarities to HSI2, and sequences conserved among three HSI2 subfamily proteins of Arabidopsis are also conserved in this rice protein (data not shown).

The EAR motif was first identified in class II ERFs, such as NtERF3, AtERF3, and AtERF4 (Fujimoto et al., 2000; Ohta et al., 2001). These class II ERFs function as active repressors that down-regulate not only basal transcription levels of a reporter gene but also the transactivation activity of other transcription factors. Unlike passive transcriptional repressors, active repressors generally contain small, active repression domains (Hanna-Rose and Hansen, 1996). Most of the repression domains of active repressors from animals are loosely categorized according to the primary amino acid content, such as Ala rich, Pro rich, or charged (Hanna-Rose and Hansen, 1996). By contrast, the EAR motif sequence is conserved not only in the

C-terminal regions of class II ERFs but also in the C-terminal regions of TFIIIA-type zinc-finger proteins from plants such as Arabidopsis ZAT1, ZAT5, ZAT10/STZ, and ZAT11 (Ohta et al., 2001). The C-terminal regions of ZAT10 and ZAT11 were shown to function as active repression domain (Ohta et al., 2001).

The presence of EAR motif-like sequences in the C-terminal region of HSI2 and its conservation among the HSI2-related proteins suggested that HSI2 functions as an active transcriptional repressor. Coexpression of HSI2 in Arabidopsis protoplasts reduced the transient expression of the *Spo*^{min}::*LUC* reporter to less than 20% of the level in the absence of effector. On the other hand, coexpression of a truncated *hsi2* mutant form of HSI2 (HSI2[Δ553-790]) only slightly repressed the LUC expression. Either deletion (ΔEAR) or mutation (mEAR) of the EAR motif-like sequence of HSI2 also significantly diminished the transrepression activity. The weak transrepression activity of the ΔEAR and mEAR effectors does not seem to be due to loss of DNA-binding activity or rapid degradation of modified effectors, since these molecules were converted into activators of *Spo*^{min}::*LUC* by fusion with the VP16 activation domain. On the other hand, the EAR motif of HSI2 strongly repressed the expression of reporter gene by the activation domain of VP16. These results indicate that HSI2 functions as a transcriptional repressor of *Spo*^{min} and the EAR motif is important for repression. The transrepression activity of HSI2 was not completely abolished by deletion or mutation of the EAR motif. Since deletion of the N-terminal C1 and C2 regions, but not the C1 region alone, reduced the repressor activity, the N-terminal region could also participate in the repressor function of HSI2.

The mechanism by which the EAR motif inhibits transcription is not known. Generally, the repression domain of active repressors inhibits the activation of transcription by interacting with basic transcription factors, activator/coactivator, or corepressor (Hanna-Rose and Hansen, 1996; Thiel et al., 2004). Since the EAR motif is present in proteins with the DNA-binding domain, these proteins are likely to inhibit selectively the transcription from the promoter to which they bind. The ERF/AP2 domain proteins include both transcriptional activators and repressors, and the ERF domains of transcriptional activators and repressors both display GCC box-specific DNA-binding activity (Fujimoto et al., 2000). Furthermore, since many class I ERF transcriptional activators are involved in various stress responses and expression of class II ERF transcriptional repressors is regulated under stress conditions such as cold, drought, and salt stress, it is suggested that class II ERF repressors might act as negative regulators during the transduction of stress signals (Ohta et al., 2001).

The B3 domains of ABI3, FUS3, and LEC2 exhibit binding to sequences containing the RY motif [CATGCA] (Suzuki et al., 1997; Kroj et al., 2003; Mönke et al., 2004). It is not known at present whether the B3 domains of the HSI2 subfamily proteins exhibit binding to sequences similar to the RY motif. Unlike seed-specific expression of ABI3, FUS3, and LEC2 (Parcy et al., 1994, 1997; Stone et al., 2001), HSI2 and HSI2-L1 are also expressed in vegetative tissues, suggesting that HSI2 subfamily proteins might regulate expression of genes that are not targets of seed-specific B3 domain transcriptional activators. HSI2 was identified as an active repressor of the *Spo*^{min}::*LUC* transgene, which is inducible by sugar or ABA. We compared levels of mRNAs for several genes that are regulated by sugar or ABA in the sGsL, ΔHSI2, OX4, and OX7 plants. Effects of disruption or overexpression of HSI2 on the expression of these genes varied depending on the gene (data not shown), and it is not clear at present whether HSI2 is selectively involved in the expression of a specific subset of genes that are regulated by sugar or ABA. Analyses on endogenous target genes of HSI2 by microarray analysis and determination of the DNA-binding properties of HSI2 are now in progress. Germination of seeds of *hsi2* and *ΔHSI2* mutant plants shows weak resistance to both sugar and ABA (data not shown). Since the three HSI2 subfamily proteins of Arabidopsis may have some overlapping functions, we are currently characterizing mutants of *HSI2-L1* and *HSI2-L2* in addition to mutants of *HSI2*, as well as double and triple mutants of them, to understand the physiological role of HSI2 subfamily transcriptional repressors.

MATERIALS AND METHODS

Plant Materials and Treatment with Sugar or ABA

The Arabidopsis (*Arabidopsis thaliana* L.) Heynh. (ecotype Col-0) harboring one copy of the *Spo*^{min}::*GUS-Spo*^{min}::*LUC* transgene on the upper arm of chromosome 5 (referred to as the sGsL line or the wild type) and the screening of mutants displaying abnormal patterns of LUC reporter expression will be described elsewhere (A. Morikami, T. Saijo, M. Yamada, H. Tsukagoshi, T. Hattori, and K. Nakamura, unpublished data). Unless otherwise indicated, seeds were sterilized in sterile water, kept at 4°C for 4 d, and sown on gellan gum plates containing Murashige and Skoog medium, pH 5.8, 100 mg/L myoinositol, 10 mg/L thiamine-HCl, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, and 1.5% Suc. Plates were incubated in a growth chamber at 22°C under continuous fluorescent light at an intensity of 65 μmol m^{-2} s^{-1}. Mature leaves of the 3-week-old plants were excised with a sharp razor blade, and the cut edges of petioles were immersed in a sterile solution of sugar or ABA and incubated at 22°C under continuous light (Mita et al., 1995).

In Planta Luminescence Imaging of LUC Activity and Determination of LUC Activity

For luminescence imaging of LUC activity, leaves treated with Suc or ABA for 48 h were sprayed with 0.8 mM D-luciferin (Molecular Probes, Eugene, OR) in 0.01% Triton X-100 and kept in the dark for 5 min before imaging. Luminescence images were taken with a 5-min exposure time by a CCD system and processed with Argus-50 (Hamamatsu Photonics, Shizuoka, Japan). For determination of LUC activity, leaves were homogenized in an extraction buffer composed of 100 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol and centrifuged at 15,000*g* for 10 min at 4°C. The resulting supernatants were used as protein extracts. The protein concentrations were determined using a protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. LUC activity was determined using a commercial assay kit (Picagene Luminescence kit; Toyo-Ink, Tokyo).

Histochemical GUS Staining

Whole plants or leaves were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-β-GlcUA in GUS staining buffer, which contained 100 mM sodium phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 0.1% Triton X-100 (Jefferson et al., 1987), and then placed under vacuum for 5 min. After incubation at 37°C for 1 d, chlorophyll was cleared by immersing the plants or leaves in 70% ethanol.

Quantification of ABA

To quantify the ABA content in leaves, 100 mg of leaves were frozen in liquid N_2 and freeze-dried before extraction with 1 mL of sterile distilled water by shaking constantly overnight at 4°C. The amount of ABA was quantified with a Phytodetek ABA kit (AGDIA, Elkhart, IN) following the protocol provided by the manufacturer.

Genetic Analysis and Cloning of the *hsi2* Mutant

For genetic mapping, the *hsi2* mutant was crossed with the ecotype Ws and L*er*, and the F_1 plants were allowed to self-pollinate. Among F_2 plants descended from crosses with Ws and L*er*, 502 and 363 homozygous *hsi2* mutant plants, respectively, were selected based on high LUC activity in leaves treated with water. The genomic DNA isolated from these homozygous *hsi2* mutants were subjected to genetic mapping using SSLP and CAPS markers. For fine mapping, new SSLP and CAPS markers were designed based on DNA sequences. The new SSLP and CAPS markers used for fine mapping were as follows (for SSLP markers, the relevant restriction enzyme and the size of fragments for Col-0/L*er* in base pairs are indicated in parentheses): TD9-4in-f, 5′-ACAATTAGAGGTACGTGGGAAT-3′; TD9-4in-r, 5′-TCATATTGATTAATGGGTTCCA-3′ (310/280); TB20-85-f, 5′-GGACCGTAGTGCTACTTGTGACG-3′; TB20-85-r, 5′-GACCTATTACTATTAGTACTACGAATG-3′ (*Tru*9I, 220/120 + 100); TB20-R32-f, 5′-GGAAAAGAGATGGAACGTGGGTG-3′; and TB20-R32-r, 5′-GAATCGAATTGAATCTAAGTTTTGTGG-3′ (*Hha*I, 120 + 110/230).

Genomic and cDNA Clones of HSI2 and Transformation of Arabidopsis

For complementation of the *hsi2* mutation, the DNA from TAC clone K06D02 was digested with *Pvu*II. A 10-kb genomic fragment covering the entire At2g30470 locus (HSI2 gene), including an approximately 2,000-bp sequence upstream from the putative initiation ATG codon, was cloned into the *Sma*I site of the binary vector pBIB-Hyg (Becker, 1990). Transformation of *hsi2* mutant plants was carried out using the vacuum-infiltration method (Bechtold and Pelletier, 1998) with *Agrobacterium tumefaciens* strain C51C1(pMP90). A full-length cDNA clone for HSI2 (RAFL07-13-K13) was obtained from RIKEN (Yokohama, Japan), and the cDNA structure was verified by sequencing.

To construct genes for GFP fusion proteins, we used Gateway cloning technology (Invitrogen, Carlsbad, CA) with pGWB2, pGWB5, or pGWB6 vectors, which was developed by Dr. T. Nakagawa (Research Institute for Molecular Genetics, Shimane University, Shimane, Japan). The resulting plasmids, pGWB2-HSI2, pGWB5-HSI2, pGWB6-HSI2, pGWB5-HSI2[Δ545-790], and pGWB6-HSI2[Δ545-790] for CaMV 35S promoter-dependent expression of HSI2, HSI2-GFP, GFP-HSI2, HSI2[Δ545-790]-GFP, and GFP-HSI2[Δ545-790], respectively, and pGWB6 carrying 35S-GFP were transferred into Agrobacterium and used to transform the sGsL line.

Subcellular Localization of HSI2-GFP Fusion Proteins

Transient expression assays of GFP localization in onion (*Allium cepa*) epidermal cells were carried out by particle bombardment (IDERA GIE III; Tanaka, Hokkaido, Japan) as described (Takeuchi et al., 1992). Typically, each assay included 5 μg of plasmid DNA carrying various GFP fusion protein genes under control of the CaMV 35S promoter. After 20 h of bombardment, GFP fluorescence in epidermal cells was observed by confocal fluorescence microscopy (FV500; Olympus, Nagano, Japan).

RNA Preparation and Expression Analysis

RNA was isolated from plant tissues using Trizol reagent (Invitrogen) and dissolved in nuclease-free water. For quantitative real-time PCR, the first-strand cDNA was synthesized from 5 μg of total RNA with oligo$(dT)_{20}$ primers using SUPERSCRIPT III (Invitrogen) and diluted with 10 volumes of RNase-free water. The real-time PCR reaction mixture was performed in 25 μL containing 5 μL of diluted cDNA solution, 12.5 μL of Cybergreen dye set (Bio-Rad), and 0.5 μL of each primer (final concentration of 200 nM). PCR was initiated with denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s. The comparative threshold cycle method was used to determine the relative mRNA levels. ACT2 was used as an internal reference, and expression levels were expressed as relative to the control treatment. The following primer sets were used: HSI2, 5′-CTTCCATATCAGCTTGAAACTCTC-3′ and 5′-TGGCTCAAGACGCCAGTGATGTTT-3′; and ACT2, 5′-CTGTTGACTACGAGCAGGAGATGGA-3′ and 5′-GACTTCTGGGCATCTGAATCTCTCA-3′. The forward and reverse primer pairs used for RT-PCR analysis of mRNA expression were as follows: HSI2-L1, 5′-ATGAGGCTTCTCCAAGCTGCAGCGT-3′ and 5′-GAACCGTGTTCTGTGCTGACCATAT-3′; and HSI2-L2, 5′-AGTGCTCATAGCCAAAATAACAAGG-3′ and 5′-CTACATGGAGCTTGTGGTGGTGGTG-3′.

Transient Expression Assay in Protoplasts

Arabidopsis protoplasts were isolated from T87 suspension-cultured cells (Axelos et al., 1992) and transfected with plasmid DNA by a modified polyethylene glycol method as described by Kovtun et al. (2000). A suspension of protoplasts (150 μL; 10^5 protoplasts per mL) was cotransfected with 15 μg of both reporter and effector DNA as well as 7.5 μg of *35S::GUS* internal control plasmid. The transfected protoplasts were incubated at 22°C for 20 h before collection and measurement of reporter expression.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank data libraries under accession numbers AB206553 and AB206554.

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