The BEL1-type homeobox gene SH5 induces seed shattering by enhancing abscission-zone development and inhibiting lignin biosynthesis

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SUMMARY
Seed shattering is an important trait that influences grain yield. A major controlling quantitative trait locus in rice is qSH1. Although the degree of shattering is correlated with the level of expression of qSH1, some qSH1-defective cultivars display moderate shattering while others show a non-shattering phenotype. Os05 g38120 (SH5) on chromosome 5 is highly homologous to qSH1. Although we detected SH5 transcripts in various organs, this gene was highly expressed at the abscission zone (AZ) in the pedicels. When expression of this gene was suppressed in easy-shattering ‘Kasalath’, development of the AZ was reduced and thereby so was seed loss. By contrast, the extent of shattering, as well as AZ development, was greatly enhanced in moderate-shattering ‘Dongjin’ rice when SH5 was overexpressed. Likewise, overexpression of SH5 in the non-shattering ‘Ilpum’ led to an increase in seed shattering because lignin levels were decreased in the basal region of spikelets in the absence of development of an AZ. We also determined that two shattering-related genes, SHAT1 and Sh4, which are necessary for proper formation of an AZ, were induced by SH5. Based on these observations, we propose that SH5 modulates seed shattering by enhancing AZ development and inhibiting lignin biosynthesis.

Keywords: abscission zone, lignin deposition, Oryza sativa, seed shattering, SH5.

INTRODUCTION
Seed shattering is an important agronomic trait. While easy shattering causes a reduction in yields when seed is lost prior to harvest, non-shattering also confounds those yields by hampering the process of harvesting (Ji et al., 2006). Therefore, cultivars with an appropriate level of shattering have been selected for domestication. In rice, indica cultivars generally display an easy-shattering phenotype, whereas most japonica cultivars are moderate- to non-shattering types (Konishi et al., 2006). Seed shattering depends upon development of an abscission zone (AZ), which is composed of small and isodiametric compacted cells (Zhou et al., 2012). In a rice pedicel, the AZ is formed between the sterile lemma and rudimentary glume at Stage 7, which occurs 16 to 20 days before heading when panicles are 5–30 mm long (Ji et al., 2010).

Differentiation of the AZ has been studied in various plants. In Arabidopsis, functionally redundant SHP1 and SHP2 MADS-box genes control differentiation of the dehiscence zone and promote lignification (Liljegren et al., 2000). The valve margin identity factor INDEHISCENT (IND) is required for an auxin minimum and coordinates auxin efflux in separation-layer cells (Sorefan et al., 2009). The FRUITFULL (FUL) MADS-box transcription factor, acting within the valve, suppresses expression of the valve margin gene, thus limiting its expression to the valve–replum boundary (Gu et al., 1998). Similarly, the REPLUMLESS (RPL) homeobox gene, a key regulator for replum development, controls preferential expression of the valve margin gene (Venglat et al., 2002; Roeder et al., 2003).

In crop plants, the wheat Q gene regulates plant architecture and seed dispersal (Simons et al., 2006). Shattering1 (Sh1), which encodes a YABBY transcription factor, controls shattering in sorghum (Lin et al., 2012). The orthologous Sh1 is present on rice chromosome 3, where
a shattering quantitative trait locus (QTL) with a minor genetic effect has been mapped. On maize chromosomes 1 and 5, Sh1-orthologous genes have been identified as major shattering QTL traits (Lin et al., 2012).

Four other genes are involved in seed shattering in rice, including the QTL Sh4, identified from a cross between Oryza sativa and Oryza nivara. An amino acid substitution in the trichel transcription factor encoded by Sh4 is responsible for a reduction in shattering (Li et al., 2006). qSH1 has also been revealed through a cross between shattering-type indica ‘Kasalath’ and the non-shattering-type japonica ‘Nipponbare’ (Konishi et al., 2006). This gene encodes a BELL-type homeobox protein that is highly homologous to Arabidopsis RPL (Konishi et al., 2006). A single nucleotide polymorphism (SNP) in its 5’-regulatory region causes a loss of expression only at the AZ (Konishi et al., 2006). Overexpression of the wheat BEL1-like gene TaqSH1 regulates floral organ abscission in Arabidopsis (Zhang et al., 2013). SHAT1, a member of APETAL2 (AP2), also functions in differentiation of the AZ (Zhou et al., 2012). Moreover, qSH1 has a role in maintaining the expression of Sh4 and SHAT1 (Zhou et al., 2012). We have previously reported that OsCPL1, which encodes a carboxy-terminal domain (CTD) phosphatase-like protein, represses development of the AZ (Ji et al., 2006).

In this work we identified a seed-shattering gene, SH5, that is highly homologous to qSH1. Both function together in controlling development of the AZ. We also determined here that cold induces expression of these shattering genes as well as differentiation of the AZ.

RESULTS
Identification of SH5
Among the 17 BEL1-like homeobox family members in rice, Os05 g38120 shows the highest homology to qSH1, with 70% identity and 77% similarity (Figure S1 in Supporting Information). Because it is located on chromosome 5 we named it SH5. The SH5 protein is also highly homologous to Arabidopsis RPL (Figure S1). All three proteins contain conserved motifs, a homeodomain for DNA binding and a MEINOX-interacting domain composed of SKY and BELL regions.

This gene was weakly expressed in seedlings, but its transcript level was increased in leaf sheaths and nodal regions in mature plants (Figures 1a and S2). During panicle development in the easy-shattering ‘Kasalath’ (indica), SH5 was highly expressed in young panicles (1-2 cm long) as well as at later maturation stages. However, in the moderate-shattering ‘Dongjin’ and non-shattering ‘lipum’ (both japonica), transcript levels in young panicles were much lower than in ‘Kasalath’ (Figure 1a). Those young panicles contained spikelets at Stages 7 and 8 when the AZ was being developed (Ji et al., 2010).

To study patterns of expression at the tissue level, we generated transgenic rice plants that expressed the GUS reporter gene under control of the SH5 promoter from ‘Kasalath’ (Figure S3a). The 3.3-kb promoter fragment included a 1.9-kb promoter region, the 714-bp first exon from ATG and the 732-bp first intron. The GUS analyses revealed expression in young spikelets from the transgenic plants, especially in the AZ and at the base of the spikelets (Figure 1k,l). A GUS signal was also detected in the leaf sheath pulvinus, lamina joint and anthers (Figure 1d,f,m).

Seed shattering is enhanced by cold temperatures (Estonell et al., 2012). Therefore, we tested whether expression of SH5 was inducible by chilling. Exposing mature panicles to temperatures of 10°C resulted in greater expression in both ‘Kasalath’ and ‘Dongjin’ (Figure 1j). Transcript levels in ‘Dongjin’ were increased two-fold after 10 h of such treatment, and cold-related induction was even higher in ‘Kasalath’. Analysis of GUS transgenic plants also showed that this stress induced expression of SH5 in the spikelets (Figure 1i,o–r), leaves (Figure 1c,e) and stems (Figure 1g). Whereas the shattering genes qSH1 and SHAT1 were also induced by cold stress, CPL1 was not (Figure S4). Because Sh4 was expressed at a very low level we were unable to examine its pattern in the cultivated rice.

Functional characterization of SH5
To elucidate its functional roles, we generated SH5 RNA interference (RNAi) transgenic plants in ‘Kasalath’, using the maize ubiquitin promoter. Because the SH5 coding region is highly homologous to qSH1, we used the 5’- and 3’-untranslated regions (UTRs) for RNAi vector construction (Figure S3b). Eight RNAi plants from the 5’-UTR and two transgenic plants from the 3’-UTR were obtained; all displayed decreased expression of SH5 (Figure 2a). Most of the RNAi plants showed a significant increase in the value for pedicel breaking tensile strength (BTS), which was inversely proportional to the degree of shattering (Figure 2b). Lines 4 and 8 were selected for confirmation of the shattering phenotype in the T2 generation. The BTS values for both lines were approximately twice as high as for their segregating wild-type (WT) plants that retained the easy-shattering phenotype (Figure 2c). Because the qSH1 transcript level was not significantly changed in the RNAi plants, we ruled out the possibility that the phenotypes were due to a suppression of qSH1 (Figure S5b).

Anatomical investigation with longitudinal sections indicated that development of the AZ was reduced in the SH5 RNAi plants (Figure 2d–f). Although WT plants showed good development across their pedicels, this process was reduced in the RNAi lines. This demonstrated that SH5 is required for formation of the AZ.
Enhancing SH5 expression induces seed shattering in a moderate-shattering cultivar

We also generated SH5 RNAi plants in moderate-shattering ‘Dongjin’ rice, but saw no significant change in the degree of shattering (Figure S5c,d). This was probably because expression of SH5 during spikelet development was weaker in that cultivar than in the easy-shattering ‘Kasalath’ (Figure 1a). Alternatively, SH5 may be defective in ‘Dongjin’. Sequencing the gene in that cultivar revealed one indel and three SNPs in the coding region, which resulted in two amino acid additions and a single amino acid substitution (Figure S6). We also found two deletions in the second intron (Figure S7). The promoter region had 22 SNPs and two indels within the 1.9-kb region from...
ATG that was used for the promoter–GUS construction (Figure S8).

We sequenced the promoter region from 17 cultivars, the shattering degrees of which had been previously determined (Konishi et al., 2006). The easy-shattering wild rice, Oryza rufipogon, was also included. Of the five indica type cultivars, three with a relatively high shattering degree had the same 22 SNPs and two indels as those occurring in the easy-shattering cultivars (Figure S9). Another cultivar, ‘Muha’, had the same two indels, but fewer SNPs. The fifth indica cultivar, ‘Tadukan’, which shows relatively low shattering, did not contain any of the SNPs or indels found in other indica cultivars. Because all of the indica rice cultivars carry the same qSH1 allele, the degree of shattering among them appears to be controlled in part by SH5 expression. Sequencing the eight japonica cultivars revealed that promoter sequences from seven of them are the same as the moderate- or non-shattering cultivars. However, ‘Dabaigu’, which has a relatively high degree of shattering among japonica cultivars, had 22 SNPs and two indels as found from most indica cultivars. The promoter regions from four tropical japonica cultivars lacked those
SNPs and indels, similar to temperate *japonica* types. These results indicated that the promoter region of SH5 is a major determinant of the propensity for seed shattering.

To examine whether ‘Dongjin’ SH5 is functional, we used a T-DNA activation tagging line, 3A-07285, in which the 3SS enhancer elements were located 4 kb downstream from the stop codon of SH5 (Figure S3d). Expression was significantly enhanced in the *SH5*-D heterozygous lines, and further increased in homozygous lines (Figure 3b). Compared with WT ‘Dongjin’, the activation lines displayed a phenotype with dramatically greater shattering (Figure 3a). Whereas the non-shattering degree of ‘Dongjin’ and segregating WT siblings was approximately 160 gravitational units of force (gf), that value was reduced to 15 to 40 gf in the *SH5*-D heterozygous lines (Figure 3c). Those heterozygous lines displayed a shattering degree similar to that of cpl1, an easy-shattering mutant (Ji et al., 2010). We were unable to measure the mechanical degree of shattering in the homozygous lines because almost all of the seeds had dropped before harvest.

Overall, our results indicated that although ‘Dongjin’ SH5 was functional its weak expression in young spikelets was responsible for the reduced amount of shattering.

To elucidate further how the shattering phenotype of the activation line might be associated with increased expression of SH5, we generated transgenic plants that expressed a full-length ‘Dongjin’ SH5 cDNA under control of the maize *ubiquitin* promoter (Figure S3c). Five independent *SH5*-overexpressing plants showed easy shattering compared with the parental line. The degree of shattering was correlated well with the magnitude of this increase in transcripts (Figure 3d,e). This finding supported our conclusion that SH5 plays an important role in seed shattering.

**Figure 3.** Phenotypes of *SH5* activation and -overexpressing lines.
(a) Seed-shattering habit of tagging line 3A-07285. Photos were taken at fully mature stage. WT, panicle from segregating wild type; hetero, panicle from heterozygous plant; homo, panicle from homozygous line. Scale bar = 2 cm.
(b) Quantitative RT-PCR analyses of *SH5* transcripts in the activation tagging line. The RNAs were prepared from seedling leaf blades. Error bars indicate standard deviations; n ≥ 3.
(c) The degree of non-shattering was obtained by measuring the breaking tensile strength required to remove seeds from pedicels. Error bars indicate standard deviations; n ≥ 10.
(d) *SH5* expression levels in ‘Dongjin’ WT and five *SH5*-overexpressing transgenic plants. The expression level is represented in log (expressed level in overexpression line/WT level).
(e) Shattering degree of ‘Dongjin’ and overexpressing plants. Error bars indicate standard deviations; n ≥ 10.

Development of an AZ is enhanced in *SH5*-overexpressing plants

We observed the AZ layer from longitudinal sections of the spikelet during panicle formation (Figure 4). Spikelet development is divided into eight stages (Itoh et al., 2005). In WT panicles, stamen primordia appeared during Stage 6 (Figure 4a). At Stage 7, carpel primordia formed on the floral meristem and stamen primordia differentiated into an anther and filaments (Figure 4b). During that stage, an AZ also started to form near sterile lemma in the pedicels. At Stage 8, development of ovules and anthers was completed, and AZ development ceased (Figure 4c). We noted that although in Stages 6 and 7 the AZ did not differ between *SH5* activation tagging lines and the WT (Figure 4b,g), that zone did begin to show difference in early Stage 8 (Figure 4c versus h). While AZ development in the WT stopped in the early part of Stage 8 (Figure 4d,e), it continued for the activation lines into late Stage 8 (Figure 4i,j).

**SH5** increases seed shattering in the absence of qSH1

The non-shattering type qSH1 allele causes complete absence of an AZ (Konishi et al., 2006). To evaluate whether *SH5* could increase seed shattering in non-shattering plants, we introduced the *SH5*-overexpression construct into *japonica* cultivar ‘Ilpum’, which has the non-shattering qSH1 allele. From several transgenic plants, we selected three lines (Ox-1, -2 and -3) that showed significant levels of transgene expression (Figure 5a). All three lines displayed an easy-shattering phenotype, in which the degree of shattering was proportional to the level of expression (Figure 5b). This result suggested that *SH5* could induce shattering in the absence of qSH1.
To confirm our observation, we crossed 'Ilpum' carrying non-shattering qSH1 and SH5 alleles and 'Dongjin' carrying active qSH1 and the activated SH5 (Figure S10). F2 progeny that had the inactive qSH1 allele from 'Ilpum' and the activated SH5 allele from 'Dongjin' exhibited easy shattering (Figure 5c, line #2). This supported our conclusion that introduction of the active SH5 allele into 'Ilpum' increases grain shattering without qSH1. Although introducing the 'Dongjin' qSH1 allele into 'Ilpum' also increased shattering (Figure 5c, line #3), the extent to which this occurred was less than for the line with activated SH5 (line #2). When both genes were introduced, the shattering degree was further increased, thereby demonstrating an additive effect between qSH1 and SH5 (Figure 5c, line #1).

**SH5 sustains the expression of Sh4 and SHAT1 in the AZ**

In the absence of active qSH1, expression of SHAT1 and Sh4 was reduced in the AZ during late Stage 8, causing formation of the AZ to fail (Zhou *et al.*, 2012). This observation suggested that qSH1 could maintain expression of SHAT1 and Sh4 in that zone. To examine whether SH5 also induces SHAT1 and Sh4, we performed RT-PCR with panicles bearing Stage 7–8 spikelets (Figure 6). Transcript levels of Sh4 were significantly increased in the SH5 activation line when compared with the WT (Figure 6d). Because overexpression of SH5 did not significantly affect qSH1 expression (Figure 6d), we discounted the possibility that SH5 influenced Sh4 via qSH1.
In contrast, the transcript level of **SHAT1** was not significantly enhanced (Figure 6c). Because **SHAT1** is universally expressed in vegetative and reproductive tissues (Zhou *et al.*, 2012), it is possible that any change would have been insignificant if expression had increased only in the AZ. To investigate this possibility, we performed *in situ* hybridization experiments, first studying whether **SH5** is expressed in the AZ of developing spikelets at Stage 8. No significant signal was found in the pedicels of spikelets from WT ‘Dongjin’ (Figure 6e,f). However, positive signals were detected in the AZ as well as vascular bundles in the **SH5** activation tagging line (Figure 6g,h). This pattern was consistent with the expression profile we had observed from GUS transgenic plants, in which the reporter gene was preferentially expressed in the AZ and the basal region of the spikelets.

*In situ* hybridization experiments with the **SHAT1** probe revealed a signal in the AZ of ‘Dongjin’ WT, as reported previously (Figures 6i,j, and S12). In the activation tagging line, **SHAT1** expression was significantly increased in the AZ (Figure 6k,l). These results indicated that **SH5** preferentially enhanced **SHAT1** expression in that zone.

**SH5** alone does not induce AZ development

Overexpression of **SH5** in ‘Ilpum’ led to easy shattering (Figure 5b). However, the phenotype of the seed separation zone for these overexpressing plants differed from that of ‘Dongjin’ (Figure 7). Whereas seeds were cleanly separated from the branches via the AZ in WT and **SH5** activation lines from ‘Dongjin’ (Figure 7a,b), this did not occur with either the WT or **SH5**-overexpressing seeds from ‘Ilpum’ (Figure 7c,d). The ‘Ilpum’ progeny that carried the activated **SH5** did not produce a clean layer at the detachment site, but instead left a portion of the pedicel attached to the seeds (Figure 7f). To investigate whether the AZ had developed in those overexpressing plants, we compared sections from them and the ‘Ilpum’ WT. In both sources, the AZ was absent from the junction region between the sterile lemma and rudimentary glume (Figure 7i,j). This suggested that introduction of active **SH5** into ‘Ilpum’ could not induce development of an AZ. In contrast, ‘Ilpum’ progeny carrying the active **qSH1** allele from ‘Dongjin’ (Figure 7g) produced seeds that were cleanly detached from the pedicels. Similarly, introduction of functional **qSH1** and **SH5** produced clean separations (Figure 7h). These observations indicated that **SH5** alone was unable to induce AZ development and that **qSH1** was necessary.

**SH5** suppresses lignin deposition in pedicels

Introducing active **SH5** into ‘Ilpum’ increased the degree of seed shattering in the absence of an AZ. We analyzed...
lignin contents to see whether the easy-shattering phenotype was due to a reduction in lignin (Figures 7, 8, and S11). Staining of the pedicels at late Stage 8 showed that lignin deposition was much lower in the SH5-overexpressing plants (Figure 7n,p) than in the ‘Ilpum’ WT (Figure 7m,n). To confirm that this deposition was suppressed by SH5, we measured the lignin content in the ‘Dongjin’ WT and the SH5 activation line. Based on panicle-staining results, there was less lignin in the AZ and surrounding pedicel tissues of the activation line (Figure 8b,d) than in the WT (Figure 8a,c). We also measured the lignin content in stems at the heading stage because SH5 is expressed in the stems. Again, deposition was decreased in the activation line compared with the WT (Figure 8e–h). These results implied that SH5 repressed lignin deposition.

We investigated levels of expression for lignin biosynthesis genes (Koshiba et al., 2013) in the junction region of developing (3–10 cm) panicles (Figure 8i–n). There, transcripts of Phenylalanine ammonia-lyase 1 (PAL1), Cinnamyl alcohol dehydrogenase 2 (CAD2), and Cinnamyl CoA reductase 19 (CCR19) were significantly less abundant in the SH5 activation lines than in the WT. Transcript levels of Caffeic acid-O-methyl transferase1 (COMT1) and Caffeoyl-Coenzyme A 3-O-Methyltransferase 1 (COA1) were also reduced in 5-cm panicles from the activation lines. These results further supported our conclusion that SH5 affected lignin deposition.

Figure 7. Phenotype of SH5-overexpressing ‘Ilpum’ plants. Phenotypes of the broken area on mature seeds from ‘Dongjin’ (a), the SH5 activation line in the ‘Dongjin’ background (b), ‘Ilpum’ (c), the overexpressing line in the ‘Ilpum’ background (d), and four progeny (e–h) from a cross between ‘Ilpum’ and ‘Dongjin’ carrying SH5-D. Progeny are inactive qSH1/inactive SH5 (e), inactive qSH1/active SH5 (f), active qSH1/inactive SH5 (g) and active qSH1/active SH5 (h).

Longitudinal sections of pedicel junction from ‘Ilpum’ WT (i,k,m,o) and SH5-overexpressing ‘Ilpum’ (j,l,n,p). (i,j) staining with toluidine blue. (k,l) Enlarged images of boxed areas in panels (i,j), respectively. (m,n) Lignin-staining using phloroglucinol. (o,p) Enlarged images of the boxed areas in panels (m,n), respectively. Scale bars = 100 μm.
DISCUSSION

SH5 enhances seed shattering

Quantitative trait loci analysis of the shattering-type indica cultivar ‘Kasalath’ and the non-shattering-type japonica cultivar ‘Nipponbare’ has identified qSH1 as a major trait for seed shattering (Konishi et al., 2006). The non-shattering allele is distinguished from the shattering allele by a single nucleotide polymorphism (G to T) in the 5'-regulatory region that causes a loss of expression only in the AZ. Although indica cultivars carry this shattering qSH1 allele, the degree of non-shattering can range from approximately 10 to 40 gf (Konishi et al., 2006). In temperate japonica cultivars, both non-shattering and shattering qSH1 alleles exist. Although the cultivars that carry the former type are generally more tolerant to shattering than

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those that have the latter, there is large variation in the non-shattering degree (40–120 gf) among cultivars. This indicates that additional factors are responsible for those differences.

Because SH5 is highly homologous to qSH1, we hypothesized that SH5 is a genetic element that also affects the degree of seed shattering. Easy-shattering *indica* ‘Kasalath’ has both. We suppressed SH5 expression by the RNAi approach and observed that this action significantly reduced the amount of shattering in that cultivar, thereby demonstrating a controlling role by SH5.

Although weak in vegetative organs, SH5 was highly expressed in ‘Kasalath’ panicles at both early and later developmental stages. Overall, this pattern of expression was similar in ‘Dongjin’ except for panicles during early development. Although both cultivars have the same active qSH1 allele, ‘Dongjin’ displays moderate shattering. Therefore, this difference in phenotype might be due to the reduced expression of SH5 in the panicles at early developmental stages. To examine this hypothesis, we increased expression and found that doing so significantly enhanced the degree of shattering, thus demonstrating that SH5 has this function.

**SH5 enhances AZ development, but requires qSH1**

Suppression of SH5 expression in ‘Kasalath’ decreased seed shattering due to a reduction in AZ development. Increasing expression in ‘Dongjin’ promoted this development, thereby proving the role of that gene. However, overexpression of SH5 in ‘Ilpum’ did not induce formation of an AZ. The major difference between ‘Dongjin’ and ‘Ilpum’ is the presence of qSH1, which is an active allele in the former but not functional in the AZ of the latter. Therefore, it appears that qSH1 is needed for AZ development. It has previously been reported that introducing active qSH1 into ‘Nipponbare’, another non-shattering rice cultivar carrying non-shattering alleles of qSH1 and SH5, can restore the AZ (Konishi et al., 2006). Similarly, we observed that the introduction of active qSH1 into ‘Ilpum’ led to seeds without a pedicel attachment. Therefore, we conclude that qSH1 induces AZ differentiation while SH5 enhances AZ development.

**SH5 represses lignin deposition to enhance AZ development**

SH5 encodes a BEL1-type homeobox protein. Because BEL1 proteins interact with KNOX proteins (Hay and Tsiantis, 2010), SH5 may have an interacting KNOX protein for rice grain shattering. Arabidopsis RPL, an ortholog of SH5, interacts with BP and STM for internode patterning during inflorescence development (Smith and Hake, 2003; Kanrar et al., 2006). The BP gene regulates cell differentiation by modulating the lignin biosynthesis pathway (Mele et al., 2003). Lignin levels are increased in the stems of bp-1 mutants whereas BP overexpression delays this deposition (Mele et al., 2003). The BP protein directly binds to promoters of two lignin biosynthesis genes – COMT1 and caffeoyl-CoA O-methyltransferase (CCoAOMT). We observed that lignin levels were decreased in developing panicles of transgenic plants that overexpressed SH5. Expression of several genes in the lignin biosynthesis pathway was also reduced in those overexpressing plants, thereby demonstrating the involvement of SH5 in that pathway.

In rice, OSH1 and OSH15 are highly homologous to BP. Both the former are required for shoot apical meristem formation; their null mutants have a severe phenotype in plant architecture, especially during internode development at the vegetative stage (Sato et al., 1996, 1999). Therefore, it will be interesting to find out whether they function in lignin formation by interacting with SH5.

**SH5 is expressed in tissues where lignin levels are low**

Analysis of the transgenic plants expressing the SH5 promoter GUS revealed that the gene was expressed in various tissues during vegetative and reproductive development. At the vegetative stage, expression was detected in the lamina joint of the leaf. The likely role for SH5 there is to inhibit lignification of the tissues to provide flexibility. In the stems, expression occurred specifically in the leaf sheath pulvinus, which is located at the base of the stem, where it provides flexibility. The pulvinus and lamina joint are specific organs in the leaves that respond to gravity (Tan et al., 2008; Wu et al., 2002). Regulatory factors that control plant architecture are expressed in those tissues. In addition to seed-shattering, the SH5 RNAi transgenics had a pleiotropic phenotype. The presence of an elongated leaf sheath pulvinus in such plants was linked to a prostrate growth habit. Therefore it is possible that SH5 is involved in the gravity response.

In the panicles, the gene was expressed in the basal regions of the spikelets. High expression in those tissues was probably related to the low levels of lignin in the cells. This gene was also expressed in the early stages of anther development but not in mature anthers. Based on this pattern of expression, we could predict that SH5 functions in suppressing the deposition of lignin during early anther development.

**SH5 is a positive regulator of SHAT1 and Sh4**

Because transcripts of SHAT1 and Sh4 are not detected in qSH1-defective rice, it is thought that qSH1 controls their expression in the AZ (Zhou et al., 2012). We also showed that SHAT1 and Sh4 expression was significantly increased in the SH5-overexpressed lines. Therefore, we concluded that both qSH1 and SH5 positively affect SHAT1 and Sh4. Transcript levels of qSH1 were not affected when SH5 expression was altered, indicating that they function independently to induce those downstream genes.
Shattering genes are inducible by cold temperatures
Development of the AZ is affected by environmental stress and senescence (Estornell et al., 2012). We showed that shattering genes SH5, qSH1 and SHAT1 are inducible by cold stress. Whereas most indica and tropical cultivars are easy-shattering due to active qSH1 and SH5, most japonica cultivars grown in temperate regions are moderate- to non-shattering because neither SH5 nor qSH1 is expressed in that zone. This difference is probably due to temperature in the harvest season. For example, field temperatures are high all year round in tropical regions, such that expression of shattering genes remains low. However, in temperate regions, field temperatures drop rapidly at harvest time, thus inducing those genes. Therefore, japonica cultivars must be domesticated so that expression of shattering genes is decreased in the AZ.

EXPERIMENTAL PROCEDURES
Plant materials, growing conditions and characterization of mutant phenotypes
The T-DNA activation tagging lines were generated in O. sativa japonica cv. Dongjin using the activation tagging vector pGA2715 (Jeong et al., 2002; Yi and An, 2013). Plants were grown in a paddy field, greenhouse or controlled environment room (12 h of light at 28°C/12 h of dark at 22°C). At 15 days after heading we estimated the degree of seed shattering by measuring the BTS, as described previously (Li et al., 2006), using a digital force gauge (SHIMPO, http://www.shimpodrives.com/). Each data point was an average of 15 samples from the upper part of the panicles.

RNA in situ hybridization
The in situ hybridization of RNA was performed as previously described (Lee et al., 2007, 2010; Lee and An, 2012). Young panicles were fixed in 4% paraformaldehyde, dehydrated, embedded, cut and attached to slides. For probe generation, we amplified the fragment using primers presented in Table S1 (Zhao et al., 2012). The PCR products were cloned into a pBluescript II SK (-) vector, linearized and used as templates for generating the digoxigenin-labeled sense and antisense RNA probes, as previously described (Lee et al., 2010; Lee and An, 2012). Briefly, tissue samples were placed on an APS-coated slide (Matsunami Glass, http://www.matsunami-glass.co.jp/english/). After rehydration, they were hybridized overnight at 57°C with the digoxigenin-labeled RNA probe. For detection of the digoxigenin-labeled probe, we used anti-digoxigenin alkaline phosphatase (Roche Molecular Biochemicals, http://www.roche.com/shop/en/us/home) and nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

RNA isolation and quantitative RT-PCR analysis
Total RNA was isolated with RNAiso (TaKaRa, http://www.takara.bio.com/). The first cDNA was synthesized using total RNA and Moloney murine leukemia virus reverse transcriptase (Promega, http://www.promega.com/) with the oligo (dT) primer and 2.5 μM deoxyribonucleotide triphosphate. Quantitative RT-PCR was performed on a Rotor-Gene Q instrument system (Qiagen, http://www.qiagen.com/). Synthesized cDNAs were amplified using SYBR Premix Ex Taq (TaKaRa). Transcript levels were normalized by rice ubi. The ΔΔCt method was applied for calculating alterations in expression (Choi et al., 2014). To ensure primer specificity, we used the data when the melting curve showed a single peak. Primers for quantitative real-time PCR are presented in Table S1.

Histochemical analysis
Samples were fixed in formalin-acetic acid-alcohol solution after vacuum infiltration, and incubated at 4°C overnight. They were then dehydrated through a graduated ethanol series (50, 70, 90 and 100%). Afterwards they were treated with a tert-Butyl alcohol series and paraffin was infiltrated. Tissues were placed in an embedding ring and the paraffin block was cut to a thickness of 10 μm with a microtome (model 2165; Leica Microsystems, http://www.leica-microsystems.com/). The cut samples were attached overnight to a coated slide on a 60°C hot-plate, then rehydrated with 100% Histochoice for clearing, using an ethanol series (100, 70, 50 and 30%) and distilled water. The samples were stained with toluidine or phloroglucinol (saturated phloroglucin in 20% HCl), and protected with a cover slide. They were then observed under a BX61 microscope (Olympus, http://www.olympus-global.com/en/).

Vector construction and rice transformation
For construction of the SH5-overexpression vector, the SH5 full-length cDNA (J023133E06) was obtained from KOME (http://cdna01.dna.affrc.go.jp/cDNA/). The cDNA was placed under the control of the maize ubiquitin 1 promoter, using the pGA3426 binary vector (Kim et al., 2009). For construction of the SH5 RNAi vector, a 208-bp fragment (28 to 236 bp from the translation start site ATG) of the 5’-UTR region was ligated into the pGA3426 binary vector with an amplicin linker (Kim et al., 2009). In addition, a 286-bp fragment (21 to 307 bp from the translation stop site TGA) of the 3’-UTR region was used for another RNAi vector construction (Kim et al., 2009). For construction of the SH5 promoter-GUS vector, the 3.3-kb promoter region (containing a 1.9-kb promoter region, the first exon and the first intron of ‘Kasalath’) was inserted into the pGA3519 binary vector. The constructs were transformed into Agrobacterium tumefaciens LBA4404 (An et al., 1988). Transgenic rice plants were generated by a stable transformation method via Agrobacterium-mediated co-cultivation, as previously reported (Lee et al., 1999).

The GUS assay and clearing method
The GUS assays were performed as previously described (Kim et al., 2013). After filtration, samples were incubated overnight at 37°C in a solution containing 0.5 m sodium phosphate (pH 7.0), 1 m potassium ferricyanide, 1 m potassium ferrocyanide, 0.1% Triton X-100, 0.5 m EDTA (pH 8.0), 0.1% X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid/cyclohexylammonium salt), 2% DMSO and 5% methanol. They were then washed several times with 70% ethanol at 65°C and stored in 95% ethanol. For young spikelets, samples were washed with PBS buffer and cleared for several minutes in a solution (2:2:2:2:1:1, lactic acid:chloral hydrate:phenol:clove oil:xylene:benzyl-benzoate) (UCHIYAMA et al. 2007). All samples were observed with a SXZ16 dissecting microscope (Olympus) and BX61 microscope.

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