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Shortened Basal Internodes Encodes a Gibberellin 2-Oxidase and Contributes to Lodging Resistance in Rice

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ABSTRACT

Breeding semi-dwarf varieties to improve lodging resistance has been proven to be enormously successful in increasing grain yield since the advent of the "green revolution." However, the breeding of the majority of semi-dwarf rice varieties in Asia has been dependent mainly on genetic introduction of the mutant alleles of *SD1*, which encodes a gibberellin (GA) 20-oxidase, OsGA20ox2, for catalyzing GA biosynthesis. Here, we report a new rice lodging-resistance gene, *Shortened Basal Internodes* (*SBI*), which encodes a gibberellin 2-oxidase and specifically controls the elongation of culm basal internodes through deactivating GA activity. *SBI* is predominantly expressed in culm basal internodes. Genetic analyses indicate that *SBI* is a semi-dominant gene affecting rice height and lodging resistance. *SBI* allelic variants display different activities and are associated with the height of rice varieties. Breeding with higher activity of the *SBI* allele generates new rice varieties with improved lodging resistance and increased yield. The discovery of the *SBI* provides a desirable gene resource for producing semi-dwarf rice phenotypes and offers an effective strategy for breeding rice varieties with enhanced lodging resistance and high yield.

Key words: *Shortened Basal Internodes*, gibberellin activity, lodging resistance, yield, rice

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INTRODUCTION

Lodging is a serious problem that results in reduced grain crop yield. Breeding lodging-resistant varieties has been highly attended to as a genetic improvement strategy to increase yield in rice, wheat, and other crops (Khush, 1995; Berry et al., 2007). Plant height, particularly the length of basal internodes, is an important component of plant architecture affecting lodging resistance and relating to crop yield (Wang and Li, 2005). Since the ''green revolution'' began in the 1960s, the introduction of lodging-resistant semi-dwarf varieties of rice and wheat has achieved great success in increasing the worldwide production of grain (Khush, 2001; Hedden, 2003; Berry et al., 2004). The recessive *sd1* was first introduced into rice for breeding of semi-dwarf varieties (Khush, 1999) and widely distributed across Asia to improve lodging resistance in rice (Hargrove and Cabanilla, 1979; Spielmeyer et al., 2002; Asano et al., 2007). However, despite the importance of breeding lodging-resistant semi-dwarf rice varieties, the *sd1* allele is the only verified semi-dwarf gene suitable for use to engineer improvements in lodging

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resistance to date. Given that extensive use of one gene may disadvantage the diversification of rice varieties and hinder the genetic improvement process (Luh, 1980; Matsuo et al., 1997) over time, the development of new genetic resources for breeding lodging-resistant varieties is highly desirable.

More than 60 recessive dwarf mutants and 10 recessive semi-dwarf mutants have been identified in rice to date (Zhang et al., 2014). Plant height in rice is regulated by a complex of major genes and minor genes (Huang et al., 1996; Yan et al., 1998). The molecular mechanism underlying height regulation in plants typically affects the biosynthesis and signal transduction of endogenous phytohormones, mostly gibberellins (GAs) (Yang and Hwa, 2008). GAs comprise a large group of diterpenoid carboxylic acids that are ubiquitously present in higher plants, including functionally active molecules that exhibit various physiological activities such as GA_1 and GA_4 as well as inactive molecules such as GA_8, GA_{29}, GA_{34}, and GA_{51}, which do not have physiological activity *in planta* (Hedden and Thomas, 2012). A number of genes that encode GA metabolic enzymes and key factors in GA signal transduction pathways have been isolated from various plants (Hedden and Phillips, 2000). A typical phenotype of these gene mutants is plant dwarfism (Hedden and Phillips, 2000; Sakamoto et al., 2004). *SD1* is a semi-dwarf recessive gene that encodes OsGA20ox2 to catalyze the conversion of GA_{12}/GA_{53} to bioactive GA precursors GA_9/GA_{20}, which is highly expressed in rice culm and leaf and has been widely used for breeding lodging-resistant rice varieties (Sasaki et al., 2002), given that its mutant *sd1* exhibits the semi-dwarf phenotype but causes no obvious defects in other agronomic traits. In addition, increase of *OsGA20ox1/GNP1* (*grain number per panicle 1*) transcripts had a positive effect on grain number and plant height in rice (Wu et al., 2016). Most of the other reported rice dwarf mutants are not used for breeding due to the defects such as extreme dwarfism, short grain, and sterility (Sakamoto et al., 2004).

In this study, we report the characterization of a new rice semi-dominant lodging-resistance gene *SBI* (*Shortened Basal Internodes*) from a rice variety originated in Hunan province, China. *SBI*, which is predominantly expressed in rice culm, encodes an OsGA2ox that reduces the content of bioactive gibberellin in culm, especially in the basal internodes. The *SBI* allele exhibiting higher activity of OsGA2ox resulted in rice height reduction, and its application in rice production led to the breeding of rice varieties with enhanced lodging resistance and increased yield.

RESULTS

Characterization of a Rice Variety with Enhanced Lodging Resistance and Reduced Basal Internodes

Zhu1S is a thermo-sensitive male sterile line commonly used for hybrid rice breeding in Hunan province, China. However, the hybrid progeny varieties from Zhu1S generally display unsatisfactory performance in lodging resistance (Yang et al., 2000). SV14 was developed from Zhu1S through tissue culture, and its hybrid progeny displayed enhanced lodging resistance with shorter stature (Liu et al., 2002). SV14 was ~20 cm shorter (Figure 1A and 1B) than Zhu1S, but apart from the height other agronomic traits and features of the plant architecture of SV14 were similar to those of Zhu1S (Supplemental Figure 1). To identify at which development stage the height difference between Zhu1S and SV14 is incurred, we recorded the height progression of the two rice varieties across an entire growth cycle. We found no height difference between Zhu1S and SV14 in the first 4 weeks of growth (Supplemental Figure 2A). Height differences appeared between the two varieties in week 6 at the start of basal internode elongation (Supplemental Figure 2B). From the 8th to 14th week, the difference expanded significantly as rice culm rapidly elongated (Supplemental Figure 2C–2E), indicating that the SV14 height-reduction phenotype is closely related to culm elongation. Although each internode of SV14 was shortened (Figure 1C), the basal internodes including the third, fourth, and fifth internodes (counting from the top) exhibited a higher percentage of length reduction in SV14 (Figure 1D). In the fifth internode, cell elongation was drastically blocked in SV14 culm (Figure 1E and 1F). Meanwhile we examined the *SD1* allele in Zhu1S and SV14, as rice *SD1* mutants also caused short stature. Zhu1S and SV14 were identified as containing the same *sd1* allele (Supplemental Figure 3A). Taken together, these results suggest that the SV14 phenotype as characterized by shortened culm basal internodes is caused by a gene different from *sd1*. The gene locus underlying this trait is named *shortened basal internodes* (*SBI*).

SBI Encodes a Gibberellin 2-Oxidase

Zhu1S was selected from hybrid breeding between *indica* and *japonica* rice varieties and SV14 was further developed through tissue culture and genetic improvement from Zhu1S (Yang et al., 2000; Liu et al., 2002). To dissect the genome composition of Zhu1S and SV14, we carried out their whole-genome resequencing. By comparing with *indica* and *japonica* rice genome sequences, we found that both Zhu1S and SV14 genomes were primarily inherited from *indica* rice with infiltration of the *japonica* rice genome in several loci (Supplemental Figure 3B and Supplemental Table 1). Zhu1S and SV14 contained basically identical genome sequences except a few regions, especially at the end of the fifth chromosome (Supplemental Figure 3B).

Next, we crossed SV14 and Zhu1S to analyze the genetic basis of the SBI phenotype. The F1 progeny exhibited a plant height at the medium range of the two parents (Supplemental Figure 4A). Segregation of a ratio of approximately 1:2:1 plant height phenotypes with SV14/F1/Zhu1S (Supplemental Figure 4B) was observed in F2 plants, suggesting that the SV14 phenotype was caused by a semi-dominant gene at a single locus. The F2 population was used as a mapping population to clone *SBI* using the Mutmap method (Abe et al., 2012). We isolated DNA from 70 F2 progeny plants with the SV14 phenotype and bulked them in an equal ratio for whole-genome sequencing. We then aligned the sequence reads to the Zhu1S genome and performed a linkage analysis with SNP index distribution. We identified a region on chromosome 5 as the putative location harboring the causal gene (Figure 2A, Supplemental Figure 5, and Supplemental Table 2). The region contained three genes, encoding a GA 2-oxidase, a hypothetical protein, and a putative expressed protein, respectively (Figure 2A and Supplemental Table 2). We examined the expression of the three genes and found that the GA 2-oxidase gene was highly expressed in culm tissue whereas expression of the other two genes was hardly detectable in culm

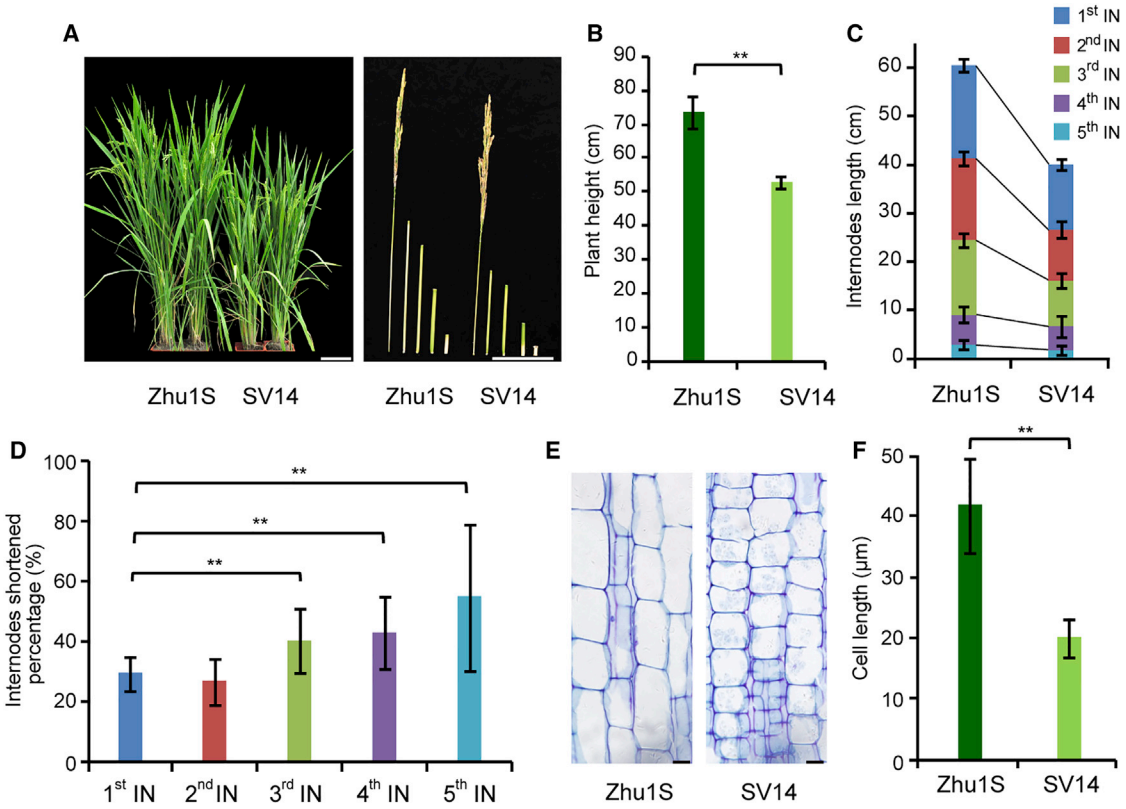

Figure 1. Rice Variety SV14 Displayed Shorter Basal Internodes than Zhu1S.

(A) Zhu1S and SV14 Phenotypes of whole plant (14 weeks old) and culm (16 weeks old). Scale bars, 10 cm.

(B) Height of Zhu1S and SV14 mature plants. Values are means ± SD (n = 30 plants).

(C) Internode length of Zhu1S and SV14 culm. Values are means ± SD (n = 30 plants).

(D) Internode shortened percentage of SV14 compared with Zhu1S. IN, internode. The number of internodes is counted from top to bottom. Values are means ± SD (n = 30 plants).

(E) Longitudinal section from the fifth internode of Zhu1S and SV14. Scale bar, 10 μm.

(F) Statistical data of cell length in **(E)**. Values are means ± SD (n = 200 cells from five plants).

Statistical significance of differences calculated based on two-tailed, two-sample Student's t-test at $^{**}p < 0.01$.

tissue (Figure 2B). This suggested the GA 2-oxidase gene may be the candidate for *SBI*. We compared the allele sequences of the candidate GA 2-oxidase (*GA2ox*) homolog in Zhu1S and SV14 and found that six SNPs were present in their exon regions. Among the SNPs, one caused an aspartic acid (D308) in Zhu1S to be substituted with asparagine (N308) in SV14; one replaced a glycine (G338) in Zhu1S with an arginine (R338) in SV14 (Figure 2A); and the others made no amino acid change. To examine whether allele variations affected its function, we transformed the cDNA of the *SBI* candidate gene from SV14 driven by its own promoter into rice variety Zhonghua 11. Phenotypes of the transgenic lines displayed a severely dwarfed phonotype (Figure 2C–2F). Meanwhile the transgenic lines expressing the Zhu1S form of *SBI* showed a dwarfed phonotype with much less severity (Supplemental Figures 6 and 7). These results suggest that the shortened basal internode phenotype is caused by this GA 2-oxidase gene in rice.

SBI Is Predominantly Expressed in Rice Culm

The rice (*Oryza sativa*) genome contains a family of 11 *GA2ox* homologs (Han and Zhu, 2011). SBI shares 17.9%–60.8% amino acid sequence identity with other members in the *GA2ox* family and was numbered as *GA2ox4* (Supplemental Figure 8A). To understand where they are expressed in the rice plant, we analyzed the expression of each member across various rice tissues or organs. The *SBI*-specific expression was confirmed by real-time qPCR measurement, which indicated that *SBI* was expressed with the highest level in culm basal internodes (Figure 2B). Consistent with this, information from analyzing public databases showed that *SBI* is also specifically expressed in rice culm (Supplemental Figure 8B). To further verify *SBI* expression, we cloned its promoter from both Zhu1S and SV14. The promoter activity was examined using a GUS (β-glucuronidase) report transformation system in rice. Both promoters showed strong GUS activity in culm basal internodes and little activity in leaves, spikelets, and roots (Supplemental Figure 9A–9F). These results suggest that *SBI* was specifically expressed in rice culm with its highest expression in basal internodes.

SBI Alleles in Zhu1s and SV14 Display Different Activities for GA Deactivation

To characterize the SBI biochemical function, we generated two SBI recombinant proteins from the Zhu1S (SBI^{Zhu1S}) and SV14

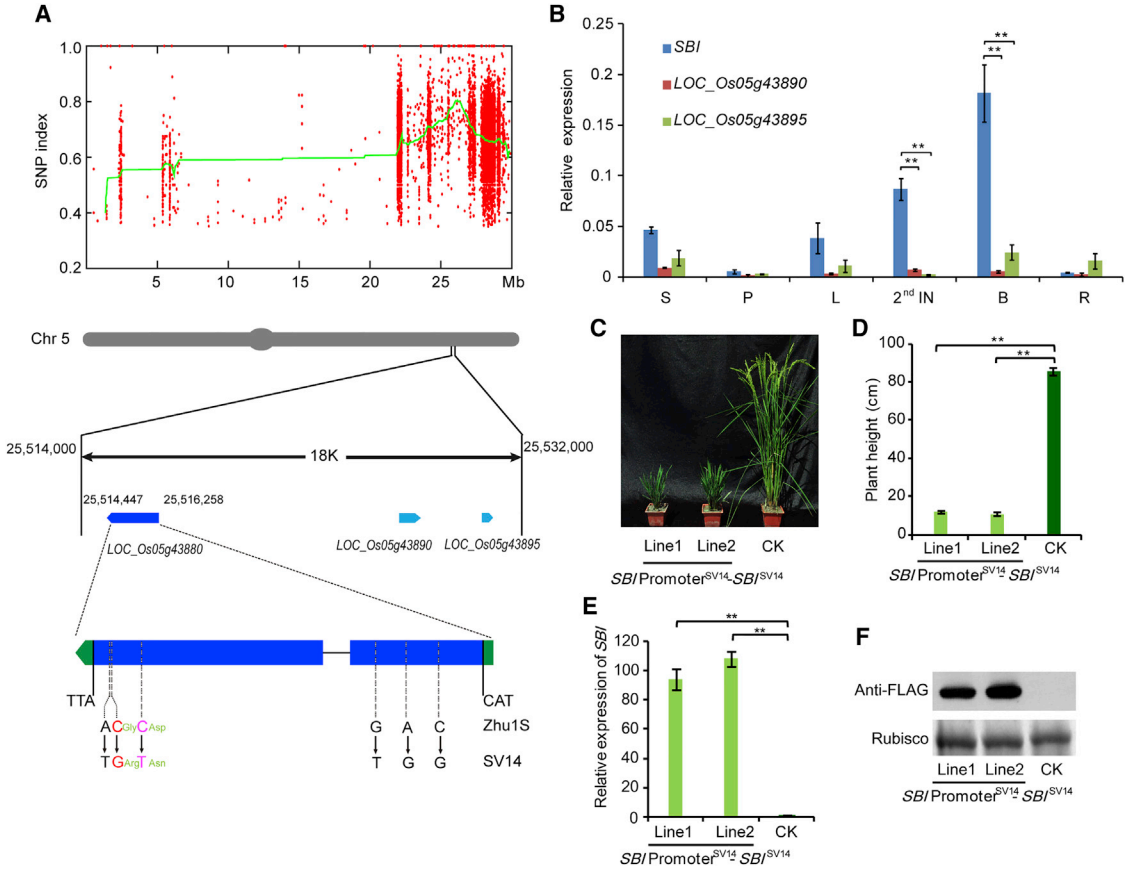

Figure 2. Identification of *SBI*.

(A) Map-based cloning of *SBI*. Top: SNP linkage analysis to localize *SBI* to a region on chromosome 5. Bottom: *SBI* structure and variations between SV14 and Zhu1S.

(B) Expression pattern of candidate genes. S, seedling; P, panicle; L, leaf blade; 2nd IN, second internode; B, basal internodes; R, root. Values are means ± SD (n = 3 biological repeats).

(C–F) Morphology of SBI^{SV14} overexpression plants. SBI^{SV14} cDNA driven by its native promoter from SV14 was transferred into ZH11. CK, transgenic negative control. Scale bar, 10 cm. Plant height **(D)**, qPCR analysis of expression of SBI^{SV14} **(E)**, and protein blot detection of exogenous SBI^{SV14}-FLAG protein in transgenic lines **(F)**. CK, transgenic negative control. Rubisco (stained with Coomassie brilliant blue) shows equal amount of total protein loading. Plant heights are means ± SD (n = 30 plants). Values are means ± SD (n = 3 biological repeats).

Statistical significance of differences calculated based on two-tailed, two-sample Student's t-test at $^{**}p < 0.01$.

(SBI^{SV14}) alleles, respectively, using an *Escherichia coli* expression system (Supplemental Figure 10). We analyzed the recombinant protein enzymatic activity using GA_1, GA_9, and GA_{20} as substrates. Results revealed that both recombinant proteins were able to convert GA_1, GA_9, and GA_{20} to their corresponding 2β-hydroxylated products GA_8, GA_{51}, and GA_{29}, respectively, as characterized by liquid chromatography combined with triple quadrupole mass spectrometry (LC-MS/MS) analysis (Supplemental Figures 11–13 and Supplemental Table 3). However, SBI^{SV14} displayed much stronger catalytic activity than SBI^{Zhu1S} (Figure 3A–3C).

To further verify SBI function *in vivo*, we determined the content of endogenous GAs in Zhu1S and SV14 basal internodes. SV14 contained significantly less active GA_1 and more inactive 2β-hydroxylated GA_8 than Zhu1S (Figure 3D–3E and Supplemental Table 4). Furthermore, in the seedlings of transgenic plants overexpressing SBI^{Zhu1S} or SBI^{SV14}, the GA_1 content was significantly reduced compared with the control (Figure 3F and Supplemental Table 4). By contrast, the content of inactive 2β-hydroxylated GA_{29} was much higher in transgenic plants (Figure 3G and Supplemental Table 4). Transgenic plants overexpressing SBI^{SV14} showed significantly more GA changes than those overexpressing SBI^{Zhu1S}. Together these results demonstrate that SBI^{SV14} encodes an enzyme with significantly stronger activity than SBI^{Zhu1S}.

To further elucidate the difference in activity of *SBI* alleles, we analyzed the two non-synonymous *SBI* variants between Zhu1S and SV14 alleles. SBI at the position 338 (Zhu1S, G338; SV14, R338) is localized at a conserved region in C_{19} OsGA2ox proteins, while position 308 (Zhu1S, D308; SV14, N308) is located in an unconserved region (Figure 4A). For validation of *SBI* variant function, we constructed SBI^{D308N} and SBI^{G338R} mutation vectors driven by the SBI^{SV14} promoter and transferred them into Zhonghua 11. All of the transgenic plants expressing SBI^{G338R} from 18 independent lines were severely dwarfed, while most of the transgenic plants expressing SBI^{D308N} were slightly shorter compared with the control (Figure 4B–4I). The endogenous active GA_1 content in these transgenic plants was

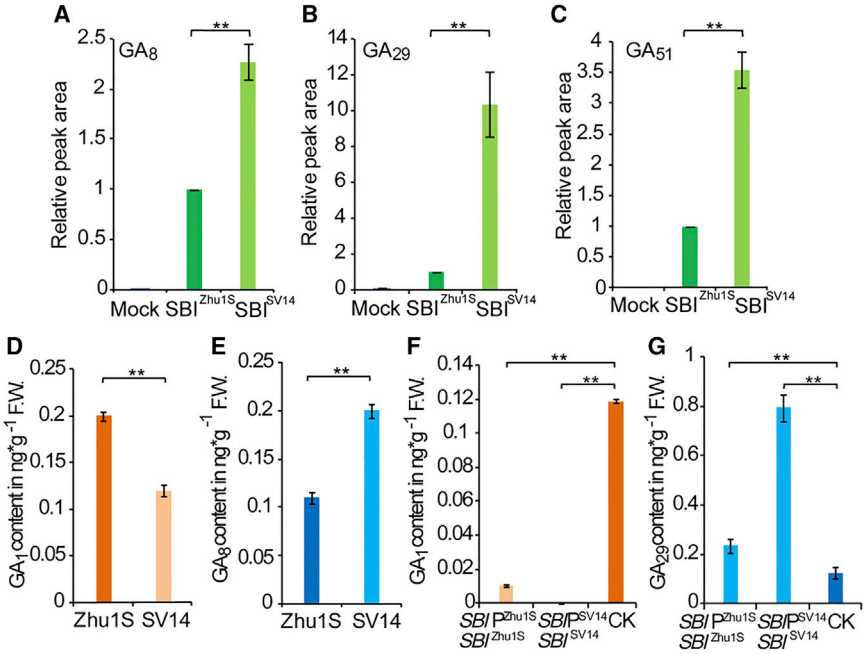

Figure 3. SBI^{SV14} Displayed Higher Catalytic Activity than SBI^{Zhu1S}.

(A–C) Conversion of GA_1 to GA_8 **(A)**, GA_{20} to GA_{29} **(B)**, and GA_9 to GA_{51} **(C)** by recombinant SBI^{Zhu1S} and SBI^{SV14} proteins. Reaction with MBP (maltose-binding protein) was used as mock group. GA content of recombinant SBI^{Zhu1S} was set to 1 for comparative analysis. Values are means ± SD (n = 3 replicates).

(D and E) GA_1 **(D)** and GA_8 **(E)** content measured in the basal internodes of Zhu1S and SV14 plants (8 weeks old). F.W., fresh weight.

(F and G) GA_1 **(C)** and GA_{29} **(D)** content measured in shoots of transgenic plants expressing *SBI*^{SV14} and *SBI*^{Zhu1S} under control of their native promoter (6 weeks old). CK, transgenic negative control; F.W., fresh weight.

Values in **(D)** to **(G)** are means ± SD (n = 3 replicates of five plants). Statistical significance of differences calculated based on two-tailed, two-sample Student's t-test at $^{**}p < 0.01$.

reduced while the endogenous inactive GA_{29} content was much higher in the transgenic plants. Furthermore, changes in GA_1 and GA_{29} content in transgenic plants expressing *SBI*^{G338R} were more significant than in *SBI*^{D308N} transgenic plants (Figure 4J–4K and Supplemental Table 4). These results revealed that SBI position 338 played a critical role in determining SBI activity in the conversion of active GA to inactive GA in transgenic plants.

SBI Alleles Affect Plant Height in Different Types of Rice Varieties

Conventional rice varieties were collected from several regions in China in order to further verify the effect of the *SBI* on rice height. Based on their genetic background, the conventional rice varieties (Huanzhan, Huanghuazhan, Meiyazhan, R1813, R608, R1128, R1141, 6439, CL161, 6377-c2662, and WYQ4) were divided into three groups with each group having a similar genetic background. The three group rice varieties were planted under the same conditions in our experimental farm and analyzed with *SBI* alleles, and phenotyped with plant height. The *SBI* allele sequence showed that SNP R338 displayed shorter plant height, while SNP G338 displayed higher plant height in all of the examined rice varieties (Figure 5A). The basal internode lengths were shorter in varieties with the SNP R338 allele than those with SNP G338 (Figure 5B). Furthermore, we integrated the *SBI*^{SV14} allele into a group of 21 rice varieties and analyzed their phenotypes in the F1 generation. The progeny with *SBI*^{SV14} integration displayed shortened plant height and basal internodes (Supplemental Table 5).

On the other hand, we analyzed the *SBI* alleles in 94 accessions of cultivated and wild rice varieties, including 20 from *Oryza indica*, 25 from *Oryza japonica*, and 49 from *Oryza rufipogon* (Huang et al., 2012) (Supplemental Table 6). Analysis indicated that the *SBI*^{SV14} allele was clustered with the *japonica* group and in association with *O. rufipogon* III type wild rice (Supplemental Figure 14), while the *SBI*^{Zhu1S} allele was associated with the cluster of *indica* group and *O. rufipogon* I type wild rice. These results suggest that *SBI* alleles in Zhu1S and SV14 are inherited from *indica* and *japonica* rice separately and that the genetic difference of *SBI* alleles between *indica* and *japonica* may result from their ancestor groups and are involved in shaping the height trait in rice breeding.

Breeding with the *SBI*^{SV14} Allele Improved Lodging Resistance and Grain Yield in Rice

Since SV14 displayed a semi-dwarf phenotype and otherwise normal agronomic traits (Liu et al., 2002), it was applied to breed rice varieties to improve lodging resistance. Integration of the *SBI*^{SV14} allele has generated more than 94 authorized new varieties that exhibit apparent enhanced lodging resistance compared with their parental varieties. To compare *SBI* alleles from Zhu1S or SV14, we carried out field trials for three pairs of the newly developed varieties, which were generated from the same chassis varieties by introducing the *SBI*^{SV14} or *SBI*^{Zhu1S} allele (Table 1) at 14–34 different field sites for trait assessment. With the introduction of the SV14 allele, the new varieties displayed significant improvements in terms of increased grain yield and enhanced height reduction and lodging resistance. Two varieties (Lingliangyou 22 and Lingliangyou 211) generated from the introduction of SV14 alleles have been widely planted across more than 366 000 hectares as they demonstrate superior performance in terms of lodging resistance in the field (Table 1). These field breeding practices validated the effectiveness of introducing the *SBI*^{SV14} allele for generating elite rice varieties with improved lodging resistance and increased grain yield.

DISCUSSION

Lodging resistance is an important trait necessary for achieving high grain yield in rice production (Khush, 1999). The success

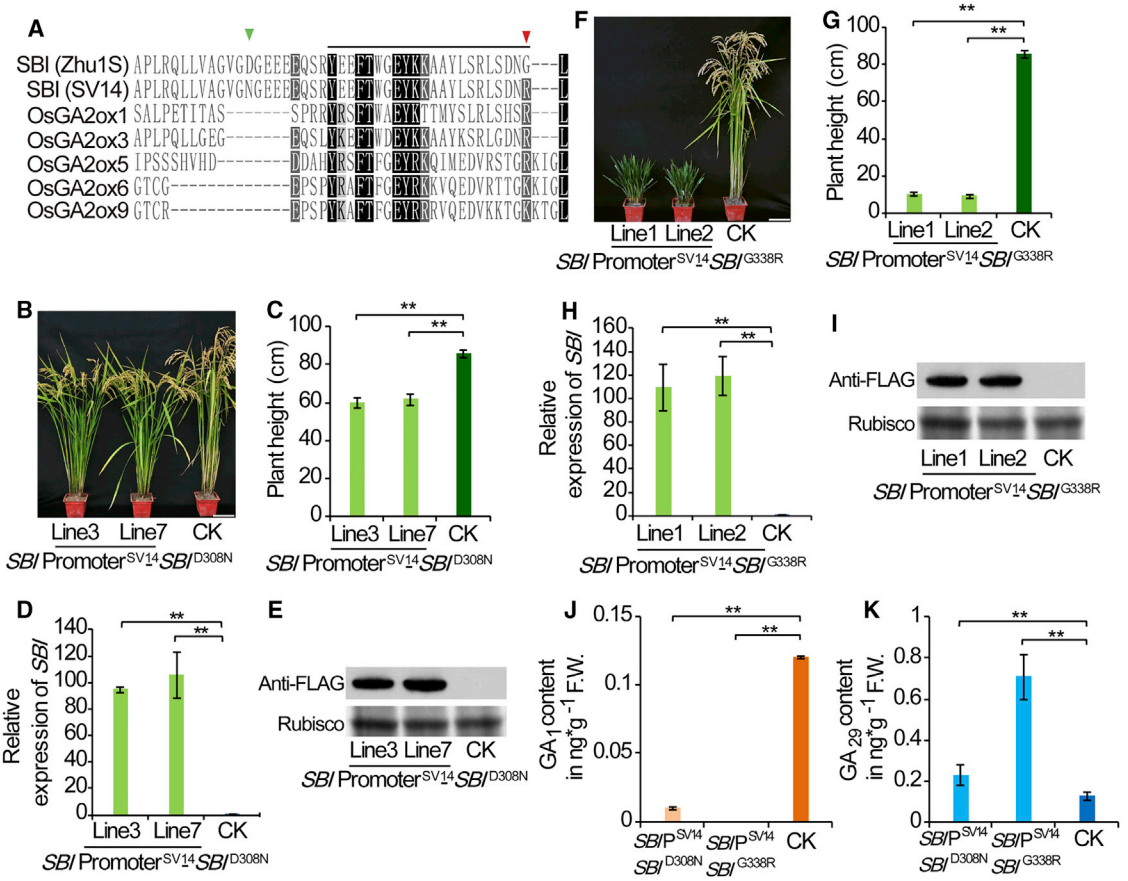

Figure 4. *SBI* Variations Regulated Rice Height and GA Deactivation *In Planta*.

(A) Alignment of SBI with other rice GA2ox proteins. The proteins were aligned with the MUSCLE method implemented in Geneious software. Black and gray boxes represent identical and similar amino acids, respectively. Black lines indicate a conserved domain. Green and red triangles represent the 308th and the 338th amino acids in the SBI protein.

(B) Morphology of SBI^{D308N} transgenic plants. *SBI* with the point mutation SBI^{D308N} was expressed in ZH11 under control of the SBI^{SV14} promoter. Scale bar, 10 cm.

(C) Plant heights are means ± SD (n = 30 plants).

(D and E) qPCR analysis of *SBI* expression level **(D)** and protein blot detection of exogenous SBI-FLAG protein **(E)** in transgenic lines of SBI^{D308N}. CK, transgenic negative control.

(F) Morphology of SBI^{G338R} transgenic plants. *SBI* with the point mutation SBI^{G338R} was expressed in ZH11 under control of the SBI^{SV14} promoter. Scale bar, 10 cm.

(G) Plant heights are means ± SD (n = 30 plants).

(H and I) qPCR analysis of *SBI* expression level **(H)** and protein blot detection of exogenous SBI-FLAG protein **(I)** in transgenic lines of SBI^{G338R}. CK, transgenic negative control.

(J and K) GA_1 **(J)** and GA_{29} **(K)** content measured in shoots of the transgenic plants (6 weeks old). F.W., fresh weight. Rubisco (stained with Coomassie brilliant blue) shows equal amount of total protein loading. Values are means ± SD (n = 3 replicates of five plants).

Statistical significance of differences calculated based on two-tailed, two-sample Student's t-test at $^{**}p < 0.01$.

of semi-dwarf rice in improving lodging resistance and increasing grain yield since the "green revolution" (Chandler and Robert, 1969; Xuchu, 1987) has resulted in a great deal of interest in finding semi-dwarf genes suitable for breeding elite rice varieties. Although more than 70 genes have been reported to manipulate plant height and cause dwarfism in rice, they are rarely applied to rice breeding due to defects caused to other agronomic traits (Sasaki et al., 2002). In this study, we mapped and cloned the semi-dwarf gene *SBI*, which particularly affected elongation of the rice basal internodes. *SBI* regulates rice height without interfering with other agronomic traits, a property that makes it highly valuable for breeding elite rice varieties with the desired lodging-resistant trait.

SBI belongs to the *GA2ox* family, which contains 11 gene members in the rice genome. These members are differentially expressed in various tissues and developmental stages. *SBI* displays preferential expression in culm, especially in the basal internodes. GA2ox members are thought to disable GA function by hydroxylating the C-2 position of active GAs or its precursors. Studies have shown that GA2oxs can be divided into two main groups according to their biochemical function: one group catalyzes the C-2 hydroxylation of C_{19}-GAs or GA precursors (such as GA_1, GA_4, GA_{20}, and GA_9) and the other group uses C_{20}-GA precursors (such as GA_{12} and GA_{53}) as substrates (Hedden and Thomas, 2012). OsGA2ox1 is reported to be able to hydroxylate GA_{44}, GA_{20}, GA_1, GA_9, and

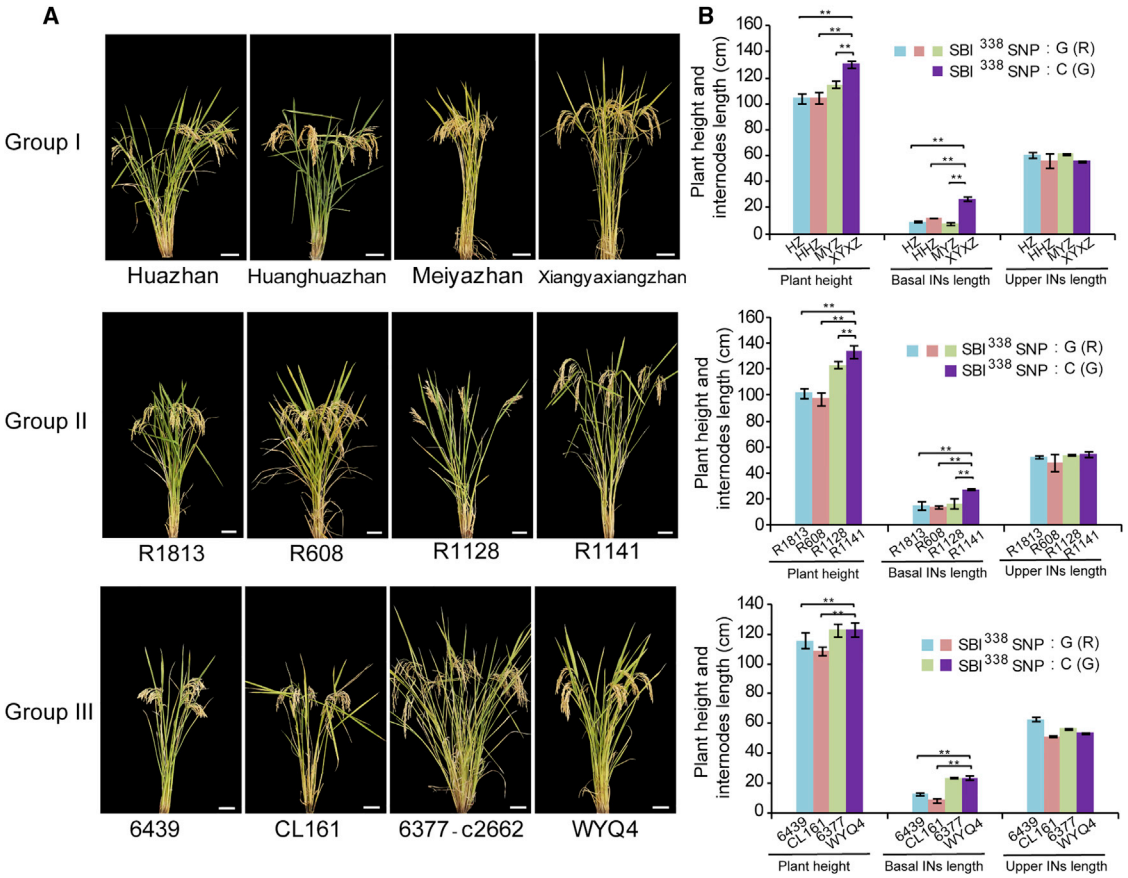

Figure 5. *SBI* Alleles Affected Rice Height Architecture.

(A) Height architecture of rice varieties grouped by similar genetic background. Scale bars, 10 cm.

(B) Plant height and length of upper internodes (1–3) and basal internodes (4–5) in association with *SBI* alleles. The corresponding amino acid is indicated in parentheses. HZ, Huazhan; HHZ, Huanghuazhan; MYZ, Meiyazhan; XYXZ, Xiangyaxiangzha; 6377, 6377-c2662. Values are means ± SD ($n = 30$ plants). Statistical significance of differences calculated based on two-tailed, two-sample Student's *t*-test at $^{**}p < 0.01$.

GA_4 (Sakamoto et al., 2001). OsGA2ox3 is shown to hydroxylate GA_{20} and GA_1 (Sakai et al., 2003). OsGA2ox6 is reported to have catalytic activities in converting GA_{12} to GA_{110} and GA_{53} to GA_{97}(Lo et al., 2008). Overexpression of several members in this family results in retardation of shoot elongation and increased numbers of tiller and root in transgenic rice (Sakai et al., 2003; Lo et al., 2008; Huang et al., 2010; Shan et al., 2014). Here, recombinant SBI (GA2ox4) displayed enzymatic activities in converting C_{19}-GA GA_1, GA_9, and GA_{20} to GA_8, GA_{51}, and GA_{29}, respectively (Figure 6). Overexpression of *SBI* in rice significantly increased GA_{29} and decreased GA_1. Transgenic rice displayed dwarf phenotypes due to reduction in active GA_1. The *SBI* allele variants displayed different GA2ox activities, which resulted in different content of GA_1 and GA_8 being accumulated in the culm basal internodes. Although *SBI* promoter from SV14 and Zhu1S showed slightly different activities, the transgenic plants of SBI^{SV14} driven by either SV14 or Zhu1S promoter displayed much more severe phenotypes than the SBI^{Zhu1S} transgenic plants. This suggests that the SBI^{SV14} format encoding the enzyme with a higher activity is the primary cause for the phenotype. Together, both *in vitro* and *in vivo* evidence revealed that the SNP G338R is a key site for modulating GA2ox activity in the process of culm elongation.

In rice, we found that G338 and R338 alleles were predominantly present in *indica* and *japonica* varieties, respectively. Two SBI variants with different enzyme activity may contribute to the modification of the culm basal internode length. Interestingly, only R338 position has been detected in other cereal species (Pearce et al., 2015). Analysis of the *SBI* alleles in 94 accessions of cultivated and wild rice showed that *SBI* alleles in *indica* may be sourced from *O. rufipogon* type I wild rice and *SBI* alleles in *japonica* may be sourced from *O. rufipogon* type III during domestication. This is consistent with the finding that *indica* rice originated from *O. rufipogon* type I wild rice while *japonica* rice originated from *O. rufipogon* type III wild rice (Huang et al., 2012). In the breeding process, introgression of *SBI* alleles from *japonica* rice into some *indica* landraces was observed and may contribute to the breeding selection of rice height structure.

sd1 alleles from IR8, a semi-dwarf rice variety, have been widely used to improve rice lodging resistance in Asia (Hargrove and Cabanilla, 1979). Semi-dwarf male sterile lines carrying the *sd1* allele have played an important role in the breeding of lodging-resistant and high-yield rice varieties (Asano et al., 2009). However, extensive use of the *sd1* may bring about genetic homogeneity and hinder rice improvement. Moreover, given

New variety	Chassis variety	Integration of SBI allele	Plant height (cm)	Culm length (cm)	1000 grains weight (g)	No. of trial sites	No. of lodging occurrence sites	Lodging occurrence (%)	Grain yield per 667 m^2 (kg)	Grain yield increase over chassis variety (%)	Cultivated area (10^3 ha)
Lingliangyou 22	Zhongzao 22	SBI^{SV14}	83.1 ± 2.2	64.1 ± 1.2	27.2 ± 1.1	28.0	0.0	0.0	516.7	8.2*	133.3
Zhuliangyou 22	Zhongzao 22	SBI^{Zhu1S}	89.3 ± 1.9	70.0 ± 0.9	27.6 ± 1.8	27.0	3.0	21.4	512.4		33.3
Lingliangyou 211	Hua 211	SBI^{SV14}	79.8 ± 1.8	61.1 ± 1.3	26.4 ± 1.8	31.0	0.0	0.0	511.4	9.3*	233.3
Zhuliangyou 211	Hua 211	SBI^{Zhu1S}	84.0 ± 1.5	65.0 ± 1.4	26.3 ± 1.7	34.0	7.0	20.6	491.5		46.7
Lingliangyou 179	Zhong 09-179	SBI^{SV14}	89.3 ± 1.4	69.3 ± 0.8	27.1 ± 2.1	14.0	0.0	0.0	543.1	5.0*	
Zhuliangyou 179	Zhong 09-179	SBI^{Zhu1S}	93.0 ± 1.5	72.7 ± 0.9	26.8 ± 1.9	14.0	3.0	11.1	526.3		

Table 1. Field Performance of the New Varieties Integrated with *SBI* Alleles.

Yield performance and lodging resistance of new varieties Lingliangyou 22, Lingliangyou 211, and Lingliangyou 179 are significantly improved compared with the chassis varieties Zhuliangyou 22, Zhuliangyou211, and Zhuliangyou 179 in the field tests of different cultivation regions during 2008–2015. Values in plant height, culm length, and 1000 grains weight are means ± SD, and asterisk indicates significant difference of yield increase in comparison with chassis varieties determined by the Student's *t*-test at $p < 0.05$ ($n = 6$).

that *sd1* is a recessive gene, both parents used for crossing should carry the same *sd1* allele for hybrid F1 plants to show a semi-dwarf trait. In contrast, as *SBI* is a semi-dominant gene there is no restriction for selecting the genotype of the hybrid male, which simplifies the acquisition of a hybrid male parent.

In traditional rice semi-dwarf varieties, the length of all internodes was reduced (Spielmeyer et al., 2002). Short length of basal internodes can help build an ideal culm structure for lodging resistance while reduction of the upper internode length may have adverse effects on panicle exertion (Zhu et al., 2006; Liu and Li, 2016). Elongation of different internodes may be regulated by different genes. Loss of function or downregulation of *eui1* (*elongated uppermost internode 1*), which encodes a P450, CYP714D1, gave rise to the elongated uppermost internode (Luo et al., 2006; Zhu et al., 2006; Gao et al., 2016). The double mutant of two GA 13-oxidase genes *cyp714b1cyp714b2* also had a longer uppermost internode (Magome et al., 2013). Here we find that *SBI* is primarily expressed in rice culm basal internodes, regulating the basal internode length (Figure 6). Finding the semi-dominant *SBI*, which enables modulation of the length of the culm basal internodes, opens a new avenue to improve rice lodging resistance. In our rice breeding program, nearly 100 rice varieties with improved lodging resistance have been developed since 2002 by introducing the SBI^{SV14} allele. Male sterile lines carrying SBI^{SV14} can be crossed with high-culm varieties to facilitate machine-assisted pollination, from which the F1 hybrids can be used to select for the SBI trait. Applications of the SBI^{SV14} allele in rice breeding have been demonstrated to be an efficient strategy to develop elite rice varieties with improved lodging resistance and increased yield.

METHODS

Plant Material and Phenotyping

Rice varieties Zhu1S and SV14 are thermosensitive male sterile lines used for breeding in the Hunan Ava Seed Research Institute. F1 progeny plants were generated by reciprocal cross between Zhu1S (female/male) and SV14 (male/female). F1 plants from the cross of Zhu1S (female) and SV14 (male) were then self-pollinated and more than 1000 F2 progeny plants were generated for phenotypic investigation and genetic analysis. Rice varieties used for phenotype analysis shown in Figure 5 were as follows: Huanzhan, Huanghuazhan, Meiyazhan, and Xiangyaxiangzhan were from Guangdong province, China; R1813, R608, R1128, R1141, 6439, CL161, 6377-c2662, and WYQ4 were from Hunan province, China. For genome analysis, the wild and cultivated rice landraces were from The National Center for Gene Research of Chinese Academy of Sciences (Huang et al., 2012). For seedling analysis, rice plants were grown in a phytotron at 28°C and 50% relative humidity under a 13-h light/11-h dark photoperiod. For field assessment of phenotypes, rice varieties were planted at the Institute's experimental farm in Shanghai (31.12° N, 121.3° E) and Lingshui (18.22° N, 110.08° E), Hainan province, China.

Plasmid Construction and Transformation

To overexpress *SBI* in rice, we amplified by PCR a fragment of 2422-bp promoter upstream of the *SBI* start code from SV14 genomic DNA and *SBI* cDNAs from Zhu1S and SV14 (Supplemental Table 7). For promoter activity analysis, The *SBI* promoter was subcloned into a pCAMBIA1301 vector upstream of *GUS* report gene. For overexpression, *SBI* cDNA was constructed in a binary vector

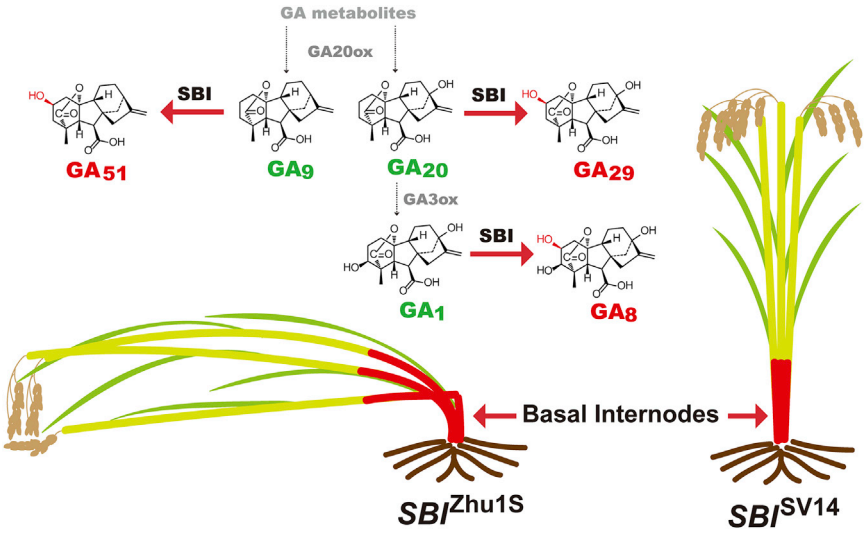

Figure 6. A Model Illustrating that *SBI* Encodes a GA 2-Oxidase for GA Deactivation and Contributes to Lodging Resistance in Rice.

SBI is primarily expressed in basal internodes of rice culm. SBI catalyzes the conversion of bioactive GA metabolites (indicated in green) to inactive GA metabolites (indicated in red). SBI^{SV14} displays much higher activity than SBI^{Zhu1S}, and leads to reduction of the basal internodes length and increase of lodging resistance. Red arrows and hydroxy group denote reactions catalyzed by SBI. Black dotted arrows indicate the GA metabolic pathways catalyzed by other enzymes.

pCAMBIA1300 with a fusion of 3×FLAG tag at the C terminus under the control of *SBI* promoter. For mutation analysis, SBI^{SV14} cDNA sequence was used as a template to generate SBI^{D308N} and SBI^{G338R} single point mutations using Hieff Mut Site-Directed Mutagenesis Kit (Yeasen Biotech). Accuracy of all binary vector constructs was confirmed by sequencing. After being moved into *Agrobacterium tumefaciens* strain EHA105, the aforementioned constructed plasmids were transferred into rice Zhonghua 11, Zhu1S, and SV14. At least 25 independent transgenic lines were identified for each construct transformed. Homozygous T3 transgenic lines for Zhonghua 11 transformation and T0 transgenic lines for Zhu1S and SV14 transformation verified by PCR were used in this study, and transgenic plant with empty vector was used as a control.

Gene Expression Analysis

Total RNA from various tissues at different growth stages was extracted using an E.Z.N.A. Total RNA Kit (Omega) following the user manual. First-strand cDNA was synthesized using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kits (Transgene). qPCR assay was conducted using an SYBR Green method (TransStart Top Green qPCR mix) with a MyiQ real-time PCR detection system (Bio-Rad). Gene-specific primers (Supplemental Table 7) were designed using the online tool Quantprimer (http://www.quantprime.de/). The rice *OsUBQ5* was selected as an internal control to normalize the data. Validation of the qPCR data was done with at least three biological replications.

To verify the overexpression of *SBI* in transgenic rice, we extracted total proteins from the culm tissue of transgenic plants with 2× SDS loading buffer (100 mM Tris–HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol, 1% 2-mercaptoethanol). The protein was separated through SDS–PAGE and immunoblotted with anti-FLAG antibodies (Abmart, M20008). For histological examination of the expression of *SBI* promoter , GUS staining and activity assays were carried out as described by Gui et al. (2011). The data were obtained from at least three biological replications.

Tissue Sectioning and Staining

Elongated mature tissue of the fifth internode was cut into ~0.2 cm length and fixed in 5% formaldehyde with 5% acetic acid and 70% ethanol solution overnight at 4°C. The samples were then dehydrated with a graded ethanol series and a graded dimethylbenzene series. The samples were then embedded in Paraplast (Sigma, P3683), cut into 10-μm sections, and mounted onto slides. The prepared sections were stained in 0.1% toluidine blue solution (0.01 M sodium acetate buffer [pH 4.5]) for 30 min, washed by clean water, and dried in a 37°C oven. The sections were then dewaxed twice in dimethylbenzene, each time for 10 min, and sealed with neutral resin size. Slides were observed with a microscope and photographed. Cell length was measured using ImageJ software, and cell number in the fifth internode was calculated based on the internode length.

Genome Sequencing and Analysis

DNA was extracted from 1 g of rice leaves using the CTAB method. DNA was quantified using Quant-iT PicoGreen dsDNA reagent and kits (Invitrogen). DNA from F2 progeny individuals was mixed in an equal ratio. One microgram of DNA was used for library construction. The DNA libraries were then used for cluster generation on a flow cell and sequenced for 100 cycles on an Illumina Hiseq 2000. Raw sequencing data were trimmed using an import module of the SHORE software package (Ossowski et al., 2008). The reference genomes of *indica* and *japonica* rice were downloaded from the public databases http://plants.ensembl.org/Oryza_indica/Info/Annotation/ and http://rgp.dna.affrc.go.jp/E/IRGSP/Build4/build4.html, respectively. For genome comparison of Zhu1S and SV14, the genome sequence of *indica* rice 93-11 was modeled as sequencing data with 60× read depth and aligned to the *japonica* rice reference genome IRGSP4.0. A total of 2 760 832 homozygous SNPs and InDels with an average quality score ≥20 were selected as the genetic different loci between *japonica* and *indica*. Paired-end sequence reads of Zhu1S and SV14 were aligned to the reference sequence IRGSP using BWA software (Li and Durbin, 2009). SNPs and InDels were called using SHORE software consensus module (Ossowski et al., 2008) and compared with the genetic different loci between *japonica* and *indica* identified above. The SNPs and InDels were divided into three categories: homozygous sites consistent with 93-11, homozygous sites consistent with Nipponbare, and heterozygous sites or other mutations, which were scored with 1, 0, and 0.5, respectively. Regressions were calculated and shown in line graphs by averaging scores from a 100-kb window size and 10-kb increment.

Map-Based Sequencing of *SBI*

To identify the causal gene underlying the dwarf phenotype, we generated a reference sequence of Zhu1S on the basis of public Nipponbare rice genome sequence. 100 062 179 reliable paired-end reads of Zhu1S were aligned to Nipponbare sequence, and 2 494 971 SNPs and InDels were identified. The Zhu1S reference sequence was then produced by replacing Nipponbare nucleotides with those sites mentioned above.

76 573 815 reliable paired-end reads from the bulked DNA of SV14 F2 progeny were aligned to the Zhu1S reference sequence. SNPs were scored using the SHORE software and divided into homozygous or heterozygous groups. Homozygous SNPs were defined as SNPs having an

SNP index ≥0.8 with a coverage of the position of more than four reads; heterozygous SNPs were defined as SNPs with an SNP index ≥0.25 and <0.8 with a minimum coverage of four reads. Allele frequency regression lines of SNP index plots were generated by averaging scores in a 2-Mb window size and sliding the window with 10-kb increments.

Determination of Endogenous GAs

Zhu1S and SV14 were grown in field conditions for approximately 8 weeks. Transgenic rice was grown in a phytotron for 6 weeks. The elongating fourth and fifth internodes of Zhu1S and SV14 and the shoots of the transgenic rice were collected for GA content analysis with both high-performance liquid chromatography (HPLC) fluorescence and LC-MS/MS methods (Supplemental Table 4) according to Wild et al. (2012) and Lu et al. (2016). Each sample was mixed with equal amounts of at least three independent plants, and three samples were used for each determination.

Assay of Recombinant SBI Enzyme Activity

The full coding length cDNA of *SBI* from Zhu1S and SV14 was subcloned into pMAL-C5X (NEB) vector using primers pMALSBIF and pMALSBIR (Supplemental Table 7). Construct was confirmed and transformed into *E. coli* Transetta (DE3) (Transgene) for recombinant protein expression. The non-carrier pMAL-C5X vector was used in the mock group. The *E. coli* transformants were incubated in 100 ml of Luria–Bertani medium containing 100 mg/l ampicillin at 37°C until reaching OD_{600} of 0.6. Induction of the recombinant protein was performed by adding 0.5 mM isopropyl-β-D-thiogalactoside and incubated at 16°C for another 12 h. After harvest by centrifugation, the cells were resuspended in lysis buffer containing 100 mM Tris–HCl (pH 7.5) and 5 mM dithiothreitol (DTT) and were disintegrated with 20 kHz ultrasonication and 200 W acoustic power for 10 min (5 s work/10 s rest). Cell debris was pelleted by centrifugation and supernatant was concentrated through a 30K centrifugal filter device (Millipore Amicon Ultra-15). Recombinant proteins were analyzed by western blotting and adjusted to approximate equal amounts in 100 μg of total protein. For enzyme activity assay, the procedure was adapted from previous studies (Sakamoto et al., 2001; Lee and Zeevaart, 2002, 2005) with slight modification. One hundred micrograms of total protein was incubated with GA metabolites (1 μg) in 100 μl of reaction mixture containing 100 mM Tris–HCl (pH 7.5), 1 mM $FeSO_4$, 10 mM 2-oxoglutarate, 10 mM ascorbate, and 5 mM DTT at 30°C for up to 6 h. After incubation, 100 μl of methanol was added and mixed. After centrifugation the supernatant was collected for analysis by LC-MS/MS (Thermo Scientific Dionex UltiMate 3000, TSQ Quantum Access MAX triple quadrupole mass spectrometer). A C18 HPLC capillary column (Thermo Scientific Hypersil GOLD, 50 mm × 2.1 mm × 1.9 μm) was used for separation. Mobile phase was composed of solvent A (0.1% formic acid) and solvent B (100% MeOH). Solvent gradient was programmed as 15% of solvent B for 2 min, increasing solvent B to 95% over 15 min, holding for 3 min, and returning back to 5% of solvent B over 2 min, followed by a 5-min re-equilibration prior to the next sample injection. Flow rate was maintained at 0.3 ml/min. Each sample injection volume was 15 μl. MS spectra were acquired in negative electrospray ionization combined with the SRM (selected-reaction monitoring) mode (Urbanová et al., 2013) and analyzed using Qual Browser software (Thermo Xcalibur 3.0.63).

Phylogenetic Analysis and Microarray Analysis

Alignment of OsGA2ox family protein sequences was conducted by ClustalW based on sequences from Phytozome v12 (https://phytozome.jgi.doe.gov/pz/portal.html). A maximum likelihood (ML) phylogenetic tree was constructed using MEGA6 (Tamura et al., 2013). DNA sequences of the *SBI* in rice accessions of cultivated and wild rice were cloned by overlapping PCR with primers SBI1 to SBI5 (Supplemental Table 7), and sequenced and subjected to haplotype analysis using the ML method implemented in MEGA6 (Tamura et al., 2013). *SBI* sequence from African rice was obtained by BLAST against http://ensembl.gramene.org/Oryza_glaberrima/Info/Index and http://ensembl.gramene.org/Oryza_barthii/Info/Index (Wang et al., 2014). Expression profiling of *OsGA2ox* family genes was retrieved from the GEO database for microarray data GEO: GSE21396. The heatmap was analyzed and downloaded from RiceXPro (http://ricexpro.dna.affrc.go.jp/) (Sato et al., 2011).

Rice Breeding Utilizing *SBI* Alleles and Field Trials

To improve lodging-resistant traits, we introduced the SBI^{SV14} allele in a number of chassis varieties by crossing with Xiangling 628S, a thermosensitive genic male sterile line derived from SV14 and harboring the SBI^{SV14} allele (Fu et al., 2010). By screening for improved lodging-resistant traits in F2 progenies, 94 new varieties have been selected and trialed in different districts for trait assessment. For verification of *SBI* allele effect, SBI^{SV14} or SBI^{Zhu1} allele was integrated into the same chassis varieties and the derived new varieties were compared with assess traits in the same trial sites.

ACCESSION NUMBERS AND GENE LOCI

Gene sequence data from this article can be found in the GenBank database under accession numbers as follows: SBI^{SV14} (MF574209), SBI^{Zhu1S} (MF574210).

The gene loci can be found in Phytozome 12.1.2 as follows:

OsUBQ5, LOC_Os01g22490; *OsGA2ox1*, LOC_Os05g06670; *OsGA2ox2*, LOC_Os01g22920; *OsGA2ox3*, LOC_Os01g55240; *SBI* (*OsGA2ox4*), LOC_Os05g43880; *OsGA2ox5*, LOC_Os07g01340; *OsGA2ox6*, LOC_Os04g44150; *OsGA2ox7*, LOC_Os01g11150; *OsGA2ox8*, LOC_Os05g48700; *OsGA2ox9*, LOC_Os02g41954; *OsGA2ox10*, LOC_Os05g11810; *OsGA2ox11*, LOC_Os04g33360.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

C.L. and L.L. designed the research; C.L., S.Z., J.G., D.S., H.Y., and J.S. performed experiments; Y.Y., C.F., P.Q., and X.L. contributed to elite variety breeding and field trials; B.H. provided rice germplasms and genome sequence analysis; C.L. and L.L. analyzed data and wrote the paper.

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