Map-Based Cloning of *Seed Dormancy1-2* Identified a Gibberellin Synthesis Gene Regulating the Development of Endosperm-Imposed Dormancy in Rice[1]

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Natural variation in seed dormancy is controlled by multiple genes mapped as quantitative trait loci in major crop or model plants. This research aimed to clone and characterize the *Seed Dormancy1-2* (*qSD1-2*) locus associated with endosperm-imposed dormancy and plant height in rice (*Oryza sativa*). *qSD1-2* was delimited to a 20-kb region, which contains *OsGA20ox2* and had an additive effect on germination. Naturally occurring or induced loss-of-function mutations of the gibberellin (GA) synthesis gene enhanced seed dormancy and also reduced plant height. Expression of this gene in seeds (including endospermic cells) during early development increased GA accumulation to promote tissue morphogenesis and maturation programs. The mutant allele prevalent in semidwarf cultivars reduced the seed GA content by up to 2-fold at the early stage, which decelerated tissue morphogenesis including endosperm cell differentiation, delayed abscisic acid accumulation by a shift in the temporal distribution pattern, and postponed dehydration, physiological maturity, and germinability development. As the endosperm of developing seeds dominates the moisture equilibrium and desiccation status of the embryo in cereal crops, *qSD1-2* is proposed to control primary dormancy by a GA-regulated dehydration mechanism. Allelic distribution of *OsGA20ox2*, the rice Green Revolution gene, was associated with the *indica* and *japonica* subspeciation. However, this research provided no evidence that the primitive *indica*- and common *japonica*-specific alleles at the presumably domestication-related locus functionally differentiate in plant height and seed dormancy. Thus, the evolutionary mechanism of this agriculturally important gene remains open for discussion.

Seed dormancy is primarily developed on the plant before maturation as an adaptive trait to regulate the timing of germination and seasonal fluctuations in plant density. Natural variation of the trait is controlled by multiple genes, which have been mapped as quantitative trait loci (QTLs) in crop (cereal, oil, vegetable, and fruit) or model plants (Anderson et al., 1993; Ullrich et al., 1993; Lin et al., 1998; Fennimore et al., 1999; Lijavetzky et al., 2000; Alonso-Blanco et al., 2003; Argyris et al., 2005; Gandhi et al., 2005; Masojć et al., 2007; Blaker et al., 2013; Schatzki et al., 2013). Allelic variants of a validated QTL for seed dormancy were naturally occurring and may have been selected for the trait itself or for dormancy-interrelated characteristics during evolution or domestication (Gu et al., 2005). Cloning and characterization of the QTLs underlying genes will provide in-depth insights into developmental and evolutionary mechanisms of seed dormancy. This knowledge is also important for an efficient use of the QTL alleles to manipulate germination capability in crop breeding.

Seed development starts with tissue morphogenesis (including double fertilization and cell division, differentiation, or expansion) followed by maturation programs (e.g. nutrient reserve deposition, dehydration, acquisition of desiccation tolerance, and programmed cell death to form protective structures). The GA and abscisic acid (ABA) hormones are involved in regulating the sequential development programs. In cereal crops, such as rice (*Oryza sativa*) varieties that were

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selected for weak or no dormancy to obtain uniform germination, the seed GA content started to decline rapidly from the highest level at a few DPA; with the GA declining, the ABA content kept increasing and reached the peak level when the endosperm cell number and grain-filling rate approached the maximum (Zhang et al., 2009). Mutagenesis analysis revealed that developing seeds require a given level of ABA to prevent viviparous germination in maize (*Zea mays*) and rice (Robertson, 1955; Agrawal et al., 2001; Fang et al., 2008). Introduction of the GA-deficient allele *dwarf1* into the maize *viviparous5* background resulted in desiccation-tolerant seeds, suggesting that the ABA-to-GA ratio (or balance) could be more important than the level of individual hormones in the induction of maturation programs (White and Rivin, 2000). The hormone balance hypothesis was often used to synthesize germination data, largely from mutagenesis analysis, to infer regulatory mechanisms of seed dormancy (Kucera et al., 2005; Yamaguchi et al., 2007; Finkelstein et al., 2008). However, it is unclear to what extent an ABA-to-GA balance generated by an artificial mutant could represent a natural balance selected for a dormant genotype and if the two hormones synthesized at different stages act antagonistically with each other, or in concert, to influence the development of seed and primary dormancy. In addition, of the genes cloned from the QTLs for primary or conditional dormancy, such as *DELAY OF GERMINATION1* and *IBO* in Arabidopsis (*Arabidopsis thaliana*; Bentsink et al., 2006; Amiguet-Vercher et al., 2015), *Seed dormancy4* (*Sdr4*) and *qSD7-1* in rice (Sugimoto et al., 2010; Gu et al., 2011), *QPhs.ocs-3A.1/pseru-3AS* in wheat (*Triticum aestivum*; Nakamura et al., 2011; Liu et al., 2013), and *High temperature germination6.1* (*Htg6.1*) in lettuce (*Lactuca sativa*; Huo et al., 2013), only *qSD7-1* (a transcription factor gene) and *Htg6.1* (an ABA synthesis enzyme gene) are known to regulate ABA accumulation in developing or mature seeds, and the other loci are not involved in GA or ABA biosynthesis or signaling. Therefore, the hormone balance hypothesis for the development of seed dormancy has yet to be tested with natural variants.

Bioactive GAs promote stem/cell elongation and seed germination through the GA signaling pathway (Sun and Gubler, 2004; Steber, 2007). During germination, GA synthesized in the embryo is released to the endosperm aleurone cells to trigger GA signaling for the synthesis of hydrolases to soften cell walls of the covering tissues and break down nutrient reserves. For dicot (e.g., Arabidopsis, *Lepidium sativum*, and tomato [*Solanum lycopersicum*]) seeds, the embryo is enclosed by one or a few layers of endospermic cells, and the micropylar portion (endosperm cap) is believed to serve as a mechanical constraint to radicle emergence; GA application could induce endosperm cap-specific gene expression to release the constraint (Müller et al., 2006; Martínez-Andújar et al., 2012). For cereal seeds, with a small lateral embryo partially surrounded by the endosperm tissue, the latter may impose dormancy through a different mechanism.

This research aimed to clone and characterize *qSD1-2*, a QTL that was identified in different mapping populations and associated with endosperm-imposed dormancy and plant height in rice (Ye et al., 2010, 2013; Mispan et al., 2013; Gu et al., 2015). In addition, the *qSD1-2* region was reported for the *semidwarf1* (*sd1*) gene, the plant height QTLs *CULM LENGTH 1a* (*qCL1a*), *qCL1b*, *ph1*, and *qPH1*, and the QTLs associated with root, seedling, and panicle growth, flowering time, and seed setting (Asano et al., 2011; Kovi et al., 2011; Mispan et al., 2013; Zhang et al., 2013). Thus, the objectives of this research were (1) to isolate the *qSD1-2* underlying gene from the multi-QTL region and confirm its functions; (2) to identify the molecular and physiological mechanisms of dormancy development regulated by the cloned and confirmed gene; and (3) to determine the origin, distribution, and functional differentiation of major alleles at the seed dormancy locus.

RESULTS

Fine-Mapping Identified *OsGA20ox2* as the Only Candidate Gene for *qSD1-2*

qSD1-2 was colocated with *qPH1* on a genomic region of approximately 400 kilo base pairs (Kb), and the QTL-containing region from SS18-2 (weedy rice) was introduced into the EM93-1 (ssp. *indica*) background to identify recombinants from an F2 population (Supplemental Fig. S1, A and D). A total of 32 recombinants between the m2 and RM11988 markers were identified from 2,592 F2 plants (5,184 gametes) and genotyped with new markers (Supplemental Table S1) to distribute the recombinant events on a partial high-resolution map (Fig. 1A). Ten recombinants (R numbers in Fig. 1B) selected from seven intervals on the map were advanced to the F3 generation to test for marker-trait associations in the progeny lines. Significant correlations between marker genotypes and trait values for germination (r_g = 0.44–0.57) and plant height (r_h = 0.96–0.99) were detected in six of the 10 F3 lines (Fig. 1B), and the associations were centered on the m2 to m6 interval of approximately 20 Kb. It was estimated, based on data from the six lines, that the 20-Kb interval had an additive effect on germination, which accounted for 19% to 32% of the phenotypic variances, and also had additive and dominance effects on plant height, which together accounted for greater than 90% of the phenotypic variances (Supplemental Table S2). The marker-trait correlations were not significant in the remaining F3 lines (Fig. 1B), as they were fixed for the *qSD1-2/qPH1* alleles from EM93-1 (R05) or SS18-2 (R03, R10, and R13; Supplemental Table S1). The absence of correlation in the four progeny lines indicates that there is no seed dormancy or plant height gene, which is differentiated between EM93-1 and SS18-2, in flanking regions of the 20-Kb interval.

Five homozygotes were selected from the F3 lines as a set of isogenic lines for single subsegments from

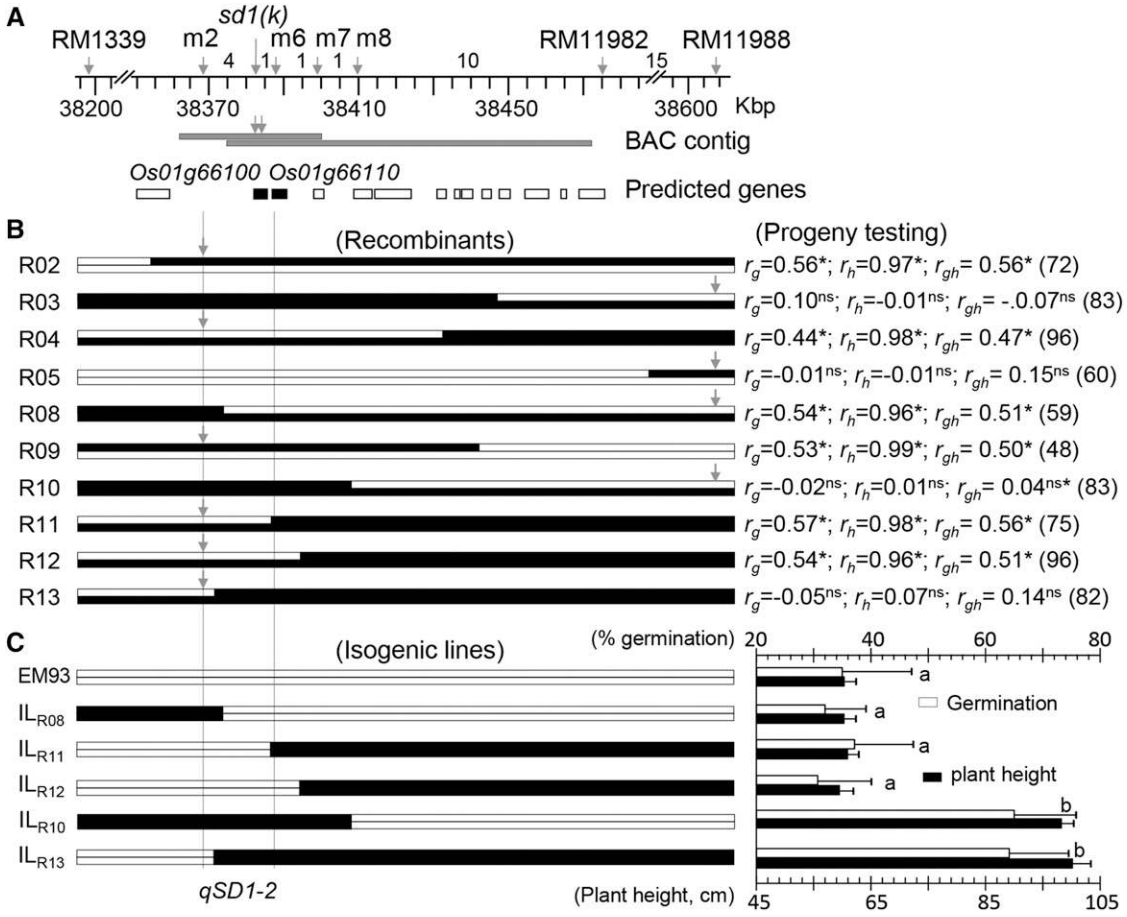

Figure 1. Fine-mapping of *qSD1-2*. A, Partial high-resolution map. Arrows indicate marker positions on the reference genome (Kawahara et al., 2013). Figures between markers are numbers of recombinant events identified from 5,184 gametes. Horizontal bars below the map are a bacterial artificial chromosome (BAC) contig screened using the probes (arrows). Boxes below the contig are predicted genes on the reference genome, and genes in the narrowed QTL region are depicted by black color. B, Recombinants and progeny testing. Recombinants (R numbers) for the QTL-containing region are represented by two alleles (chromosomal segments) from SS18-2 (black bars) and/or EM93-1 (white bars). Arrows indicate markers used to genotype the progeny lines. At right, correlation coefficients between marker genotypes and trait values for germination (r_g) or plant height (r_h) in the lines of *n* plants are shown. Superscripts indicate the correlations significant at $P < 0.001$ (*) or not significant (ns). Vertical dotted lines delimit the narrowed *qSD1-2* region. Additional statistical and genetic parameters for the lines are listed in Supplemental Table S2. C, Genotypic differences in seed dormancy and plant height among isogenic lines. The isogenic lines were selected from the progeny lines. At right, genotypic means ± SD of 20 to 24 plants for germination (%) and plant height (cm) are shown. The letters a and b indicate results from Duncan's multiple comparison test for not significant (same letter) and significant (different letter) at $P < 0.0001$.

SS18-2 in the EM93-1 background. The five isogenic lines fell into two groups based on phenotypes evaluated under the same conditions (Fig. 1C). Group I lines (IL_{R08}, IL_{R11}, and IL_{R12}) were similar to EM93-1 for germination percentage and plant height and displayed stronger dormancy and shorter stature than group II lines (IL_{R10} and IL_{R13}). Results from these isogenic lines confirmed the pleiotropic effects of the 20-Kb interval and that the allele from EM93-1 inhibits germination and stem elongation.

The 20-Kb interval encompasses the locus *Os01g66100*, its flanking intergenic sequences, and part (less than 1 Kb of the 3′ end) of the locus *Os01g66110* (Fig. 1A), as predicted based on the genome sequence for the *japonica* cv Nipponbare (Kawahara et al., 2013). We sequenced the 3′ end of *Os01g66110* from EM93-1 and SS18-2 and found no sequence variation between the parental lines. Therefore, *Os01g66100*, which was annotated as the GA synthesis gene *OsGA20ox2*, was identified as the only candidate gene for *qSD1-2*.

Loss-of-Function Mutations of *OsGA20ox2* Enhanced Seed Dormancy

The *Os01g66100* allele from SS18-2 ($SD_{1\text{-}2}^{S}$) was isolated from a BAC library (Gu et al., 2010). A BAC contig was aligned across the 20-Kb interval (Fig. 1A) and

used to sequence $SD_{1\text{-}2}{}^{S}$. The sequence was predicted to be 3,183 bp in length and consist of three exons, with untranslated regions (UTRs) in exons 1 (5′ UTR) and 3 (3′ UTR; Fig. 2A). To confirm the gene model, we cloned and sequenced two overlapped complementary DNA (cDNA) fragments (Fig. 2B) from IL_{R10} (Fig. 1C), an isogenic line for $SD_{1\text{-}2}{}^{S}$ ($IL_{SD1\text{-}2}{}^{S}$). An assembly of the cDNA fragments is identical to the predicted mRNA sequence for the three exons. The protein sequence deduced from the coding sequence consists of 389 amino acid residues and contains two domains for dioxygenese or oxygenese functions (Fig. 2C). The sequence data suggest that $SD_{1\text{-}2}{}^{S}$ could be a functional GA 20-oxidase.

The *Os01g66100* allele of 2,803 bp from EM93-1 ($SD_{1\text{-}2}{}^{E}$) was cloned by PCR and direct sequencing. Alignment of the $SD_{1\text{-}2}{}^{E}$ with the $SD_{1\text{-}2}{}^{S}$ sequence detected eight point mutations, including a 382-bp deletion (Fig. 2A). This deletion removed intron 1 (102 bp) and its flanking sequences of exons 1 (262 bp) and 2 (18 bp; Fig. 2A), reduced the 5′ end cDNA fragment size (Fig. 2B), and generated a premature stop codon in exon 2, resulting in loss of the two predicted domains (Fig. 2C). The sequence variation suggests that the dormancy-enhancing allele $SD_{1\text{-}2}{}^{E}$ is a loss-of-function allele.

To prove that the loss-of-function mutation could result in enhanced seed dormancy, we analyzed a transfer DNA (T-DNA) insertion line, which has an insertion fragment (approximately 8 Kb) in the 5′ UTR of *Os01g66100* (Fig. 3A) in the *japonica* cv Dongjin (Jeong et al., 2006). Sequence analysis revealed that the cv Dongjin allele $SD_{1\text{-}2}{}^{D}$ is 3,196 bp in length and is different from $SD_{1\text{-}2}{}^{S}$ at 17 sites, including three single-nucleotide polymorphisms (SNPs) in the exons (Fig. 2A). However, the SNPs do not alter the deduced protein length and predicted domains (Fig. 2C), suggesting that $SD_{1\text{-}2}{}^{D}$ could also be functional. Phenotypic identification revealed that both cv Dongjin and the mutant line are similar in seed morphology, but *OsGA20ox2* was not transcribed in the mutant (Fig. 3A), suggesting that the T-DNA insertion knocked out the gene function. As expected, the knockout line displayed reduced germinability and plant height, as compared with cv Dongjin, in two independent experiments (Fig. 3B). Thus, the natural ($SD_{1\text{-}2}{}^{E}$) and induced ($SD_{1\text{-}2}{}^{KO}$) mutants have a similar effect on delay of germination.

Mutation of *OsGA20ox2* Altered Transcription Patterns of GA Metabolic and Signaling Genes in Seeds at Early Development

To determine the regulatory role of *qSD1-2* in seed development, genes known for GA synthesis (*OsGA20ox2*

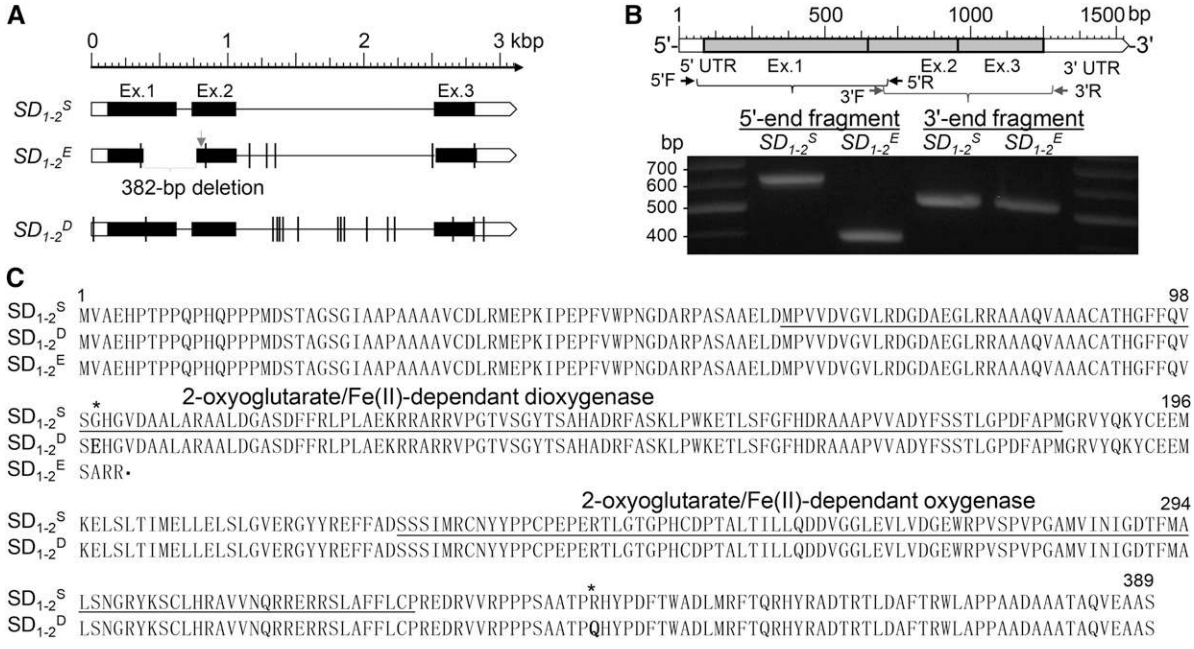

Figure 2. Allelic variants of *qSD1-2*. A, Gene structure. $SD_{1\text{-}2}{}^{S}$, $SD_{1\text{-}2}{}^{E}$, and $SD_{1\text{-}2}{}^{D}$ are alleles from SS18-2, EM93-1, and cv Dongjin, respectively. Boxes indicate 5′/3′ UTRs (white) or exons (Ex.; black), and line segments indicate introns. Vertical lines indicate positions of point mutations as compared with $SD_{1\text{-}2}{}^{S}$. The gap in $SD_{1\text{-}2}{}^{E}$ represents a deletion resulting in a premature stop codon at the arrow-pointed position. B, cDNAs. The scale indicates lengths of UTRs (white boxes) and exons (gray boxes). Arrows indicate positions of PCR primers used to amplify the overlapped fragments on the gel image. C, Protein sequences. Amino acid sequences were deduced based on the cDNA sequences for $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{E}$ or on the genomic DNA sequence for $SD_{1\text{-}2}{}^{D}$. Underlined sequences are predicted functional domains, asterisks indicate residues different between $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{D}$, and $SD_{1\text{-}2}{}^{E}$ is a truncated protein.

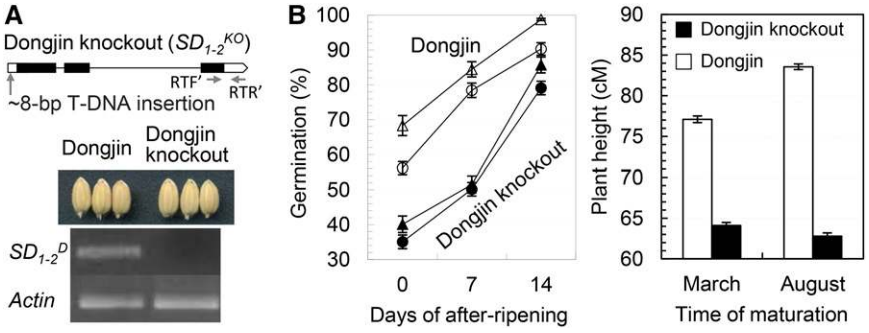

Figure 3. Mutant analysis of SD_{1-2}^{KO}. A, Seed morphology and transcription. The knockout allele SD_{1-2}^{KO} was generated by a T-DNA insertion at the 5′ UTR of SD_{1-2}^{D} (Fig. 2A) in the cv Dongjin background. Images show the morphology of seeds and semiquantitative reverse transcription PCR products for the segment (horizontal arrows) and *ACTIN* control from Dongjin and Dongjin knockout M2 plants. Horizontal arrows indicate positions of forward (RTF′) and reverse (RTR′) primers for the semiquantitative and real-time PCR. B, Genotypic differences in seed dormancy and plant height. Seed dormancy was evaluated by germination of seed samples after-ripened for different days. Data shown are means ± SE of 20 to 24 plants harvested in early March or late August.

and its paralog *OsGA20ox1*), catabolism (*OsGA2ox1*), or signal transduction (*OsGAMyb*; Gubler et al., 1997; Sakamoto et al., 2004; Tsuji et al., 2006) were quantified for transcription levels in developing caryopses from the isogenic lines. A caryopsis is a true seed united with the covering tissue pericarp. The four selected genes were all transcribed in the caryopses, with the abundance decreased from 3 to 9 DPA (Fig. 4). However, the transcription level was higher for the GA synthesis genes but lower for the catabolism and signaling genes in IL_{SD1-2}^{E} than in IL_{SD1-2}^{S} (Fig. 4). The contrasting expression levels between the synthesis and catabolism genes in the isogenic lines strongly suggest that an *OsGA20ox* feedback mechanism (Middleton et al., 2012) existed in the developing seeds, presumably to regulate GA homeostasis, signal transduction, and responses.

The *OsGA20ox2* transcripts in the developing caryopses (Fig. 4A) may include those from the endospermic cells, as *qSD1-2* was associated with endosperm-imposed dormancy. To prove this hypothesis, a transgenic line with the SD_{1-2}*promoter*::*GUS* reporter system was used to pollinate cv Nipponbare (the recipient of the transgene) to produce hybrid F_1 seeds. GUS activity was visualized on caryopses self-pollinated by the transgenic plants and also on the F_1 caryopses at 3 to 9 DPA, and the staining intensity decreased dramatically after 6 DPA (Fig. 5A). Quantitative real-time (qRT)-PCR analysis detected transcripts of the reporter gene in the embryo-removed F_1 caryopses (Fig. 5B), which consist of the endosperm and maternal (testa and pericarp) tissues. Because the maternal tissues did not receive the reporter gene from the pollen parent, the transcripts detected in the embryoless F_1 caryopses must be expressed in the endosperm. These transcriptional data demonstrated that *OsGA20ox2* was expressed in developing seeds, including the endosperm tissue, and is likely involved in the regulation of dormancy induction through the GA signaling pathway.

Loss-of-Function Mutation of *OsGA20ox2* Reduced the Seed GA Level, Resulting in Delay of Tissue Morphogenesis, ABA Accumulation, and Subsequent Maturation Programs

To gain deep insight into *qSD1-2*-regulated mechanisms for dormancy development, a series of experiments were conducted to compare seed hormone, morphological, histological, and physiological profiles between the isogenic lines. In the first experiment, caryopses at 5 DPA were quantified for the GA, ABA, auxin, and cytokinin metabolites. Several GA forms on the early 13-hydroxylation (GA_{53}, GA_{44}, GA_{19}, and GA_8)

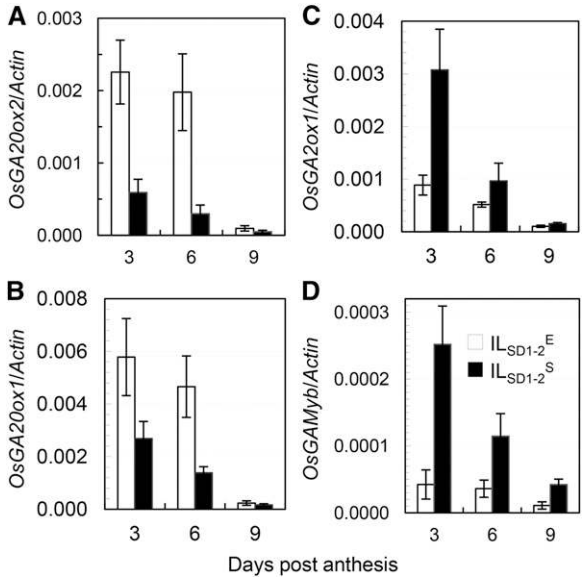

Figure 4. Gene expression in developing seeds from IL_{SD1-2}^{E} and IL_{SD1-2}^{S}. A and B, GA biosynthesis enzyme genes (*OsGA20ox2* and *OsGA20ox1*). C, GA catabolic enzyme gene (*OsGA2ox1*). D, GA signaling gene (*OsGAMyb*). RNA samples were prepared from caryopses at 3, 6, and 9 DPA and quantified by qRT-PCR. Data shown are mean transcription levels ± SD, relative to the *ACTIN* control, for three biological replicates.

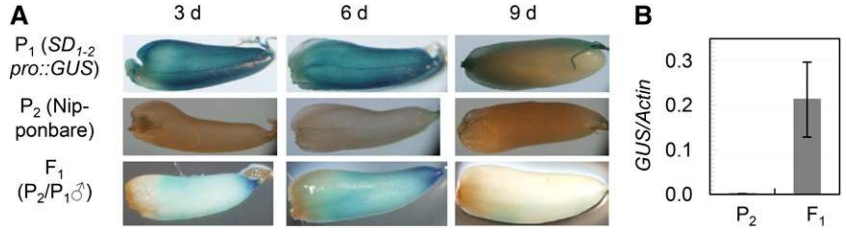

Figure 5. Histochemical localization of GUS reporter activity in developing seeds. A, GUS-stained caryopses. Caryopses were produced by self-pollinating the *$SD_{1\text{-}2}$pro::GUS* transgenic (P_1) and cv Nipponbare (P_2) plants, or by crossing P_2 with the pollen parent P_1 (F_1), and sampled at 3, 6, and 9 DPA. B, Expression levels of the GUS reporter gene in embryoless P_2 and F_1 caryopses. RNA samples were prepared from 6-d caryopses after the embryo portion was removed and quantified by qRT-PCR. Data shown are means ± SD, relative to the *ACTIN* control, of two biological replicates.

and nonhydroxylation (GA_{24}, GA_9, GA_{51}, and GA_{34}) pathways were detected in both lines, and trivial amounts of bioactive GAs were present in $IL_{SD1\text{-}2}{}^{S}$ (GA_3 and GA_7) or $IL_{SD1\text{-}2}{}^{E}$ (GA_1; Supplemental Table S3). The presence of the GA 20-oxidase-catalized metabolites in $IL_{SD1\text{-}2}{}^{E}$ indicates that *OsGA20ox2*'s paralogs, such as *OsGA20ox1* (Fig. 4B), were functional in the genetic background. Genotypic differences were also observed for the other three hormones or their metabolites. For example, the ABA and indole acetic acid contents were relatively higher, while the cytokinin metabolites were relatively lower, in $IL_{SD1\text{-}2}{}^{E}$ than in $IL_{SD1\text{-}2}{}^{S}$ (Supplemental Table S3). Thus, the functional mutation of *OsGA20ox2* may affect seed development at the early stage, as the indole acetic acid, cytokinin, and ABA quantities were correlated with endosperm cell-division and grain-filling rates in rice (Zhang et al., 2009).

In the second experiment, caryopses sampled at 3-d intervals from 6 to 30 DPA were quantified for GA_3 and ABA contents to model temporal distribution patterns. The GA distribution fits to an inverse first-order polynomial, with the highest level at approximately 6 DPA in $IL_{SD1\text{-}2}{}^{S}$, while the GA level was reduced by up to approximately 2-fold in $IL_{SD1\text{-}2}{}^{E}$ during the 30-d period (Fig. 6A). The ABA distribution was unimodal, with the peak value at approximately 10 DPA in $IL_{SD1\text{-}2}{}^{S}$ or at approximately 15 DPA in $IL_{SD1\text{-}2}{}^{E}$ (Fig. 6B). Interestingly, the isogenic lines were similar in the peak concentration, indicating that the ABA synthesis/accumulation was delayed, but not repressed or promoted, in $IL_{SD1\text{-}2}{}^{E}$.

In the third experiment, caryopses sampled at 3 to 30 DPA were examined for morphological and histological changes to visualize the mutant effect on seed development. An intact developing caryopsis has a green color because of the presence of chlorophyll in the pericarp; as the development proceeds, the green color disappears gradually due to chlorophyll decomposition in the maternal tissue. In this experiment, the green pigment disappeared at approximately 12 d in $IL_{SD1\text{-}2}{}^{S}$ and at approximately 18 d in $IL_{SD1\text{-}2}{}^{E}$ (Fig. 7A). A similar genotypic difference in the green pigment was also observed in leaves of flowering plants, as shown by higher chlorophyll meter readings in $IL_{SD1\text{-}2}{}^{E}$ than in $IL_{SD1\text{-}2}{}^{S}$ during seed development (Fig. 7B). Thus, *OsGA20ox2* was involved in the regulation of chlorophyll metabolism, and the functional mutation delayed the pigment decomposition in green tissues. Histological analysis demonstrated that cell development and differentiation were slower in $IL_{SD1\text{-}2}{}^{E}$ than in $IL_{SD1\text{-}2}{}^{S}$ (Fig. 7C). For example, the aleurone cell layer and the aleurone cell protein bodies became visible at approximately 7 and 10 d, respectively, in $IL_{SD1\text{-}2}{}^{S}$, and at approximately 10 and 15 d, respectively, in $IL_{SD1\text{-}2}{}^{E}$. The developmental delay of $IL_{SD1\text{-}2}{}^{E}$ seeds from early to middle stages did not affect the fresh weight of caryopses at approximately 24 DPA (Fig. 7D), but it postponed

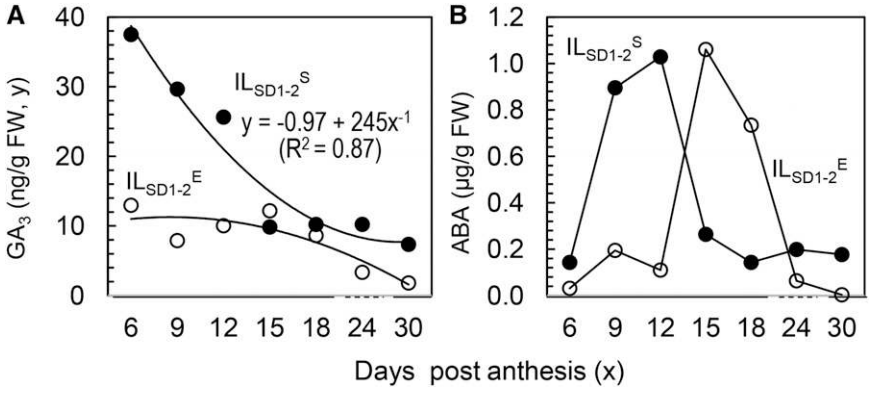

Figure 6. Temporal distributions of seed GA (A) and ABA (B) contents in $IL_{SD1\text{-}2}{}^{S}$ and $IL_{SD1\text{-}2}{}^{E}$. The nonlinear regression equation was developed to model the GA distribution in $IL_{SD1\text{-}2}{}^{S}$. FW, Fresh weight.

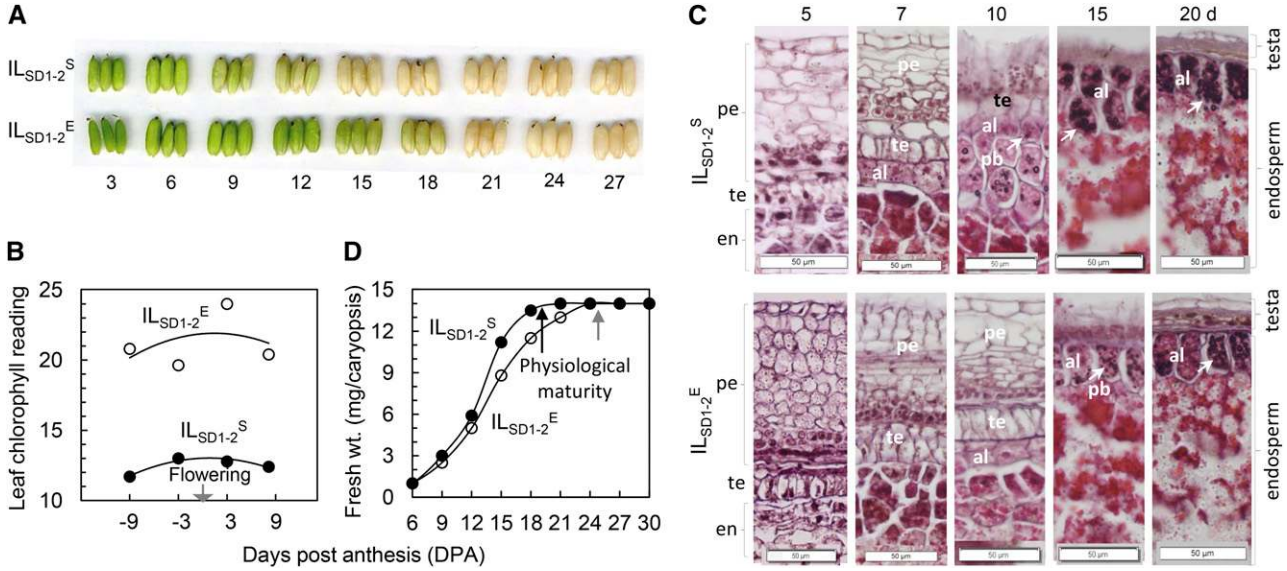

Figure 7. Genotypic differences in seed development and chlorophyll content between $IL_{SD1\text{-}2}{}^{S}$ and $IL_{SD1\text{-}2}{}^{E}$. A, Caryopsis morphology at 3 to 30 DPA. The green color shows the presence of chlorophyll in the pericarp tissue. B, Temporal distributions of leaf chlorophyll meter readings. The measurement was made for the flag leaves of flowering plants. C, Histological profiles of developing caryopses. Images show the tissues pericarp (pe), testa (te), and endosperm (en) cells. Arrows point to protein bodies (pb) in aleuronic (al) cells. Bars = 50 μm. D, Temporal distributions of caryopsis fresh weight. Arrows indicate estimated time points of physiological maturity.

the physiological maturity. Physiological maturity is the time point when the seed dry mass reaches the maximum weight and is followed by a rapid decline in seed moisture (or dehydration) to gain germinability and longevity (Bewley et al., 2013). The above results demonstrated that the mutation of *qSD1-2* slowed down the sequential steps of seed development from tissue morphogenesis to maturation.

In the fourth experiment, seeds sampled at 5-d intervals from 20 to 45 DPA were measured for moisture content immediately after sample collection and germination percentage after 7 d of air drying to model temporal distribution patterns for dehydration and germinability development. The seed moisture kept declining in an approximately linear manner from approximately 26% at 20 d to 21% at 45 d for $IL_{SD1\text{-}2}{}^{S}$ and was approximately 2% higher for $IL_{SD1\text{-}2}{}^{E}$ during this period (Fig. 8). The $IL_{SD1\text{-}2}{}^{S}$ seeds started to gain germinability at approximately 20 d (approximately 4%); then, the capability increased in an approximately normal distribution pattern, with 50% germination at approximately 32 d (Fig. 8). In contrast, for $IL_{SD1\text{-}2}{}^{E}$ seeds, it took 25 d of development to gain germinability (less than 2%) and approximately 38 d to reach 50% germination. The germinability was negatively correlated with the moisture content in each of the two lines ($r > 0.97$). Similar distribution patterns for and high correlations ($r = -0.98$) between germinability and moisture content were also observed in a preliminary experiment, in which seeds from the isogenic lines were sampled at 3-d intervals from 24 to 45 DPA (Supplemental Fig. S2). These two independent observations demonstrated that *qSD1-2* acted as a regulator of dehydration and desiccation (maturation drying), which is critical for orthodox seeds to switch from the development to the germination program (Angelovici et al., 2010).

OsGA20ox2 Was Expressed Only in the Embryo of Germinating Seeds, and GA Application Accelerated the Release of Primary Dormancy Associated with *qSD1-2*

Both GUS activity and *OsGA20ox2*'s transcripts were detected in the embryo, but not the endosperm, of

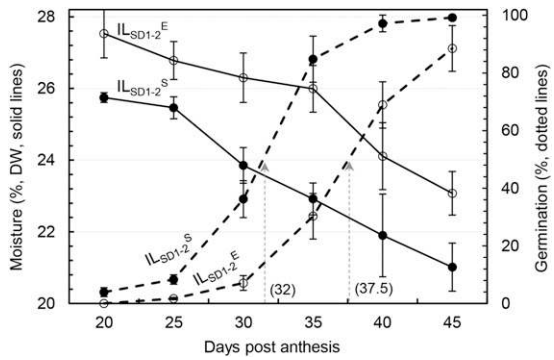

Figure 8. Temporal distribution patterns of seed moisture content and germination capability in $IL_{SD1\text{-}2}{}^{S}$ and $IL_{SD1\text{-}2}{}^{E}$. Arrows indicate the time of development required to reach 50% germination. Data shown are means ± SD of three biological replicates. DW, Dry weight.

germinating seeds. The *$SD_{1\text{-}2}$promoter::GUS* reporter system was activated in the embryo tissue after imbibition, and the activity became visible after 12 h and was extended to the rapidly growing radicle after the completion of germination (Fig. 8). The transcription level of *OsGA20ox2* in the embryo tissue of seeds imbibed for 24 h was higher in $IL_{SD1\text{-}2}{}^{E}$ than in $IL_{SD1\text{-}2}{}^{S}$ (Fig. 8). This genotypic difference implies that *OsGA20ox2* does not express in the aleuronic cells where the GA signaling pathway is triggered to promote germination.

The loss-of-function mutation of *OsGA20ox2* lowered the GA content in the developing seeds (Fig. 6A) and is also expected to reduce the hormone in the embryo of germinating seeds. Thus, the mutant effect on delayed germination can be caused by the primary dormancy and/or by the reduced GA production in the embryo after imbibition. The effect due to the GA reduction in the embryo could be compensated by a GA treatment with the concentration equivalent to the endogenous GA level. To test this hypothesis, non-after-ripened seeds from $IL_{SD1\text{-}2}{}^{S}$ and $IL_{SD1\text{-}2}{}^{E}$ were germinated in 0.0, 0.1, 1, and 10 μM GA_3. Genotypic differences in germination distribution pattern and germination percentage at day 7 were observed at 0.1 and 1 μM; with the increase in GA concentration, the difference diminished and finally disappeared at 10 μM (Fig. 9C). Provided that the GA concentration of 0.1 or 1 μM was higher than, or equivalent to, the embryonic GA level in $IL_{SD1\text{-}2}{}^{S}$, the observed genotypic difference in germinability could be an effect of the primary dormancy imposed by the tissue(s), including the endosperm. The requirement for a high GA concentration (approximately 10 μM) to completely offset the effect of *qSD1-2* on germination was similar to the GA response of the GA-deficient mutant *ga-1* in Arabidopsis (Koornneef and van der Veen, 1980). It is noted that the germination response of *qSD1-2* to the 10 μM GA application occurred only in the genetic background where the *qSD7-2* locus was fixed for the dormancy-reducing allele (Ye et al., 2013).

Origin, Distribution, and Functional Differentiation of $SD_{1\text{-}2}{}^{E}$, $SD_{1\text{-}2}{}^{S}$, and $SD_{1\text{-}2}{}^{N}$

The dormancy-enhancing allele $SD_{1\text{-}2}{}^{E}$ was traced back to the semidwarf line IR1545339-2-2 through the pedigree of the donor parent EM93-1 (Supplemental Fig. S1A). Like the other semidwarf varieties developed by the International Rice Research Institute, IR1545339-2-2 carries the Green Revolution gene *sd1* initially introduced from the traditional cv Dee-Geo-Woo-Gen (IRRI, 1981). Because *sd1* has been used to develop high-yield semidwarf varieties worldwide since the 1960s, $SD_{1\text{-}2}{}^{E}$ should be common in modern cultivars.

Phylogenetic analysis based on *OsGA20ox2* genomic DNA sequences from 183 accessions (Supplemental Table S4) revealed that the allele $SD_{1\text{-}2}{}^{S}$ from SS18-2 represents a primitive one in wild (*Oryza rofipogon*), *indica*-type cultivated, and *indica*-like weedy rice (Supplemental Fig. S3). The allele $SD_{1\text{-}2}{}^{D}$ from cv Dongjin is identical to the allele from cv Nipponbare ($SD_{1\text{-}2}{}^{N}$) and belongs to a clade consisting of *japonica* cultivars and *japonica*-like weedy rice (Supplemental Fig. S3). Based on deduced protein sequences, the 183 accessions consist of six alleles, and the primitive ($SD_{1\text{-}2}{}^{S}$) and *japonica* specific ($SD_{1\text{-}2}{}^{N}$) alleles are present in 68% and 28% of the accessions, respectively (Fig. 10A).

Five of the six protein sequences (excluding $SD_{1\text{-}2}{}^{E}$) differentiated at 10 residues. The 100th (G) and 340th (R) residues in $SD_{1\text{-}2}{}^{S}$ were substituted with E and Q, respectively, in $SD_{1\text{-}2}{}^{N}$ (Fig. 10A). To determine if there is a functional differentiation between the two common alleles, we introduced $SD_{1\text{-}2}{}^{N}$ into the EM93-1 background (Supplemental Fig. S1, C and D). The isogenic lines $IL_{SD1\text{-}2}{}^{N}$, $IL_{SD1\text{-}2}{}^{S}$, and $IL_{SD1\text{-}2}{}^{E}$ were evaluated for seed dormancy and plant height under controlled conditions. Genotypic differences in both traits were present between $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ or $IL_{SD1\text{-}2}{}^{N}$ but absent between $IL_{SD1\text{-}2}{}^{S}$ and

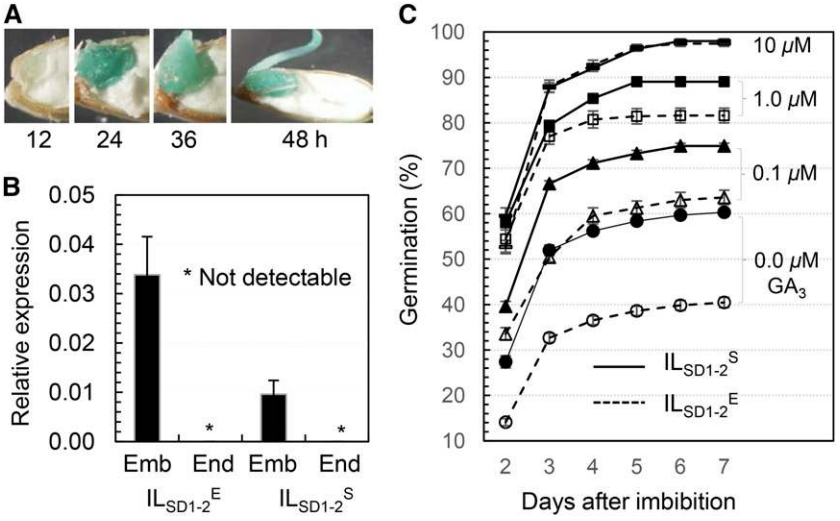

Figure 9. Gene expression in germinating seeds and germination responses to GA applications. A, Histochemical localization of GUS reporter activity. Seeds from *$SD_{1\text{-}2}$promoter::GUS* transgenic plants were imbibed for 12 to 48 h. B, Relative expression levels of *OsGA20ox2* in embryo and endosperm tissues. Seeds from $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ plants were imbibed for 24 h; RNA samples prepared from the embryo (Emb) and endosperm (End) tissues were quantified for transcription levels by qRT-PCR. Data shown are means ± SD, relative to the *ACTIN* control, of three biological replicates. C, Genotypic differences in the GA response of germination. Seeds from $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ were germinated in the indicated GA_3 solutions. Data shown are means ± SE of 10 plants.

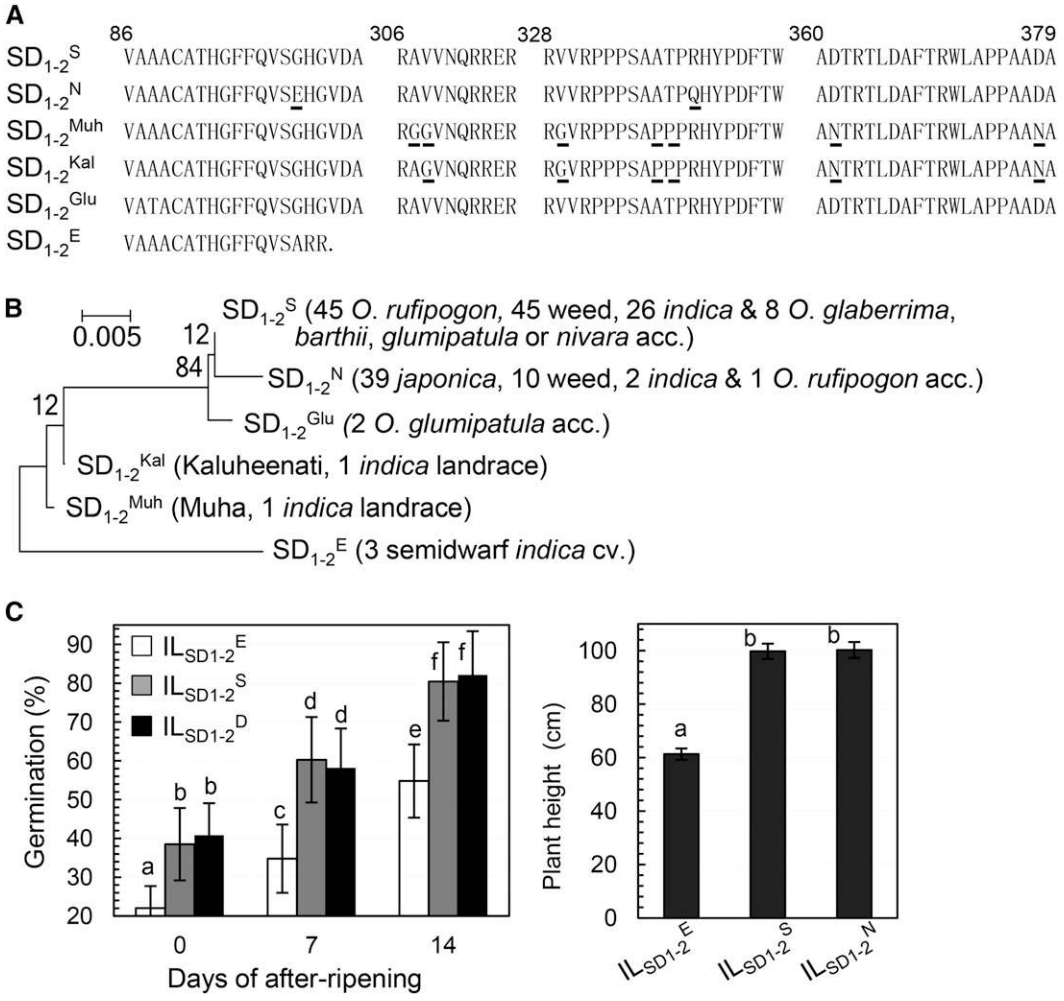

Figure 10. Allelic variants of *OsGA20ox2* in protein sequences and phenotypes. A, Partial protein sequences. The six sequences represent deduced proteins in 183 accessions of wild, weedy, or cultivated rice (Supplemental Table S4; Supplemental Fig. S3) and are represented by the alleles from SS18-2 ($SD_{1\text{-}2}^{S}$), cv Nipponbare ($SD_{1\text{-}2}^{N}$), cv Muha ($SD_{1\text{-}2}^{Muh}$), cv Kaluheenati ($SD_{1\text{-}2}^{Kal}$), *Oryza glumipatula* ($SD_{1\text{-}2}^{Glu}$), and EM93-1 ($SD_{1\text{-}2}^{E}$). Underlined residues are different from the primitive allele $SD_{1\text{-}2}^{S}$. B, Protein dendrogram. Shown in parentheses are numbers of accessions (acc.) from *Oryza* spp., rice ssp. *indica* and *japonica*, or weed type. C, Genotypic differences in germinability and plant height. $IL_{SD1\text{-}2}^{E}$, $IL_{SD1\text{-}2}^{S}$, and $IL_{SD1\text{-}2}^{N}$ are isogenic lines for the $SD_{1\text{-}2}^{E}$, $SD_{1\text{-}2}^{S}$, and $SD_{1\text{-}2}^{N}$ alleles, respectively (Supplemental Fig. S1D). Data shown are means ± SD of 20 plants. The letters (a–f) indicate results of Duncan's multiple comparison test for nonsignificance (same letter) or significance (different letter) at $P < 0.0001$.

$IL_{SD1\text{-}2}^{N}$. The data from the isogenic background indicate that the GR-EQ differentiation between $SD_{1\text{-}2}^{S}$ and $SD_{1\text{-}2}^{N}$ has no effect on seed dormancy and plant height.

DISCUSSION

Natural Variants of GA Synthesis Genes Contribute to Genetic Variation in Seed Dormancy

The *qSD1-2* QTL was identified as the *OsGA20ox2* locus by map-based cloning and mutant analysis. There are four paralogs encoding GA 20-oxidase catalyzing the next to last steps of bioactive GA synthesis in rice (Sakamoto et al., 2004). Of these loci, *OsGA20ox2* was reported for natural variants based on the mutant effect on reduced plant height, such as the allele *sd1* widespread in semidwarf cultivars. This research demonstrated that a loss-of-function mutation of the GA synthesis gene, naturally occurring or induced, also enhances seed dormancy.

Discovery of the dormancy function explained the perpetuation of *sd1* in populations of wild and weedy rice and also revealed that the Green Revolution gene has contributed to the resistance to preharvest sprouting (PHS) in crop production. The *sd1* mutant originally occurred in an *O. rufipogon* population and may have been hidden in the genetic background of tall plants before introgression into traditional varieties (e.g. cv Dee-Geo-Woo-Gen), because short-statured genotypes are less competitive in natural populations (Nagano et al., 2005). However, the selective disadvantage that *sd1* carries in plant height could be compromised by a

gain in adaptability from the enhanced seed dormancy in a wild population. Similarly, because of the gained adaptability, if *sd1* escapes from semidwarf cultivars to accompanying plants of weedy rice by cross-pollination, it would promote the persistence of *sd1*-containing weeds in the local ecosystems. As expected, *sd1* has been found in newly established populations of weedy rice in the United States (Reagon et al., 2011). It is noticed that *sd1* is also present in male-sterile lines of hybrid rice, and these lines usually have panicles partially enclosed in the leaf sheath (Virmani et al., 1997). Seed producers have to apply GA_3 to promote the peduncle elongation, which could offset *sd1*'s dormancy effect and increase the susceptibility to PHS. Thus, additional dormancy genes are required to improve male-sterile lines for resistance to PHS.

Some other GA synthesis or signaling genes may also influence seed dormancy. Dozens of dwarf mutants assigned to the GA biosynthesis pathways in rice (Sakamoto et al., 2004) have not been determined for an effect on seed dormancy. Reasons may include that these mutants usually have detrimental effects on gametophyte development or seed setting, some may not express in developing seeds, and research was focused on postgermination events (Kaneko et al., 2003). Natural mutants used to develop semidwarf varieties in the barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), and wheat crops usually have a mild negative effect on plant development, and some of them are GA sensitive (Gale and Marshall, 1975; Franckowiak and Pecio, 1992; Rodríguez et al., 2012). Some seed dormancy QTLs in barley and wheat have been associated with GA metabolism genes by comparative genomics research using rice as the reference (Li et al., 2004; Cabral et al., 2014). Thus, the information from this research would encourage exploring new functions of GA mutants to enrich knowledge on seed development and to test if orthologous genes are involved in the regulation of seed dormancy in different species.

A Developmental Mechanism of Seed Dormancy

qSD1-2 controls the variation of primary dormancy through a GA-regulated dehydration mechanism outlined in the working model (Fig. 11). The expression of *OsGA20ox2* in seeds at early development increases the GA content to stimulate cell development and tissue morphogenesis, which in turn accelerates ABA accumulation to promote dehydration and the likely acquisition of desiccation tolerance. The loss-of-function mutation reduces the GA level, which slows down tissue morphogenesis, delays ABA accumulation and subsequent maturation programs, and eventually results in increased dormancy at harvest. The GA and ABA molecules signal and induce the early (cell elongation of the maternal tissues and endospermic cell differentiation) and late development events, respectively, rather than act antagonistically with each other, to influence moisture content and germinability. Given that dormancy is an adaptive mechanism for the embryo to survive in a dehydrated environment after physiological maturity to prevent vivipary or PHS, seed moisture would be critical for the acquisition of desiccation tolerance and the onset of after-ripening to release primary dormancy. However, in previous research, we detected no difference in the seed moisture content at 40 DPA between the isogenic lines for the *qSD7-1* dormancy QTL in the EM93-1 background (Gu et al., 2011). *qSD7-1* encodes a basic helix-loop-helix family transcription factor, which promotes ABA accumulation and activates flavonoid biosynthesis in the lower epidermal cell layer of the pericarp tissue to control maternal tissue-imposed dormancy (Gu et al., 2011). Therefore, the model (Fig. 11) represents an additional mechanism for the development of primary dormancy.

The association of *qSD1-2* with endosperm-imposed dormancy was demonstrated by four distinct germination distribution patterns, each for one of the four triploid genotypes of seeds (F2) from the same plant (F1) that is heterozygous ($SD_{1\text{-}2}{}^{S}SD_{1\text{-}2}{}^{E}$) for the QTL (Gu et al., 2015). From a genetic point of view, the dormancy gene must express in the endosperm and have an additive effect on germination to display the four distribution patterns. As expected, $SD_{1\text{-}2}{}^{S}$ was expressed in the triploid tissue (Fig. 5), promoted endosperm cell differentiation (Fig. 8C), and had only an additive effect on germination (Supplemental Table S2). From a physiological point of view, the endosperm tissue at the middle to late development stages accounts for a vast majority of the seed fresh weight and, accordingly, dominates the dehydration and desiccation programs to influence germination potential at harvest. Thus, the GA-regulated dehydration mechanism (Fig. 11) may apply only to endosperm-imposed dormancy.

Allelic Differentiation of *OsGA20ox2* between the *indica* and *japonica* Subspecies

This research provided no evidence that the *indica*- and *japonica*-specific alleles at *OsGA20ox2* are functionally differentiated. The allelic distribution was associated

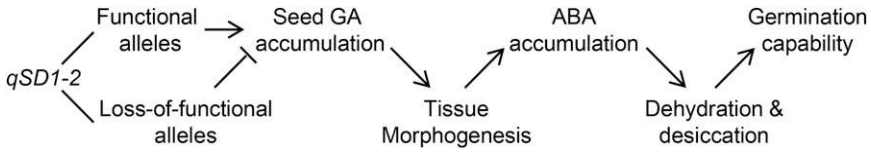

Figure 11. A working model for the development of primary dormancy regulated by *qSD1-2*.

with the subspeciation of *indica* and *japonica* rice (Supplemental Fig. S3; Nagano et al., 2005; Asano et al., 2011). A discussion was focused on whether the nonrandom distribution arose from an artificial selection for reduced plant height early during the domestication of *japonica* rice (Asano et al., 2011; Paterson and Li, 2011). $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{N}$ are prevalent in the *indica* and *japonica* subspecies, respectively (Supplemental Fig. S3), and $SD_{1\text{-}2}{}^{S}$ (GR) differs from $SD_{1\text{-}2}{}^{N}$ (EQ) in two residues (Fig. 2, A and C). The similarity between the $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{N}$ isogenic lines in plant height and seed dormancy (Fig. 10C) suggests that both alleles are functional, and the two-residue substitution may not cause a discernible change in the gene function in the EM93-1 background. Considering the additive effect of *qSD1-2* on plant height (Supplemental Table S2), it was surprising that the addition of an $SD_{1\text{-}2}{}^{N}$ as a transgene to the cv Nipponbare background did not change the plant height (Asano et al., 2011). In any event, $SD_{1\text{-}2}{}^{S}$ is identical to the allele (GR) from the *indica* landrace Kasalath, based on the genomic DNA sequence for $SD_{1\text{-}2}{}^{K}$ reported by Reagon et al. (2011). The reported association between plant height and the *sd1*-containing segment of approximately 300 kb in the advanced generation of the cv Nipponbare/Kasalath cross (Asano et al., 2011) may arise from a functional differentiation of the *OsGA20ox2* locus (including the noncoding sequences) between $SD_{1\text{-}2}{}^{K}$ and $SD_{1\text{-}2}{}^{N}$ or from a genetic differentiation outside the locus through an uncharacterized molecular mechanism. It is noticed that $SD_{1\text{-}2}{}^{S}_{K}$ represents a more primitive/frequent allele than $SD_{1\text{-}2}{}^{S}$ in the clade that consists of wild and weedy rice and *indica* cultivars (Supplemental Fig. S3). It is likely that $SD_{1\text{-}2}{}^{K}$, together with the rare alleles $SD_{1\text{-}2}{}^{Kal}$ and $SD_{1\text{-}2}{}^{Mhl}$ in the clade (Fig. 10, A and B), differentiated from $SD_{1\text{-}2}{}^{S}$ after the divergence between $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{N}$. Thus, Kasalath is not an ideal genotype to be used as the wild-type control to elucidate the evolutionary mechanism of the putatively domestication-related gene in *japonica* rice. Consequently, additional research is needed to address driving forces for the fixation of the *japonica*-specific allele and to determine if the rare alleles are functionally differentiated from $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{N}$.

MATERIALS AND METHODS

Plant Materials and Cultivation

The rice (*Oryza sativa*) BC_5F_3 line used to develop the F2 population was selected from a recombinant that has only one chromosomal segment from SS18-2 in the EM93-1 background (Ye et al., 2013). The pedigree of EM93-1 (Supplemental Fig. S1A) was made based on information from Gu et al. (2004) and from Hanfu Qian, who developed the parent line Wujinxianxian at the Jiangsu Wujin Rice Research Institute. $IL_{SD1\text{-}2}{}^{N}$ was developed by recurrent backcrossing with marker-assisted selection (Supplemental Fig. S1C) and the backcross initiated to introduce the *Rc/SD7-1* transgene from cv Nipponbare into the EM93-1 background (Gu et al., 2011).

To develop plant populations, after-ripened seeds were germinated at 30°C for 5 d before being cultured with nutrition solution (Yoshida et al., 1976) in 200-cell plug trays for 2 to 3 weeks to sample the leaf tissue for marker genotyping. Seedlings with known genotypes for *qSD1-2* or its flanking regions were transplanted into pots in a greenhouse to harvest seeds.

Progeny Testing and Seed Dormancy Assessment

Seedlings (F3) from selected recombinants (F2) were sorted based on marker genotypes and grown under temperature (29°C/21°C for day/night)-controlled conditions. Plants were tagged for flowering date when the first panicle of a plant emerged from the leaf sheath and measured for plant height at harvest. Seeds were harvested at 40 d after flowering and dried in the greenhouse for 3 d before being stored at −20°C in a freezer to maintain the dormant status.

Seed dormancy was evaluated by germination testing. Prior to a test, seed samples from the freezer were partially after-ripened at room temperature (approximately 24°C) for several hours to 30 d, depending on the experiment. A sample of approximately 50 well-developed seeds was distributed on filter paper in a 9-cm petri dish, soaked with 8 mL of water, and incubated at 30°C and 100% relative humidity in the dark. Germination was evaluated visually by protrusion of the radicle from the hull by more than 3 mm and counted daily from day 2 to day 7 or at day 7. A test for a plant at a time point was replicated three times, and germination percentages were averaged for genetic analysis.

Marker Development and Genotyping

The high-resolution map (Fig. 1A) was developed with simple sequence repeat (SSR) and SNP markers (Supplemental Table S1). Nine new SSRs were selected from the reference genome and screened for polymorphism between EM93-1 and SS18-2. Six genomic DNA fragments were sequenced from EM93-1 and SS18-2 and their sequences aligned to identify SNPs. Genomic DNA preparation, sequencing and sequence alignment, and genotyping with SSR markers were performed using previously described methods (Gu et al., 2011). Recombinant genotypes for the SNP loci were determined by PCR and direct sequencing.

Marker-Trait Association Analysis and Estimation of Genic Effects

Linear correlation analysis was used to determine the marker-trait associations in progeny lines. Genotypes for a marker locus were coded as i ($i = 1, 2,$ and 3 for EM93-1-like homozygote, heterozygote, and SS18-2-like homozygote, respectively) for the correlation analysis. Additive and dominance effects of the locus on germination or plant height were estimated using the linear regression model:

$$y_{ij} = \mu + \alpha x + dz + \varepsilon_{ij}$$

where y_{ij} is the trait value for the jth plant of the ith marker genotype; μ is the model mean; x is the dummy variable for the additive component and was coded as -1, 0, and 1 for $i = 1$, 2, and 3, respectively; z is the dummy variable for the dominance component and was coded as 0.5 for $i = 2$ or -0.5 for $i = 1$ or 3; a and d are regression coefficients and estimates of the additive and dominance effects, respectively; and ε_{ij} is the error term of the model. Correlation and regression analyses were implemented using SAS (SAS Institute, 2011).

T-DNA Insertion Mutant Analysis

The mutant 3A-14927 (M1) and its recipient cv Dongjin were introduced from the Crop Biotech Institute, Kyung Hee University, in Korea. The M1 seedlings and thermal asymmetric interlaced-PCR were used to confirm the T-DNA insertion site at the 5′ UTR of *Os01g66100* (Jeong et al., 2006). The knockout effect on *Os01g66100*'s transcription was determined by semiquantitative PCR. The PCR primers are listed in Supplemental Table S1. The M2 and M3 generations of plants were grown in the greenhouse and evaluated for seed dormancy and plant height.

BAC Library Screening, DNA and cDNA Cloning, Sequencing, and Sequence Analysis

Southern blotting was used to screen the BAC library constructed specifically for SS18-2 (Gu et al., 2010). Two ^{32}P-labeled Overgo sequences designed to target the narrowed *qSD1-2* region (Supplemental Table S2) were used to probe the library membranes. Positive clones were end sequenced for approximately 500 bp and sequences aligned against the reference genome to develop the contig. A positive clone covering the 20-Kb region was sequenced for the entire *Os01g66100* and partial *Os01g66110* loci with the listed primers (Supplemental

Table S1). These primers were also used to clone *Os01g66100* from EM93-1 and cv Dongjin by PCR to assemble $SD_{1\text{-}2}{}^{E}$ and $SD_{1\text{-}2}{}^{D}$, respectively. The sequence assemblies were annotated with the RiceGAAS annotation system (Sakata et al., 2002) to predict the gene structures and also aligned by the SeqMan program (DNASTAR) to identify the allelic variation among $SD_{1\text{-}2}{}^{S}$, $SD_{1\text{-}2}{}^{E}$, and $SD_{1\text{-}2}{}^{D}$.

To isolate full-length cDNAs for the $SD_{1\text{-}2}{}^{S}$ and $SD_{1\text{-}2}{}^{E}$ alleles, total RNA samples were prepared from the $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ seedlings using TriReagent (Sigma-Aldrich) and reverse transcribed using SuperScript III First-Strand (Invitrogen). Two pairs of primers (Supplemental Table S2) were designed to clone the overlapped cDNA fragments (Fig. 2B), the fragments were sequenced, and the sequences were assembled to obtain full-length cDNAs. The cDNAs were aligned against their genomic DNA sequences to confirm the gene structures and also used to predict functional domains in deduced proteins using the Conserved Domain Database (Marchler-Bauer et al., 2013).

Transcriptional Analysis by qRT-PCR

To compare transcriptional profiles of the GA metabolism and signaling genes between the isogenic lines, three biological replicates of the $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ plants were grown in the greenhouse, and spikelets were labeled for anthesis dates to collect fresh caryopses at 3, 6, and 9 DPA in liquid N_2. A sample of approximately 50 caryopses was used to prepare total RNA, and 1 μg of the RNA was reverse transcribed into cDNAs. PCR primers were designed based on the cv Nipponbare genome annotation release 7 (Kawahara et al., 2013). The primers and Power SYBR Green PCR Master Mix (Applied Biosystems) were used to amplify the cDNA templates in the ABI 7900HT Fast Real-Time PCR System. The *ACTIN* gene was used as the control.

Development of the $SD_{1\text{-}2}$*promoter*::*GUS* System and Detection of the Reporter Gene in Endosperm

A sequence of 1.6 Kb covering the promoter and 5′ UTR of $SD_{1\text{-}2}{}^{S}$ was ligated into pCXGUS-P vector (Chen et al., 2009), and the construct was delivered to the cv Nipponbare background using the *Agrobacterium tumefaciens*-mediated transformation system (http://agron-www.agron.iastate.edu/ptf/protocol/rice.pdf). To identify the reporter gene expression in the endosperm tissue, T1 plants as the pollen parent were crossed with cv Nipponbare to obtain hybrid F1 seeds. Developing seeds at 3, 6, and 9 DPA and mature seeds imbibed for 12, 24, 36, and 48 h were stained and destained following the protocol of Jefferson et al. (1987) and photographed using a stereomicroscope.

Measurement of Plant Hormone Metabolites in Developing Seeds

To have a snapshot of metabolic profiles of the plant hormones involved in early seed development, 200 spikelets sampled from $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ plants at 5 DPA had their hulls removed and fresh caryopses freeze dried to measure the ABA, auxin, cytokinin, and GA metabolites. The measurement was conducted using an HPLC-electrospray ionization-tandem mass spectrometry system following published procedures (Ross et al., 2004; Lulsdorf et al., 2013) at the National Research Council Plant Biotechnology Institute, Canada.

To identify temporal distribution patterns of the GA and ABA hormones, spikelets from $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ plants were sampled at the indicated DPA to collect caryopses at −80°C. A sample of 50 caryopses was used to prepare and purify extracts using methods modified from Chen and Yang (2005). The hormones were quantified using HPLC-mass spectrometry with an external standard. The chromatographic analysis was performed in a Dubhe C18 column (4.6 mm × 250 mm, 5 μm) at 30°C with detection wavelength at 254 nm. A solution containing 5% (v/v) acetonitrile, 50% (v/v) methanol, and 0.27% (v/v) acetic acid was used as the mobile phase in the analytical system. The washing procedure was 0.6% (v/v) acetic acid at 0 min and 0.3% (v/v) acetic acid in 50% (v/v) methanol at 10 and 30 min. For a test, 10 μL of the extract was injected, and the recovery rate was 96.5% ± 2.6%. The test was repeated four times.

Histochemical Analysis

The histological experiment was conducted basically following the protocol of Luna (1968). Intact seeds from $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ plants were sampled at the indicated DPA, and fresh caryopses were fixed with 4% (w/v) paraformaldehyde solution before embedding in Paraplast Plus (Sigma-Aldrich). Longitudinal microtome sections of 8 to 10 μm thick were stained with hematoxylin and eosin (Sigma-Aldrich) and imaged using an Olympus AX70 upright compound microscope.

Tests of Moisture Content and Germinability of Developing Seeds

To identify the genotypic differences in dehydration and germinability acquisition during seed development, three biological replicates of the $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$ plants, 24 plants per replicate, were grown in the greenhouse, spikelets on the main tillers were marked for anthesis dates, and 200 to 250 seeds were sampled from each replicate at the indicated DPA and allocated into two bags. One bag of seeds was weighed immediately after harvesting and then dried at 105°C for 72 h to measure dry weight to calculate the moisture content. The other bag of seeds was air dried for 7 d and germinated at 30°C and 100% relative humidity in the dark.

Phylogenetic Analysis

The phylogenetic tree (Supplemental Fig. S3) was generated based on genomic DNA sequences of *OsGA20ox2* from 183 accessions of wild (*Oryza* spp.), weedy, or cultivated rice. Information about geographical origins and types of these accessions and sequence identifiers are listed in Supplemental Table S4. Protein sequences were deduced from coding sequences of the gene model (Fig. 2C). Dendrograms for genomic or protein sequences were generated using the maximum likelihood method with partial deletion after ClustalW alignment with MEGA 6.06 software (Tamura et al., 2013). Bootstrap values (%) were obtained by 500 replicates.

Sequence data used for this article were submitted to GenBank under accession numbers KT354861 to KT354865.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Pedigrees and development of plant genotypes for this research.

Supplemental Figure S2. Temporal distribution patterns for seed moisture content and germination capability in $IL_{SD1\text{-}2}{}^{S}$ and $IL_{SD1\text{-}2}{}^{E}$.

Supplemental Figure S3. Dendrogram of 183 rice accessions for the *OsGA20ox2* locus.

Supplemental Table S1. List of PCR primers used in this research.

Supplemental Table S2. Summary of genic effects of *qSD1-2* on germination and plant height in recombinant-derived progeny lines.

Supplemental Table S3. Summary of hormone metabolites in 5-d developing caryopses from $IL_{SD1\text{-}2}{}^{E}$ and $IL_{SD1\text{-}2}{}^{S}$.

Supplemental Table S4. List of accessions of rice germplasm for phylogenetic analysis of *OsGA20ox2*.

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