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WEAK SEED DORMANCY 1, an aminotransferase protein, regulates seed dormancy in rice through the GA and ABA pathways

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ABSTRACT

Seed dormancy is a critical trait that enhances plant survival by preventing seed germination at the wrong time or under unsuitable conditions. Lack of seed dormancy in rice can lead to pre-harvest sprouting on mother plants leading to reduced yield and seed quality. Although some genes have been identified, knowledge of regulation of seed dormancy is limited. Here, we characterized a weak seed dormancy mutant named *weak seed dormancy 1* (*wsd1*) that showed a higher seed germination percentage than the wild-type following the harvest ripeness. We cloned the *WSD1* encoding an aminotransferase protein using a MutMap approach. *WSD1* was stably expressed after imbibition and its protein was localized in the endoplasm reticulum. A widely targeted metabolomics assay and amino acid analysis showed that *WSD1* had a role in regulating homeostasis of amino acids. PAC treatment and RNA-seq analysis showed that *WSD1* regulates seed dormancy by involvement in the GA biosynthesis pathway. GA_1 content and expression of GA biosynthesis-related genes were increased in the *wsd1* mutant compared with the wild-type. The *wsd1* mutant had reduced sensitivity to ABA. Our overall results indicated that *WSD1* regulates seed dormancy by balancing the ABA and GA pathways.

1. Introduction

Seed dormancy is a survival strategy that determines the timing of germination under optimal conditions in complex natural environments. Many crop species were selected for reduced seed dormancy during domestication to ensure rapid and uniform germination after sowing. However, inadequate seed dormancy can lead to sprouting prior to harvest. Pre-harvest sprouting in rice often occurs under high temperature and humidity conditions, the consequence of which is reduced yield and seed quality. Moderate levels of seed dormancy ensure higher seedling emergence rates as well as avoiding pre-harvest sprouting on the mother plants. Many quantitative trait loci (QTL) related to seed dormancy in rice have been reported, but only a few genes are cloned. For example, *Seed dormancy 4* (*Sdr4*) which is polymorphic between varieties Nipponbare and the Kasalath encodes a protein with unknown function, and its expression is regulated by OsVP1 (Sugimoto et al., 2010; Chen et al., 2021). *qSD7-1*, also named *qSDr7-2*, is a pleiotropic gene that controls seed dormancy and pericarp color, regulates seed dormancy by promoting expression of some abscisic acid biosynthesis genes at the early stage of seed development (Gu et al., 2011; Nguyen et al., 2019). The OsGA20ox protein Seed Dormancy1-2 (qSD1-2) plays a positive role in seed dormancy by reducing the seed GA content (Ye et al., 2015). Overall, our knowledge about the mechanism of seed dormancy in rice is limited.

Seed dormancy is a complex agronomic trait controlled by many factors, including environment, phytohormones and specific seed dormancy genes (Shu et al., 2016; Nee et al., 2017). Among of those factors, phytohormones, especially GA and ABA, have important roles in regulation of seed dormancy. GA and ABA are antagonistic in controlling seed dormancy (Graeber et al., 2012; Shu et al., 2016). Specific seed

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dormancy genes such as *Sdr4* and *OsDOG1L-3* positively regulate seed dormancy by involving in ABA signaling pathway (Sugimoto et al., 2010; Wang et al., 2020; Chen et al., 2021). Seed Dormancy 6 (SD6), a basic helix-loop-helix (bHLH) transcription factor, antagonistically worked with another bHLH transcription factor, Inducer of C-repeat Binding Factors Expression 2 (ICE2) to control seed dormancy when responding to temperature signals by regulating ABA content (Xu et al., 2022). And *qSD1-2* and *miR156* promote seed dormancy by suppressing the GA content (Ye et al., 2015; Miao et al., 2019). Meanwhile, the balance between ABA and GA determines the seed fate transition between dormancy and germination (Shu et al., 2016). For example, ABI4 can fine-tune the ABA/GA balance by inhibiting the expression of ABA catabolism genes and GA biosynthesis genes to promote primary seed dormancy in *Arabidopsis* (Shu et al., 2013). A recent study reported that *OsGA2ox9*, encoding a GA 2-oxidase, positively regulates seed dormancy by altering GA metabolism and reducing ABA signaling (Xing et al., 2023). Therefore, identifying new seed dormancy factors mediating the ABA/GA balance will be helpful to enrich our understanding of the regulatory mechanism of seed dormancy.

During seed germination, glycolysis and the TCA cycle are activated to provide energy and nutrition (Bewley, 1997; Bewley et al., 2013). Amino acid biosynthesis is essential for seed germination (Arc et al., 2012). Amino acids can be used for the synthesis of storage proteins or can be catabolized into the TCA cycle to produce energy (Galili et al., 2014). The concentrations of many amino acids are increased during transition of Arabidopsis seeds from vernalization to germination (Fait et al., 2006). Rice isopropylmalate synthase 1 (OsIPMS1) regulates seed vigour by inducing the accumulation of the amino acids. Amino acid treatment induced the expression of GA biosynthesis-related genes in an *osipms1* mutant compared to the control (He et al., 2019). Ketogenic amino acids including leucine (Leu), isoleucine (Ile), lysine (Lys), phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr) and threonine (Thr), can be catabolized to acetyl-CoA, the precursor of GA biosynthesis (Rios-Iribe et al., 2011). *QTL FOR SEED* DORMANCY1 (*Qsd1*), a major seed dormancy gene in barley, encodes an alanine aminotransferase (AlaAT) and negatively regulates seed dormancy but its function is unclear (Sato et al., 2016; Hisano et al., 2022). However, the relationship of seed dormancy with amino acid pathways is largely unknown.

In this study, we identified a *wsd1* mutant with weakened dormancy in showing a higher germination percentage than its wild-type. We cloned the *WSD1* by a MutMap approach and found that it encodes a protein containing a P-loop NTPase domain in the N-terminus and an aminotransferase domain in the C-terminus. WSD1-GFP was localized to the endoplasm reticulum (ER) in *N. benthamiana* leaves. A widely targeted metabolomics analysis indicated that the *WSD1* mutation caused changes in 111 metabolites, 33 of which were amino acids or their derivatives. RNA-seq assays showed that *WSD1* regulated seed dormancy by affecting expression of GA biosynthesis and ABA signaling-related genes. GA contents were changed and ABA signaling was reduced in the *wsd1* mutant. Hence, the *WSD1* mutation disrupted the balance of ABA and GA. Our results provide new insights into the function of WSD1 in regulating seed dormancy.

2. Materials and methods

2.1. Plant materials and growth conditions

The *wsd1* mutant was obtained following ^{60}Co gamma-radiation of the strongly dormant variety *Oryza sativa* L. spp. *indica* cultivar (cv) N22. The wild-type, *wsd1* mutant, F_1, F_2 population, and transgenic plants were grown at the Tuqiao experimental field of Nanjing Agricultural University.

2.2. Identification of seed dormancy

Seed dormancy assays were performed as previously described method (Wang et al., 2020). When the first panicle emerged from the leaf sheath, the date was recorded and all seeds of per plant were harvested at 35 days after the emergence date of first panicle. Freshly harvested seeds at 35 days after heading (DAH) were used in germination experiments. Fifty seeds of each plant were placed in a 9 cm Petri dished containing doubled sheets of moistened filter papers and incubated for 7 days at 30 °C in darkness. The criterion for seed germination was that the length of a radicle or plumule reached half the grain length.

2.3. MutMap analysis

For MutMap analysis, a cross between the *wsd1* mutant and wild-type was used to generate a F_2 population. Germination tests were performed using seed at 35 DAH from the wild-type, *wsd1* mutant, F_1 plants, and F_2 population. Equal amounts of DNA from 20 F_2 plants with the *wsd1* mutant phenotype and 20 F_2 plants with the wild-type phenotype were separately pooled. DNA from the two pools, wild-type and *wsd1* mutant were sequenced on an Illumina HiSeq2500 platform. The MutMap assay was performed following the previously described method (Abe et al., 2012). SNP/INDEL and Δ(SNP/INDEL) indices were analyzed by Genepioneer Biotechnologies Company (www.genepioneer.com) following a previously described method (Lu et al., 2014).

2.4. Vector construction and plant transformation

For complementation test, we constructed a complementation vector by inserting both the 1.6 kb promoter sequence and the *WSD1* CDS sequence into the *pCUbi1390* binary vector. This construct was introduced into Agrobacterium strain EHA105 and transformed into *wsd1* mutant callus by Agrobacterium-mediated transformation (Hiei et al., 1994). For RNA interference vector construction, a specific fragment of the *WSD1* (596–1204 bp) CDS sequence was inserted into LH-1390-RNAi, and that construct was introduced into EHA105 and transformed into wild-type N22 callus by Agrobacterium-mediated transformation approach.

2.5. RNA extraction and quantitative RT-PCR analysis

Total RNA from seeds at different development stages and different times of imbibition were isolated using an RNA prep Pure Plant Kit (TIANGEN, China). A Takara PrimeScript 1st Strand cDNA Synthesis Kit was used to reverse transcribe the first-strand cDNA of 1 μg total RNA. An SYBR Premix Ex TaqTM Kit was used to perform a quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis on a Bio-Rad CFX96™ real-time system with the *ACTIN1* gene used as an internal control. The qRT-PCR primers are listed in Supplementary Table 1.

2.6. RNA sequencing (RNA-seq) analysis

When the spikelet hulls were flowering, the spikelet hulls were marked using black marker. Marked seeds of the wild-type and *wsd1* mutant were harvested at 24 days after flowering (DAF) for total RNA extraction using a mirVana miRNA Isolation Kit (Ambion, AM1561) following the manufacturer's protocol. RNA libraries were constructed and sequenced on an Illumina HiSeq™ 2500 platform. About 47 million clean reads were obtained for each sample and used in subsequent analyses. Genes matching the criterion of P-value <0.05 and $|\log_2(\text{fold change})| > 1$ were defined as differentially expressed genes (DEGs). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Gene Ontology (GO) enrichment assays of DEGs were carried out using a previous method (Kanehisa et al., 2008; Young et al., 2010).

2.7. Widely targeted metabolomic profiling

Seeds of wild-type and *wsd1* mutant at 24 DAF were freeze-dried in a

vacuum freeze-dryer (Scientz-100 F), and ground to powers in a grinding apparatus (Retsch, MM 400). Samples (0.1 g) of each powder were added into 1 mL 70% aqueous methanol and extracted overnight at 4 °C. The samples were centrifuged at 10,000 rpm for 10 min and supernatants were collected and extracted by filtration (pore size, 0.22 μm) prior to LC-MS/MS analysis. Widely targeted metabolomic profiling was performed by MetWare Biotechnology (www.metware.cn) according to standard procedures. Differential metabolites were selected using principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) methods. The value of Variable Importance in Project (VIP) was calculated based on PLS-DA methods. Differential metabolites were identified by the parameters of $|\log_2(\text{fold change})| \geq 1$ and VIP ≥ 1. KEGG analysis of differential metabolites was performed following a previously described method (Kanehisa and Goto, 2000).

2.8. Subcellular localization of WSD1 protein

To determine the subcellular localization of WSD1, the coding sequence of *WSD1* was inserted into the *pCAMBIA1305.1* vector with the GFP protein marker in the C-terminus. The mCherry ER-rk CD3-959 was used as the ER-localization marker. This construct was introduced into EHA105 and transformed into tobacco mesophyll cells, and the tobacco incubated 2 day at 28 °C. Fluorescence signals were observed on a confocal laser scanning microscope (Leica SP8).

2.9. Amino acid assay

Freshly harvested seeds of wild-type and *wsd1* mutant at 35 DAH were ground into powders that were added 1.5 mL distilled water and amino acids were extracted at 4 °C for 17 h before centrifugation at 12,000 rpm for 20 min; 0.8 mL supernatant and 0.8 mL 5% 5-sulfosalicylic acid dihydrate solution were transferred to 2 mL Eppendorf tubes, and the mixture was centrifuged at 12,000 g at 4 °C for 20 min. The supernatant was filtered through a 0.22 μm membrane filter prior to total amino acid assay using an AAA L-8900 auto-amino acid analyser (Hitachi Ltd., Japan).

2.10. Endogenous GAs, ABA and salicylic acid (SA) contents assays

The freshly harvested 35 DAH seeds of wild-type and *wsd1* mutant were freeze-dried in a vacuum freeze-dryer (Scientz-100 F), and ground into fine powders using a Retsch MM 400 apparatus. Fifty mg of each powder were added to 0.5 mL of acetonitrile water solution for extracting GAs. The supernatant was added to 10 μL TEA and 10 μL BPTAB, and the mixture was allowed to react for 1 h at 90 °C. The mixture was dried using a nitrogen gas stream, reconstituted with 100 μL acetonitrile water solution, and filtered through a 0.22 μm membrane filter for LC-MS analysis. The sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD; MS, Applied Biosystems 6500 Triple Quadrupole). The analytical conditions were as follows, LC: column, Waters ACQUITY UPLC HSS T3 C18 (100 mm × 2.1 mm i.d., 1.8 μm); solvent system, water with 0.04% formic acid (A), acetonitrile with 0.04% formic acid (B); gradient program, started at 5% B (0–1 min), increased to 95% B (10 min), 95% B (10.1 min–11 min), finally ramped back to 5% B (11.1 min), 5% B (11.1 min-14min); flow rate, 0.35 mL/min; temperature, 40 °C; injection volume 10 μL. Applied Biosystems (AB) 6500+ QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 550 °C; ion spray voltage (IS) 5500 V; curtain gas (CUR) was set at 35.0 psi. Declustering potential (DP) and collision energy (CE) for individual multiple reaction monitoring (MRM) transitions were done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the plant hormones eluted within this period. An internal standard method was used to determine hormone contents. Analyses of GA contents were performed by MetWare Biotechnology (www.metware.cn). Three biological repeats were made for each sample.

For determination of ABA and SA content, seeds of harvested wild-type and *wsd1* mutant were similarly ground into powder. The sample extracts were analyzed by using an LC-ESI-MS/MS system (HPLC: Agilent 1290; MS, AB 6500 Triple Quadrupole). The analytical conditions were as follows, LC: column, Agilent Poroshell 120 SB-C18 (150 mm × 2.1 mm i.d., 2.7 μm); solvent system, methanol with 0.1% formic acid (A), water with 0.1% formic acid (B); gradient program, started at 20% A (0 min–1 min), increased to 90% A (1 min–9 min), 90% A (9 min–10.5 min), finally ramped back to 20% A (10.5 min–10.6 min), 20% A (10.6 min–13.5 min); flow rate, 0.2 mL/min; temperature, 40 °C; injection volume 2 μL. The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 400 °C; ion spray voltage (IS) 4.5 kV; curtain gas was set at 15.0 psi; Gas1 was set at 65.0 psi; Gas2 was set at 70.0 psi; monitor model: MRM. The internal standard method was used to determine hormone contents. Analyses of ABA and SA were assayed by Convinced-Test Company (www.convinced-test.com). Three biological repeats were made for each sample.

2.11. Measurement of alpha-amylase activity

Alpha-amylase activity assays were performed on seeds of wild-type and *wsd1* mutant freshly harvested at 35 DAH using an alpha-amylase assay kit (Megazyme Cat. No. K-CERA 09/11) following the manufacturer's instructions.

2.12. ABA and paclobutrazol (PAC) treatment

Seeds of wild-type and *wsd1* mutant (35 DAH) for ABA or PAC treatments were exposed to 50 °C for 7 days to break dormancy. Fifty seeds of each genotype were placed in 9 cm Petri dishes containing doubled sheets of filter papers. Different concentrations of ABA (Aladdin, A100493) or PAC (PhytoTech, P687) water solution were added to the dishes prior to incubation in darkness for 7 days at 30 °C. Germination percentages of wild-type and *wsd1* mutant were monitored daily.

2.13. Data analysis

Three replicates in each experiment were performed for all samples and the experiments data were expressed as mean values ± SD (standard deviation). The statistically significant differences of the mean values in different groups were determined by using Student's *t*-test (Microsoft Excel software). Statistical significance differences at $P < 0.05$ and $P < 0.01$ were indicated by asterisks * and **, respectively.

3. Results

3.1. Characterization of the wsd1 mutant

The weak seed dormancy mutant, named *weak seed dormancy 1* (*wsd1*) was identified from the ^{60}Co radiation mutagenesis mutant library of cv N22. Freshly harvested seeds of *wsd1* at 35 DAH had lower dormancy (~54% germination) than the wild-type N22 parent (near 0% germination) following imbibition for 7 days (Fig. 1A–C). In addition, *wsd1* mutant plants had reduced plant height, reduced grain length but increased grain width, and lower 1000-grain weight than the wild-type (Supplementary Figs. S1A–D). The number of tillers and days to flowering of *wsd1* mutant were not significantly different from the wild-type (Supplementary Figs. S1E and F). These results suggested that *WSD1* played an important role in controlling seed dormancy in rice.

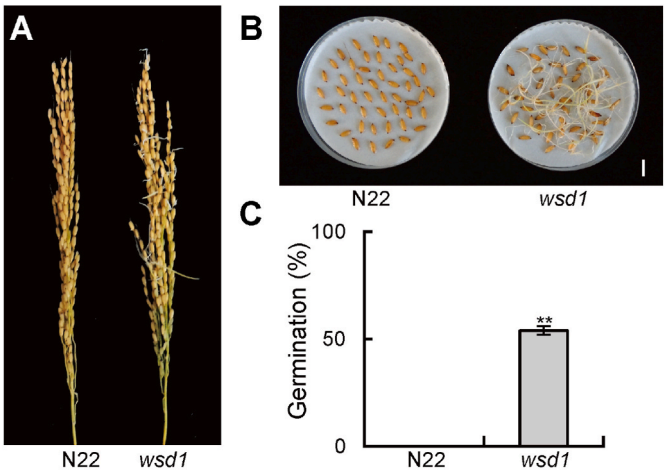


Fig. 1. Weak dormancy phenotype of the *wsd1* mutant. (A) Seed germination phenotypes of seeds from freshly harvested panicles of the wild-type and *wsd1* mutant at 35 days after heading (DAH), after 7 days of imbibition in water. (B) Germinated 35 DAH seeds of wild-type (N22) and *wsd1* mutant in Petri dishes at 7 days after imbibition. Scale bar, 1 cm. (C) Statistical analysis of germination percentage in (B). Values in (C) are means ± SD (n = 3). $^{**}P < 0.01$ compared with the wild-type by Student's *t*-tests.

3.2. Cloning of WSD1 and complementation analysis

To isolate the mutation gene in *wsd1* mutant, we crossed the *wsd1* mutant with wild-type and generated an F_1 plants. We found that the germination percentage of seed from F_1 plants was similar to that of the wild-type (Supplementary Fig. S2). Then we performed seed dormancy assay in the F_2 population, and found that the ratio of wild-type and mutant phenotype plants was closed 3:1 (102 wild-type plants, 44 *wsd1* plants; $\chi^2 = 2.05$, $P > 0.05$). These results indicated that the *wsd1* mutant phenotype was due to a single recessive locus.

To map the *WSD1* gene, we chose the MutMap approach to identify the *WSD1* locus. The *wsd1* phenotype pool, wild-type phenotype pool, *wsd1* mutant and wild-type (N22) were performed whole-genome re-sequencing, respectively. The SNP/INDEL index was calculated between wild-type, *wsd1* mutant, the wild-type phenotype pool and the mutant phenotype pool in the whole genome. From the combined MutMap analysis and the characteristics of radiation mutagenesis, we detected 10 INDELs (INDEL index = 1) in chromosome 1, 3, 4, 9 and 12 (Supplementary Fig. S3A, Supplementary Table 2). The sequence comparison of wild-type and *wsd1* mutant detected only one indel was in a coding region. The gene involved, LOC_Os03g24460, had a 19 bp deletion (10 bp deletion in the fourth exon and 9 bp in fourth intron) in the *wsd1* mutant relative to the wild-type (Fig. 2A). This deletion caused splicing errors in the mRNA, and leaded to the frame shift and premature termination of protein translation (Fig. 2B, Supplementary Figs. S3B and C). Therefore, we speculated that LOC_Os03g24460 is the *WSD1* gene.

To confirm this assumption, we constructed a transgenic complementary vector containing the coding sequence of the *WSD1* gene and 1600 bp of promoter, and transformed it into *wsd1* mutant callus. The positive transgenic complementary lines complemented the weak seed dormancy phenotype of the *wsd1* mutant (Fig. 2C and D). The *WSD1* RNA interference (*RNAi*) lines exhibited weak seed dormancy phenotypes, similar to the *wsd1* mutant (Fig. 2 E and F). The *RNAi* lines had lower *WSD1* transcript levels than the wild-type (Fig. 2G). These results demonstrated that the 19 bp deletion in LOC_Os03g24460 caused the *wsd1* mutant phenotype.

The *WSD1* encodes a 459-amino acids protein with a P-loop NTPase domain in the N-terminus and an aminotransferase domain containing a BCAT-like N-terminal domain and a BCAT-like C-terminal domain in the C-terminus (Supplementary Fig. S4A). Phylogenetic tree analysis

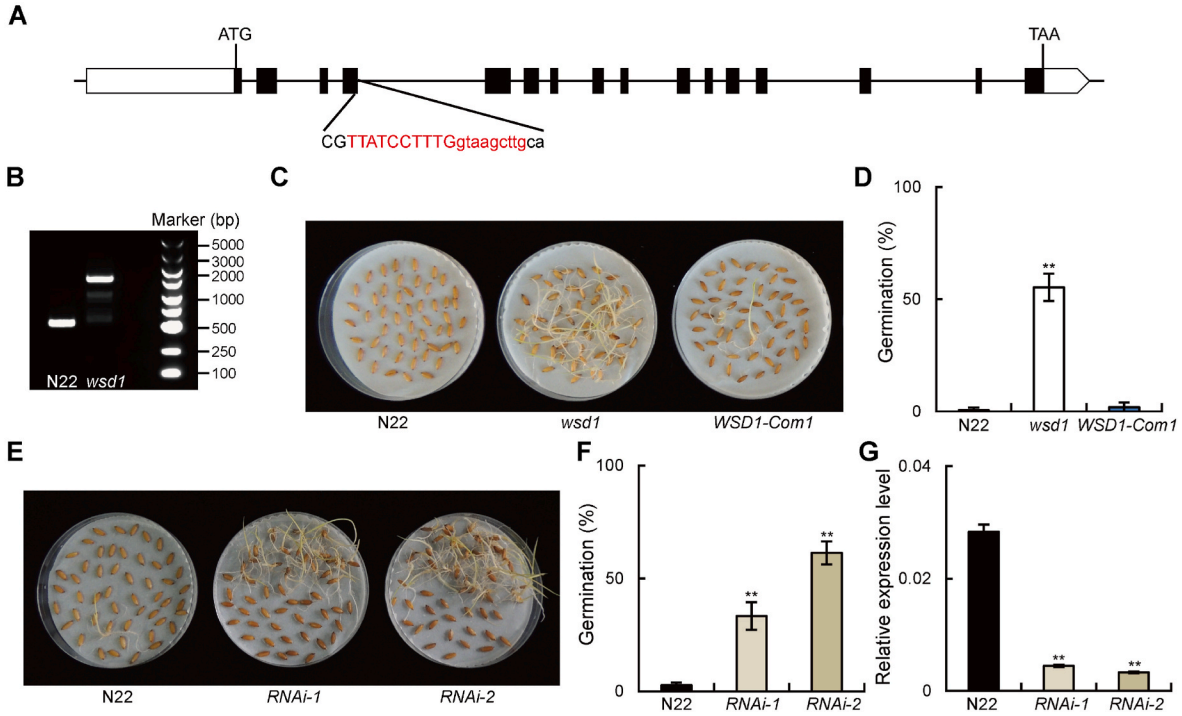


Fig. 2. Cloning and functional verification of *WSD1*. (A) Gene structure of the *WSD1* locus. The *WSD1* mutation site is shown in red. Black boxes represent exons. The start and stop codons are shown. (B) Size verification of the coding sequence between wild-type and *wsd1* mutant alleles. (C) Germinated seeds of wild-type (N22), *wsd1* mutant and a *WSD1* transgenic complementation line after 7 days of imbibition. The seeds were harvested at 35 days after heading (DAH). (D) Statistical analysis of germination percentages in (C). (E) Germinated 35 DAH seeds of wild-type (N22) and two the *WSD1* RNA interference lines after imbibition for 7 days. (F) Statistical analysis of germination percentage in (E). (G) The qRT-PCR analysis of the *WSD1* expression in wild-type (N22) and two the *WSD1* RNA interference lines. Values in (D), (F) and (G) are means ± SD (n = 3). $^{**}P < 0.01$ compared with the wild-type by Student's *t*-tests.

indicated that WSD1-like proteins differed between dicots and monocots, and that WSD1 belonged to the monocot group (Supplementary Fig. S4B).

3.3. Expression pattern and subcellular localization of WSD1

To better understand the biological functions of *WSD1*, we found that *WSD1* was expressed in all tissues and at various developmental stages according to the Rice Expression Profile Database (https://ricexpro.dna.affrc.go.jp). The qRT-PCR assays were performed to analyze the *WSD1* expression in the seeds of different developmental stages. And the expression of *WSD1* decreased in seeds from 4 d to 12 d, and was low in seeds of 24 d and 28 d (Fig. 3A). In addition, *WSD1* maintained stable expression in seeds after imbibition (Fig. 3B). Transient expression of

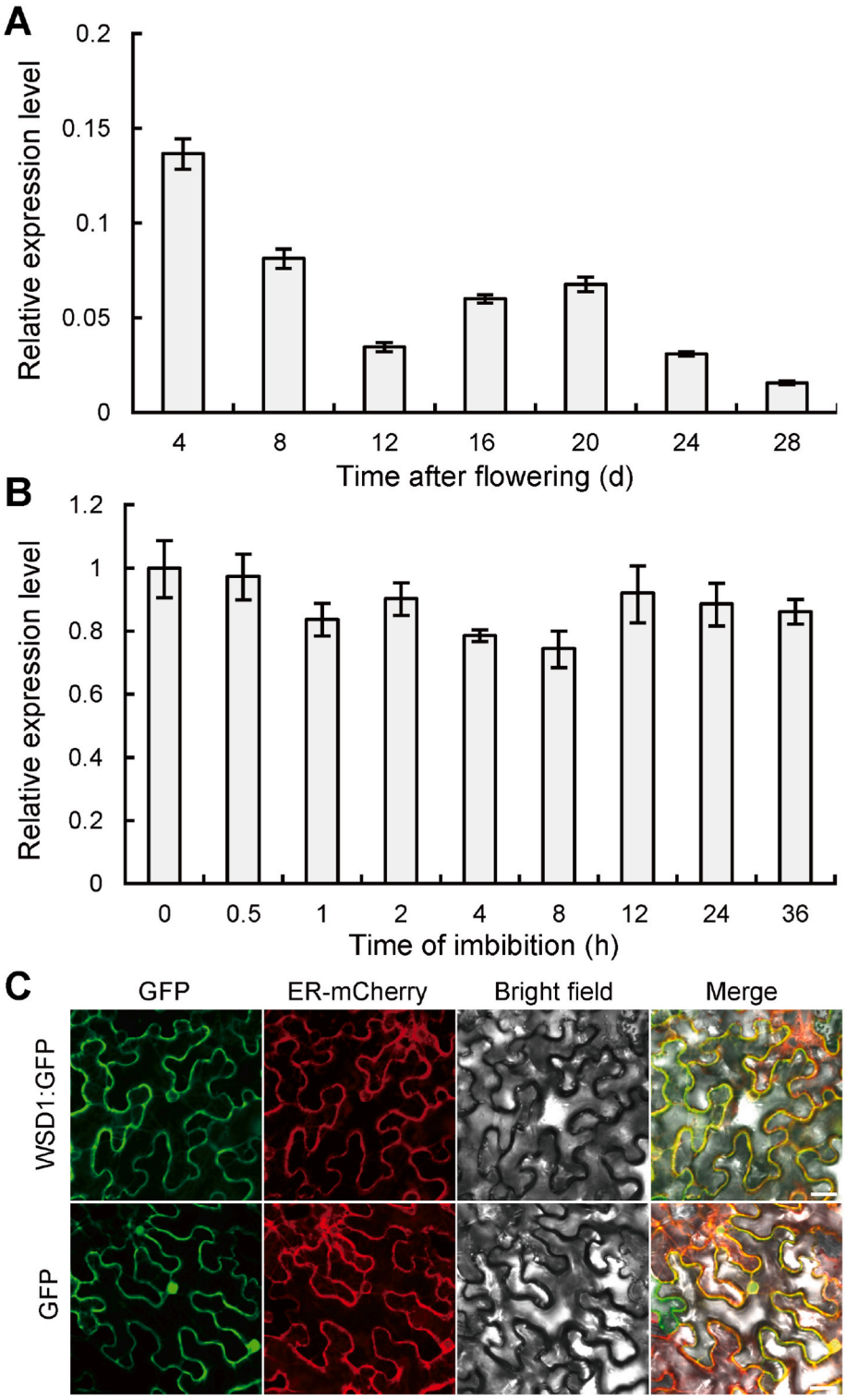
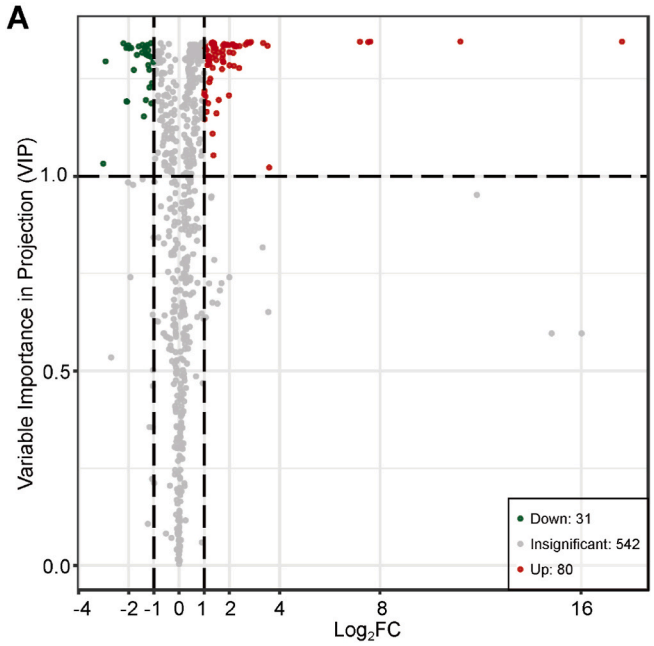


Fig. 3. Expression pattern and subcellular localization of WSD1. (A) The qRT-PCR analysis of *WSD1* expression in seeds at different times after flowering. (B) The qRT-PCR analysis of *WSD1* expression at different times of seed imbibition. (C) WSD1-GFP fusion protein localized in the endoplasm reticulum in *N. benthamiana* leaves. GFP protein was used as the control. Scale bar, 25 μm. Values in (A) and (B) are means ± SD (n = 3).

WSD1-GFP vector in *N. benthamiana* leaves showed that WSD1-GFP was localized to the endoplasm reticulum (ER) (Fig. 3C).

3.4. Mutation of WSD1 altered the amino acids contents in seeds

Since *WSD1* encoded a protein containing an aminotransferase domain, it likely participated in metabolism or biosynthesis of amino acids. Therefore, we performed widely targeted metabolomics assays to investigate the function of *WSD1*. Metabolomics analysis showed that 653 metabolites were detected between wild-type and *wsd1* mutant, involving amino acids and derivatives, lipids, organic acids and its derivatives, alkaloids, alcohols, nucleotides, carbohydrates, flavonol, phenylpropanoids, and vitamins and derivatives in seeds at 24 DAF (Supplementary Table 3). Orthogonal partial least squares discriminant analysis (OPLS-DA) identified 111 metabolites with differences between *wsd1* mutant and wild-type of fold change ≥2 or ≤ 0.5 and Variable



B

Group name Class	*wsd1* vs WT	
	up	down
Amino acid and its derivatives	31	2
Alkaloids	4	4
Alcohols	1	5
Carbohydrates	1	3
Flavonol	2	3
Lipids	11	1
Nucleotide and its derivatives	5	0
Organic acids and its derivatives	8	4
Phenylpropanoids	4	2
Vitamins and derivatives	4	0

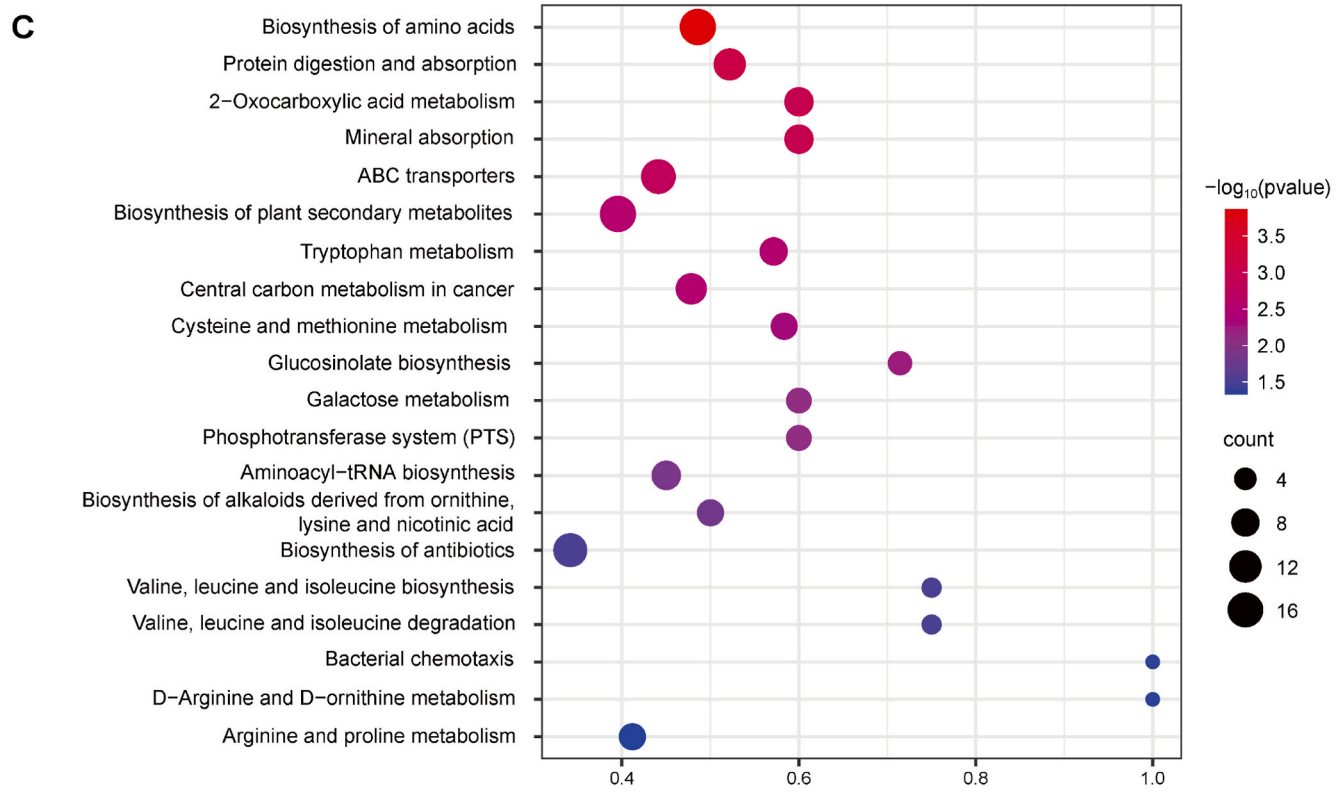


Fig. 4. Widely targeted metabolomic profiling of wild-type and *wsd1* mutant. (A) Volcano plot of 111 differential metabolites between the seeds of wild-type and *wsd1* mutant at 24 days after flowering (DAF). The parameters of differential metabolites were | $\log_2$(fold change) | ≥1 and Variable Importance for Projection (VIP) ≥1. (B) Classification of some differential metabolites in the wild-type and *wsd1* mutant. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the 111 differential metabolites in the wild-type and *wsd1* mutant.

Importance in Project ≥1 (Fig. 4A, Supplementary Table 3). Thirty-one metabolites were decreased and 80 metabolites were increased in the *wsd1* mutant compared with wild-type. Among them, 33 amino acids or derivatives showed differences, 31 up-regulated and 2 down-regulated (Fig. 4B). KEGG enrichment analysis indicated that major differential metabolites were enriched in several pathways including biosynthesis of amino acids, protein digestion and absorption, 2-Oxocarboxylic acid metabolism, mineral absorption, ABC transporters, biosynthesis of plant secondary metabolites, and tryptophan metabolism (Fig. 4C). These results suggested that *WSD1* plays an important role in biosynthesis of amino acids.

To further verify the result of metabolomics assay, we measured the levels of free amino acids in the 35 DAH seeds of *wsd1* and wild-type. Free amino acids levels in 35 DAH seeds were significantly increased in the *wsd1* mutant relative to the wild-type, especially Arg, Asp, Lys, Leu (Supplementary Fig. S5). The levels of nearly all amino acids were increased in *wsd1* mutant. Amino acids are used for the synthesis of proteins, hormones and energy for seed germination at the early stages (He et al., 2019). These results suggested that the function of *WSD1* in seed dormancy might be homeostatic control of amino acids.

3.5. Expression of GA biosynthesis-related genes is up-regulated in the *wsd1* mutant

To reveal the effect of *WSD1* mutation in transcriptional levels, we performed the RNA-seq analysis on the seeds of wild-type and *wsd1* mutant at 24 DAF. Compared with the wild-type, there were 3537 differentially expressed genes (DEGs) including 1458 up-regulated DEGs and 2079 down-regulated DEGs in *wsd1* mutant (Fig. 5A and B, Supplementary Table 4). Verification based on expression levels of randomly selected genes by qRT-PCR gave results consistent with those from RNA-seq analysis (Fig. 5C and D). KEGG enrichment analyses

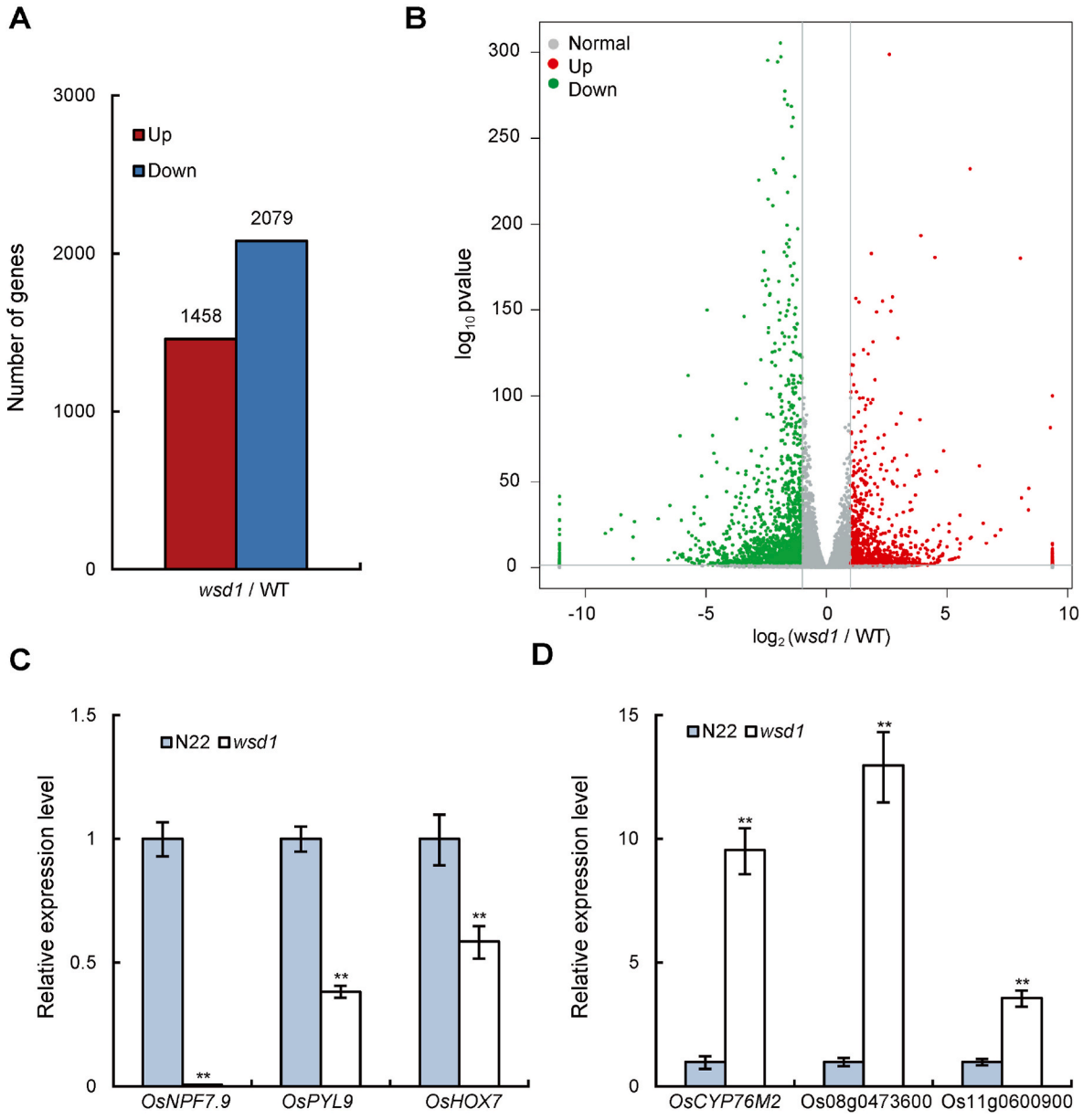


Fig. 5. RNA sequencing analysis in wild-type and *wsd1* mutant. (A) Number of differentially expressed genes (DEGs) in the seeds of the wild-type and *wsd1* mutant at 24 days after flowering (DAF). (B) Volcano plot of 3537 DEGs between the wild-type and *wsd1* mutant in seeds of 24 DAF. (C–D) The qRT-PCR assays of DEGs in RNA-seq. Three up-regulated DEGs and three down-regulated DEGs were verified. Values in (C) and (D) are means ± SD (n = 3). $^{**}P < 0.01$ compared with the wild-type by Student's *t*-tests.

indicated that up-regulated DEGs were enriched in starch and sucrose metabolism, diterpenoid biosynthesis, carotenoid biosynthesis and phenylpropanoid biosynthesis, and down-regulated DEGs were enriched in photosynthesis, photosynthesis-antenna proteins, plant hormone signal transduction and flavonoid biosynthesis (Supplementary Figs. S6A and B). Specifically, some genes involved in GA biosynthesis were significantly expressed in the *wsd1* mutant, such as *OsCPS1*, *OsKS3* and *OsGA2ox3* (Supplementary Fig. S6C). Several genes involved in plant hormone signal transduction, especially those for ABA signaling, were decreased including *OsPYL7*, *OsbZIP17* and *OsPYL8* (Supplementary Fig. S6C). Thus, these results suggested that *WSD1* might affect seed dormancy through control of GA biosynthesis or ABA signaling-related genes expression.

3.6. WSD1 controls seed dormancy by regulating GA levels

Gibberellin is the key phytohormone in control of seed dormancy and germination. RNA-seq analysis indicated that expression of some GA biosynthesis-related genes was increased in the *wsd1* mutant (Supplementary Fig. S6C). To verify the relationship between *WSD1* and the GA pathway, we firstly performed PAC (a GA synthesis inhibitor) treatment assays. Firstly, we found that seeds of the wild-type and *wsd1* had similar germination rates when dormancy was compromised by heat treatment (Supplementary Fig. S7A). Then we treated the non-dormant seeds of *wsd1* mutant and wild-type with 10 μM PAC for 7 days. PAC assays showed that the germination percentages of both wild-type and *wsd1* mutant under PAC treatment were markedly decreased at 3 days post imbibition compared to a mock treatment, but the germination rates were not significantly different between wild-type and *wsd1* mutant (Supplementary Figs. S7A and B). These results suggested that the *WSD1* mutation have no obvious effect on GA signaling pathway or GA biosynthesis pathway.

GA content analysis indicated that the contents of GA_{19} and GA_{20} were similar in the wild-type and *wsd1* mutant, but the GA_{29} and GA_5 levels were lower in the *wsd1* mutant than the wild-type (Fig. 6A–C, Supplementary Figs. S8A and B). The levels of GA_6, as a GA_5 product, was similar between wild-type and *wsd1* mutant, but GA_3 was not detected in either wild-type or *wsd1* mutant (Fig. 6D). The bioactive form GA_1 was significantly increased and the content of GA_8 was decreased in *wsd1* mutant compared to the wild-type (Fig. 6E and F). And the content of GA_4 was similar between wild-type and *wsd1* mutant (Supplementary Fig. S8C). Expression levels of GA biosynthesis-related genes, including *OsCPS1*, *OsKS1*, *OsKO2* and *OsGA3ox2*, were significantly increased in the *wsd1* mutant (Fig. 6G). These results indicated that *WSD1* regulates seed dormancy by control of GA levels and GA-related gene expression.

Alpha-amylase plays an important role in seed germination and GA positively regulates its expressions (Damaris et al., 2019). Then, we performed alpha-amylase activity assay and found that alpha-amylase activity in *wsd1* mutant was higher than that in the wild-type (Fig. 6H). The qRT-PCR analysis indicated that the expression of amylase-related genes was significantly up-regulated (Fig. 6I). These results suggested that both activity and expression level of alpha-amylase were increased in the *wsd1* mutant.

3.7. ABA signaling is reduced in the wsd1 mutant

ABA and GA have antagonistic roles in control of seed dormancy

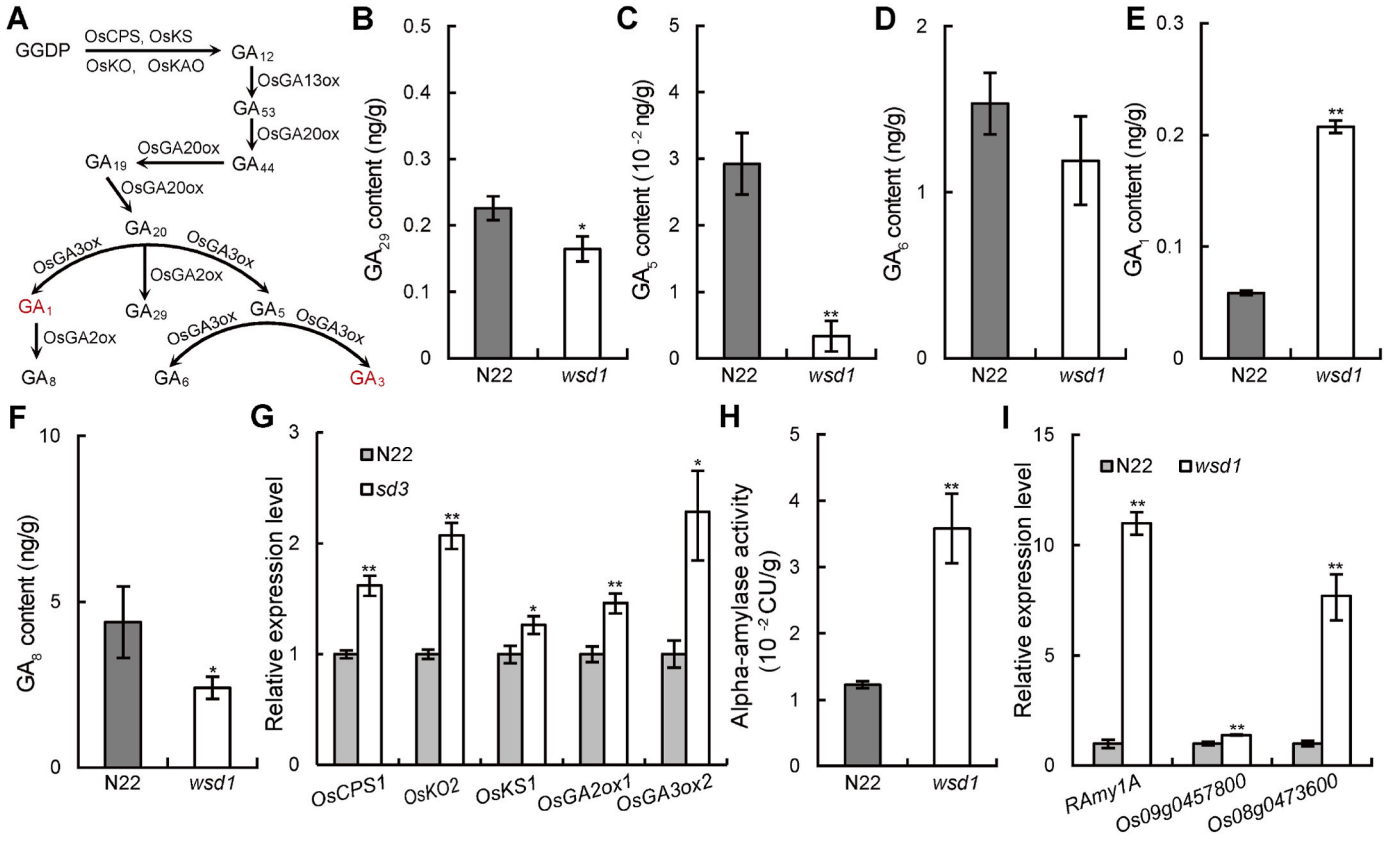


Fig. 6. GA_1 content and alpha-amylase activity are increased in the *wsd1* mutant. (A) Conversion of GGDP to the GA_1 or GA_3 pathways in rice based on previous studies. (B–F) The GA_{29} (B), GA_5(C), GA_6 (D), GA_1 (E) and GA_8 (F) contents in seeds of wild-type and *wsd1* mutant at 35 days after heading (DAH). (G) The qRT-PCR analysis of expression of *OsCPS1*, *OsKO2*, *OsKS1*, *OsGA2ox1* and *OsGA3ox2* after 24 h of imbibition by seeds at 35 DAH. (H) Alpha-amylase activity of wild-type and *wsd1* mutant seeds at 35 DAH. (I) The qRT-PCR analysis of expression of *RAmy1A*, Os09g0457800 and Os08g0473600 using seeds sampled 35 DAH seeds. Values in (B–I) are means ± SD (n = 3). $^{**}P < 0.01$ compared with the wild-type by Student's *t*-tests.

(Shu et al., 2016). To reveal the effect of *WSD1* in ABA signaling, we performed the ABA treatment to test the ABA response. We firstly treated the seeds of wild-type and *wsd1* mutant under 50 °C for 7 days to break seed dormancy, and found the germination percentages of wild-type (89%) was similar to *wsd1* (92%) (Fig. 7A). ABA treatment assays showed that both wild-type and *wsd1* mutant responded similarly to low concentrations of ABA (0.5 μM and 1 μM), but the *wsd1* mutant exhibited remarkably reduced sensitivity compared to the wild-type when subjected to higher ABA concentrations (Fig. 7B). The germination percentage of *wsd1* mutant was 71% under 5 μM ABA treatment whereas that of the wild-type was 17% (Fig. 7B). The ABA content of *wsd1* mutant was increased relative to the wild-type (Fig. 7C). Furthermore, we examined the expression level of ABA signaling-related genes. In *wsd1* mutant, we found that the expression levels of ABA receptor-related genes and OsABI5 were markedly reduced (Fig. 7D). The results of a subsequent qRT-PCR analysis were consistent with those from the ABA treatment assays. These results suggested that the weak seed dormancy phenotype of *wsd1* mutant was largely caused by decreased sensitivity to ABA.

4. Discussion

Seed dormancy in rice is required to prevent pre-harvest sprouting, but knowledge about the molecular mechanisms of regulating seed dormancy is limited. In this study, we identified a previously unidentified locus (*WSD1*) that positively regulates seed dormancy. *WSD1* encodes a protein with an aminotransferase domain. Metabolomics assays showed that *WSD1* mutation disrupted homeostatic control of amino acids. In *wsd1* mutant, the GA_1 content was increased and the ABA sensitivity was reduced compared with wild-type. Our findings revealed that *WSD1* positively regulates seed dormancy by affecting GA biosynthesis and ABA signaling.

WSD1 has an aminotransferase domain and includes BCAT-like N-terminal and C-terminal domains (Supplementary Fig. S4A). By means of MutMap analysis and transgenic experiments, we confirmed that the *WSD1* mutation caused the weak dormancy phenotype of the *wsd1* mutant (Fig. 3). Previous studies showed that branched-chain aminotransferases are involved in branched-chain amino acid (BCAA) synthesis and catabolism (Binder et al., 2007). For example, AtBCAT-1 located in mitochondria in *Arabidopsis* may be responsible for BCAA catabolism (Diebold et al., 2002). Our metabolomics analysis indicated differences in 33 amino acids or derivatives between the *wsd1* mutant and wild-type; BCAA contents were increased in *wsd1* mutant (Fig. 4, Supplementary Table 3). The result of a free amino acids assay was consistent with those from the metabolomics analysis (Supplementary Fig. S5). Although these results suggest that *WSD1* has a role in

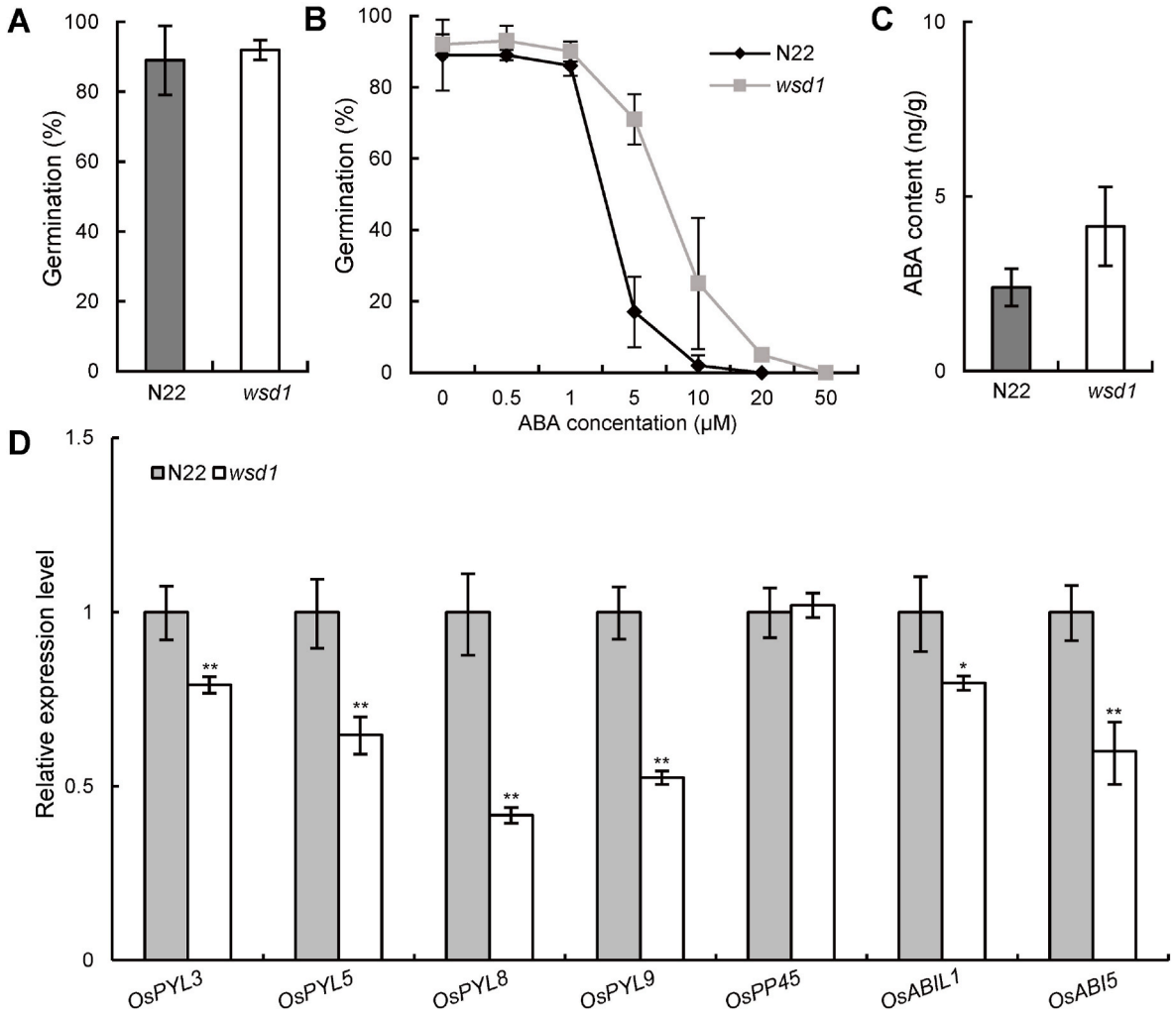


Fig. 7. The *wsd1* mutant has reduced sensitivity to ABA. (A) Germination percentages of seeds of wild-type and *wsd1* mutant sampled at 35 days after heading and following 50 °C treatment for 7 days. (B) Germination percentages of non-dormant seeds of wild-type and *wsd1* mutant following treatment with various concentrations of ABA. (C) ABA contents of wild-type and *wsd1* mutant seeds at 24 days after flowering. (D) The qRT-PCR analysis of the expression of ABA signaling-related genes in seeds at 35 days after heading. Values in (C–D) are means ± SD (n = 3). $^*P < 0.05$, $^{**}P < 0.01$ compared with the wild-type by Student's *t*-tests.

regulating amino acids homeostasis, it will be necessary to clarify whether WSD1 has aminotransferase activity in future studies. Some genes encoding aminotransferases have multiple roles in regulating agronomic traits in crop species. For example, *Qsd1*, encoding an alanine aminotransferase, negatively controlled seed dormancy in barley (Sato et al., 2016); *TaBCAT1*, encoding a branched-chain amino acid aminotransferase, positively regulated wheat rust susceptibility, and the disruption mutants had elevated BCAA content and increased levels of SA (Corredor-Moreno et al., 2021). And we found that the SA content in *wsd1* mutant was reduced compared to that in wild-type (Supplementary Fig. S9). In RNA-seq analysis, the KEGG enrichment analyses indicated that down-regulated DEGs were also enriched in phenylalanine metabolism (Supplementary Fig. S6B). In phenylalanine metabolism pathway, some SA biosynthesis-related genes were reduced expression that may lead to decrease the SA content, such as *OsPAL4* and *OsPAL7* (Supplementary Table 4). Therefore, *WSD1* and *TaBCAT1* may function differently in regulating the SA content. Here, we found that *WSD1* positively regulates seed dormancy. The *wsd1* mutant plants displayed significantly higher germination percentages than the wild-type after imbibition for 7 days at 30 °C (Fig. 1). Days to flowering and grain size of the *wsd1* mutant were similar to those of the wild-type (Supplementary Fig. S1). Therefore, WSD1 is more useful on moderating seed dormancy than other agronomic traits. Phylogenetic tree analysis showed that WSD1 belongs to a monocot group (Supplementary Fig. S4B). The functions of other WSD1-like proteins are unknown. Further studies are needed to explore whether other WSD1-like proteins are involved in regulation of seed dormancy.

GA is the primary phytohormone group promoting seed germination (Shu et al., 2016). For example, *qSD1-2* encodes GA synthesis enzyme OsGA20ox2, and negatively regulates seed dormancy by increasing GA content (Ye et al., 2015). KEGG enrichment analyses in RNA-seq indicated that some up-regulated DEGs were enriched in diterpenoid biosynthesis, and some GA biosynthesis-related genes had increased expression in the *wsd1* mutant (Supplementary Figs. S6A and C). GA_1 level was significantly increased in the *wsd1* mutant (Fig. 6E). It is known that GA positively regulates the alpha-amylase expression (Sun and Gubler, 2004; Damaris et al., 2019). Activity and expression of alpha-amylase in the *wsd1* mutant were increased relative to the wild-type (Fig. 6H and I). These results indicated that *WSD1* positively regulated seed dormancy by reducing GA content. Previous studies showed that the free amino acids have a role in seed germination; ketogenic amino acids can be degraded into acetyl-CoA, the precursor in GA biosynthesis (Muntz et al., 2001; Rios-Iribe et al., 2011). A recent study reported that *IPMS1* encodes an isopropylmalate synthase that catalyses Leu biosynthesis, and that IPMS1 was involved in GA synthesis by promoting the synthesis of GA biosynthesis-related amino acids (He et al., 2019). Therefore, *OsIPMS1* regulates amino acid contents to affect GA biosynthesis in a similar way to *WSD1*. Our metabolomics analysis and free amino acids assays showed that the levels of some ketogenic amino acids, including Leu, Lys, Ile and Tyr, were higher in the *wsd1* mutant (Fig. S5, Supplementary Table 3). Thus, the increased levels of amino acids in the *wsd1* mutant might promote the GA biosynthesis, but future study is required.

The ABA/GA balance plays a crucial role in determining seed germination at the appropriate time (Shu et al., 2016). In rice, some reported seed dormancy regulators are mainly involved in the ABA pathway, such as *Sdr4*, *SD6*, *OsDOG1L-3* and *qSD7-1* (Sugimoto et al., 2010; Gu et al., 2011; Wang et al., 2020; Chen et al., 2021; Xu et al., 2022). And *qSD1-2* and *miR156* are principally involved in GA biosynthesis pathway to regulate seed dormancy (Ye et al., 2015; Miao et al., 2019). However, our knowledge about genes controlling ABA/GA balance is still limited in seed dormancy regulation. In our study, we found that *WSD1* regulates seed dormancy by affecting GA biosynthesis pathway and ABA signal pathway. In the *wsd1* mutant, the GA_1 content and the expression of GA biosynthesis-related genes were increased, and ABA sensitivity was reduced compared with wild-type (Figs. 6 and 7B). *OsGA2ox9* encodes a GA 2-oxidase that can inactivate endogenous bioactive GAs, and its knockout mutant *OsGA2ox9*-Cas9 increased the bioactive GA content, alpha-amylase activity and the expression of alpha-amylase encoding genes. And GA-promoted starch hydrolysis resulted in reduced ABA signaling in *OsGA2ox9*-Cas9 mutant (Xing et al., 2023). So, the regulatory mechanism of *WSD1* in ABA signaling may be similar to that of *OsGA2ox9*. In addition, WSD1 was localized in endoplasmic reticulum in tobacco (Fig. 3C). A previous study reported that co-localization of OsABIL2 and OsPYL1 was also in the endoplasmic reticulum, and the bimolecular fluorescence complementation (BIFC) signal of OsPYL1-OsABIL2 interaction was detected in the endoplasmic reticulum in tobacco (Li et al., 2015). Thus, it needs further study to explore the molecular mechanism of how *WSD1* regulates ABA/GA balance and ABA signaling in the future.

5. Conclusions

We identified a new seed dormancy regulator *WSD1* that controls seed dormancy by affecting GA biosynthesis and ABA signaling. The WSD1 protein contains an aminotransferase domain, and mutation of *WSD1* leads to alter amino acids homeostasis. Compared with the wild-type, GA_1 content was increased and ABA signaling was reduced in the *wsd1* mutant. The present findings about the function of WSD1 in regulating seed dormancy provide new insights for the strategy by moderating seed dormancy to optimize pre-harvest sprouting control in rice.

Author contributions

J.M.W. and L.J. supervised the project. Y.S.H. and L.J. designed the research. Y.S.H., J.W.S., Q.X.H., C.L.M., H.M.W., F.L.Z., Z.Y.Z., P.W., T. F.M., K.F., Y.P.C. and T.N. performed the experiments. S.J.L. cultivated the transgenic plants in the field. Y.S.H. S.R.Z.and L.J. analyzed the data. Y.S.H. drafted the manuscript. L.J. and J.M.W. revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.107923.

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