Research article

Rice OsBT1 regulates seed dormancy through the glycometabolism pathway

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ABSTRACT

Seed dormancy and germination in rice (Oryza sativa L.) are complex and important agronomic traits that involve a number of physiological processes and energy. A mutant named h470 selected from a60Co-radiated indica cultivar N22 population had weakened dormancy that was insensitive to Gibberellin (GA) and Abscisic acid (ABA). The levels of GA4 and ABA were higher in h470 than in wild-type (WT) plants. The gene controlling seed dormancy in h470 was cloned by mut-map and transgenesis and confirmed to encode an ADP-glucose transporter protein. A 1 bp deletion in Os02g0202400 (OsBT1) caused the weaker seed dormancy in h470. Metabolomics analyses showed that most sugar components were higher in h470 seeds than the wild type. The mutation in OsBT1 affected glycometabolism.

1. Introduction

Rice (Oryza sativa L.) is one of the most important food resources for humankind (Hong et al., 2019), and the level of production affects food security. Increased yield and superior processing quality are major foci of research (Wang et al., 2015). Seed dormancy in rice is an important agricultural trait on account of its association with pre-harvest sprouting (PHS), seedling vigor, quality and yield as in many different cereals (Nonogaki et al., 2018).

Seed dormancy is affected by many factors and is controlled by multiple proteins (Allen et al., 2018; Nonogaki et al., 2018). Numerous studies have shown a close correlation between seed dormancy and phytohormones. Among them, GA and ABA are particularly important in regulating seed dormancy (Urbanova and Leubner-Metzger, 2018). Mutants ga1 and ga2 in Arabidopsis cause defective GA biosynthesis that results in strong dormancy and sensitivity to exogenous GA (Holdsworth et al., 2008). Overexpression of GID1 (GA INSSENSITIVE DWARF1, GA receptor) significantly decreased seed dormancy (Hauvermale et al., 2015). A SnRK2.2SnRK2.3SnRK2.6 (Sucrose Non-fermenting1–Related Protein Kinase, activated by ABA) triple mutant was insensitive to ABA and showed weak seed dormancy in humid conditions (Fujii and Zhu, 2009). A number of quantitative trait loci (QTLs) controlling seed dormancy in rice have also been reported, but only three, named Sdr4, qSD7-1/qPC7 and qSD1-2 have been isolated (Sugimoto et al., 2010; Gu et al., 2011; Ye et al., 2015). Sdr4 was the first map-based cloned gene in rice, but the function of the protein was largely undetermined although possibly regulated by OsVP1 (positive regulators in the ABA signaling pathway). Sdr4 positively regulates expression of OsDOG1-like (DELAY OF GERMINATION 1, orthologous to Arabidopsis thaliana DOG1) genes during seed maturation, suggesting that it acts as an intermediate regulator of seed dormancy (Sugimoto et al., 2010). qSD7-1/qPC7 defined as the Os07g11020 or Rc gene encoding a basic helix-loop-helix (bHLH) transcription factor, functions in the regulation of seed dormancy through ABA biosynthesis and pigmentation pathways (Gu et al., 2011). qSD1-2 encodes GA20ox2, which is involved in GA metabolism and regulates seed dormancy (Ye et al., 2015).

Abbreviations: WT, wild-type; PHS, pre-harvest sprouting; ABA, abscisic acid; QTLs, quantitative trait loci; DPH, days post heading; QRT-PCR, quantitative real-time PCR; MCF, mitochondrial carrier family; HAI, hours after imbibition; SNPs, single nucleotide polymorphisms; InDels, insertions/deletions; Nip, nipponbare; PP2C, protein phosphatase 2C; TCA, tricarboxylic acid

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2015). It is important to explore more genes that regulate seed dormancy in rice.

The BT1 gene was first identified in a brittle-leaf maize (Zea mays) mutant (Sullivan et al., 1991). BT1 proteins in cereal endosperms were proposed to be ADP-glucose transporters, major amyloplast membrane proteins containing three mitochondrial carrier family (MCF) domains (Haferkamp, 2007). BT1 was also characterized in many other species including Arabidopsis thaliana (Kircherger et al., 2008) and Triticum aestivum (Bowsher et al., 2007). Other research confirmed that BT1 is important in starch biosynthesis where it affects the formation of composite starch granules in rice seeds (Cakir et al., 2016; Li et al., 2017). However, there was no report suggesting that BT1 affected seed dormancy.

Here, we characterized a weakly dormant mutant, h470, which was insensitive to applied GA and ABA. GA and ABA contents were both increased in h470 relative to the WT. Mut-map and transgenesis studies showed that a mutation in OsBT1 caused the h470 phenotype. Metabolomics studies showed that the mutation affected seed dormancy by its effects on glycometabolism.

2. Materials and methods

2.1. Plant materials and growth conditions

The weakly dormant mutant h470 was selected from the strongly dormant indica cultivar N22 following 60Co irradiation. The h470 mutant line with stable inheritance was established after several generations of self-crossing. A cross was made between h470 and N22 to produce an F2 population for subsequent mut-mapping. Rice seedlings were grown in a paddy field at Nanjing Agricultural University in 2018.

2.2. Germination test and trait measurement

Seeds of N22 and h470 and individual F2 plants were harvested at 7, 14, 21, 24, 28, and 35 days post heading (DPH) for the germination experiment. Fifty seeds of each parent and F2 plant were put on filter paper in a 9 cm Petri dish to which 10 mL of water was added. The dishes were held in darkness at 30 °C and each germination test was replicated three times. Germination percentages were determined on the seventh day. Grain lengths and widths were measured by a Wansen automatic rice test analyser (Hangzhou). Plant height, numbers of tillers and grain number per panicle were recorded at 35 DPH. There were three biological repeats for each trait.

2.3. Evaluation of sensitivity to exogenously applied GA and ABA and quantification of endogenous GA4 and ABA

N22 and h470 seeds at 24 DPH were used for endogenous treatment experiments. Germination trials were set up as described above but different concentrations of GA4 (0, 1, 10 μM) and ABA (0, 1, 4 μM) were added to the dishes. Germination percentages were recorded each day for 7 day at 30 °C. GA4 and ABA levels were measured in WT and h470 seeds collected at 24 DPH. Assays were carried out by ZhongDing Biological Company (Nanjing). GA4 and ABA were extracted from sampled seeds using an isopropl alcohol/water/hydrochloric acid extraction method, and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS) (Izumi et al., 2009). Three biological repeats were made for each sample.

2.4. Identification the mutant gene in h470

Thirty F2 plants from the h470 × N22 cross were selected and placed into weak and strong dormancy groups according to phenotype. Young leaves from each type were cut and pooled with equal numbers for whole-genome resequencing by Genepeonie Biotechnologies Company (Nanjing) using Illumina HiSeqXTen (Illumina, http://www.illumina.com/), the mut-map was performed according to a previous method based on whole-genome resequencing of pooled DNA from a segregating population of plants that showed significantly different phenotypes. The SNP/InDel ratio was determined as described previously (Abe et al., 2012). The 58.76 Gb of data showed 300 single nucleotide polymorphisms (SNPs) and 68 insertions/deletions (InDels).

2.5. Plasmid construction and isolation of transgenic lines

Among 368 potential mutant locations, there was only one InDel that showed a SNP/InDel ratio of 1. This InDel has a 1-bp deletion in the first exon of Os02g0202400. To test whether Os02g0202400 was responsible for h470 mutation, ~2 kb of promoter and full-length DNA of OsBT1 were cloned into the pCUbi1390 binary vector. Fusion vector was transformed into h470 callus by Agrobacterium-mediated transformation. The CDNA of OsBT1 from Nipponbare (Nip) was cloned and fused into the binary vector LH-FAD2-1390RNAi under the control of the maize UBQUITIN1 promoter to acquire an RNAi vector. The recombination plasmid then introduced into Agrobacterium tumefaciens strain EHA105 and transformed into Nipponbare calli. The transgenic
plants were grown in a paddy field. All primer sequences are listed in Supplementary Table 2.

2.6. RNA extraction, reverse transcription and qRT-PCR analysis

N22 and h470 seeds were harvested at 24 DPH and total RNA was extracted using an RNApure Plant Kit (DNase I) (CWBIO, Beijing). A SuperScript II Kit was used for reverse transcription (TaKaRa, Beijing). Real-time PCR was conducted using a SYBR Premix Ex TaqTM Kit (TaKaRa) on an ABI 7500 Real-Time PCR Analyser. The 2$^{−ΔΔCT}$ method was used to calculate relative expression levels of genes (Livak and Schmittgen, 2001). The rice ubiquitin gene (Os03g0234200) was used as a reference. The PCR procedure was: 95 °C for 30s, 40 cycles of 95 °C for 5s and 60 °C for 34s, then a melting curve was generated and analyzed.

2.7. Measurement of alpha-amylase activity

Alpha-amylase activity in seeds of N22 and h470 harvested at 24 DPH was measured with a Megazyme Cat. No. K-CERA 09/11 kit (McCleary and Sheehan, 1987). Three biological repeats were measured for each sample.

2.8. Metabolomics and determination of starch, sugar and glucose contents

Metabolic assays on N22 and h470 seeds harvested at 24 DPH were performed by UPLC-MS/MS at Metware Technology Company, Wuhan (Chen et al., 2013). Total starch contents were measured by a Megazyme assay kit (Wicklow, Ireland). Soluble sugar contents were assayed using a kit from the Nanjing Jiancheng Bioengineering Institute. Glucose contents were measured specifically by a kit from Pribolab (Beijing). Germination trials were set up as described above but 100 μM glucose was added with or without 5 μM of ABA to the dishes.
biological repeats were made for each sample.

2.9. Data analysis

Three replicates for each experiment refers to the number of technical replicates that were used in statistical analysis. All values in figures were means ± SD (standard deviation). Results were subjected to Student’s t-test to compare the statistically significant differences of the mean values between WT and h470 (Microsoft Excel software). The mass spectrometry data of metabolites were analyzed by the software of Analyst 1.6.3. Statistically significance differences at P < 0.05 and P < 0.01 were indicated by asterisks * and **, respectively.

3. Results

3.1. h470 exhibits weak seed dormancy

To identify the differences in dormancy levels of N22 and h470, we detected the germination percentages after imbibition for seven days, the germination percentages of h470 seeds harvested at 35 DPH and stored at room temperature (about 25 °C) for 30 days were about 40% higher than N22 (Fig. 1a). We also determined germination percentages after seven days of imbibitions using seeds harvested at 7, 14, 21 and 28 DPH. The seed dormancy of h470 was broken at 28 DPH whereas N22 maintained strong dormancy (Fig. 1b). Then germination percentages of different storage periods after heading 35 days were detected. At 35 DPH N22 completed germination to about 8%, compared to 60% for...
3.2. The weak dormancy phenotype of h470 was probably not regulated by the GA or ABA pathways

As h470 had a high germination typical of many GA overexpressors or ABA-related mutants, we speculated that h470 might involve the GA or ABA pathways. We therefore monitored germination over seven days of imbibition using different concentrations of GA3 and ABA. Neither N22 or h470 responded to GA treatment with germination percentages maintained at 0 and 20%, respectively (Fig. 2a–c). When dormancy was broken by a 7-day, 50 °C treatment the germination percentages of both N22 and h470 exceeded 90% (Fig. 2d). Germination percentages of both lines was also inhibited at < 20% with 4 μM ABA (Fig. 2e–f).

Assays of endogenous GA or ABA contents at 24 DPH showed that the ABA level in seeds of h470 was twofold higher than N22 (Fig. 2h), and the GA4 content had increased nearly 13-fold relative to N22 (Fig. 2g). In regard to GA- and ABA-related genes the expression levels of PYL5 (PYROBACTIN RESISTANT LIKE5, an ABA receptor), SAPK8 (SUCROSE NONFERMENTING1-RELATED PROTEIN KINASE8), ABI2 (a negative regulator in the ABA signaling pathway), and D1 (a G protein α subunit, and positive regulator of the GA signaling pathway) were unchanged in h470 relative to N22. These results were consistent with phenotypes following exogenous hormone treatments. KO1 (ENT-KAURENE OXIDASE) and NCED2 (9-CIS-EPOXYCAROTENOID DIOXYGENASE), which regulate synthesis of both GA and ABA, were more highly expressed in h470 (Fig. 2i). The overall results suggested that the weaker dormancy of h470 was not regulated by the GA or ABA pathways.

3.3. Identification of the h470 gene

To identify the gene responsible for the phenotype of h470, the reciprocal cross between N22 and h470 was made to produce a F2 segregation population adapted for mut-map re-sequencing. Germination percentages of F2 segregation ratio implied that a single gene determined the phenotype of h470 (weak dormancy plants n = 255, strong dormancy plants n = 150, $\chi^2_{2,1} = 1.11 < \chi^2_{2,0.05} = 3.84$). Defined the SNP/InDel index as the ratio between the number of reads of a mutant SNP and the total number of reads corresponding to the SNP, this index would approach 1 near the mutant gene and 0.5 for unlinked location (Abe et al., 2012). Because the two pools of extreme individuals were selected from the F2 population, the numerical value of SNP/InDel index of mutant site should be 1 (Fig. 3a). A total of eight putative genes were identified, but only Os02g0202400 had one InDel in exon 1. This was a 1 bp deletion (Fig. 3a–b). Moreover, these mutations between weak and strong dormancy groups existed in co-segregation assays (Fig. 3c). On the basis of these results we hypothesized that Os02g0202400 was the mutated gene in h470.

Os02g0202400 encodes an ADP-glucose transporter protein containing 425 amino acids, and annotated as OsBT1. Further bioinformatic analysis indicated that the single base deletion caused premature termination of protein translation (Fig. 3b). For further confirmation that OsBT1 was responsible for the mutant phenotype, we constructed a 4076 bp fragment containing the entire OsBT1 DNA sequence and promoter region, cloned it into the pCUbi1390 complementation binary vector and transformed it into h470. Germination percentages of 10 independent positive transgenic lines were zero, similar to that of the WT (Fig. 3d–e). An RNAi knock-down plasmid (Ri) containing the cDNA of OsBT1 was transformed into japonica cv. Nipponbare (Nip) and all 4 independent Ri transgenic lines exhibited increased germination.
3.4. Expression analysis of OsBT1 and genes related to dormancy

We analyzed the temporal expression patterns of OsBT1 at 0, 0.5, 1, 3, 12, 24, 48 and 72 h after imbibition (HAI). Expression of OsBT1 at 0.5 h was significantly elevated but subsequently reverted to a level slightly higher than that at 0 h (Fig. 4a). Although expression levels of many genes regulating seed dormancy, including Amy3 (Alpha-Amylase3), VP1, ABI5 (positive regulators in the ABA signaling pathway), DOG1L-1, DOG1L-2, DOG1L-3 and Sdr4, were not significantly different between N22 and h470, expression of others such as Amy1A (Alpha-Amylase1A) and EXP83 were increased in h470 compared to N22 (Fig. 4b). The α-amylase enzyme activity in h470 seeds was approximately four-fold higher than that in the WT (Fig. 4c). We concluded that OsBT1 might regulate seed dormancy by affecting the expression of amylase-related genes.

3.5. OsBT1 influences glycometabolism in rice

The above results indicated that the weaker dormancy of h470 was associated with changes in starch metabolism. Metabolites in seeds of N22 and h470 at 24 DPH were qualitatively and quantitatively analyzed by UPLC-MS/MS. Among 811 metabolites, 386 were increased and 40 were decreased in h470 relative to N22 (Supplementary Table 3). Using KEGG functional annotation and enrichment analysis, the differentially generated metabolites in h470 were mostly involved in carbohydrate metabolism. These included melibiose, DL-arabinose, D(+)-sucrose, D(+)-melezitose, N-acetyl-D-glucosamine, ribulose-5-phosphate, galactitol, trehalose 6-phosphate and D-sedoheptulose 7-phosphate, indicative of increased sugar contents (Fig. 5b) and decreased total starch (Fig. 5a–c). These results suggested that OsBT1 was involved in glycometabolism to regulate seed dormancy in rice. Previous studies showed that sugar acted as a signaling molecule with ABA to inhibit seed germination in rice (Zhu et al., 2009). We examined the effects of 100 μM of exogenous glucose with or without 5 μM of ABA on germination of N22 and h470. Addition of glucose alone had no effect (Fig. 6d), but in combination with ABA there was a 20% increase in germination percentage of N22 whereas h470 remained unchanged (Fig. 6e).

4. Discussion

Seed dormancy is a complex quantitative trait regulated by many proteins. Despite identification of many genes, there are more to be characterized. Our lab has been focusing research on rice seed dormancy for decades, most of the detected sites were from different populations using strongly dormant indica cultivar N22. We previously reported reduced dormancy mutants Q4359 and Q4646 in N22 following 60Co irradiation. QTL analysis showed that Q4359 lacked qSdn-1 and Q4646 lacked qSdn-5 (Lu et al., 2011; Wu et al., 2016). At present, we report mutant h470 with reduced dormancy but other agronomic traits were not different from N22 wild type (Supplemental Table 1). The germination percentages of h470 seeds after seven days of imbibitions were higher than N22 after 28 DPH and storage periods (Fig. 1a–c).

It is well-known that GA and ABA play an antagonistic role in regulating seed dormancy. Many genes related to GA and ABA have been cloned and reported to regulate seed dormancy. GA2ox (GA2-oxidases) are involved in GA metabolism, and loss of function decreases seed dormancy (Yamauchi et al., 2007). RGL2 (RGA-like 2) and SPY (SPINDLY) are DELLA proteins that negatively regulate the GA signaling pathway, both expression up-regulated during seed imbibition (SPINDLY) are DELLA proteins that negatively regulate the GA signaling pathway, both expression up-regulated during seed imbibition. Many genes related to GA and ABA have been cloned and reported to regulate seed dormancy.
harvested seeds of these both genotypes. Endogenous ABA content was increased to a small extent, along with a significantly increased level of GA4 (Fig. 2g–h). Expression levels of PYL5, SAPK8, ABI2 and D1 were almost invariably, but KO1 and NCED2 expression levels were markedly higher in h470 (Fig. 2i). These results were consistent with the previous consequences and phenotype (Miura et al., 2009; Kim et al., 2011; Li et al., 2015; Zhu et al., 2009). We assayed previously reported dormancy-related genes, including Alpha-Amylase1A, Alpha-Amylase3, EXPB3 (an expansin gene), VP1, ABI5, DOG1L-1, DOG1L-2, DOG1L-3 and Sdr4, there were no differences, except for Alpha-Amylase1A and EXPB3 (Huang et al., 2000; Lee and Kende, 2001) (Fig. 4b). These latter changes in h470 were consistent with the weaker dormancy phenotype and we concluded that the weak dormancy phenotype of h470 was not regulated by the GA or ABA pathways.

Specific regulatory genes, such as AlaAT (ALANINE AMINOTRANSFERASE) in barley (Sato et al., 2016) and Sdr4 (Seed Dormancy 4) in rice (Sugimoto et al., 2010), and metabolism-associated factors affecting dormancy were identified. In barley, Thioredoxin h (Thioredoxin of the h-type) converted from an oxidized to a partially reduced state during seed germination. Overexpressing Thioredoxin h increased the alpha-amylase activity, soluble protein and GA1 content, ultimately regulated seed dormancy (Wong et al., 2002). PHS8, which encodes an isoamylase (ISA1), influenced the accumulation of low molecular weight sugars in the seed endosperm and reduced seed dormancy. Mutation of PHS8 caused inhibited expression of OsVP1 and OsABIS, two important transcription factors in the ABA signaling pathway causing PHS. Furthermore, overexpression of OsABI3 or OsABIS could partially alleviate the PHS of phs8 (Du et al., 2018). OsPMS1 (ISOPROPYLMALATE SYNTHASE) affects the biosynthesis of free amino acids during seed germination and promotes GA synthesis, enhancing the glycolysis and tricarboxylic acid (TCA) reactions and provides more energy for during the seed germination and seedling growth (He et al., 2019). By mut-map, complementation and RNAi knock-down, we confirmed that mutation of OsBT1 was the cause of the h470 mutant phenotype (Fig. 3a–g). There are reports on the role of BT1 protein in various species such as maize, Arabidopsis, barley and wheat. Previous studies have shown that ZmBT1 (maize), HvNST1 (barley) and TaBT1 (wheat) are able to transport ADPG into amyloplasts and used for starch biosynthesis. OsBT1 encodes an ADP-glucose transporter that functions in starch synthesis (Cakir et al., 2016; Li et al., 2017). Analysis of metabolic differences between N22 and h470 showed that OsBT1 involved in the glycometabolism physiology processes, impacted the total starch, soluble sugar and glucose contents (Fig. 6). How OsBT1 regulates seed dormancy through the glycometabolism pathway remains unclear.

5. Conclusions

We identified a mutant allele of OsBT1 that caused reduced seed dormancy independent of the GA and ABA pathways and implied a glycometabolism disorder. This study provides important insights into the function of OsBT1 on seed dormancy in rice and the underlying metabolic mechanisms might be beneficial to cultivate new varieties resistance to PHS and increase quality in crops.

Contributions

Weihan Song, Ling Jiang and Jianmin Wan were responsible for establishing and designing the experiments. Weihan Song, Qixian Hao, Mengying Cai, Yihua Wang, Xingjie Zhu, Xi Liu, Yunshuai Huang, Nguyen Thanhliem, Chunyan Yang, Jiangfeng Yu and Hongming Wu operated the experiments. Liaoming Chen and Yunlu Tian planted and managed the materials in the fields. Weihan Song and Ling Jiang wrote the manuscript and analyzed the data. All authors have read and approved the manuscript.
Declaration of competing interest

The authors declare no conflicts of interest.

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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.2020.03.055.

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