Genome-wide association study reveals that the cupin domain protein OsCDP3.10 regulates seed vigour in rice

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Summary

Seed vigour is an imperative trait for the direct seeding of rice. In this study, we examined the genetic regulation of seedling percentage at the early germination using a genome-wide association study in rice. One major quantitative trait loci qSP3 for seedling percentage was identified, and the candidate gene was validated as qSP3, encoding a cupin domain protein OsCDP3.10 for the synthesis of 52 kDa globulin. Disruption of this gene in OsCdp3.10 mutants reduced the seed vigour, including the germination potential and seedling percentage, at the early germination in rice. The lacking accumulation of 52 kDa globulin was observed in the mature grains of the OsCdp3.10 mutants. The significantly lower amino acid contents were observed in the mature grains and the early germinating seeds of the OsCdp3.10 mutants compared with those of wild-type. Rice OsCDP3.10 regulated seed vigour mainly via modulating the amino acids e.g. Met, Glu, His, and Tyr that contribute to hydrogen peroxide (H2O2) accumulation in the germinating seeds. These results provide important insights into the application of seed priming with the amino acids and the selection of OsCDP3.10 to improve seed vigour in rice.

Introduction

Seed vigour is an important characteristic of seed quality that determines the rapid and uniform germination, and then the good seedling growth, as well as influences stress tolerance (Fujino et al., 2008; He et al., 2019a,b; Sun and Wang, 2007). In recent years, the direct seeding method of rice has become popular due to its advantages of cost- and labour-saving (Liu et al., 2015). However, the poor seed germination, seedling establishment, and growth in field are the major constraints in the production of direct seeding in rice. Rice varieties with high seed vigour will improve seed germination and seedling growth in field under various stress conditions (He et al., 2019a,b; Wang et al., 2010). Thus, mining genes involved in seed vigour and exploring their regulatory mechanisms are important objectives of rice breeding.

Seed vigour is a complex quantitative trait in rice. A large number of quantitative trait loci (QTL) for seed vigour have been detected using the traditional biparental mapping approach (Cui et al., 2002; Fujino et al., 2004; Wang et al., 2010; Xie et al., 2014; Zeng et al., 2021), but only few genes have been cloned (Fujino et al., 2008; He et al., 2019b). For example, qTG3-3-1 associated with low-temperature germination has been map-based cloned, and its expression is tightly associated with vacuolation of the tissues covering the embryo (Fujino et al., 2008). Rice qSE3 for seed germination and seedling establishment under salinity stress has been map-based cloned, and it encodes a K+ transporter gene OsHAK21 that regulates seed vigour via abscisic acid (ABA) and reactive oxygen species (ROS) pathways (He, et al., 2019b). Recently, the genes associated with seed vigour have been explored using genome-wide association studies (GWAS) approach in rice. For example, a total of 11 loci associated with seed vigour have been detected under salt stress using GWAS approach in rice, and two candidate genes OsNRT2.1 and OsNRT2.2 encoding nitrate transporter have been identified (Shi et al., 2017). A total of 53 QTLs for low-temperature germination have been detected, and one causal gene Stress-Associated Protein 16 (OsSAP16) has been functionally validated (Wang et al., 2018a). A total of 17 and 179 candidate genes contributing to salt and cold tolerance during seed germination have been identified using GWAS approach by Yu et al. (2018) and Yang et al. (2020), respectively. These results indicate that GWAS is a powerful approach to determine the causal genes associated with seed vigour in rice.

Cupin domain proteins were originally identified as the wheat protein germin that usually comprise both enzymatic and non-enzymatic partners, such as helix-turn-helix transcription factor, AraC type transcription factor, plant oxalate oxidases (germins), and seed storage globulins (Cechin et al., 2008). The cupin domain proteins have been reported involving in diversity functions such as plant development and plant defence. For example, the Arabidopsis cupin domain protein AtPirin1 interacts with the G protein α-subunit GPA1 and regulates seed germination and early seedling development (Lapik and Kaufman, 2003). Germin-like proteins have been shown to involve in the basal host resistance against powdery mildew in barley and wheat...
in the regulation of ROS such as hydrogen peroxide (H$_2$O$_2$) that exceed the significance threshold value of 1 (Figure 1c,d). Regions were considered as QTLs when the SNPs of polymorphism (SNP) associations with seedling percentage (Figure 1a), and the indica accessions that had significantly higher seedling percentage than the japonica accessions (Figure 1b). To investigate the genetic architecture of the natural variation in seedling percentage, a GWAS was conducted. Manhattan plots showed that expression was progressively increased with grain filling from 0 to 28 DAF and followed by decline after 28 DAF. Based on this criterion, one major QTL qSP3 for seedling percentage was identified at the early germination using GWAS approach in rice, and the candidate gene encoding cupin domain protein (Oscdp3.10) was functionally validated. We observed that Oscdp3.10 mutants resulted in low seed vigour compared with wild-type (WT) rice line. Unlike that in WT, the accumulation of 52 kDa globulin is lacking in the seeds of Oscdp3.10 mutants. We explored why Oscdp3.10 could regulate seed vigour through modulation of amino acid and H$_2$O$_2$ levels in the early germination. Based on these results, we conclude that application of seed priming with amino acids and the use of natural variation of Oscdp3.10 will be useful for improvement of seed vigour in rice.

**Results**

**Identification of qSP3 for seedling percentage by GWAS**

The seeds of 203 rice accessions were germinated in water at the normal condition (Table S1), and the seedling percentage was measured at 4 days germination. A wide range of variation in seedling percentage was observed across the accessions (Figure 1a), and the indica accessions had a significantly higher seedling percentage than the japonica accessions (Figure 1b). To investigate the genetic architecture of the natural variation in seedling percentage, a GWAS was conducted. Manhattan plots were generated to identify the significant single-nucleotide polymorphism (SNP) associations with seedling percentage (Figure 1c,d). Regions were considered as QTLs when the SNPs exceeding the significance threshold value of $1 \times 10^{-5}$ occurred within a 200-kb interval of the leading SNP. Based on this criterion, one major QTL qSP3 with the lowest $P$-value was identified for seedling percentage on chromosome 3. The region of qSP3 is similar to the previous QTLs for imbibition rate and germination percentage (Cheng et al., 2013), and then it was focused on the further investigation.

A total of 22 SNPs were found to be in linkage disequilibrium (LD) block on qSP3 associated with seedling percentage, and seven tag SNPs (highlighted in red lines) were identified to form haplotypes that explained the range of seedling percentage (Figure 1e,f). Haplotypes C, D, E, F, and G represented accessions that had high seedling percentage, while Haplotypes A and B represented accessions that had low seedling percentage (Figure 1f). The potential candidate genes of qSP3 were analysed within 100-kb upstream and 100-kb downstream of the leading SNP of qSP3. However, those annotated as ‘retrotransposon’ and ‘transposon’ were excluded from the analysis. To further narrow down the numbers, the expression patterns of the candidate genes were analysed in the developing grains and germinating seeds using the database in rice (Rice eFP Browser and Genevestigator). A total of 16 positional candidate genes with differential expression (fold changes $>4.0$) were identified for qSP3 during seed development and germination (Figure 1g; Figure S1). In which, we observed that the expression of the candidate gene encoding the cupin domain protein (LOC_Os03g57960) was the most significantly changed (Figure S1B,C). Meanwhile, three tag SNPs (highlighted in red number in Figure 1e) are located in the region of LOC_Os03g57960. In which, tag SNP (33020245) as the leading SNP of qSP3 is located in the coding region of LOC_Os03g57960 (Figure 1g) to cause A→C nonsynonymous amino acid substitution from Leu→Phe. Thus, the candidate gene LOC_Os03g57960 was focused on further analysis.

**Oscdp3.10 is the qSP3 candidate gene regulating seed vigour**

As mentioned above, the qSP3 candidate gene encodes the cupin domain protein (LOC_Os03g57960). We identified 49 genes encoding cupin domain proteins with the conserved motifs in the rice genome (http://rice.plantbiology.msu.edu/), and these cupin domain proteins can be divided into two classes (Figure S2; Table S2). Then the LOC_Os03g57960 was named as the cupin domain protein Oscdp3.10. To further confirm the function of Oscdp3.10 in seed vigour, the CRISPR/Cas9 system was employed to generate mutants named Oscdp3.10-1, Oscdp3.10-2, and Oscdp3.10-3 in the japonica Nipponbare background (Figure 2a,b; Figure S3). The progeny of these homozygous mutants was used in the following experiments. Non-dormancy was observed in the freshly harvested seeds at 35 days after flowering (DAF) among Oscdp3.10 mutants and WT plants, and their germination percentage was more than 85% at 7 days germination (Figure S4). However, the disruption of Oscdp3.10 caused the vigour decline in the dried seeds harvested at 35 DAF under various conditions in rice. The seedling establishment of Oscdp3.10 mutants was significantly reduced compared with that of WT in direct seeding in soils (Figure 2c). The germination potential and seedling percentage of Oscdp3.10 mutants were significantly lower than those of WT plants under normal and salt stress (150 mM NaCl) conditions (Figure 2d–h). However, the seed vigour of Oscdp3.10 overexpression lines was not significantly different from that of WT plants (Figure S5), indicating that the function of Oscdp3.10 may require cofactors.

We next examined the expression patterns of Oscdp3.10 using quantitative RT-PCR and found much higher expression in developing grains and germinating seeds compared with root, stem, leaf, and node (Figure S6A). More detailed examination showed that expression was progressively increased with grain filling from 0 to 28 DAF and followed by decline after 28 DAF. Meanwhile, the higher expressions of Oscdp3.10 were progressively decreased during seed germination. Examination of transgenic lines expressing GUS under the control of the Oscdp3.10 promoter showed that Oscdp3.10 was strongly expressed in...
Figure 1  Phenotypic diversity of seedling percentage and identification of QTLs by GWAS in rice. (a) Variation and of (b) box-plots of seedling percentage among the 203 accessions of the rice population studied. (c) Manhattan plots and (d) quantile–quantile (Q-Q) plots for the whole population of rice accessions. The green dots indicate the qSP3 identified. (e) Identification of haplotype block of qSP3 using the 22 SNPs most significantly associated with seedling percentage. Seven tag SNPs are highlighted in red lines. Pairwise LD was determined by calculation of $r^2$ (the square of the correlation coefficient between SNP states). (f) Seven haplotypes formed from tag SNPs where phenotypic ranges for seedling percentage are explained by haplotypes. (g) Identification of candidate genes with differential expression during seed development and germination in the leading SNP (tag SNP 33020245) region of qSP3. The leading SNP located in the coding region of candidate gene LOC_Os03g57960. Different letters above the column indicate significant difference at the 1% level according to an analysis of variance (ANOVA) test.
developing shoot and radicle (Figure S6B). Overall, these results suggested that OsCDP3.10 is likely to be the causal gene of qSP3 modulating seed vigour in rice. 

OsCDP3.10 regulates the globulin and amino acids accumulation

As described above that the higher expressions of OsCDP3.10 were observed in filling grains in rice. We speculated that OsCDP3.10 regulating seed vigour might be through influencing grain qualities. We observed that there were no significant differences in grain length, width, grain weigh, starch, and protein content between WT and Oscdp3.10 mutants (Figure 3a–e,g,h), while the soluble sugar content was significantly lower in the mature grains of Oscdp3.10 mutants (Figure 3f). OsCDP3.10 functions as the synthesis of seed storage protein 52 kDa globulin (Ogo et al., 2014), and then the globulin contents were compared between Oscdp3.10 mutants and WT. The lacking accumulations of 52 kDa globulin were observed in the Oscdp3.10 mutants (Figure 3i). Seed storage proteins later become the amino acids for the embryonic and seedling development. Thus, the amino acid levels were further compared between Oscdp3.10 mutants and WT during seed germination. Results showed that the contents of most amino acids, including Tyr, Ile, His, Met, Val, Glu, Thr, Gly, Lys, and Pro, were significantly reduced in mature grains and germinating (36 and 72 h imbibition) seeds in Oscdp3.10 mutants compared with those of WT (Figure 4). It suggests that the OsCDP3.10 regulated seed vigour might be involving in amino acids in rice.
OsCDP3.10 regulates seed vigour involving in the amino acids level

It has been well reported that the activation of amino acid biosynthesis and/or recycling pathways is essential for seed germination (Gipson et al., 2017; He et al., 2019a). Therefore, we speculated that the lower amino acids in the mature seeds and the germinating seeds of the Oscdp3.10 mutants might cause lower seed vigour compared with WT. To confirm this, the above-mentioned amino acids including Tyr, His, Met, Val, Glu, Thr, Lys, and Pro that were significant differences between Oscdp3.10 mutants and WT were used for exogenous treatments. We observed that the germination potential and seedling percentage in Oscdp3.10 mutants were significantly enhanced especially by Met, Glu, His, and Tyr treatments compared with those under water conditions (Figure 5). The relative seed vigour (i.e. the ratio mutant/WT), including the relative germination potential and seedling percentage, was significantly larger under the exogenous Met, Glu, His, and Tyr treatments that it was in the control (treated with water only; Figure S7). We therefore predicted that the lower amino acids content, especially Met, Glu, His, and Tyr, in the Oscdp3.10 mutants might explain their low seed vigour.

OsCDP3.10 regulates seed vigour involving in the ROS level

It has been reported that the amino acids involve in the regulation of ROS level in plants (Batista-Silva et al., 2019). Plant ROS such as H₂O₂ in seeds as a signal can promote germination (Katsuya-Gaviria et al., 2020). We speculated that the OsCDP3.10 regulated seed vigour might be through ROS pathway that influenced by amino acids during seed germination. To confirm this, the comparison of H₂O₂ contents was firstly conducted between Oscdp3.10 mutants and WT during seed germination. We observed that the H₂O₂ contents were significantly higher in the dry mature seeds and the later germinating (72 h imbibition) seeds of Oscdp3.10 mutants compared with those of WT but the significantly lower H₂O₂ contents in the early germinating (36 h imbibition) seeds (Figure 6a). Meanwhile, we observed that the H₂O₂ contents were significantly enhanced in germinating (36 and 72 h imbibition) seeds of WT and Oscdp3.10 mutants by Met, Glu, His, and Tyr treatments that it was in the control (treated with water only; Figure S7). We therefore predicted that the lower amino acids content, especially Met, Glu, His, and Tyr, in the Oscdp3.10 mutants might explain their low seed vigour.
Figure 4  Amino acids level in the mature grains and germinating seeds of Nipponbare wild-type (WT) and Oscdp3.10 mutants. Data are means (±SD), n = 3. Significant differences compared with the Oscdp3.10 mutants were determined using Student’s t-test: *P < 0.05; **P < 0.01.

Figure 5  Effects of amino acids on seed vigour in Nipponbare wild-type (WT) and Oscdp3.10 mutants under normal condition. (a) Representative images are shown of the effects of exogenous applications of Met, Glu, His, and Tyr. Scale bars are 10 mm. Comparison of the germination potential (b), and seedling percentage (c) between the WT and Oscdp3.10 mutants under the H2O and amino acids treatments conditions. The numbers above the box-plots indicate the relative value of the mutant compared with that of the WT. Data are means (±SD), n = 3. Significant differences compared with the WT were determined using Student’s t-test: *P < 0.05; **P < 0.01; n.s., not significant.
exogenous Met, Glu, His, and Tyr treatments compared with those of the control (H$_2$O; Figure 6b, c). It suggests that the OsCDP3.10 will enhance the accumulation of amino acids in the early germinating seeds contributing ROS production as an important signal to promote seed germination in rice.

**Seed priming with amino acids improves seed vigour**

Seed priming techniques limit the availability of water to the seed (Heydecker and Higgins, 1973), which is an effective way to improve seed vigour under stress condition (Chen et al., 2021). As mentioned above, the Met, Glu, His, and Tyr that have important roles for the improvement of seed vigour in rice. Thus, the effects of seed priming with Met, Glu, His, and Tyr were firstly conducted in Oscdp3.10 mutants and WT plants in direct seeding in soils (Figure S8). The seedling establishment was significantly enhanced by seed priming with amino acids compared with those of non-primed seeds in both Oscdp3.10 mutants and WT plants. To further reveal its potential application, the effects of seed priming with Met, Glu, His, and Tyr were conducted using one salt sensitive variety Huahang 31 that popularly cultivated in Guangdong Province of China under salt stress. We observed that the seedling establishment of Huahang 31 was significantly enhanced by priming treatments in direct seeding in soils under 150 mM NaCl condition compared with those of non-primed seeds (Figure 7a). Moreover, the traits of seed vigour, including germination potential and seedling percentage, were significantly improved by seed priming with Met, Glu, His, and Tyr in Huahang 31 variety under 150 mM NaCl condition compared with those of non-primed seeds (Figure 7a–c). Especially, the traits were significantly increased by seed priming with Met, Glu, and His compared with those of non-priming and priming with water. These results indicated that the Met, Glu, His, and Tyr are the useful priming agents to improve seed vigour in rice.

**Nature allelic variation of OsCDP3.10 contributes to seed vigour**

As noted above, the indica accessions in our population had higher seedling percentage than the japonica accessions, and we therefore investigated whether variation in the OsCDP3.10 allele contributed to this difference. To examine the natural allelic
variation in OsCDP3.10 associated with seed vigour, we analysed the SNP polymorphisms that occurred in the untranslated region, the coding sequence (CD5), and the region 2 kb upstream of the gene. A total of two haplotypes of LOC_Os03g57960 were identified (Figure 8a). The elite haplotype Hap 1 was associated with higher seedling percentage in both indica and japonica accessions, while haplotype Hap 2 was associated with lower seedling percentage mainly occurred in japonica accessions (Figure 8b). Additionally, we observed that the variations of SNP 4 in the coding region were associated with seedling percentage (Figure 8c). To further investigate whether the variation in OsCDP3.10 caused the differences between the subspecies, we examined SNP 4 using RiceVarMap (Zhao et al., 2015) and found that variations mainly existed in japonica accessions (Figure 8d).

We randomly selected the accessions containing Hap 1 that had higher seed vigour together with the accessions containing Hap 2 that had lower seed vigour in direct seeding in soils (Figure 8e) and in Petri dishes (Figure 8f), and examined the globulin accumulation in seeds. We found that that the accessions containing Hap 1 had higher 52 kDa globulin accumulation than those of accessions containing Hap 2 (Figure 8g). Taken together, the natural allelic variation in OsCDP3.10 appeared to be associated with the differential seed vigour observed among the rice accessions.

**Discussion**

The rapid seedling establishment under various conditions is an important agronomic trait for direct seeding in rice. Previously, the cupin domain proteins such as Arabidopsis AtPirin1 has been reported involving in seed germination and seedling growth (Lapik and Kaufman, 2003). However, the regulation of the cupin domain protein on seed vigour has not been investigated in rice. In this study, one major QTL qSP3 for seedling percentage was detected at the early germination, and its region was similar to previous QTLs identified for imbibition rate and germination percentage (Cheng et al., 2013). We confirmed that the candidate gene of qSP3 encoding the cupin domain containing protein OsCDP3.10 with the function of 52 kDa globulin synthesis regulates seed vigour in rice. The disruption of OsCDP3.10 caused low seed vigour, including germination speed and seedling establishment, under normal and salt stress conditions in rice. The mechanism of seed vigour regulation by OsCDP3.10 was investigated in this study.

Seed vigour is established during seed development and maturation stages, in which maturation stage is characterized by storage activity. In rice, seed storage proteins are mainly composed of three groups, including alcohol-soluble prolamins, acid/alkaline-soluble glutelins, and saline-soluble globulins, according to their solubility (Tian et al., 2013). Of them, glutelins and prolamins are the major seed storage proteins of rice, accounting for 60%–80% and 20%–30% by weight of the total seed protein content, respectively (Saito et al., 2012), and globulin accounts for only 2%–8% (Cho et al., 2016). In this study, qSP3 encodes the cupin domain protein OsCDP3.10 with the function of 52 kDa globulin synthesis. Our results showed that the OsCDP3.10 exhibited especially relative higher expression at the late mature stage (28 DAF) and the early germination (4 h imbibition) stage. It suggested that OsCDP3.10 regulated seed vigour might be through influencing seed development and seed germination in rice. Previous study observed aberrant and loosely packed structures in the storage organelles of Globulin-RNAI seeds of rice, which may be attributable to the reductions in seed storage proteins (Lee et al., 2015). In this study, we observed that the 52 kDa globulin was lacked in Oscdp3.10 mutants in mature seeds compared with those of WT. Here, the influence of seed germination by OscDP3.10 was focused on analysis, and that of seed storage reserves during seed development needs further investigation in future. We speculated that OscDP3.10 regulated seed vigour might be influencing globulin accumulation in rice.

Seed storage proteins later become the amino acid and organic nitrogen reserves for the embryonic and seedling development. The globulin mobilization during seed germination has been investigated in few plant species. For instance, the storage globulins are firstly mobilized in the embryonic axis during seed germination in legumes and rape (Tiedemann and Neubohn, 2000). In rice, the rates of sprouting and reducing sugar accumulation during germination were found to be delayed in the deficiency transgenic plants suppressing all glutelins, prolamins, and globulin genes compared with the wild-type (Cho et al., 2016). In this study, we observed that ten amino acids, including Tyr, Ile, His, Met, Val, Glu, Thr, Gly, Lys, and Pro, were significantly reduced in Oscdp2.10 mutants in the mature grains and the germinating seeds compared with those of WT. It is well known that seed germination is linked to numerous amino acid metabolic pathways. For example, the Asp-derived amino acids (Lys, Met, and Thr) involve in the germination efficiency and post-germination growth of maize (Anzala et al., 2006). Metabolism of His, Pro, Iso, Val, Thr, Phe, and Tyr is important in the energy production for seed germination in rice (He et al., 2019a). Glu takes part in seed germination by counteracting the effect of ABA and/or its receptor ABA-insensitive 4 (ABI4) (Kong et al., 2015). We therefore predicted that OsCDP3.10 contributing to seed vigour might be due to the enhancement of amino acids in germinating seeds. It was further confirmed by exogenous amino acids treatments that the seed vigour in Oscdp3.10 mutants was rescued especially by Met, Glu, His, and Tyr treatments. Meanwhile, our results showed that seed priming with amino acids improves seed vigour in rice. It suggests that the OsCDP3.10 regulated seed vigour might be involving in amino acids accumulation in the mature grains and germinating seeds.

It has been assumed for a long time that ROS accumulation is detrimental to seed viability because of oxidative stress imposed during seed desiccation or seed ageing (Bailly, 2004). However, it has also been reported that ROS as cell signal promotes dormancy release and seed germination (Katsuya-Gavira et al., 2020). The H2O2 accumulation may increase the rates of protein carbonylation and protein turnover, and decrease the electron pressure in the mitochondrial electron transport chain, which is involved in seed dormancy and germination (Barba-Espin et al., 2011; Job et al., 2005; Oracz et al., 2007). Additionally, the H2O2 accumulation may influence the hormone balance by increasing gibberellins (GAs) and decreasing ABA and ethylene, which is important for the regulation of seed dormancy and germination (Bahin et al., 2011; Barba-Espin et al., 2010, 2011; Jeevan Kumar et al., 2015). In this study, we observed that the H2O2 levels were significantly reduced in the early germinating (36 h imbibition) seeds of Oscdp3.10 mutants compared to that of WT. Therefore, we predicted that OsCDP3.10 contributing to seed vigour might be due to the enhancement of H2O2 levels in the early germinating seeds. Meanwhile, amino acids treatments improved the H2O2 accumulation in the early germinating seeds of Oscdp3.10 mutants and WT in this study and then promoted seed vigour. We thus speculated that OsCDP3.10 improved seed
vigour might be through enhancing amino acids to promote H$_2$O$_2$ accumulation in the early germination. However, the mechanisms of underlying OsCDP3.10 regulate seed vigour through ROS pathway need to be further investigated. The production of ROS may result from the Amadori and/or Maillard reactions and lipid peroxidation in dry seeds, whereas the mitochondria, glyoxysomes, and plasma membrane NADPH oxidases act as major sources for ROS in the imbibed seeds (Jeevan Kumar et al., 2015; Leubner-Metzger, 2005; McDonald, 1999; Zhang et al., 1995). The relationships between amino acids, for example Met, Glu,

Figure 7  Seed priming with amino acids improves seed vigour of salt sensitive variety Huahang 31 under salt stress (150 mM NaCl) in rice. (a) Images are shown of the effects of seed priming with amino acids Met, Glu, His, and Tyr on seedling establishment in direct seeding in soils and on seed germination in Petri dishes. Scale bars are 10 mm. Comparison of the germination potential (b), and seedling percentage (c) in various priming treatments. Data are means (±SD), n = 3. Different letters above the column indicate significant difference at the 5% level according to an analysis of variance (ANOVA) test.
Figure 8  Haplotypes of OsCDP3.10 associated with seed vigour in rice. (a) Haplotypes of OsCDP3.10 identified in the coding sequence (CDS) and the region 2 kb upstream of the gene. (b,c) Box-plots of seedling percentage of accessions containing the different haplotypes and SNP 4. (d) The variations in SNP 4 of OsCDP3.10 as determined using RiceVarMap. (e) Images of the seedling establishment from randomly selected accessions containing either Hap 1 or Hap 2 in direct seeding in soils. Scale bars are 10 mm. (f) Images of seed germination from randomly selected accessions containing either Hap 1 or Hap 2. Scale bars are 10 mm. (g) Comparison of 52 kDa globulin between two haplotypes groups.
His, and Tyr, and H$_2$O$_2$ accumulation during seed germination need to be further investigated.

In summary, we validated one candidate gene for the major QTL qgSP2, which encodes cupin domain containing protein OsCDP3.10. Disruption of this gene in rice Oscdp3.10 mutants reduced seed vigour at the early germination stage. The underlying mechanisms for the regulation of seed vigour by OsCDP3.10 appear to be mainly through influencing amino acids and ROS levels. This study provides important insights into the roles of Oscdp3.10 on seed vigour, and the application of seed priming with amino acid agents to improve seed vigour in rice. Previously, the Arabidopsis cupin domain protein AtPrin1 interacts with the G protein to regulate seed germination (Lapik and Kaufman, 2003). Further studies are needed to confirm that whether OsCDP3.10 has the similar interaction with G protein to regulate seed vigour in future.

Materials and methods

Plant materials

A total of 203 accessions were randomly selected from the Rice Diversity Panel 1 (RDP1, https://www.ars.usda.gov/; Table S1). Three homozygous mutants (Oscdp3.10-1, Oscdp3.10-2, and Oscdp3.10-3) were generated in the japonica Nipponbare background using the CRISPR/Cas9 system according to the protocol as described by Cong and Zhang (2015). The mutants were generated using the target guide sequences (S’-AGGCCGTACCA CTTAGGGGAGG-3’) in the first coding exon of Oscdp3.10. Meanwhile, the lines of overexpression OsCDP3.10 were generated in the japonica Nipponbare background. All the plants were grown in an experimental field at South China Agricultural University (Guangzhou, Guangdong, China) as described by Zhao et al. (2021). All seeds were harvested at their maturity stage and dried at 42 °C for 7 days and storage at 4 °C, and then randomly selected well-filled seeds for experiments.

Evaluation of seed vigour

Twenty seeds per replicate of each genotype were directly sowed in soils under water or 150 mM NaCl at 25 °C conditions. Meanwhile, 30 seeds per replicate of each genotype were imbibed in Petri dishes (diameter 9 cm) with 10 mL distilled water or 150 mM NaCl at 25 °C conditions for 7 and 12 days, respectively. Seeds were considered germinated when the radicle protruded (1 mm) through the seed coat, and seedlings were considered established when the root length reached seed length. The traits of germination potential and seedling percentage were calculated. Three biological replications were performed.

Genome-wide association study

A GWAS was conducted with a linear mixed model in EMMAX using germination phenotype and a marker set of 700 000 SNPs as described by McCouch et al. (2016). The marker set was filtered using a nucleotide variation missing rate <0.25 and a minor allele frequency >0.05 (Yano et al., 2016). With the number of SNPs analysed (n = 399 537), the threshold for significance was estimated to be approximately $P = 1.0 \times 10^{-5}$ (Crowell et al., 2016). Only associations that exceeded a $P$-value threshold of $<1.0 \times 10^{-5}$ within the 200-kb region around the lead SNP were considered as one association locus (Zhao et al., 2011). Linkage disequilibrium block analysis was performed using the confidence intervals in Haploview (Barrett et al., 2005). To explain phenotypic variation of seedling percentage, haplotypes across the GWAS panels were built with tag SNPs (Butardo et al., 2017). Candidate genes were predicted within a genomic region of ±100 kb of the leading SNP for QTL (Lv et al., 2016) using Rice Genome Annotation Project MSU7 database (http://rice.plantbiology.msu.edu). The haplotypes of candidate genes were determined using the database of http://www.ricediversity.org/ (McCouch et al., 2016) and RiceVarMap (Zhao et al., 2015). Only haplotypes represented by more than ten accessions were considered.

Characterization of rice cupin domain proteins

The information and sequences of rice cupin domain proteins were downloaded from the National Rice Database (https://www.ridgedata.cn/). Multiple sequence alignment was conducted with ClustalW (Neighbor-joining, ND) in Mega v6.0, and phylogenetic analysis was performed with default parameters (Tamura et al., 2013). Gene structure map was conducted using Online Gene Structure Display Server (http://gsds.gao-lab.org/).

Quantitative RT-PCR

Total RNA was extracted from the plants using a HP Plant RNA Kit according to the manufacturer’s instructions (Omega, Atlanta, GA). The first-strand cDNA was synthesized by HiScript Reverse Transcriptase (Vazyme, Nanjing, China), and quantitative RT-PCR was carried out according to Zhao et al. (2021). All reactions were performed using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) with the rice Actin gene as internal controls. The PCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. The primers used for quantitative RT-PCR are listed in Table S3. Normalized transcript levels were calculated using the comparative Ct method (Livak and Schmittgen, 2001). Three biological replications were performed.

GUS staining

Transgenic plants carrying the OscDP3.10 promoter-GUS fusion construct were constructed in the japonica Nipponbare. Briefly, a 1.9-kb genomic DNA fragment of the 5’ upstream region of OscDP3.10 was amplified by PCR using primers OscDP3.10-Promoter-F and OscDP3.10-Promoter-R (Table S3). These fragments and the GUS gene were cloned into the pCAMBIA 1304 plasmid vector. The post-germinated seeds of the positive transgenic plants were stained with GUS Staining kit (Coolaber, Beijing, China) and were observed using a digital camera (Nikon Corporation, Tokyo, Japan).

Total protein, starch, and soluble sugar assay

The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China). The grains at 35 DAF were harvested for the physiological assay. The content of starch and soluble sugar were detected using kits (Suzhou Keming Bioengineering Company, Suzhou, China), and the content of protein was extracted and determined using kit (Tianjin Sino-Pharm Biotech Co. Ltd., Tianjin, China).
10 min for the protein isolation on 15% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For protein staining, the gels were soaked in Coomassie Brilliant Blue (CBB) staining solution (0.1% (v/v) CBB R-250, 45% (v/v) methanol and 45% (v/v) glacial acetic acid) for 3 h, and then washed in destaining solution (1% methanol, 1% glacial acetic acid, and 8% distilled H2O).

**Amino acid assay**

Thirty seeds per replicate of each genotype were imbibed in Petri dishes (diameter 9 cm) with 10 mL distilled water after 0, 36 and 72 h imbibition at 25 °C condition. After that, seeds were frozen with liquid nitrogen and grounded into powder. Amino acids were extracted according to He et al. (2019a) with minor revisions. Thirty seeds per replicate were imbibed in Petri dishes (diameter 9 cm) with 0.1 mL amino acid solutions under normal (10 mL distilled H2O) condition. The mixture was centrifuged at 4000 rpm for 10 min, and then the absorbance of the supernatant was measured as described above. Three biological replicates were used.

**Evaluation of H2O2 level**

The H2O2 level was carried out using the commercial assay kits according to the manufacturer’s instructions (Suzhou Keming Bioengineering Company, Suzhou, Jiangsu, China). Approximately 0.1 g fresh weight (FW) of each sample was rapidly frozen in 1 mL of cold acetone (4 °C) and homogenized into a powder. After that, the reaction solutions were added to the homogenate. The mixture was centrifuged at 4000 g at 25 °C for 10 min, and then the absorbance of the supernatant was determined immediately at 415 nm. The H2O2 content was expressed as μmol/g FW. Three biological replicates were used.

**Seed treatments with amino acids**

The Oscp10 mutants and WT Nipponbare were used to evaluate the seed vigour influenced by amino acid treatments. Thirty seeds per replicate were imbibed in Petri dishes (diameter 9 cm) with 0.1 mL amino acid solutions under normal (10 mL distilled H2O) condition at 25 °C for 7 days. Seeds germinated under 10 mL distilled H2O condition without amino acids added were used as control. Meanwhile, the Oscp10 mutants, WT Nipponbare, and indica Huahang 31 were used for seed priming with amino acids described by He et al. (2019a) with minor revisions. Thirty seeds per replicate were imbibed in Petri dishes (diameter 9 cm) with 0.1 mL amino acid solutions, including His, Met, Tyr, and Glu, in 10 mL distilled H2O for 12 h in the dark. After that, the imbibed seeds were dried to their original weights at 25 °C for 5 days and then germinated under water or 150 mM NaCl at 25 °C condition. The traits of seed vigour were measured as described above. Three biological replicates were performed.

**Data analysis**

Experimental data were analysed using the Graphpad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA), and significant differences between samples were compared using Student’s t-test or an analysis of variance (ANOVA) test.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

Z.W. planned the research. L.P. and S.S. performed all important experiments. B.Y. and J.Z. performed GWAS assay. W.L. performed physiological assay. Z.H. and Z.L. performed seed germination experiments. Z.W., Y.H., and L.P. analysed the data and wrote the paper.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** The candidate genes of qSP3 and its expression pattern in the embryo of developing grains and germinating seeds using database (Rice eFP Browser and Genevestigator) in rice.

**Figure S2** Characterization of the cupin domain proteins in rice.

**Figure S3** Comparison of amino acid sequences of OsCDP3.10 protein between Nipponbare wild-type (WT) and Oscdp3.10 mutants in rice.

**Figure S4** Comparison of seed dormancy in freshly harvested seeds at 35 days after flowering between Nipponbare wild-type (WT) and Oscdp3.10 mutants in rice.

**Figure S5** Seed vigour in the Nipponbare wild-type (WT) and OsCDP3.10 overexpression lines under normal conditions.

**Figure S6** Expression patterns of OsCDP3.10 in rice determined by quantitative RT-PCR and histochemical staining for GUS activity.

**Figure S7** Effects of amino acids Pro, Val, Lys, Thr, and Gly on seed vigour in Nipponbare wild-type (WT) and Oscdp3.10 mutants under normal condition.

**Figure S8** Effects of seed priming with Met, Glu, His, and Tyr on seedling establishment of Oscdp3.10 mutants and Nipponbare wild-type (WT) in direct seeding in soils.

**Table S1** Information of the 203 rice accessions used for the GWAS.

**Table S2** Information of the cupin domain proteins in rice.

**Table S3** List of primer pairs used in this study.