Influence of isopropylmalate synthase OsIPMS1 on seed vigour associated with amino acid and energy metabolism in rice

Yongqi He1, Jinping Cheng1, Ying He1, Bin Yang1, Yanhao Cheng1, Can Yang1, Hongsheng Zhang1 and Zhoufei Wang1,2,*

1The Laboratory of Seed Science and Technology, State Key Laboratory of Crop Genetics and Germplasm Enhancement, Jiangsu Collaborative Innovation Center for Modern Crop Production, Nanjing Agricultural University, Nanjing, China
2The Laboratory of Seed Science and Technology, Guangdong Key Laboratory of Plant Molecular Breeding, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou, China

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*Correspondence (Tel 8602584399532; fax 8602584399532; emails wangzf@scau.edu.cn (Z.W.), hzhang@nau.edu.cn (H.Z.)) Yongqi He and Jinping Cheng contributed equally to this work.

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Summary
Seed vigour is an imperative trait for the direct seeding of rice. Isopropylmalate synthase (IPMS) catalyses the committed step of leucine (Leu) biosynthesis, but its effect on seed vigour remains unclear. In this study, rice OsIPMS1 and OsIPMS2 were cloned, and the roles of OsIPMS1 in seed vigour were mainly investigated. OsIPMS1 and OsIPMS2 catalyse Leu biosynthesis, and Leu feedback inhibits their IPMS activities. Disruption of OsIPMS1 resulted in low seed vigour under various conditions, which might be tightly associated with the reduction of amino acids in germinating seeds. Eleven amino acids that associated with stress tolerance, GA biosynthesis and tricarboxylic acid (TCA) cycle were significantly reduced in osipms1 mutants compared with those in wide type (WT) during seed germination. Transcriptome analysis indicated that a total of 1209 differentially expressed genes (DEGs) were altered in osipms1a mutant compared with WT at the early germination stage, wherein most of the genes were involved in glycolysis/gluconeogenesis, protein processing, pyruvate, carbon, fructose and mannose metabolism. Further analysis confirmed that the regulation of OsIPMS1 in seed vigour involved in starch hydrolysis, glycolytic activity and energy levels in germinating seeds. The effects of seed priming were tightly associated with the mRNA levels of OsIPMS1 in priming seeds. The OsIPMS1 might be used as a biomarker to determine the best stop time-point of seed priming in rice. This study provides novel insights into the function of OsIPMS1 on seed vigour and should have practical applications in seed priming of rice.

Introduction
Rice (Oryza sativa L.) is one of the most important food crops in the world. Recently, the direct-seeding method of rice is becoming increasingly popular in China because of its low cost and operational simplicity (Wang et al., 2011). High seed vigour, including rapid, uniform germination and vigorous seedling growth, is essential for the direct seeding of rice (Mahender et al., 2015). Seeds with speed and uniform germination may significantly improve field emergence, lead to better suppression of weed growth and produce high yield under various conditions (Foolad et al., 2007; Wang et al., 2010). Therefore, the mining of key genes controlling seed vigour and illuminating their molecular mechanisms are important objectives of rice breeding.

Seed germination is a quantitative trait controlled by multiple genes and environmental factors during seed development and germination stages. To date, several quantitative trait loci (QTLs) for seed germination have been reported in rice (Cheng et al., 2013; Fujino et al., 2004, 2008; Hsu and Tung, 2015; Li et al., 2013; Miura et al., 2002; Wang et al., 2010, 2011). Of those, several QTLs for germination speed are likely to coincide with QTLs for seed weight, seed size and seed dormancy in rice (Wang et al., 2010). The first clone-related QTL, qLTG3-1, which controls germination speed under various conditions, is tightly associated with vacuolation of tissues covering the embryo (Fujino et al., 2008); qLTG3-1 plays an important role in the weakening of seed tissues during germination through programmed cell death (Fujino and Matsuda, 2010). The second clone-related QTL Sdr4 is associated with seed dormancy, which is positively regulated by OsVP1 in rice (Sugimoto et al., 2010). OsVP1 is orthologous to Arabidopsis ABI3, which is a central component of the abscisic acid (ABA) signalling pathway during seed germination (Graeber et al., 2012; Holdsworth et al., 2008). The gibberellin (GA) biosynthesis-related gene OsGAs20ox1 has been reported as a candidate gene for a major QTL controlling seedling vigour (Abe et al., 2012). It is widely accepted that the balance between ABA and GA is important for seed germination (Graeber et al., 2012; Nambara et al., 2010). These results suggest that germination speed is associated with seed weight, size and seed dormancy and is also influenced by endosperm weakening, hormones and storage metabolism (Bethke et al., 2007; Catusse et al., 2008; Fait et al., 2006).

Seed germination and subsequent seedling growth need large amounts of energy and nutrition, which are provided only by seed reserves, because the germinating seeds lack a mineral uptake system and photosynthetic apparatus (Bewley, 1997). When quiescent dry seeds imbibe water, their oxygen uptake increases (Pergo and Ishii-Iwamoto, 2011) and three respiratory pathways, including glycolysis, pentose phosphate pathway and tricarboxylic acid (TCA) cycle, are activated in the imibed seeds (Bewley et al., 2013).
2013). Glycolysis operates under aerobic and anaerobic conditions to produce pyruvate. In the presence of O$_2$, further utilization of pyruvate occurs within the mitochondria; that is, oxidative decarboxylation of pyruvate produces acetyl-CoA, which is completely oxidized to CO$_2$ and water via the TCA cycle to yield up to 30 ATP molecules per glucose molecule respired (Bewley et al., 2013). In the glycolysis pathway, glyceraldehyde-3-phosphate (Gly-3-P) dehydrogenase converts Gly-3-P into 1,3-bisphosphoglycerate, and pyruvate dehydrogenase converts pyruvate into acetyl-CoA (Xu et al., 2016a). Accumulation of glycolytic enzymes in several species, such as Gly-3-PDH (Han et al., 2014; Kim et al., 2009) and pyruvate dehydrogenase (Kim et al., 2008) in rice, fructose-1,6-bisphosphatase in Arabidopsis (Rajjou et al., 2008), and Gly-3-PDH in sugar beet (Catussi et al., 2011) and Arabidopsis (Rajjou et al., 2008), is positively correlated with seed vigour. Taken together, these results indicate that glycolysis and TCA cycle provide most of the energy for seed germination.

Amino acids are not only used for the synthesis of storage proteins but are also catabolized for the TCA cycle to generate energy (Gallili et al., 2014). Leucine (Leu) is one of the essential branched-chain amino acids (BCAA; leucine, valine and isoleucine), serving as an alternative energy source for mammals (Harper et al., 1984) and plants (Binder, 2010). For example, Leu promotes energy metabolism (glucose uptake, mitochondrial biogenesis and fatty acid oxidation) for improving protein synthesis while inhibiting protein degradation in mammals (Duan et al., 2016). Catabolism of Leu produces energy-rich intermediates, such as acetyl-CoA and propionyl-CoA (Anderson et al., 2008); the expression patterns of OsIPMS1 and OsIPMS2 were observed at all of the developmental stages and in various tissues (Figure 2a,b). To expand our understanding of the physiological function of OsIPMS1 and OsIPMS2, the expression patterns of OsIPMS1 and OsIPMS2 were determined using qRT-PCR approach. Transcripts of OsIPMS1 and OsIPMS2 convert acetyl-CoA and α-ketoisovalerate into 2-isopropylmalate and CoASH (Figure 1c,d). OsIPMS1 and OsIPMS2 activities were significantly declined with the increasing concentrations of Leu (Figure 1e,f), suggesting that the OsIPMS1 and OsIPMS2 enzymes are subject to Leu feedback inhibition.

Expression pattern of OsIPMS1 and OsIPMS2

Based on the publicly available microarray database (http://www.genevestigator.com), the higher transcript abundances of OsIPMS1 and OsIPMS2 were observed at all of the developmental stages and in various tissues (Figure 2a,b). To expand our understanding of the physiological function of OsIPMS1 and OsIPMS2, the expression patterns of OsIPMS1 and OsIPMS2 during seed development and seed germination were further analysed using qRT-PCR approach. Transcripts of OsIPMS1 and OsIPMS2 were significantly increased in the filling grain from 0 to 7 days after flowering (DAF) and then sharply decreased after the 14th DAF (Figure 2c). Additionally, the transcript levels of OsIPMS1 and OsIPMS2 were slightly altered during the 4- to 36-h imbibition stage; however, the transcript was strongly detected at 48-h imbibition when seeds begin germinating (Figure 2d). Similar spatial-temporal expression patterns were observed in OsIPMS1 and OsIPMS2, suggesting both genes might behave similar functions in seed germination of rice.

Disruption of OsIPMS1 resulted in low seed vigour under various conditions

To study the role of OsIPMS1 on seed vigour, a T-DNA mutant osipms1 was subsequently obtained from Rice Mutant Database (http://trmd.ncpgr.cn/; Miyao et al., 2003). The T-DNA insertion was located in the sixth intron of OsIPMS1 (Figure 3a). Using specified primer sets (Table S1), a homozygous insertion mutant individual was isolated and named osipms1a. Furthermore, RT-PCR analysis demonstrated that the T-DNA insertion resulted in a complete suppression of OsIPMS1 expression in the osipms1a mutant (Figure 3b). To further confirm OsIPMS1 function, we employed the CRISPR/Cas9 system to generate mutants, which were named osipms1b and osipms1c. The osipms1b and
Osipms1c mutant plants contained a ‘T’ and ‘G’ insertion in the first exon of OsIPMS1 (Figure 3c). The levels of OsIPMS1 expression were much lower in osipms1b and osipms1c mutants than those in WT plants (Figure S3a). The amino acid sequence of OsIPMS1, predicted based on these nucleotide sequences, contains only 172 amino acids in osipms1b and osipms1c and is caused by premature termination (Figure S3b). These results indicate that the osipms1b and osipms1c mutant lines lacked OsIPMS1. The progeny of these homozygous mutants were used in subsequent experiments.

Phenotype evaluation demonstrated that the disruption of OsIPMS1 resulted in low germination speed and seedling growth under various conditions. The GP and GI of mutant lines (osipms1a, osipms1b and osipms1c) were significantly reduced while T50 was significantly increased compared to those of WT plants under normal conditions (Figure 3d-g). The early seedling growth, including the length, fresh and dry weight of shoots and roots, in mutants were also significantly reduced compared with those of WT plants (Figure 3h-j). Similar results were observed in the osipms1t mutant lines under salt, drought and cold stress conditions (Figure S4). Moreover, seedling emergence and seedling growth were significantly decreased in osipms1t mutant lines compared to those in WT plants under the direct-seeding method in soils (Figure S5). Similarly, the significant decreases in germination speed and seedling growth were also observed in osipms2 mutant lines compared to those in WT plants under normal condition (Figure S6). It further confirmed that OsipMS1 and OsipMS2 might behave the similar functions in seed germination. Here, the following analyses were mainly focused on the physiological roles of OsipMS1 in seed vigour.

OsipMS1 increased amino acid biosynthesis during seed germination

We predicted that the disruption of OsipMS1 affects amino acid biosynthesis due to its effect on the IPMS enzyme through Lue biosynthesis. Therefore, the levels of free amino acids in osipms1 mutants (osipms1a, osipms1b and osipms1c) and WT lines were measured during seed germination (Figure 4). By comparison, the levels of amino acids Thr and cysteine (Cys) were significantly increased in mature unstratified seeds of osipms1 mutants compared to those in WT plants. However, the decreases in amino acid levels were generally observed in osipms1 mutants compared to the levels in WT plants during seed germination. In which, the levels of eleven amino acids were significantly decreased in osipms1 mutant lines compared to those in WT plants under the direct-seeding method in soils (Figure S5). Similarly, the significant decreases in germination speed and seedling growth were also observed in osipms2 mutant lines compared to those in WT plants under normal condition (Figure S6). It further confirmed that OsipMS1 and OsipMS2 might behave the similar functions in seed germination. Here, the following analyses were mainly focused on the physiological roles of OsipMS1 in seed vigour.
decreased in 60-h imbibed seeds of three osipms1 mutants simultaneously compared to the levels in WT plants. Approximately 10%–50% lower levels of Leu, Ile, Phe, Tyr, Thr, Cys, serine (Ser), glycine (Gly), valine (Val), histidine (His) and proline (Pro) were observed in mutants than those in WT plants. Overall, the levels of all amino acids were progressively enhanced during seed germination, wherein the amino acids might be used for the synthesis of proteins, hormones and energy for seed germination at the early stages. Higher levels of eleven amino acids in the WT might contribute towards seed germination and vigorous seedling growth in this study. To confirm this hypothesis, the effects of 10 mM amino acids, including Leu, Ile, Phe, Ser, Gly and Val, treatment on seed germination of osipms1 mutants were determined. We observed that seedling growth of osipms1 mutants was nearly rescued by exogenous application of amino acids compared to that in WT plants (Figure 5a). Meanwhile, the root lengths of three osipms1 mutants were significantly increased by amino acids treatment compared to those in untreated seeds (Figure 5b–e). Ketogenic amino acid, for example Leu, Ile, and Phe, can be degraded directly into acetyl-CoA for GA biosynthesis (Rios-Iribe et al., 2011). Here, we predicted that amino acid treatment contributing to seed vigour of osipms1 mutants might be through stimulating GA biosynthesis during seed germination. Expectedly, the expressions of GA biosynthesis-related genes in osipms1 mutants were significantly increased after amino acid treatment compared with those in the control (Figure S7). These results suggest that the regulation of OsIPMS1 on seed germination might be through altering amino acids associated with GA biosynthesis in germinating seeds.

Disruption of OsIPMS1 altered gene expression during seed germination

To further understand the OsIPMS1 function, genomewide transcriptional levels were compared between osipms1a and WT at the 8-h imbibition stage. A total of 1209 differentially expressed genes (DEGs) were identified between osipms1a and WT (Figure S8; Table S2). Of those, 424 and 785 genes were significantly up-regulated and down-regulated, respectively, in osipms1a compared to WT. GO and KEGG pathway categories of DEGs were examined. Most overrepresented GO categories were classified as biological processes, including single-organism process, single-organism metabolic process and carbohydrate metabolic process (Figure S8). Most overrepresented KEGG pathway categories were related to glycolysis/gluconeogenesis, protein processing and carbon, pyruvate and fructose metabolism, etc (Table S3). Of these, 27 and 31 genes were associated with glycolysis/gluconeogenesis and proteins processing, respectively, and 13 and 29 genes were associated with pyruvate and carbon metabolism.

GO and KEGG analysis indicated that the altering of glycolysis process is an important factor in OsIPMS1 regulation of seed germination. To confirm this hypothesis, qRT-PCR was used to determine the transcript levels of OsPFK and OsPK

Figure 2 Expression patterns of OsIPMS1 and OsIPMS2 in rice. Expression pattern of OsIPMS1 and OsIPMS2 in various developmental stages (a) and tissues (b) of rice based on the publicly available microarray data (http://www.genevestigator.com). Transcription levels of OsIPMS1 and OsIPMS2 in filling grains (c) and germinating seeds (d) were conducted using qRT-PCR approach. The expression of OsIPMS1 and OsIPMS2 was normalized to that of OsActin gene control. The relative expression levels were represented by fold change relative to the expression level of OsIPMS1 at 0 DAF (c) or 4-h imbibition stage (d). Each column represents the means ± standard deviation.
genes, which are two key regulated genes in glycolysis (Figure 6a). Consistent alterations in the levels of these genes were observed through RNA sequencing and qRT-PCR approaches. The transcripts of *OsPKs*, *OsPFKs* and *OsPEPCK* were significantly reduced in *osipms1* compared to those in WT at 8-h seed germination stage (Figure 6b). These data suggest that germination regulated by *OsIPMS1* is tightly associated with glycolytic metabolism.

**OsIPMS1 enhanced starch mobilization during seed germination**

Imbibition is the critical first step to induce starch hydrolysis for glycolysis during seed germination. Therefore, dynamic imbibition was compared between mutants and WT during seed germination (Figure S9a). A significant decrease in the imbibition rate was only observed at the seed germinating stage (60 h) when the radicle protruded in mutants compared to that of WT. This outcome suggests that the altering of germination by *OsIPMS1* is not through the influence of imbibition rate at the early germination stage. The initial imbibition is primarily affected by protein and starch levels in mature seeds. We observed that there were no significant differences in total protein and starch levels in mature seeds of mutants compared to those in WT, whereas the total soluble sugar levels were significantly decreased in mutants (Figure S9b–d). These results indicate that the disruption of *OsIPMS1* reduced seed vigour might be partly due to decreasing total soluble sugar levels in mature seeds.

Starch hydrolysis by amylase is an important contributing factor for seed germination and vigorous seedling growth. The α-amylase biosynthesis is induced by GA during seed germination. As described above, the amino acids associated with GA biosynthesis, including Leu, Ile, Phe, Thr and Tyr, were...
growth of osipms1 application of GA3 compared to those in WT (Figure 7d,e,f). The levels of GA3 were significantly reduced in osipms1 mutants compared to those in WT (Figure 7b,c). It was also confirmed by the analysis of GA3 treatments on seed germination (Figure 7a). The expressions of GA biosynthesis-related genes, including OsKS, OsKO and OsGA20ox1, were significant decreased in osipms1 mutants compared to those in WT (Figure 7b,c). This outcome suggests that glycolytic activity was reduced in osipms1 mutants compared to those in WT during seed germination. Generally, the levels of pyruvate and acetyl-CoA were significantly decreased in osipms1 mutants during seed germination. Expectedly, the intermediate metabolites of glycolysis were assayed during seed germination in osipms1 mutants. Thus, we predicted that GA biosynthesis might be regulated by OsIPMS1 in germinating seeds. Expectedly, the levels of ATP, AMP and energy charge were associated with energy production during seed germination. We observed that seedling growth of osipms1 mutants was nearly rescued by exogenous application of GA3 compared to those in WT (Figure 7d,e,f). Furthermore, we observed that the activities of α-amylase and β-amylase (Figure 8a,b), and the levels of glucose and fructose were generally and significantly lower in mutants than those in WT during seed germination (Figure 8c,d). These results indicate that OsIPMS1 regulates seed vigour might be through altering starch hydrolysis in germinating seeds.

OsIPMS1 improved glycolytic activity and ATP level during seed germination

To further examine the regulation of glycolytic activity by OsIPMS1, the intermediate metabolites of glycolysis were assayed during seed germination. Generally, the levels of pyruvate and acetyl-CoA were significantly decreased in osipms1 mutants compared to those in WT (Figure 9a,b). This outcome suggests that glycolytic activity was reduced in osipms1 mutants compared to that in WT. The intermediate metabolites of glycolysis are associated with energy production during seed germination. We observed that the levels of ATP, AMP and energy charge were significantly reduced in osipms1 mutants compared to those in WT during seed germination (Figure 9c–f). Notably, the ATP and AMP levels in osipms1 mutants were decreased by approximately 50% compared to those in WT at the late germination stage (48 and 60 h). These results further demonstrate that OsIPMS1 is involved in the regulation of glycolysis and energy production during seed germination.

Application of OsIPMS1 for seed priming

Seed priming is a technique used to improve seed vigour, which allows imbibition to a certain extent but prevents radicle emergence. Thus, an important requirement for successful seed priming is to stop the priming treatment and dehydrate the seed at the right moment (Paparella et al., 2015). To determine whether OsIPMS1 expression influencing priming effect, the different duration of priming treatments were conducted and compared between osipms1 mutants and WT. The significant higher GI and lower T50 were observed in 12-h primed seeds compared to those in unprimed seeds (0 h) in WT plants (Figure S10). Less adverse effects of priming were observed in 24- and 36-h primed seeds in WT; however, the adverse effects of priming were observed in 12-, 24- and 36-h primed seeds in osipms1 mutants. Generally, the significant lower GP, GI and SP but higher T50 were observed in 12-, 24- and 36-h primed seeds in osipms1 mutants compared to those in unprimed seeds. It suggests that the disruption of OsIPMS1 will reduce the priming effects in rice.

To further determine the correlation between priming effects and the mRNA levels of OsIPMS1 during seed priming, various durations of priming treatments were conducted using rice cv. Ningdao NO.1 and Wuyungeng NO.7. Generally, the better seedling growth was observed in 4-, 8- and 12-h primed seeds compared to that in unprimed seeds, but the adverse effects of priming were observed in 12-, 24- and 36-h primed seeds in osipms1 mutants. Generally, the significant lower GP, GI and SP but higher T50 were observed in 12-, 24- and 36-h primed seeds in osipms1 mutants compared to those in unprimed seeds. It suggests that the disruption of OsIPMS1 will reduce the priming effects in rice. To determine whether OsIPMS1 expression influencing priming effect, the different duration of priming treatments were conducted and compared between osipms1 mutants and WT. The significant higher GI and lower T50 were observed in 12-h primed seeds compared to those in unprimed seeds (0 h) in WT plants (Figure S10). Less adverse effects of priming were observed in 24- and 36-h primed seeds in WT; however, the adverse effects of priming were observed in 12-, 24- and 36-h primed seeds in osipms1 mutants. Generally, the significant lower GP, GI and SP but higher T50 were observed in 12-, 24- and 36-h primed seeds in osipms1 mutants compared to those in unprimed seeds. It suggests that the disruption of OsIPMS1 will reduce the priming effects in rice.

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best stop time-point of seed priming might be at the time before the mRNA levels of OsIPMS1 significantly reduced in priming seeds.

Discussion

Seed vigour is an important agronomic trait that consists of seed longevity, germination and seedling growth, etc. However, the trait of seed vigour has not been selected in conventional breeding due to its complex nature and quantitative inheritance in rice. Identification and utilization of vigour-related genes are important for the improvement of seed vigour in rice. Previous studies demonstrated that IPMS catalyses the committed step of Leu biosynthesis (Zhang et al., 2014). There have been few reports regarding the phenotype of IPMS disruption in plants (Field et al., 2006). The present study demonstrated that the disruption of OsIPMS1 and OsIPMS2 reduced seed vigour in rice. To the best of our knowledge, this is the first report highlighting the involvement of IPMS regulation in seed vigour of plants. The roles of OsIPMS1 influencing seed germination were analysed in this study, and the application of OsIPMS1 as a biomarker for seed priming was also discussed in rice.

Isopropylmalate synthase contains two core domains – an N-terminal catalytic region and a C-terminal allosteric regulatory

Figure 5 Comparison of seedling growth between WT and osipms1 mutants under amino acids treatments. (a) Seedling growth in WT and osipms1 mutants under normal and amino acids treatment conditions for 5 days. Bars = 10 mm. Comparison of shoot and root length between normal and amino acids treatment in WT (b) and osipms1 mutants (c, d, e). Each column represents the means ± standard deviation. ** indicates the significant difference compared to normal condition at 1% level. n.s. represents not significant.
domain – which mediate Leu feedback inhibition in plants and bacteria (de Kraker and Gershenzon, 2011). Similar results were observed in this study. OsIPMS1 and OsIPMS2 catalyse Leu biosynthesis, and generally their IPMS activities were gradually inhibited with the increase in Leu concentration in rice. However, the OsIPMS2 activity was activated by 2 mM Leu treatment in this study, which similar with the observation in Arabidopsis (de Kraker et al., 2007). OsIPMS1 and OsIPMS2 might have similar but not identical biochemical characteristics, and this prediction need to be further confirmed. We observed that eleven amino acids, including Leu and Val, were significantly decreased in the three osipms1 mutants during seed germination. This outcome was in contrast to the results involving Arabidopsis (de Kraker et al., 2007). OsIPMS1 and OsIPMS2 might have similar but not identical biochemical characteristics, and this prediction need to be further confirmed. 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Cys and Pro are important features of seed vigour owing to their general implications in metabolism and antioxidative potential in plants (Rajjou et al., 2008). Additionally, we observed that the branched-chain amino acids (BCAAs), including Leu, Ile and Val, were significantly reduced in osipms1 mutants compared to those in WT. BCAAs biosynthesis plays an important role in gametophyte and root development, and BCAA homeostasis contributes to stress tolerance in plants (Zhang et al., 2015). We therefore predicted that OsIPMS1 contributing to seed vigour might be due to the enhancement of amino acids associated with stress tolerance in germinating seeds. The relationships between amino acids, for example Cys, Pro, Ile, Val and Leu, and stress tolerance during seed germination need to be further analysed.

Initial seed imbibition is characterized by physical water uptake (Bewley et al., 2013). Usually, proteinaceous seeds have higher imbibition capacities than those of starch and oily seeds at the early germination stage. In this study, similar imbibition rates
were observed at the early germination stage in osipms1 mutants and WT due to the similar protein and starch levels in their mature seeds. These results indicated that OsIPMS1 regulates seed vigour but not by altering the imbibition rate. GA biosynthesis upon seed imbibition is necessary for seed germination and seedling growth (Vieira et al., 2002). In this study, we observed that the amino acids related to GA biosynthesis were significantly reduced in osipms1 mutants compared to those in WT. Further amino acid and GA treatments confirmed that the regulation of OsIPMS1 on seed vigour was significantly associated with GA biosynthesis in germinating seeds. GA stimulates the expression of α-amylase genes for hydrolysis of carbohydrate reserves, and then nourishes seed germination and seedling growth (Vieira et al., 2002). In this study, we observed that the expression of OsKAO gene control. The relative expression levels were represented by fold change relative to the expression level of OsKAO in WT. (d) Seedling growth in WT and osipms1 mutants under normal and GA3 treatment conditions for 5 days. Bars = 10 mm. Comparison of shoot length (e) and root length (f) between WT and osipms1 mutants under normal and GA3 treatment conditions. Each column represents the means ± standard deviation. * and ** indicate the significant difference compared to WT at 5% and 1% levels, respectively. n.s. represents not significant.

Figure 7 OsIPMS1 altering seedling growth involved in GA biosynthesis. (a) GA3 content in WT and osipms1 mutants during seed germination. (b) General overview of GA biosynthesis pathway in rice according to previous reports. (c) Relative expression levels of GA biosynthesis-related genes in germinating seeds. The expression of genes was normalized to that of OsActin gene control. The relative expression levels were represented by fold change relative to the expression level of OsKAO in WT. (d) Seedling growth in WT and osipms1 mutants under normal and GA3 treatment conditions for 5 days. Bars = 10 mm. Comparison of shoot length (e) and root length (f) between WT and osipms1 mutants under normal and GA3 treatment conditions. Each column represents the means ± standard deviation. * and ** indicate the significant difference compared to WT at 5% and 1% levels, respectively.
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Amino acids serve as energy donors in the TCA cycle under nighttime, developmental and stress conditions in plants (Angelovici et al., 2011; Araújo et al., 2010, 2011; Kirma et al., 2012). The aspartic acid (Asp) family pathway synthesizes the amino acids Lys, Thr, Ile and methionine (Met), which are further catabolized by the TCA cycle to generate energy under energy shortage conditions (Galili, 2011). The relationship between amino acids, for example Lys and Asp, and the TCA cycle has been observed during the early seed germination (Araújo et al., 2011). In this study, we observed that the ketogenic amino acids, including Leu, Ile, Phe, Gly, Thr and Tyr, were significantly reduced in osipms1 mutants compared to those in WT. These ketogenic amino acids are important for pyruvate and acetyl-CoA synthesis, which are the starting substrates of the TCA cycle. Metabolism of His and Pro is important for the synthesis of the TCA cycle metabolite, α-ketoglutarate. Similarly, metabolism of Ile, Val and Thr also has important effects on the synthesis of the TCA cycle metabolite, succinate, whereas Phe and Tyr metabolism are important for fumarate synthesis. However, these amino acids, including His, Pro, Ile, Val, Thr, Phe and Tyr, were significantly decreased in osipms1 mutants compared to those in WT in this study. These amino acids altered by OsIPMS1 might behave important effects on the TCA cycle during seed germination in rice.

As description above, we hypothesized that the OsIPMS1 may exert its effects on seed vigour by altering the energy level during seed germination. This hypothesis was firstly confirmed by the analysis of pyruvate and acetyl-CoA levels during seed germination. Pyruvate is the product of glycolysis, which is converted into acetyl-CoA, which in turn is the primary input for the TCA cycle (Schwender et al., 2003). We observed that the levels of pyruvate and acetyl-CoA were significantly decreased in osipms1 mutants compared to those in WT during seed germination. Pyruvate dehydrogenase catalysing the conversion of pyruvate to acetyl-CoA is a step linking the glycolytic pathway to the TCA cycle (Wang et al., 2015). In this study, decreased expression of pyruvate dehydrogenase was also observed in osipms1 mutants, suggesting that the carbon flux through the TCA cycle declined. Finally, the levels of ATP and AMP dropped approximately 30% in osipms1 mutants compared to those in WT at the late germination stage. These data implied that disruption of OsIPMS1 will reduce energy levels in germinating seeds, potentially causing low seed vigour in osipms1 mutants.

The positive effects of priming on seed vigour are mainly due to triggering the metabolic processes when primed seeds rehydrate, including the activation of DNA repair and antioxidant mechanisms, de novo synthesis of nucleic acids and proteins and ATP production (Paparella et al., 2015). Our data demonstrated that the expression of OsIPMS1 contributes to seed germination associated with ATP production. This raises the question as to the association between priming effects and OsIPMS1 expression during priming. It is difficult to choose and monitor the correct time-point of stop priming and dehydrate the seeds (Paparella et al., 2015). Meanwhile, the success of seed priming is strongly associated with plant species, genotype, seed lot and vigour (Parera and Cantliffe, 1994). Thus, two varieties Ningdao NO.1 and Wuyungeng NO.7 that popularly cultivated in Jiangsu Province of China were used to confirm the association between priming effects and OsIPMS1 expression during priming. We observed that the best stop time-point of seed priming is likely at the time before the mRNA levels of OsIPMS1 significantly reduced during priming, suggesting a suitable extent of germination-related metabolism existed in primed seeds at that moment. Prolonged priming duration caused a significantly low mRNA level of OsIPMS1 stored in primed seeds that will cause low seed vigour when primed seeds rehydration. Previously, prolonged priming treatment reduced seed vigour has been ascribed to the loss of seed desiccation tolerance (Sliwinska and Jendrzejczak, 2002) and an unpredicted enhancement of oxidative DNA injury (Balestrazzi et al., 2010). In this study, we predicted that prolonged priming treatment reduced the priming effects is

![Figure 8](image-url)
possibly due to low ATP production in primed seeds when rehydration. The confirmation of this hypothesis is now in progress. The OsIPMS1 might be used as a biomarker to determine the best time-point of stop priming in rice. However, the different expression of OsIPMS1 was also observed among varieties. The applications of OsIPMS1 in seed priming need to be further investigated using more varieties at more time-points of priming in rice.

In summary, the regulation of OsIPMS1 in seed vigour might be involved in the changes of amino acids that associated with stress tolerance, GA synthesis and TCA cycle in germinating seeds (Figure 11). The improvement of amino acids associated with stress tolerance might be contributing to seed germination under various conditions. Meanwhile, OsIPMS1 can promote the synthesis of amino acids related to GA biosynthesis in germinating seeds, which results in the enhancement of soluble sugars for glycolysis during seed germination. After that, the ATP levels will be promoted through TCA cycle with the increases in glycolysis and TCA cycle metabolites, which contribute to rapid germination and vigorous seedling growth. This study provides important insights into the function of OsIPMS1 on seed vigour in rice.

**Materials and methods**

**Plant materials and growth conditions**

The osipms1 (osipms1a, osipms1b and osipms1c) and osipms2 mutants used in this study were in the Japonica background (Oryza sativa L. cv. Nipponbare). The T-DNA mutant osipms1a (accession number: 05NPBMT72) was obtained from Rice Mutant Database (http://rmd.ncpgr.cn/; Miyao et al., 2003). osipms1b, osipms1c and osipms2 mutants were generated using the CRISPR/Cas9 system. All plants were grown in an experimental field at the Nanjing Agricultural University. Field management was performed in accordance with the local standard methods.

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(Cheng et al., 2014). All seeds were harvested at their maturity stage and dried at 42 °C for 7 days (~13% moisture content) to break seed dormancy (Wang et al., 2011).

T-DNA mutant identification

The T-DNA insertion site and homozygous line of osipms1a was identified by PCR analysis of genomic DNA using gene-specific primers (Table S1). PCR was conducted according to Xu et al. (2017). PCR products were directly sequenced and compared using NCBI BLAST of the rice genome database (http://www.ncbi.nlm.nih.gov/Blast/). Quantitative real-time PCR (qRT-PCR) analysis, as described below, was conducted to detect the mRNA levels of OsIPMS1 in 48-h imbibed seeds of osipms1a compared to that in the wild type (WT, Nipponbare).

Mutant generation and identification

The CRISPR/Cas9 system was used to generate mutants. The CRISPR/Cas9 plasmid was designed according to the protocol described previously (Cong and Zhang, 2015). The first coding exon of OsIPMS1 and OsIPMS2 was selected for the guide RNA design. Double-stranded DNA, generated by annealing the oligo pairs, was cloned into the p1300 + pX330-U6-Chimeric_BB-CBh-hSpCas9 vector. Genomic DNA was extracted from mutant seedlings for PCR using specific primers (Table S1). Mutations in the PCR products were detected through direct sequencing methods. Next, the PCR products were identified by comparing the 20-bp gRNA target sequences to the rice reference genome (sequence is in Table S1; Liu et al., 2015). qRT-PCR analysis, as described below, was conducted to detect the mRNA levels in 48-h imbibed seeds of mutants compared to those in WT. Three independent homozygous mutants, osipms1b, osipms1c, and osipms2, were identified.

Expression and purification of GST-Tag Protein

The sequence data of OsIPMS1 and OsIPMS2 were obtained from the Institute for Genomic Research (TIGR) database. The coding sequence of OsIPMS1 and OsIPMS2 was amplified from the
reverse-transcribed RNA isolated in rice Nipponbare seedlings using the primers IPMS1-1S, IPMS1-1A, IPMS2-1S and IPMS2-1A (Table S1). The cDNA fragments were ligated into the pGEM-T easy vector (Promega, Madison, WI). The desired OsIPMS1 and OsIPMS2 fragments were amplified by PCR using the primers IPMS1-2S, IPMS1-2A, IPMS2-2S and IPMS2-2A (Table S1), and subcloned into pGEX-2T using the restriction sites BamHI and EcoRI. The glutathione-S-transferase (GST)-IPMS fusion proteins were expressed in E. coli BL21 (DE3) cells and grown in LB medium at 37 °C until they reached an OD600 of 0.5. The GST-IPMS1 proteins were then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated with constant shaking at 15 °C for 24 h. Protein expression was checked by SDS-PAGE analysis. Proteins were purified using a GST-BindTM Kit (Novagen, Germany) and quantified with gel densitometry using a bovine serum albumin (BSA, Sigma-Aldrich) protein standard.

Figure 11 Hypothetical model of the role of OsIPMS1 on seed vigour in rice. The expression of OsIPMS1 in germinating seeds induces the accumulation of the amino acids associated with stress tolerance, GA biosynthesis and TCA cycle. Increased GA biosynthesis enhances starch hydrolysis for the accumulation of soluble sugars, thus increasing the glycolytic activity. The ATP levels will be promoted through TCA cycle with the increases of glycolysis and TCA cycle metabolites, which contribute to rapid germination and vigorous seedling growth. Solid arrows indicate the major effects, while dashed arrows indicate the predicted minor effects.

OsIPMS enzyme assay
The OsIPMS1 and OsIPMS2 enzyme assay was conducted according to the methods of de Kraker et al. (2007) with minor modifications. Briefly, 5 μL enzyme preparation was added into 150 μL reaction mixture, including 500 mM acetyl-CoA, 10 mM 2-oxoisovalerate, 4 mM MgCl2 and 100 mM Tris, pH 8.0, and incubated at 30 °C for 10 min. Reactions were stopped using liquid nitrogen. Next, 200 μL 1 mM fresh DTNB (Sigma-Aldrich) in 100 mM Tris (pH 8.0), and 200 μL ethanol was added into the reaction mixture. The mixture was left at room temperature (20–25 °C) until no further reaction occurred between the free thiol group of CoA with DNTB to develop a yellow-coloured 3-carboxy-4-nitrothiophenol anion. The mixture was centrifuged, and the absorbance was detected against water at 412 nm. The amount of OD412 was used to indicate enzyme activity. Three biological replications were performed.

Seed germination
Fifty seeds per replicate were imbibed in Petri dishes (diameter 9 cm) with 10 mL distilled water, 10% PEG or 100 mM NaCl at 30 ± 1 °C for 7 days, as well as with 10 mL distilled water at 15 ± 1 °C for 14 days. Thirty seeds per replicate were sowed in 1 cm deep soils under natural conditions (25–32 °C) for 7 days. Meanwhile, seed germination of osipms1 mutants was also conducted under GA3 and amino acids treatments. Germination ability was observed daily. Seeds were considered as germinated when the radicle protruded (2 mm) through the seed coat. Seedlings were considered to be established when the root length reached seed length and the shoot length reached half of the seed length. The percentage of germinated seeds at 3 days was referred to as germination potential (GP). T50 is the time for 50% of the germination, and it was calculated by the GERMINATOR software (Joosen et al., 2010). Germination index (GI) was calculated as follows: GI = ∑[Gt]/Gtt, where Gt is the number of the germinated seeds on Day t (Wang et al., 2010). Three replications were performed.

Expression analysis
Total RNA was extracted from various plant tissues of Nipponbare, as well as from developing grains (0, 7, 14, 21, 28, 35 and 42 days after flowering; DAF) and germinating seeds (4-, 8-, 12-, 18-, 24-, 36- and 48-h imbibition), using the TransZol Plant kit (Transgen, www.transgen.com), according to the protocol by the manufacturer. The first-strand cDNA was synthesized with random oligonucleotides using the HiScript® II Reverse Transcriptase system (Vazyme Biotech Co., Ltd). qRT-PCR was carried out in a total volume of 20 μL containing 2 μL of cDNA, 0.4 μL gene-specific primers (10 μm), 10 μL SYBR Green Mix and 7.2 μL of RNase free ddH2O, using the Roche LightCycler480 Real-time System (Roche, Swiss Confederation). The PCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The rice OsActin and 18S rRNA genes were used as internal controls. Primers used for qRT-PCR are listed in Table S1. Normalized transcript levels were calculated using the comparative CT method (Livak and Schmittgen, 2001). Three biological replications were performed.

Differentially expressed genes analysis
Total RNA was extracted from approximately 80–100 mg powder of WT and osipms1 seeds after 8-h imbibition using the TransZol Plant kit (Transgen, www.transgen.com) according to the manufacturer’s protocol. Construction of cDNA libraries and HiSeq2500 sequencing were performed at Novogene Biotechnology Co., Ltd., Beijing, China. FastQC was performed to estimate the quality of raw reads (http://www.plob.org/2013/07/16/5987.html). The adapter sequences were trimmed, and the low-quality reads with Q ≤ 20 from the 5′ and 3′ ends of the remaining reads were filtered. Then, the clean reads with 21–49 bp length were mapped onto the Nipponbare reference genome (MSU Rice Genome Annotation Project Release 7) using Tophat version 2.0.12 (Kim et al., 2012). Levels of gene expression were quantified in terms of FPKM (fragments per kilo base of exon per million) using RSEM version 1.1.11 (Li and Dewey, 2011). The log2-fold changes of gene FPKM were calculated in 8-h imbibed seeds and comparisons were made between osipms1a and WT. The differentially expressed genes (DEGs) with a padj (P-adjusted) < 0.05 were selected for further pathway analysis. Gene Ontology (GO) and KEGG pathway analyses were performed.
through G0seq (Young et al., 2010) and KOBAS (2.0) with a significant level of false discovery rate (FDR < 0.05; Xie et al., 2011). Three biological replications were performed.

### Samples harvested for physiological assays

Total protein, starch and sugar content were detected in the dry mature seeds. Seeds were imbibed in Petri dishes (diameter 9 cm) with 10 mL distilled water at 30 ± 1 °C for 3 days. The weight of the imbibed seeds was recorded every 12 h to calculate the moisture content of seeds. Following 0-, 12-, 24-, 36-, 48- and 60-h imbibition, the seeds were harvested to detect the levels of free amino acids, GA3, glucose, fructose, amylase activity, acetyl-CoA, pyruvic acid and energy. Approximately 0.5 g of each sample was rapidly frozen in liquid nitrogen and homogenized into a powder. Three biological replications were performed for each index.

#### Amino acid assay

Amino acids were extracted from the powder with 2.0 mL distilled water at 4 °C for 17 h. The extraction mixture was centrifuged at 12 000 g at 4 °C for 20 min, following which 0.8 mL supernatant was transferred to 2-mL Eppendorf tubes and was passed through C18 columns (C18 Sep-Park/C2261.7 μm 2.1 × 50 mm C18 column was used for HPLC (Waters Instruments Inc.). Identification of ATP, ADP and AMP in the samples was conducted by comparison of the retention time with that of standards. ATP, ADP and AMP levels were determined using the external standard method and were expressed as μg/g DW. The energy charge (EG) was calculated as follows: EG = ([ATP] + 1/2 [ADP]) ÷ ([ATP] + [ADP] + [AMP]).

#### Hormone quantification

Hormone GA3 was extracted from each sample using 5 mL 80% (v/v) precooling methanol at 4 °C for 12 h and centrifuged at 8000 g at 4 °C for 10 min. The supernatant was collected and added 0.1 g Poly to adsorb the phenolic compounds and pigments at 4 °C for 1 h, and then, the mixture was centrifuged at 12 000 g at 4 °C for 10 min. Next, the supernatant was washed with 5 mL 100% (v/v) and 5 mL 80% (v/v) methanol and was passed through C18 columns (C18 Sep-Park® cartridges, Waters Corp., Milford, MA). The extraction was freeze-dried and dissolved in 2 mL of 75% aqueous methanol, and followed by filtration through 0.22-μm filters, the final 2 μL filtrate solution was carried out using a high-performance liquid chromatography (HPLC) system (Waters Instruments Inc., Rochester, MN). The content of GA3 was determined using the external standard method and was expressed as μg/g DW.

#### Protein, starch, sugar, amylase activity, acetyl-CoA and pyruvic acid assays

Protein, starch, sugars, amylase activity, acetyl-CoA and pyruvic acid were measured using commercial assay kits following the manufacturer’s instructions (Suzhou Keming Bioengineering Company, China). The levels of protein, starch, glucose, fructose and pyruvic acid were expressed as mg/g DW. Acetyl-CoA levels were expressed as nmol/g DW. One unit (U) of amylase is defined as 1 mg of reducing sugar produced by enzyme in 1 g DW sample in 1 min at 40 °C. The activities of α- and β-amylase were expressed as U/g DW.

#### Energy level assays

Extraction of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) from the powder was conducted according to the method of Liu et al. (2006) with minor modifications. Briefly, 1.5 mL of 600 mM perchloric acid was added into the powder placed in an ice bath for 1 min. After the extraction mixture was centrifuged at 12 000 g at 4 °C for 10 min, 1.2 mL supernatant was taken and quickly neutralized to pH 6.5–6.8 with 1000 mM KOH. After the mixture was centrifuged at 12 000 g at 4 °C for 10 min followed by filtration through 0.22-μm membrane filters, the final 2 μL filtrate solution was measured by HPLC (Waters Instruments Inc.). The ACQUITY UPLC® BEHC18 1.7 μm 2.1 × 50 mm C18 column was used for HPLC (Waters Instruments Inc.). Identification of ATP, ADP and AMP was conducted by comparison of the retention time with that of standards. ATP, ADP and AMP levels were determined using the external standard method and were expressed as μg/g DW. The energy charge (EG) was calculated as follows: EG = ([ATP] + 1/2 [ADP]) ÷ ([ATP] + [ADP] + [AMP]).

#### Seed priming

Seed priming treatments were conducted according to the method described by Cheng et al. (2017) with minor modifications. Briefly, 50 seeds were surface sterilized with 0.1% HgCl2 for 5 min and then placed in a Petri dish (diameter 9 cm) with 10 mL of distilled water. Seeds were incubated at 30 °C for 0–36 h in the dark for priming treatment, following which the imbibed seeds were dried at 30 °C for 7 days to their original moisture content (~13%). Seed germination was conducted as described above. The expression of OsIPMS1 during seed priming was analysed by qRT-PCR as described above. Unprimed dry seeds were used as controls. Three biological replications were performed.

#### Data analysis

Experimental data were analysed using the SAS software (Cary, NC), and the percentage data were transformed according to $y = \arcsin(\sqrt{(x/100)})$. The significant differences were tested using Student’s t-test or Fisher’s least significant difference (LSD) test at the 5% and 1% levels of probability.

#### Availability of supporting data

The RNA sequencing data have been submitted to the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under accession number SRP134159.

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#### Conflict of interest

The authors declare no conflict of interest.

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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 Characterization of OsIPMS1 and OsIPMS2 in rice.
Figure S2 Comparison of amino acid sequences between OsIPMS1 and OsIPMS2.
Figure S3 Confirmation of osipms1b and osipms1c mutants.
Figure S4 Comparison of seed germination between WT and osipms1 mutants under stress conditions.
Figure S5 Comparison of seed germination between WT and osipms2 mutants under direct-seeding conditions.
Figure S6 Comparison of seed germination between WT and osipms1 mutants under stress conditions.
Figure S7 Amino acid treatment improved the expression of GA biosynthesis related genes in germinating seeds of osipms1 mutants.
Figure S8 GO enrichment analysis for differentially expressed genes (DEGs) in osipms1a compared to WT.
Figure S9 Comparison of imbibition rate and seed reserves between WT and osipms1 mutants.
Figure S10 Comparison of priming effects on seed vigor between WT and osipms1 mutants.
Table S1 The primer pairs used in this study.
Table S2 Differently expression genes (DEGs) between WT and osipms1 in 8 h-imibed seeds.
Table S3 Differently expression genes (DEGs) involved in biological pathways by KEGG pathway analysis.