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Indole-3-acetate beta-glucosyltransferase *OsIAGLU* regulates seed vigour through mediating crosstalk between auxin and abscisic acid in rice

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Summary

Seed vigour is an important trait for direct seeding in rice. In this study, indole-3-acetate beta-glucosyltransferase *OsIAGLU* was cloned in rice, and its roles on seed vigour were mainly investigated. Disruption of *OsIAGLU* resulted in low seed vigour in rice. Quantitative RT-PCR analysis showed that the expressions of *OsIAGLU* were relatively higher in the late developing and the early germinating seeds and were significantly induced by indole-3-acetic acid (IAA) and abscisic acid (ABA). Transcriptome analysis revealed that the IAA- and ABA-related genes were involved in the *OsIAGLU* regulation of seed vigour in rice. The higher levels of free IAA and ABA were identified in germinating seeds of *osiaglu* mutants compared to wild-type (WT) plants. When treated with exogenous IAA and ABA, the *osiaglu* mutants and WT plants showed sensitivity to ABA while not IAA, but the exogenous IAA amplified ABA-induced reduction of seed vigour in rice. The continuously higher expressions of *ABA-INSENSITIVE 3* (*OsABI3*) and *OsABI5* occurred in germinating seeds of *osiaglu* mutants compared to WT plants. The regulation of seed vigour by *OsIAGLU* might be through modulating IAA and ABA levels to alert *OsABIs* expression in germinating seeds in rice. Based on analysis of single-nucleotide polymorphism data of rice accessions, two haplotypes of *OsIAGLU* that positively correlated with seed vigour were identified in *indica* accessions. This study provides important insights into the roles of *OsIAGLU* on seed vigour and facilitates the practical use of *OsIAGLU* in rice breeding.

Introduction

Rice is one of the oldest cultivated crops in China. In recent years, the direct seeding method of rice has been widely applied due to its advantages of cost- and labour-saving (Liu *et al.*, 2015a). Seed germination and seedling establishment as key events in plant life activities are the important factors influencing the yield of direct seeding in rice (Gommers and Monte, 2018). Seeds with high vigour will behave the characteristics of speed and uniform germination and vigorous seedling growth in fields, which will lead to better weed competitiveness and reduce yield losses (Chen *et al.*, 2019). The varieties with high seed vigour are required for the direct seeding of rice (Mahender *et al.*, 2015). The mining of key genes regulated seed vigour and illuminating their mechanisms are important objectives of rice breeding for direct seeding production.

Abscisic acid (ABA) as the major endogenous factor has been widely investigated involving in the inhibition of seed germination (Penfield, 2017). The ABA level in plants is maintained in strict homeostasis, including biosynthesis, degradation and conjugation, during various physiological processes (Palaniyandi *et al.*, 2015; Seiler *et al.*, 2011). The increase of ABA level in seeds is controlled by ABA biosynthesis genes zeaxanthin epoxidase (*ZEP*) and 9-cis-epoxycarotenoid dioxygenases (*NCEDs*; Eiji *et al.*, 2010). The decrease of ABA level is regulated by ABA catabolism *CYP707A* family genes (Kushiro *et al.*, 2004). Moreover, the conjugation of ABA with glucose catalysed by ABA-specific glucosyltransferases represents another ABA inactivation mechanism. For example, the *Arabidopsis* UGT71B6 exhibits glucosylation activity towards ABA for the conversion of free ABA to inactive form ABA-glucose (Priest *et al.*, 2006). It is well known that ABA acts through the PYR/RCAR-PP2C-SnRK2 signalling cascade (Cutler *et al.*, 2010). ABSCISIC ACID-INSENSITIVE 3 (ABI3) is a major downstream component of ABA signalling, and it has been reported as a major regulator of seed dormancy and ABA inhibition of seed germination (Liu *et al.*, 2013).

Auxin recently has also been reported to play essential roles in the regulation of seed dormancy in plants. In *Arabidopsis*, auxin controls seed dormancy through the stimulation of ABA signalling (Liu *et al.*, 2013). When auxin signalling activated, auxin binds to its receptor transport inhibitor resistant 1/auxin signalling F-box (TIR1/AFB) proteins and promotes the degradation of auxin/indole-acetic acid (AUX/IAA; Dharmasiri *et al.*, 2005). This degradation releases the activity of auxin response factor (ARF10/16) and maintains the expression of ABI3, which inhibits seed germination and maintains seed dormancy (Liu *et al.*, 2013).

The active auxin level is tightly controlled by the processes of synthesis, inactivation and transport (Korasick *et al.*, 2013). In which, cellular auxin level can be altered by auxin glycosyltransferases usually through catalysing the transfer of activated sugars to auxin in plants. For example, the maize UDP glucosyltransferase IAGLU can conjugate IAA to glucose (Szerszen *et al.*, 1994). The *Arabidopsis* UDP glucosyltransferase UGT84B1 has the glycosylation activities against IAA and indole-3-butyric acid (IBA; Jackson *et al.*, 2001), and the glycosylation activities of *Arabidopsis* UGT74E2 and UGT75D1 are prominent towards IBA (Tognetti *et al.*, 2010; Zhang *et al.*, 2016).

Glycosylation of hormones plays important roles in plant development and growth (Palaniyandi *et al.*, 2015; Shang *et al.*, 2016; Zhang *et al.*, 2016). Several studies have reported that auxin glycosyltransferases involve in plant development and stress tolerance in *Arabidopsis*. For example, the overexpression lines of auxin glycosyltransferase *UGT84B1* and *UGT74D1* behave plant dwarfing and leaf curl phenotype in *Arabidopsis* (Jackson *et al.*, 2002; Jin *et al.*, 2013). The IBA and IAA homeostasis is perturbed in the overexpression lines of *UGT74E* in *Arabidopsis*, that causes the increase of shoot branching, the alteration of rosette shape and the improvement of stress tolerance (Tognetti *et al.*, 2010). Plants overexpressing *UGT75D1* increased seed germination under stress conditions might be through modulating ARF16-ABI3 signalling in *Arabidopsis* (Zhang *et al.*, 2016). The phenotypes of enlarged leaf angle, dwarf plants and shorter panicles are observed in transgenic rice overexpressing *OsIAAGLU* (Yu *et al.*, 2019). However, the regulation roles of auxin glycosyltransferase on seed vigour are few reported in rice.

Our previous RNA-Seq data showed that the expression of indole-3-acetate beta-glucosyltransferase *OsIAGLU* was significantly induced at the early seed imbibition stage in rice, suggesting it might be involved in the regulation of seed vigour. In this study, rice *OsIAGLU* was cloned and its regulatory functions on seed vigour were mainly investigated. Disruption of *OsIAGLU* resulted in low seed vigour in rice. The higher free IAA and ABA levels were observed in germinating seeds of *osiaglu* mutants compared with wild-type (WT) plants in rice. The possible mechanisms of *OsIAGLU* involving in the regulation of seed vigour might be through modulating *OsABIs* expressions under the interactions of IAA and ABA in germinating seeds of rice. The application of *OsIAGLU* might be useful to genetic improvements of seed vigour for direct seeding of rice in the future.

Results

Disruption of *OsIAGLU* resulted in low seed vigour

Rice *OsIAGLU* was located on Chromosome 11, which was predicted to contain approximately 504 amino acids (Figure S1a). The phylogenetic tree indicated that the closer genetic relationships of IGAU exist among rice and maize (Figure S1b). To further confirm *OsIAGLU* function, we employed the CRISPR/Cas9 system to generate mutants, which were named *osiaglu-1*, *osiaglu-2* and *osiaglu-3* (Figure 1a). The *osiaglu-1*, *osiaglu-2* and *osiaglu-3* mutant plants contained a 59-, 58- and 5-bp deletion, respectively, in the exon of *OsIAGLU* (Figure 1b). The amino acid sequence of *OsIAGLU*, predicted based on these nucleotide sequences, contains only 39, 100 and 57 amino acids in *osiaglu-1*, *osiaglu-2* and *osiaglu-3,* respectively, and is caused by premature termination (Figure S2). These results indicate that the *osiaglu-1*, *osiaglu-2* and *osiaglu-3* mutant lines lacked OsIAGLU. The progeny of these homozygous mutants were used in subsequent experiments.

Seed quality and seed dormancy established during seed development will influence the performances of seed vigour during seed germination. Our data showed that there were no significant differences of grain traits, including grain length, width, thickness and grain weight, between three *osiaglu* mutants and wild-type (WT) (Figure S3). There were no seed dormancy occurred in three *osiaglu* mutants and WT with more than 90% germination percentage after 10 days of germination by dormancy breaking treatments in this study (Figure S4). Then, the seeds of three *osiaglu* mutants and WT were subsequently used for the evaluation of seed vigour. Phenotype evaluation demonstrated that the disruption of *OsIAGLU* resulted in low seed vigour, mainly including low germination speed and seedling growth, under various conditions in rice. The times for 50% germination of *osiaglu-1*, *osiaglu-2* and *osiaglu-3* were significantly increased compared to those of wild-type (WT) plants, while the germination potential, germination index and seedling vigour index were significantly reduced under normal conditions (Figure 1c–g). Moreover, the seedling percentage and seedling growth, including root length, shoot length and seedling dry weight, were significantly decreased in *osiaglu-1*, *osiaglu-2* and *osiaglu-3* lines compared to those in WT plants under the direct seeding method in soils (Figure 1h–l). All these results suggest that *OsIAGLU* might play important roles on the regulation of seed vigour in rice.

Expression pattern of *OsIAGLU* and subcellular localization

To expand our understanding of the physiological function of *OsIAGLU*, the expression patterns of *OsIAGLU* during seed development and seed germination were further analysed using quantitative RT-PCR approach. Transcripts of *OsIAGLU* were dynamically increased in the filling grain from 0 to 28 days after flowering (DAF) and sharply increased after the 28 DAF (Figure 2a). Additionally, the transcript of *OsIAGLU* was strongly detected at 4-h imbibition time-point during the early seed germination stage (Figure 2a). We also investigated whether the expression of *OsIAGLU* was induced by IAA, ABA and GA during seed germination. The results indicated that the expression of *OsIAGLU* could be obviously induced by both IAA and ABA while not GA within different duration of treatments from 12 to 72 h, with a stronger induction by IAA and ABA than GA (Figure 2b).

To investigate the tissue-specific expression of *OsIAGLU*, histochemical staining for GUS activity of the OsIAGLU promoter::GUS transgenic lines was carried out. It showed that OsIAGLU was strongly expressed in embryos and shoots of germinating seeds during 12–72 h under normal, IAA and ABA treatment conditions (Figure 2c). To determine the subcellular localization of OsIAGLU, we constructed a recombinant OsIAGLU tagged at the C-terminus with GFP and expressed it transiently in rice protoplasts. The GFP-tagged OsIAGLU was found to distribute throughout the cytoplasm (Figure 2d), suggesting the catalytic activity of OsIAGLU is present in the cytoplasm. The specific expression of OsIAGLU in germinating seeds suggested that it may play a role in modulating seed vigour in rice.

OsIAGLU regulates IAA and ABA levels during seed germination

To further understand the *OsIAGLU* function, genome-wide transcriptional levels were compared between *osiaglu-2* and WT at the 72-h imbibition stage. By comparison, the data of RNA sequencing were high consistent (Pearson correlation >0.997)

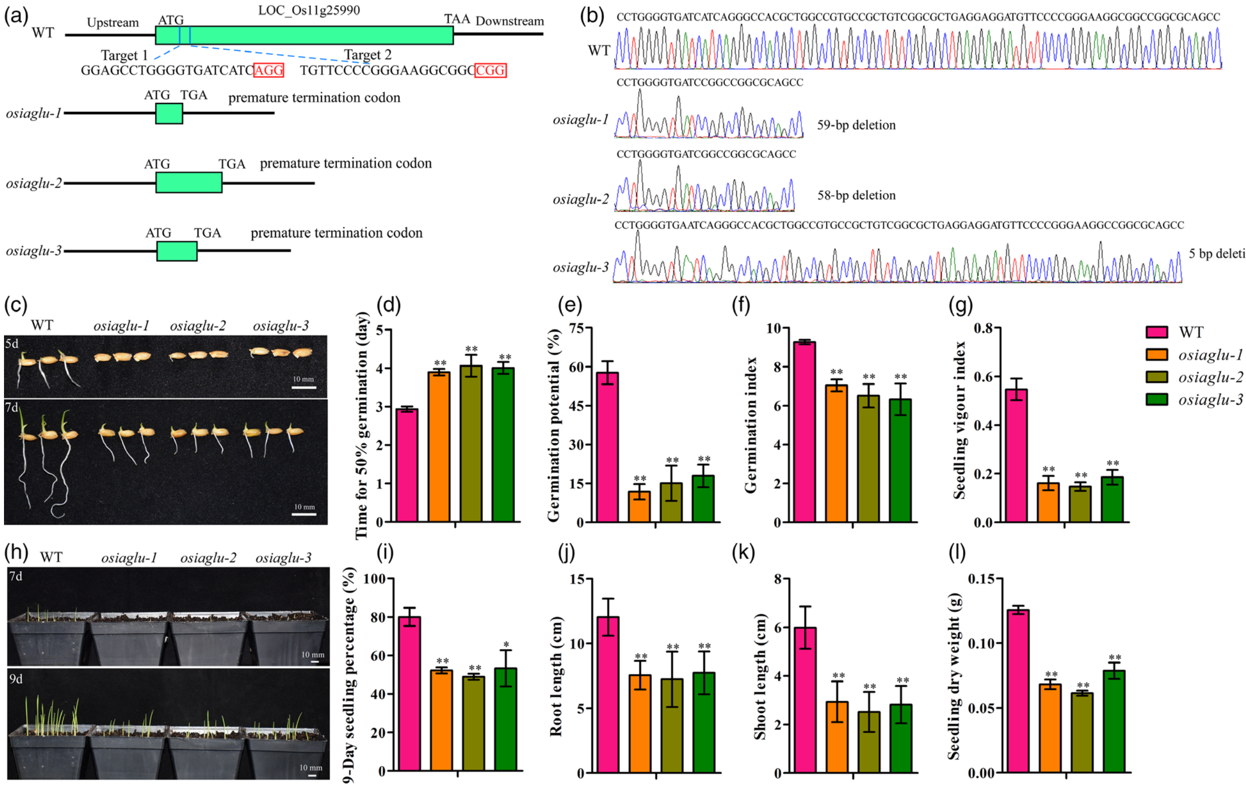


Figure 1 Comparison of seed vigour between WT and *osiaglu* mutants in rice. (a) Gene structures of WT and *osiaglu* mutants. Green box represents the *OsIAGLU* gene region. (b) A total of 59-, 58- and 5-bp nucleotides were deleted in the *osiaglu-1*, *osiaglu-2* and *osiaglu-3*, respectively. (c) Seed germination of WT and *osiaglu* mutants after 5 and 7 days. Bars = 10 mm. (d) Time to 50% germination percentage; (e) germination potential; (f) germination index; (g) seedling vigour index. (h) Seedling establishment of WT and *osiaglu* mutants after 7 and 9 days under direct seeding conditions. Bars = 10 mm. (i) seedling percentage; (j) root length; (k) shoot length; (l) seedling dry weight. Each column represents the means ± SD. * and ** indicate the significant difference compared to WT at 5% and 1% levels, respectively, according to Student's *t*-test.

between samples in this study, and then the mean FPKM of three biological repeats was used for further analysis. A total of 9121 differentially expressed genes (DEGs) were identified between *osiaglu-2* and WT (Table S1). Of those, 3148 and 5973 genes were significantly up-regulated and down-regulated, respectively, in *osiaglu-2* compared to WT (Figure 3a). Through MapMan analysis, 222 differentially expressed genes were significantly enriched in hormone metabolism. In which, the majority of hormone-related DEGs were involved in IAA (77), ABA (23), jasmonic acid (JA) (22) and ethylene (ETH) (60) metabolism (Figure 3b).

It has been reported that glycosyltransferases usually involved in IAA and ABA homeostasis in plants (Priest *et al.*, 2006; Yu *et al.*, 2019). Our above RNA-Seq data indicated that *OsIAGLU* might involve in the regulation of IAA and ABA metabolism during seed germination in rice. We therefore speculated that *OsIAGLU* might regulate IAA and ABA levels during seed germination in rice. To confirm this presumption, the levels of endogenous IAA and ABA in mature and germinating seeds of *osiaglu* mutants and WT plants were quantified using an ultra-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) approach. By comparison, the levels of IAA and ABA were significantly increased in germinating seeds of *osiaglu* mutants compared to those in WT plants during 12–72 h after germination (Figure 3c-d). Meanwhile, the increases of IAA and ABA levels were generally observed in *osiaglu* mutants compared to WT plants in mature seeds. Overall, the levels of endogenous IAA and ABA regulated by *OsIAGLU* in germinating seeds were confirmed.

OsIAGLU regulates seed vigour associated with the interaction of ABA and IAA

As descripted above, rice *OsIAGLU* can influence the free IAA and ABA levels during seed germination, and thus, we predicted that the higher accumulation of free IAA and ABA in *osiaglu* mutants might cause low seed vigour. Previous study showed that the ABA-sensitive phenotypes during seed germination were observed at 5 to 50 µM ABA treatments in rice (Liu *et al.*, 2015b), while no IAA-sensitive phenotypes were observed at 5 to 50 µM IAA treatments according to our previous data. To further confirm the above prediction, the impacts of 5 µM and 10 µM exogenous IAA and ABA on *osiaglu* mutants and WT plants were analysed (Figure 4a). In the presence of exogenous IAA, there were no significant differences in seed vigour, including times for 50% germination, germination percentage and germination index, between control and treatments among *osiaglu* mutants and WT plants (Figure 4b-d). However, in the presence of exogenous ABA, the seed vigour of *osiaglu* mutants and WT plants was significantly reduced by treatments (Figure 4e–g). Moreover, *osiaglu* plants exhibited more ABA-

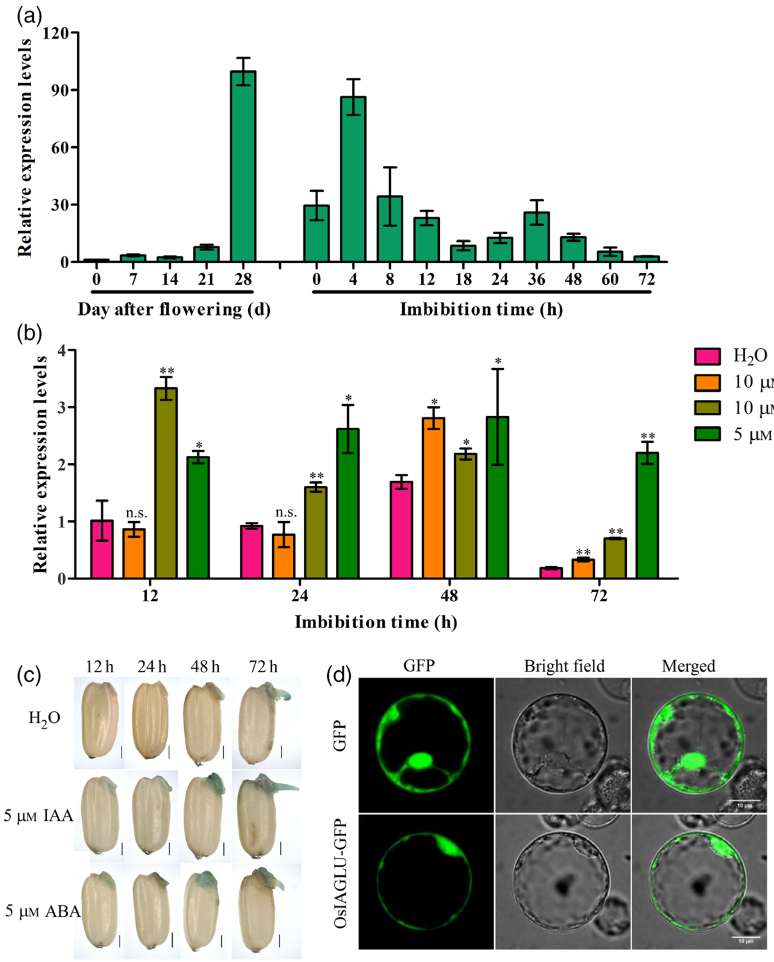


Figure 2 Expression patterns of *OsIAGLU* and subcellular localization in rice. (a) Expression pattern of *OsIAGLU* in various developmental and germination stages in rice using quantitative RT-PCR approach. (b) Transcription levels of *OsIAGLU* response to GA_3, ABA and IAA treatments in germinating seeds conducted using quantitative RT-PCR approach. The expression of *OsIAGLU* was normalized to that of *OsActin* gene control. The relative expression levels were represented by fold change relative to the expression level of *OsIAGLU* at 0 day after flowering or 12-h imbibition stage under H_2O treatment. Each column represents the means ± SD. * and ** indicate the significant difference compared to WT at 5% and 1% levels, respectively, according to Student's *t*-test. n.s. represents not significant. (c) Histochemical staining for GUS activity of OsIAGLU during seed germination under normal, IAA and ABA treatment conditions in rice. (d) Subcellular localization of OsIAGLU tagged at the C-terminus with GFP in rice protoplasts.

sensitive phenotype with lower seed vigour compared with WT plants.

Previous study indicated that auxin-mediated seed dormancy is dependent on ABA in *Arabidopsis* (Liu *et al.*, 2013). We, therefore, examined the interaction of IAA and ABA on the regulation of seed vigour in rice. The seed vigour of WT plants was tested under various concentrations of exogenous IAA (5 μM and 10 μM) treatments in the presence of exogenous ABA (5 μM; Figure 5). Our results indicated that the exogenous IAA can enhance the ABA-mediated reduction of seed vigour in rice. Seed vigour was significantly reduced by IAA + ABA treatments compared with ABA treatments. Similarly, the exogenous IAA enhanced the ABA-sensitive phenotype of *osiaglu* plants with lower seed vigour compared with WT plants (Figure S5). These results suggest that knockout of *OsIAGLU* results in low seed vigour because of excessive ABA and IAA accumulation and their interaction in rice.

OsIAGLU regulates seed vigour involved in *OsABIs* expression

How the *OsIAGLU* regulated seed vigour though influencing the IAA and ABA levels during seed germination? Our RNA-Seq also showed that the majority of IAA-related DEGs, including TIR1 (1), AUX/IAA (15) and ARFs (9), were involved in IAA signalling processes (Figure 6a), and the majority of ABA-related DEGs, including ABA synthesis/degradation (10), ABA signal transduction (5) and ABA-responsive gene (8), were involved in ABA signalling processes (Figure 6b). The consistent change trends were observed in the majority of IAA- and ABA-related DEGs between RNA-Seq and quantitative RT-PCR approaches in germinating seeds of mutants compared to WT seeds (Figure S6; Figure S7). Interestingly, the transcripts of several identified ARFs (*OsARF1*, *OsARF8* and *OsARF10*) and ABA signalling-related genes (*OsABI3*, *OsABI5*, *OsZIP23*, *OsTRAB1* and *OsABL1*) were significantly increased in *osiaglu* plants compared with those of WT plants at the 72-h imbibition stage (Figure 6a,b). These results suggested that the regulation of *OsIAGLU* on seed vigour might involve in IAA and ABA signalling during seed germination in rice.

It has been well studied that the auxin and ABA acted synergistically to inhibit seed germination is largely dependent on the ABI3 function in *Arabidopsis* (Liu *et al.*, 2013). ABIs are the downstream factors of ABA signalling pathway, which play the key roles on seed germination. Thus, the expressions of *OsABI3* and *OsABI5* regulated by *OsIAGLU* were focused on detection during seed germination in rice. We observed that the expressions of *OsABI3* and *OsABI5* were significantly induced by IAA and ABA treatments during seed germination in rice (Figure 7a-d). As descripted above, the higher levels of IAA and ABA were accumulated in *osiaglu* mutants than WT plants in rice. We therefore speculated that the higher expressions of *OsABI3* and *OsABI5* will be occurred in *osiaglu* mutants compared with WT plants during seed germination in rice. It was confirmed by our quantitative RT-PCR analysis (Figure 7e,f). Firstly, we observed that the expressions of *OsABI3* and *OsABI5* were generally down-

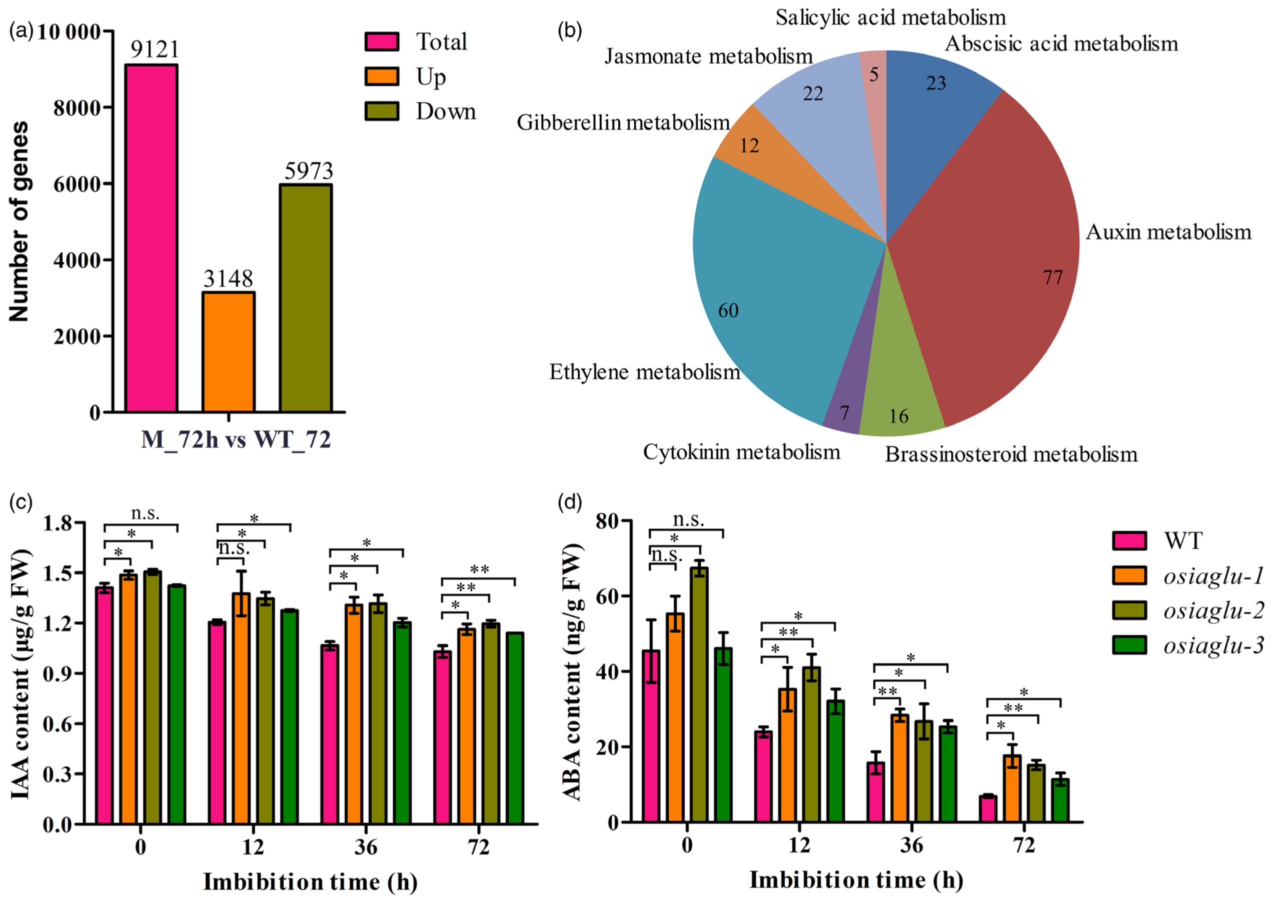


Figure 3 *OsIAGLU* altering the contents of IAA and ABA in germinating seeds in rice. (a) Differentially expressed genes (DEGs) with $p < 0.001$ between WT and *osiaglu-2* in germinating seeds (72 h) in rice. (b) DEGs involved in hormone metabolism. (c-d) The contents of IAA and ABA in germinating seeds of WT and *osiaglu* mutants in rice. Each column represents the means ± SD. * and ** indicate the significant difference compared to WT at 5% and 1% levels, respectively, according to Student's *t*-test. n.s. represents not significant.

regulated in WT plants during seed germination, while those of *osiaglu* mutants exhibited similar higher expression patterns especially at 12- to 48-h imbibition stages. Moreover, the significant higher expressions of *OsABI3* and *OsABI5* were observed in *osiaglu* mutants compared with WT plants during seed germination especially at 36- to 72-h imbibition stages. These results indicated that the continuously higher expressions of *OsABI3* and *OsABI5* in germinating seeds caused low seed vigour in *osiaglu* mutant plants in rice.

Association of *OsIAGLU* haplotypes with seed vigour

In general, *indica* accessions have stronger seed vigour than *japonica* accessions surveyed in this study (Figure 8a,b; Table S2). Therefore, to investigate whether the variation of *OsIAGLU* allele was associated with the difference of seed vigour between two subspecies, SNPs in the region from ~ 2 kb upstream to ~ 1 kb downstream of *OsIAGLU* were analysed using the SNP data of rice accessions (McCouch *et al.*, 2016). Five haplotypes of *OsIAGLU* were identified among these accessions (Figure 8c). The elite haplotypes Hap 1 and Hap 2 associated with high seed vigour were mainly existed in the *indica* accessions, while the haplotypes Hap 3 and Hap 5 mainly existed in the *japonica* accessions and Hap 4 existed in both *indica* and *japonica* accessions were associated with low seed vigour (Figure 8c-e). Several elite *indica* accessions with high seed vigour (seed germination percentage after 2 days more than 80%) were identified harbouring Hap 1 or Hap 2 (Table S3). We speculated that the SNPs of *OsIAGLU* affect its expression during seed germination to regulate seed vigour in rice. Thus, the expressions of *OsIAGLU* in accessions harbouring different haplotypes were detected during seed germination in rice. The significant higher expressions of *OsIAGLU* were observed in accessions harbouring Hap 1 or Hap 2 haplotypes (high seed vigour) compared to those of accessions harbouring Hap 3, Hap 4 or Hap 5 haplotypes (low seed vigour) in rice (Figure 8f–g; Table S4). Taken together, the variations in *OsIAGLU* seem to be associated with the differential seed vigour between *japonica* and *indica* subspecies in rice.

Discussion

Seed vigour, including speed germination and vigorous seedling growth, is an important agronomic trait for direct seeding in rice. Glycosylation is thought to be an important process in the regulation of hormone homeostasis in plants (Palaniyandi *et al.*, 2015). Previously, the plants overexpressing IAA glucosyltransferase *OsIAAGLU* exhibited typical phenotypes associated with a reduction in free IAA content with enlarged leaf angle, dwarf plants and shorter panicles in rice (Yu *et al.*, 2019). However, the regulation of seed vigour by IAA glucosyltransferase was not investigated previously in rice. In this study, we observed that the

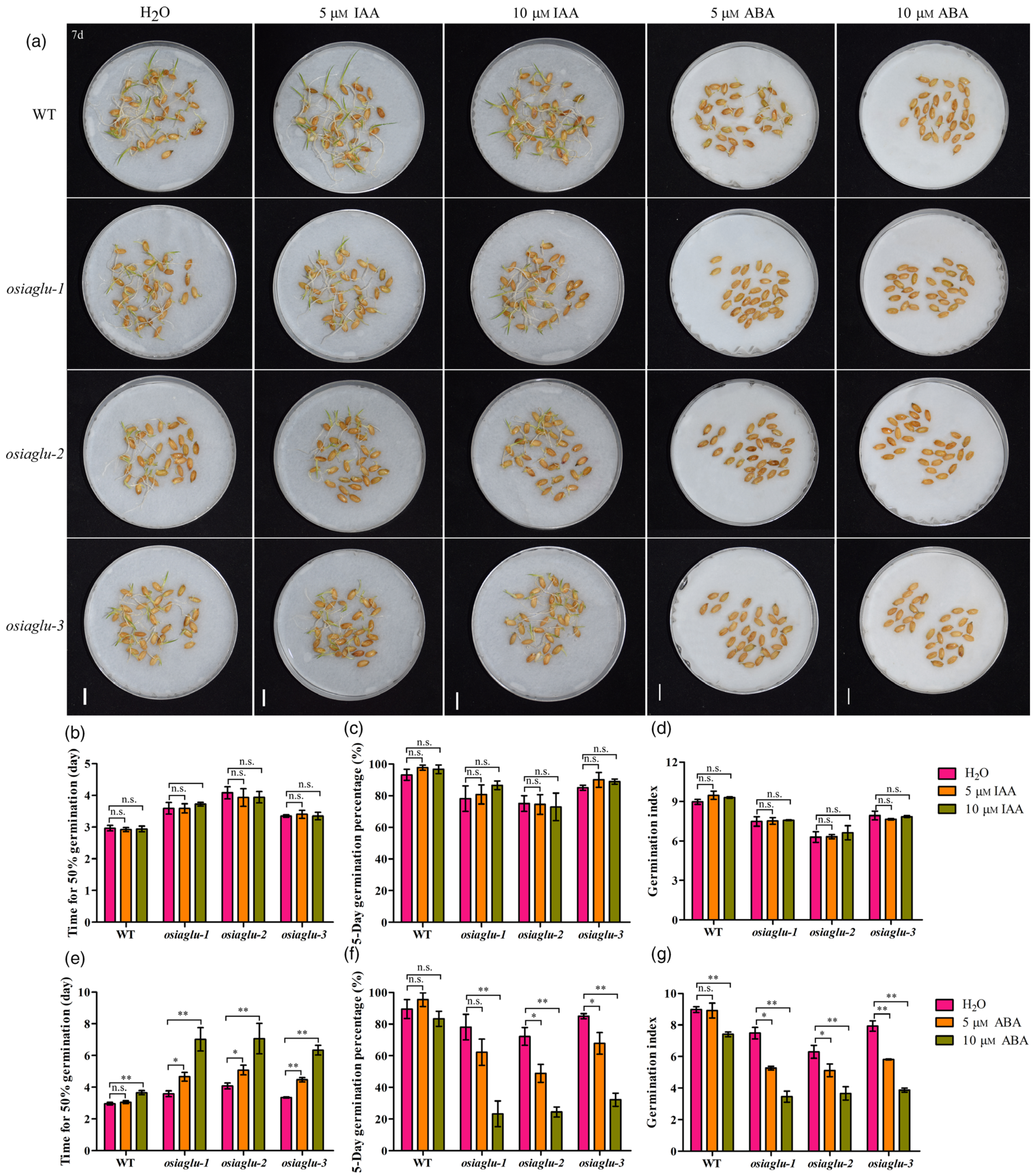


Figure 4 Effects of IAA and ABA treatments on seed vigour among WT and *osiaglu* mutants in rice. (a) Seed germination of WT and *osiaglu* mutants under IAA and ABA treatments for 7 days. Bars = 10 mm. (b-d) Comparison of time to 50% germination percentage, germination potential, germination index between normal and IAA treatments in WT and *osiaglu* mutants. (e-g) Comparison of seed vigour including time to 50% germination percentage, germination potential, germination index between normal and ABA treatments in WT and *osiaglu* mutants. Each column represents the means ± SD. * and ** indicate the significant difference compared to normal condition at 5% and 1% levels, respectively, according to Student's *t*-test. n.s. represents not significant.

disruption of *OsIAGLU* caused low seed vigour, including reducing germination speed and seedling growth, in rice. To the best of our knowledge, this is the first report on the regulation of seed vigour by *OsIAGLU* in rice. The mechanism underpinning the regulation of seed vigour by *OsIAGLU* was investigated in this study.

It has been reported that auxin glucosyltransferase was involved in seed germination in *Arabidopsis*. Ectopic expression

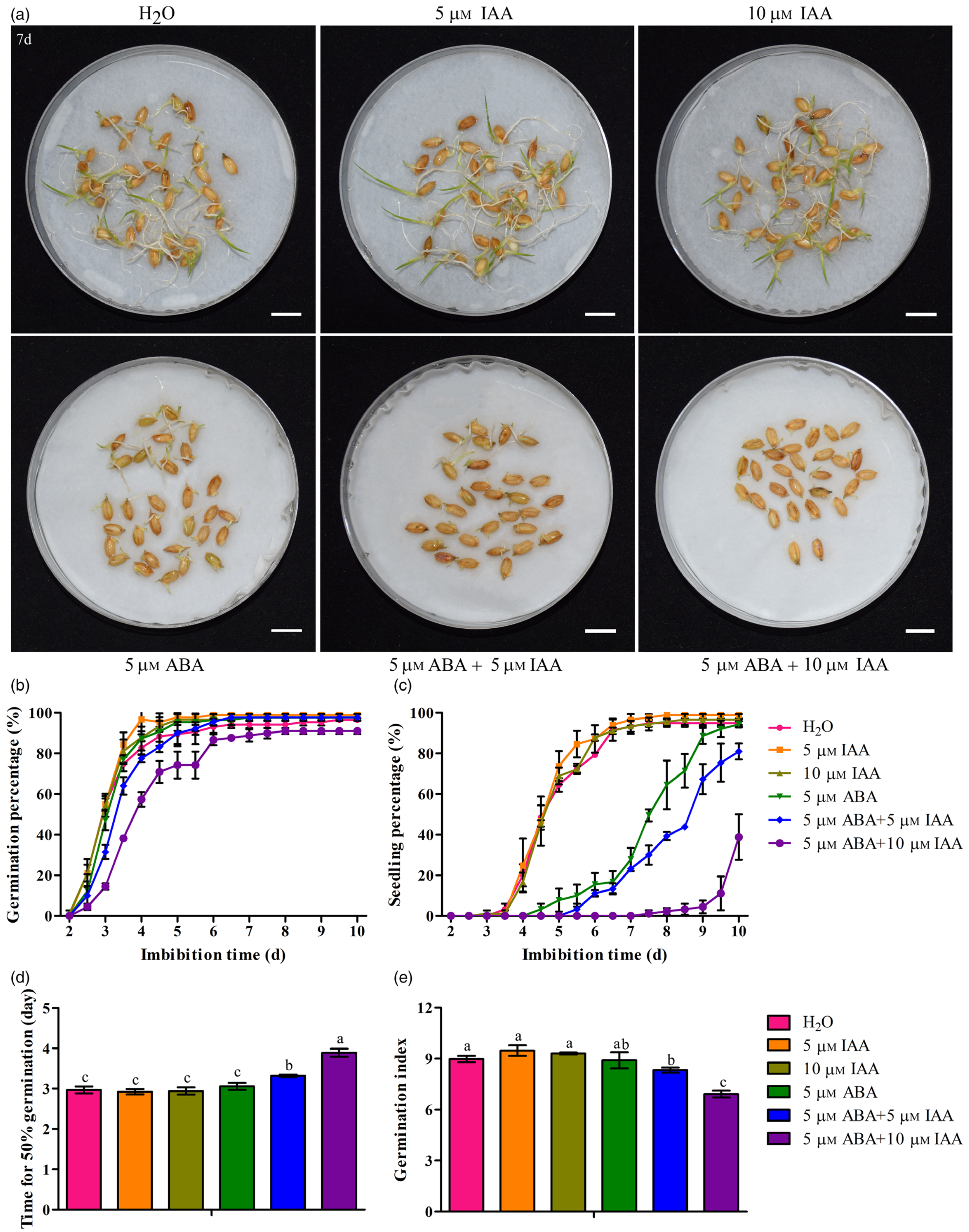


Figure 5 Interactions of IAA and ABA on seed vigour in rice. (a) Seed germination of WT under normal, IAA, ABA and IAA + ABA treatments for 7 days. Bars = 10 mm. (b-c) Comparison of dynamic changes of germination percentage and seedling percentage in WT among normal, IAA, ABA and IAA + ABA treatments during seed germination. Each point represents the means ± SD. (e-g) Comparison of time to 50% germination percentage and germination index in WT among normal, IAA, ABA and IAA + ABA treatments. Each column represents the means ± SD. Different lowercase letters represent significant difference at 5% level according to least significant difference (LSD) test.

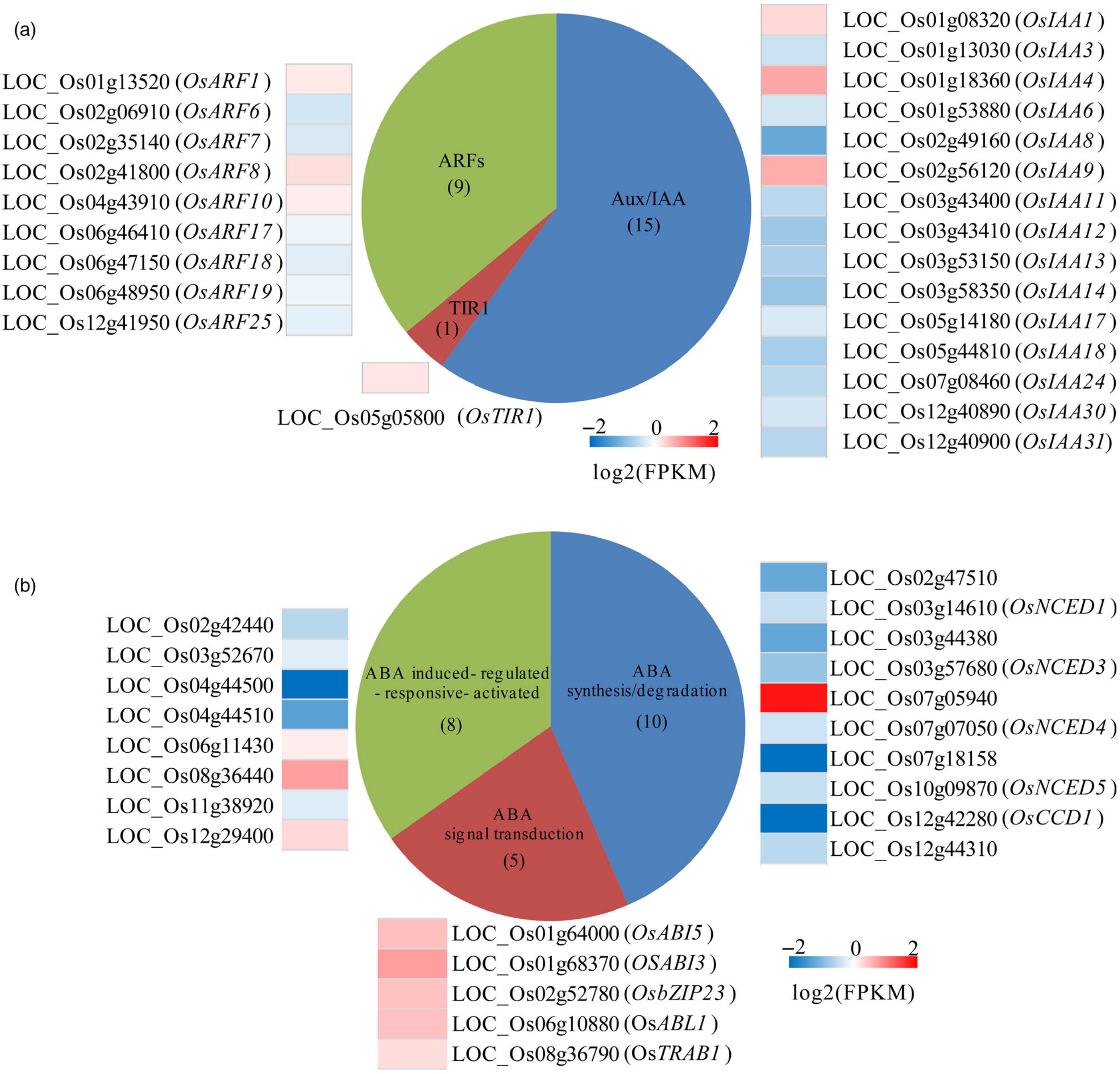


Figure 6 Rice *OsIAGLU* altering expressions of genes involved in IAA and ABA signalling during seed germination. Differentially expressed genes (DEGs) with $p < 0.001$ between WT and *osiaglu-2* mutant involved in IAA (a) and ABA (b) signalling in germinating seeds (72 h) of rice. Red, up-regulation; grey, no change; blue, down-regulation.

of *Arabidopsis UGT75D1* modulates cotyledon development and stress tolerance in seed germination, and its expression was strongly induced in germinating seeds and cotyledons during 3–14 days after germination (Zhang *et al.*, 2016). In this study, our results showed that the *OsIAGLU* exhibited especially relative higher expression at the late mature stage (28 DAF) and the early germination (4-h imbibition) stage. Further GUS staining for OsIAGLU in the present study was detected in germinating seeds and young seedlings. It suggested that *OsIAGLU* regulated seed vigour might be through influencing seed development and seed germination in rice. Here, the influence of seed germination by *OsIAGLU* was focused on analysis and that of seed development needs further investigation in the future. Moreover, the expression of *OsIAGLU* were up-regulated in response to hormones, such as GA, IAA and ABA, especially by IAA and ABA during whole germination stages, which are known to involve in seed germination. These results indicated that the regulation of seed vigour by *OsIAGLU* might be involved in IAA- and ABA-mediated processes.

It has been reported that glycosyltransferases usually involved in IAA and ABA homeostasis in plants (Tognetti *et al.*, 2010; Yu *et al.*, 2019). Meanwhile, our RNA-Seq data also showed that *OsIAGLU* regulated seed vigour might involve in IAA- and ABA-mediated processes. Thus, the regulation of IAA and ABA contents by *OsIAGLU* was firstly focused to study its regulatory mechanism on seed vigour in this study. We observed that the free IAA and ABA contents in the germinating seeds of *osiaglu* mutants were significantly higher than those of WT plants. It suggested that *OsIAGLU* plays important roles in IAA and ABA homeostasis in germinating seeds of rice. The biochemical study

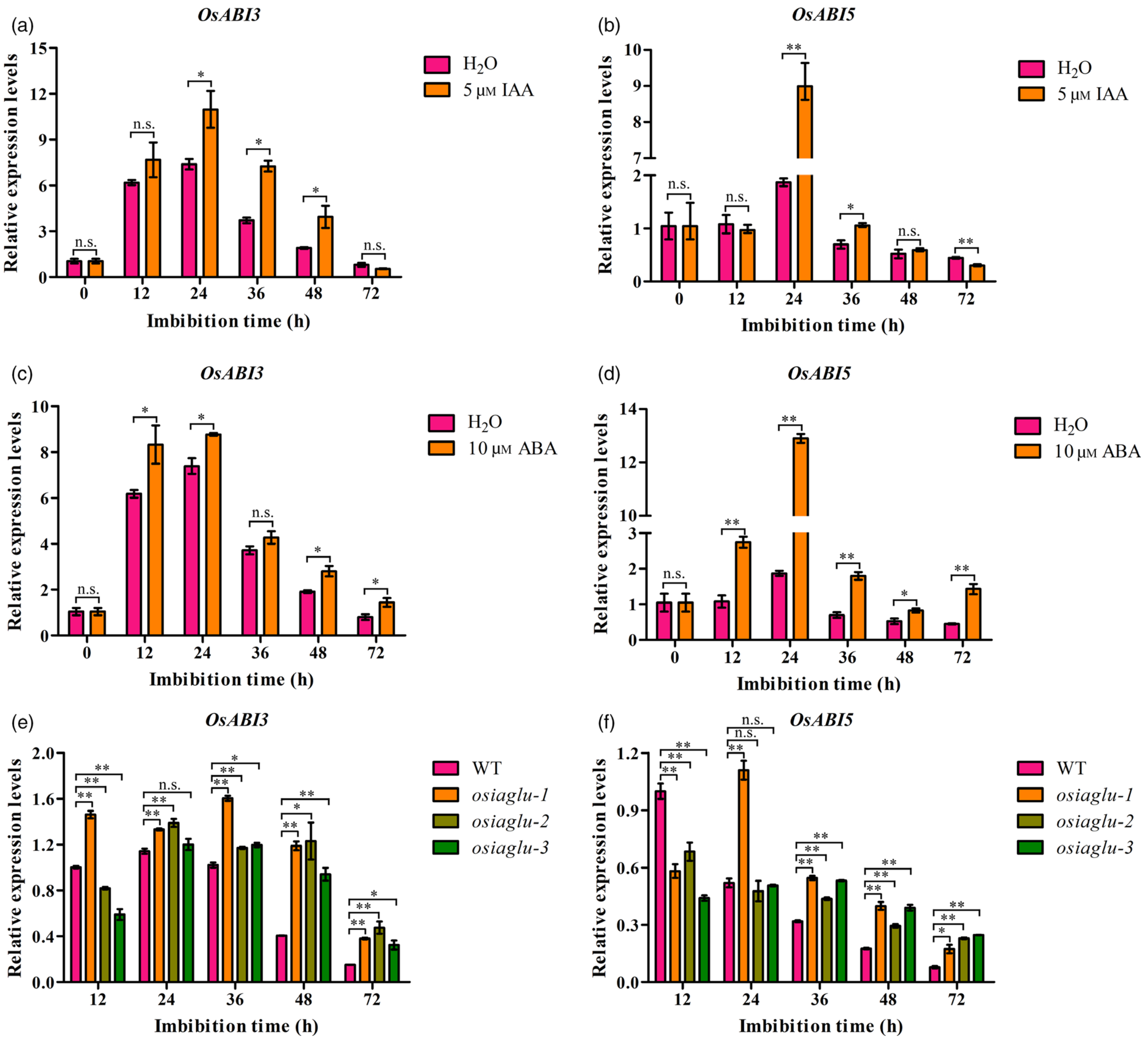
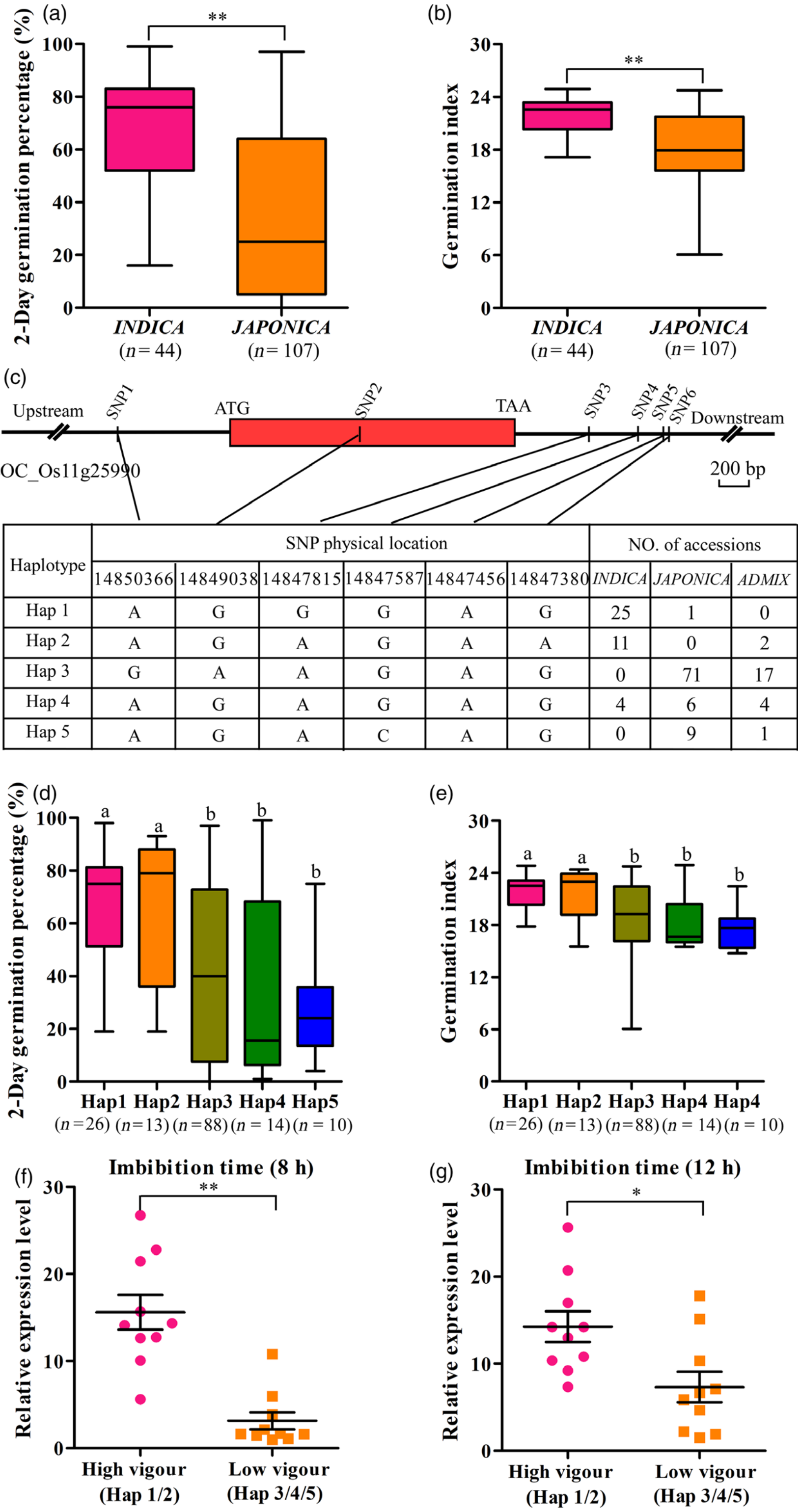


Figure 7 Rice *OsIAGLU* altering expressions of ABA signalling genes *OsABIs* during seed germination. Expressions of *OsABI3* and *OsABI5* in germinating seeds induced by various IAA (a-b) and ABA (c-d) concentrations using quantitative RT-PCR approach. Comparison of *OsABI3* (e) and *OsABI5* (f) expression in germinating seeds between WT and *osiaglu* mutants during seed germination. 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 levels of H_2O condition or WT. Each column represents the means ± SD. * and ** indicate the significant difference compared to normal condition at 5% and 1% levels, respectively, according to Student's *t*-test. n.s. represents not significant.

of *OsIAGLU* glucosylating activity towards different hormones, such as IAA, IBA, ABA and jasmonic acid (JA), is on progress. The actions of IAA and ABA are highly dependent on their concentrations, and high concentrations of IAA and ABA generally inhibit seed germination. It was, therefore, hypothesized that the low seed vigour in *osiaglu* mutants might be due to the higher accumulation of free IAA and ABA in mutants compared to WT plants. However, our further results showed that the reductions of seed vigour among *osiaglu* mutants and WT plants were only observed under ABA while not IAA treatments. We therefore preliminarily speculated that the increased ABA levels while not IAA in *osiaglu* mutants caused low seed vigour in this study.

Several reports indicated that the crosstalk of auxin and ABA is essential for the regulation of seed germination (Brady *et al.*, 2003; Chen *et al.*, 2014; Liu *et al.*, 2013; Zhang *et al.*, 2016). Auxin and ABA act synergistically to inhibit seed germination in *Arabidopsis* (Liu et al., 2013). Thus, we speculated that the effects of *OsIAGLU* on seed vigour might be tightly associated with the crosstalk between IAA and ABA in this study. This hypothesis was confirmed by the results that the exogenous IAA can amplify ABA-induced reduction of seed vigour in this study. How is the regulation of *OsIAGLU* on seed vigour involved in the IAA- and ABA-mediated processes in this study? Our global analysis of transcripts in imbibed seeds indicated that the IAA- and ABA signalling-related genes, such as *OsAFRs* and *OsABIs*, were significantly induced in *osiaglu* mutants compared to those of WT plants in rice. Previously, it has been reported that the auxin controls seed dormancy through stimulation of ABA signalling by inducing ARF mediated ABI3 activation in *Arabidopsis* (Liu *et al.*, 2013). Therefore, we supposed that *OsIAGLU* regulated seed



Haplotype	SNP physical location						NO. of accessions		
	14850366	14849038	14847815	14847587	14847456	14847380	*INDICA*	*JAPONICA*	*ADMIX*
Hap 1	A	G	G	G	A	G	25	1	0
Hap 2	A	G	A	G	A	A	11	0	2
Hap 3	G	A	A	G	A	G	0	71	17
Hap 4	A	G	A	G	A	G	4	6	4
Hap 5	A	G	A	C	A	G	0	9	1

Figure 8 Haplotypes of *OsIAGLU* associated with seed vigour in rice. Comparison of seed germination percentage (a) and germination index (b) between *indica* and *japonica* accessions. (c) Haplotypes of *OsIAGLU* identified in the region from ~2 kb upstream to ~1 kb downstream of the gene. Box represents the *OsIAGLU* gene region. (d-e) Germination phenotype of accessions harbouring different haplotypes. Number of rice accessions listed in brackets. (f-g) Relative expression levels of *OsIAGLU* during seed germination in rice accessions harbouring different haplotypes identified by quantitative RT-PCR approach. Each column represents the means ± SD. * and ** indicate the significant difference compared to normal condition at 5% and 1% levels, respectively, according to Student's *t*-test. Different lowercase letters represent significant difference at 5% level according to least significant difference (LSD) test.

vigour might be depending on OsABIs functions in this study. This hypothesis was confirmed by our following analyses of gene expressions. Firstly, the expressions of *OsABI3* and *OsABI5* in germinating seeds were significantly induced by exogenous IAA and ABA treatments in rice. The responses of *OsABI3* and *OsABI5* to IAA might be depending on the up-regulation of *OsARFs* in this study (Liu *et al.*, 2013). Moreover, the continuously higher expressions of *OsABI3* and *OsABI5* were observed in *osiaglu* mutants compared to those of WT plants during seed germination in rice. Thus, we confirmed that the higher endogenous IAA and ABA in germinating seeds of *osiaglu* mutants will induce higher expressions of *OsABI3* and *OsABI5*, which leading to the decreases of seed vigour in mutants.

Previous studies have shown that the *indica* varieties have higher seed vigour than *japonica* varieties (Lai *et al.*, 2016; Liu *et al.*, 2014; Wang *et al.*, 2010). Similar results were observed in this study. To reveal whether *OsIAGLU* contributing to the differences of seed vigour between *indica* and *japonica* rice, the allelic diversity of *OsIAGLU* was analysed using randomly selected 180 accessions, including *indica* and *japonica* accessions, in rice (McCouch *et al.*, 2016). After analysing the SNP data of rice accessions, the Hap 1 and Hap 2 haplotypes of *OsIAGLU* were identified as positively correlated with seed vigour in rice. Interestingly, we observed that the elite Hap 1 and Hap 2 haplotypes mainly existed in *indica* accessions while not in *japonica* accessions. Meanwhile, the significant higher expressions of *OsIAGLU* were observed in accessions harbouring Hap 1 or Hap 2 haplotypes during seed germination compared with accessions harbouring other three haplotypes. It suggested that the higher *OsIAGLU* expressions during seed germination might contribute to seed vigour in rice. However, how the SNPs of *OsIAGLU* affecting its expression needs to be further investigated. In addition, the proper expression of *OsIAGLU* contributing to seed vigour needs to be further investigated in the future. The determination of *OsIAGLU* allelic diversity with a focus on newly identified elite rice accession is of interest. Several accessions from China that harbouring Hap 1 or Hap 2, that is Ai-Chiao-Hong, Pao-Tou-Hung, TeQing, Chang Ch'Sang Hsu Tao and Zhenshan 97B, were identified as elite accessions in this study. Additionally, we observed that the *OsIAGLU* may not involve in the regulation of grain yield in rice. It suggested that the *OsIAGLU* is a potential candidate gene for increasing seed vigour without influencing grain yield in rice. The identified elite haplotypes and accessions might be useful for improving seed vigour in rice. We speculated that the seed vigour of *japonica* could be improved by introducing Hap 1 or Hap 2 from *indica* into *japonica* rice. The confirmation of this hypothesis is now in progress.

In summary, our results provide new insights into the regulation of *OsIAGLU* on seed vigour (Figure 9). The phenotype of low seed vigour in *osiaglu* mutants in this study implies that *OsIAGLU* may play a positive role in modulating seed vigour in rice. The expressions of *OsABIs* will be reduced in germinating seeds under the low levels of endogenous free IAA and ABA conditions regulated by *OsIAGLU*, thus leading to an enhance of seed vigour in rice. However, what other factors regulating *OsIAGLU* expression in germinating seeds and how *OsIAGLU* influencing the expressions of *OsABI3* and *OsABI5* need to be further investigated. The expression of *OsIAGLU* during seed development was also observed in this study, but its functions on modulating the establishment of seed dormancy or seed vigour during seed development warrant investigation in the future.

Materials and methods

Plant materials

The *osiaglu* mutants were generated using the CRISPR/Cas9 system in the *japonica* Nipponbare background. The generation and identification of mutants were conducted according to He *et al.* (2019a) using specific primers (Table S5). Three independent homozygous mutants, *osiaglu-1*, *osiaglu-2* and *osiaglu-3*, were identified in rice. All plants were grown in an experimental field at the South China Agricultural University. All seeds were harvested at their maturity stage and dried at 42°C for 7 days to break seed dormancy (He *et al.*, 2019b).

Seed germination

Thirty seeds per replicate of *osiaglu* mutants and wild-type (WT) Nipponbare were imbibed in 9-cm-diameter Petri dishes with 10 mL distilled water at 25 ± 1°C for 10 days. Meanwhile, thirty seeds per replicate were sowed in about 1-cm deep soils under natural conditions (25–30°C) for 10 days. Seed germination of *osiaglu* mutants and WT was also conducted under various concentrations of IAA and ABA (5 μM and 10 μM) treatments. The traits of seed vigour, including germination potential, time for 50% of the germination, germination index and seedling vigour index, were tested according to Fu *et al.* (2019) and He *et al.* (2019a). Three replications were performed.

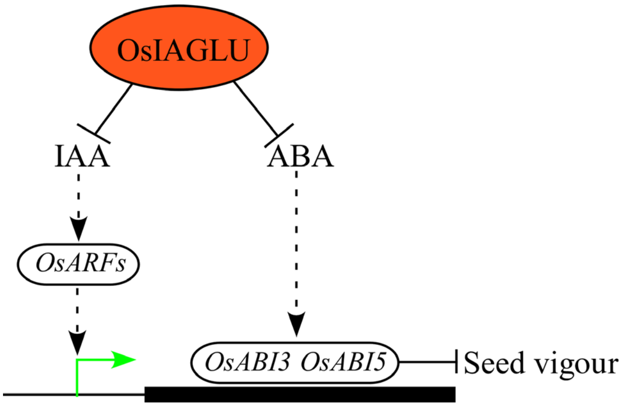


Figure 9 Hypothetical model of the *OsIAGLU* roles on seed vigour in rice. The expression of *OsIAGLU* in germinating seeds reduces the free IAA and ABA levels during seed germination. The decreases of free IAA and ABA accumulation in germinating seeds regulated by *OsIAGLU* will inhibit the expressions of *OsARFs* and *OsABIs*, which contributes to the enhancement of seed vigour. Arrows and lines with slanted dashes indicate positive and negative effects, respectively, and the dotted lines indicate indirect regulation. The black box indicates the *OsABI3*/*OsABI5* gene region.

Expression analysis

Total RNA was extracted from the developing grains (0, 7, 14, 21 and 28 days after flowering; DAF) and germinating seeds (0-, 4-, 8-, 12-, 18-, 24-, 36-, 48-, 60- and 72-h imbibition) of Nipponbare, using the HP Plant RNA Kit (Omega, Atlanta, USA), according to the manufacturer's instructions. The first-strand cDNA was synthesized, and quantitative RT-PCR was carried out according to He *et al.* (2019a). The quantitative RT-qPCRs were performed in a CFX96 Real-Time System (Bio-Rad, USA) with the rice *OsActin* gene as internal control. The PCR conditions were as follows: 95 °C for 25 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. Primers used for quantitative RT-PCR are listed in Table S5. Normalized transcript levels were calculated using the comparative CT method (Livak and Schmittgen, 2001). Three biological replications were performed. Meanwhile, transgenic plants carrying the OsIAGLU promoter-GUS fusion construct in Nipponbare were used for a GUS staining assay. GUS staining of tissues from the positive transgenic plants was performed as previously described (Fujino *et al.*, 2008).

Hormone quantification

Approximately 1.0 g of each sample was rapidly frozen in liquid nitrogen and homogenized into a powder. The extraction and quantification of endogenous IAA and ABA were conducted according to the manufacturer's instructions (Wuhan Metware Biotechnology Co., Ltd., Wuhan, China). The quantification of IAA and ABA was conducted using an ultra-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) system (UPLC, Shim-pack UFLC SHIMADZU CBM30A system, Kyoto, Japan; MS, Applied Biosystems, Foster City, CA). The content of IAA and ABA was determined using the external standard method and is expressed as μg/g fresh weight (FW) and ng/g FW, respectively. Three biological replications were performed.

Differentially expressed gene analysis

RNA-Seq approach and differentially expressed gene analysis were conducted according to He *et al.* (2019a). Total RNA was extracted from approximately 80–100 mg powder of WT and *osiaglu-2* seeds after 72-h imbibition using the HP Plant RNA Kit (Omega, Atlanta, GA) according to the manufacturer's instructions. Construction of cDNA libraries and BGISEQ-500RS sequencing were performed at BGI-Wuhan Co., Ltd., Wuhan, China. 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 differentially expressed genes (DEGs) with a *P*-adj (*P*-adjusted) < 0.001 were selected for further analysis. A metabolic pathway overview was depicted by MapMan version 3.6.0RC1 (http://mapman.gabipd.org) and shown using colour intensity (Usadel *et al.*, 2009). Three biological replications were performed.

Haplotype analyses

To determine the haplotypes of *OsIAGLU*, 700,000 SNP markers of rice accessions listed at https://ricediversity.org/data/index.cfm were used (McCouch *et al.*, 2016). Seed germination was conducted in 9-cm-diameter Petri dishes with 10 mL distilled water using randomly selected 180 accessions as descripted above for 10 days (Table S2). Haplotypes represented at least ten investigated accessions that were previously used for a comparative analysis of phenotypes.

Data analysis

Experimental data were analysed using the SAS software (Cary, NC), and significant differences among samples were compared using Student's *t*-test or Fisher's least significant difference (LSD) test at the 5% and 1% levels of probability.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Zhoufei Wang and Yongqi He planned the research. Yongqi He and Jia Zhao performed all important experiments. Bin Yang performed haplotype analyses. Shan Sun and Liling Peng performed seed germination experiments. Zhoufei Wang, Yongqi He and Jia Zhao 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 Characterization of OsIAGLU in rice.

Figure S2 Comparison of amino acid sequences between WT and *osiaglu* mutants in rice.

Figure S3 Comparison of grain traits between WT and *osiaglu* mutants in rice.

Figure S4 Dynamic changes of seed germination after dormancy breaking treatments among WT and *osiaglu* mutants in rice.

Figure S5 Comparison of IAA and ABA interactions on seed vigor between WT and *osiaglu* mutants in rice.

Figure S6 The expression confirmation of IAA-related differently expression genes in 72 h-imbibed seeds using quantitative RT-PCR approach in rice.

Figure S7 The expression confirmation of ABA-related differently expression genes in 72 h-imbibed seeds using quantitative RT-PCR approach in rice.

Table S1 Differently expression genes (DEGs) between WT and *osiaglu-2* in 72 h-imbibed seeds in rice.

Table S2 Information of 180 accessions used for haplotype analyses in rice.

Table S3 Information of the elite accessions harboring Hap 1 and Hap 2 of *OsIAGLU* with high seed vigor in rice.

Table S4 Information of the accessions harboring different haplotypes used for the analysis of *OsIAGLU* expression in rice.

Table S5 The primer pairs used in this study.