

Rice *GERMIN-LIKE PROTEIN 2-1* Functions in Seed Dormancy under the Control of Abscisic Acid and Gibberellic Acid Signaling Pathways¹

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Seed dormancy is a natural phenomenon in plants. It ensures that seeds complete the grain-filling stage before germination and prevents germination in unsuitable ecological conditions. In this study, we determined the previously unknown function of the rice (*Oryza sativa*) gene *GERMIN-LIKE PROTEIN 2-1* (*OsGLP2-1*) in seed dormancy. Using artificial microRNA and CRISPR/CAS9 approaches, suppression of *OsGLP2-1* expression in rice resulted in the release of dormancy in immature seeds. Conversely, overexpression of *OsGLP2-1* driven by the *OsGLP2-1* native promoter led to greater seed dormancy. Seed scutellum-specific expression of *OsGLP2-1* was increased by exogenous abscisic acid, but decreased with gibberellic acid treatment. We provide evidence that *OsGLP2-1* is antagonistically controlled at the transcriptional level by *ABA INSENSITIVE5* and *GAMYB* transcription factors. We conclude that *OsGLP2-1* acts as a buffer, maintaining appropriate equilibrium for the regulation of primary dormancy during seed development in rice.

A proper level of seed dormancy is important for crop cultivars. Seeds that show weak dormancy are susceptible to preharvest sprouting, which causes substantial loss in grain yield and quality. In contrast, strong dormancy inhibits germination of the seed. Published investigations provide evidence that the seed dormancy or germination developmental outcomes are mainly determined by the cooperative action of abscisic acid (ABA)- and gibberellic acid (GA)-associated signal pathways (Fang and Chu, 2008; Fang et al., 2008; Holdsworth et al., 2008a; Shu et al., 2013; Abraham et al., 2016; Wang et al., 2018). Both ABA and GA are important plant growth regulators. ABA mainly promotes seed dormancy, inhibits root growth, and facilitates

abiotic stress tolerance, whereas, in contrast, GA initiates seed germination and stimulates plant growth. Other phytohormones, such as ethylene, indoleacetic acid, strigolactone, and brassinosteroid (Yang et al., 2011; Toh et al., 2012; Wang et al., 2013; Zhao et al., 2019), as well as external environmental factors such as temperature and light intensity, and internal developmental signals such as nitric oxide and reactive oxygen species, also play roles in the processes of seed dormancy or germination through cooperation with ABA or GA (Alboresi et al., 2005; Liu et al., 2010; Toorop et al., 2012). In addition, epigenetic mechanisms (histone modification, DNA methylation, and chromatin remodeling) also regulate seed dormancy (Gao and Ayele, 2014).

ABA biosynthetic and catabolic pathways have been elucidated by ¹⁸O labeling experiments, molecular genetic analysis of auxotrophs, and biochemical studies (Nambara and Marion-Poll, 2005). The ABA signal transduction model was established based on studies of the mechanism of the ABA receptors of the REGULATORY COMPONENTS OF ABA RECEPTOR/PYR-ABACTIN RESISTANCE1/PYL1-LIKE protein family (Zhu, 2016). Binding of ABA to these receptors leads to inactivation of type 2C protein phosphatases, such as ABA INSENSITIVE1 (ABI1) and ABI2. This in turn activates SUC NONFERMENTING 1-RELATED PROTEIN KINASES, which regulates activation of downstream transcription factors through phosphorylation (Fujii et al., 2009; Melcher et al., 2009; Miyazono et al.,

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2009; Santiago et al., 2009; Sheard and Zheng, 2009; Raghavendra et al., 2010). ABI5 (a basic region/Leu-zipper protein) and ABI3 (a B3 domain transcription factor) are two key ABA-responsive transcription factors that regulate downstream target genes by binding to the ABA-responsive element (ABRE) of ACGTG in the promoters of the genes (Gao and Ayele, 2014).

Gibberellins regulate a variety of growth and developmental programs in plants, including cell differentiation and elongation, reproductive development, and germination. Gibberellins are often considered to exert antagonistic roles to ABA to control seed germination. The major effect of GA is degradation of the DELLA protein, allowing the GAMYB transcription factor to activate the α -amylase genes during seed germination (Gubler et al., 1995; Gubler et al., 1999). The α -amylase in the aleurone layer catalyzes hydrolysis of starch to Glc, thus providing energy for seed germination. In addition to its role in germination, GA is involved in regulation of seed storage protein genes during seed development (Diaz et al., 2002). The exact molecular target of GA on seed dormancy during seed development is unclear.

The transition from seed dormancy to germination is a dynamic process. Freshly harvested mature water-permeable dormant seeds show primary dormancy, which has been induced through the involvement of ABA during seed maturation on the mother plant (Hilhorst, 1995; Miyoshi et al., 1999; Manz et al., 2005). During the period of after-ripening, seed dormancy is gradually relieved (Holdsworth et al., 2008a). As one of the most important ABA-responsive transcription factors, ABI5 plays a crucial role in seed dormancy via the ABA signaling pathway. However, in contrast with germination-related genes, only a small number of dormancy-related genes (such as *EARLY MET-LABELLED6* and *MOTHER OF FT AND TFL1 [MFT]*) regulated by ABI5 and ABI3/VIVIPAROUS1 have been identified and functionally studied (Hattori et al., 1995; Lopez-Molina et al., 2002; Xi et al., 2010; Nakamura et al., 2011). Previous studies also show that ABA content and expression of ABA metabolic genes and signal-related genes exhibit no apparent differences between whole imbibing dormant and after-ripened wheat (*Triticum aestivum*) seeds (Liu et al., 2013), which suggests that wheat seed dormancy and after-ripening may be controlled by separate genetic pathways.

Seed constituents are also associated with dormancy. The embryo and/or a seed coat component (such as the testa or endosperm) are reportedly associated with seed dormancy (Hilhorst, 1995; Finch-Savage and Leubner-Metzger, 2006; Müller et al., 2006; Gu et al., 2015). Assuming to be the primary determinant of seed dormancy, the endosperm surrounding the embryo inhibits germination in intact Arabidopsis (*Arabidopsis thaliana*) seeds (Bethke et al., 2007). During seed germination, ABA delays endosperm weakening and rupture, whereas GA, acting as an early embryo signal, promotes this process (Müller et al., 2006). The *qSD7-1* and *qSD12* quantitative trait loci were determined to

be associated with maternal and offspring tissue-imposed dormancies, respectively (Gu et al., 2010). However, the mechanisms by which the embryo and endosperm coordinately control seed dormancy, especially during seed development, are not well understood.

The scutellum, which is part of the seed located between the embryo and endosperm, functions as conductive tissue between the endosperm and embryo. Several important metabolic events occur in the scutellum. In germinating wheat seeds, following degradation of endosperm starch, the resulting hexoses are converted to Suc in the scutellum and eventually transported to the embryo axis (Aoki et al., 2006; Asakura et al., 2007). In the germinating grain, the storage proteins in the endosperm are hydrolyzed into peptides and free amino acids, which was subsequently taken by the scutellum (Salmenkallio and Sopanen, 1989). Above all, the scutellum is a prospective site for the control of seed germination/dormancy mediated by the ABA/GA-response pathways (Asakura et al., 2007).

Germin-like proteins (GLPs) are a group of proteins that show a similar amino acid sequence to that of wheat germin, which are expressed during seed germination. In plants GLPs constitute a multigene family (Kim and Triplett, 2004), and perform a variety of biological functions in plant development, stress response, and pathogen resistance (Berna and Bernier, 1997; Membré et al., 1997; Liu et al., 2016). The promoter sequences of GLPs are responsive to environmental stresses and growth factors (Breen and Bellgard, 2010; Mahmood et al., 2010; Li et al., 2016). Among isolated GLPs, a portion of the proteins exhibit oxalate oxidase or superoxide dismutase activity. However, the function of most GLPs remains unknown.

Although the germins were first isolated from germinating wheat embryos since 1980s, and an increasing number of germins and GLPs were identified in the defense functions, so far, only one case (*ZmGLP*) is related to germination vigor of maize (*Zea mays*) seed (Lapik and Kaufman, 2003; Fu et al., 2016). *ZmGLP* helps to keep seed germination vigor, but its mechanism is unclear. In this study we show that a rice (*Oryza sativa*) germin-like protein, which is designated *OsGLP2-1* according to a recent nomenclature (Li et al., 2016), played a key role in dormancy regulation of developing seeds. *OsGLP2-1* is located on chromosome 2 (LOC_Os02g29000) and is expressed exclusively in the seed scutellum and minor veins of the leaf. Unlike other isolated GLPs, which are usually localized in the extracellular matrix, *OsGLP2-1* was localized in the cytosol and showed rapid movement within the cell. When compared with the control, suppression of *OsGLP2-1* in rice led to dormancy release and displayed a higher frequency of germination, whereas overexpression of *OsGLP2-1* resulted in deep dormancy. Transcription of *OsGLP2-1* was directly regulated by ABI5 and GAMYB through binding to the corresponding cis-regulatory elements in the promoter. This work shows that *OsGLP2-1* is dual-targeted by ABA

and GA signals involved in seed dormancy in opposite ways. Elucidation of the function of *OsGLP2-1* in dormancy of developing seeds will help to clarify the mechanism of seed dormancy coregulated by ABA and GA.

RESULTS

Expression Pattern of *OsGLP2-1*

OsGLP2-1 (LOC_Os02g29000) encodes a germin-like protein of 216 amino acids containing a single cupin domain that comprises two conserved amino acid sequence motifs to form a β -barrel structure (Dunwell et al., 2004). To determine the expression pattern of *OsGLP2-1*, the promoter fragment of *OsGLP2-1* (P_{GLP2-1}) spanning from position $-2,086$ to -1 relative to the translation initiation site was amplified and cloned. The reporter expression vector $P_{GLP2-1}:GUS$, in which the gene *GUS* was driven by P_{GLP2-1} , was constructed and transformed into rice callus. Staining with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide of tissues from $P_{GLP2-1}:GUS$ rice plants showed intense GUS signal predominantly in the minor veins of the leaf in the vegetative stage (Fig. 1A). In the reproductive stage, only weak GUS signal was observed in the leaf sheath, and no signal was detected in the other organs examined except the seed scutellum (tissue located between the embryo and endosperm), which showed strong expression of GUS, forming a blue crescent in both immature and mature seeds (Fig. 1, B and C; Supplemental Fig. S1A). These results indicate that a potential role of *OsGLP2-1* played in developing rice seed and seed dormancy.

OsGLP2-1 Positively Regulated the Dormancy of Developing Seed

To check the involvement of *OsGLP2-1* in seed dormancy regulation, a complementary DNA fragment (737 bp) harboring the open reading frame (ORF) of the gene was amplified and cloned from rice ssp. *japonica* 'Nipponbare'. Cauliflower mosaic virus (CaMV) 35S promoter-driven *OsGLP2-1* ectopic overexpression (OE-*GLP2-1*) and suppression (amiR-*GLP2-1*) transgenic rice plants (Supplemental Fig. S2) as well as empty vector-transformed plants were generated. The homozygous lines for each type of transgenic rice plants were selected, and their seed dormancy phenotypes were compared using T_3 seeds. The T_3 immature seeds of OE-*GLP2-1* and amiR-*GLP2-1* (35 and 45 d after flowering [DAF]) exhibited marked different dormancy. When compared with the seed dormancy of empty vector control, the T_3 seeds (35 DAF) of the majority of amiR-*GLP2-1* independent lines (10/16) displayed dormancy releasing phenotype and a high germination percentage, and the germinated seeds developed into seedlings subsequently (Supplemental

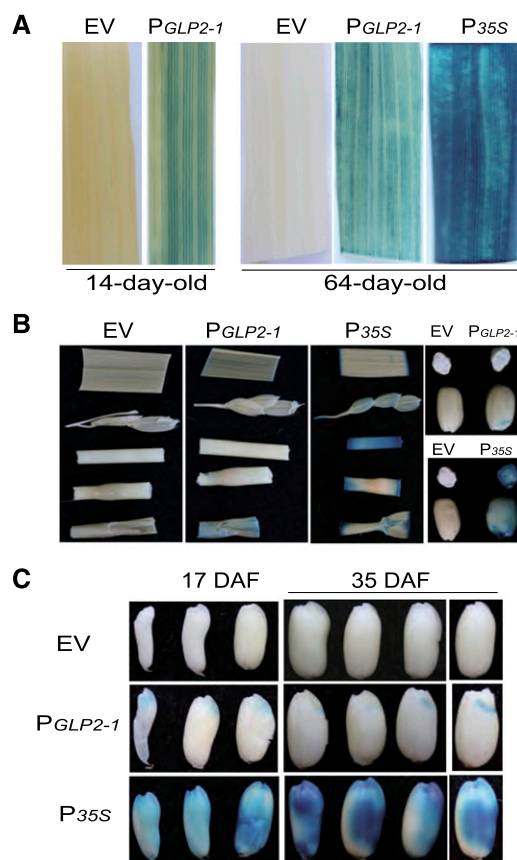


Figure 1. Expression pattern of $P_{GLP2-1}:GUS$ in transgenic rice plants at different growth stages. A, Specific expression in minor veins of the leaf in the vegetative phase. Leaves from 14-d-old seedlings: left, negative control (empty vector transformant [EV]) lacking signal; right, $P_{GLP2-1}:GUS$ leaf with strong blue GUS signal in minor veins. Leaves from 64-d-old plant: left, negative control; center, $P_{GLP2-1}:GUS$ leaf showing staining in the minor veins; right, CaMV 35S promoter (P_{35S}) driving constitutive expression of GUS in the leaf. B, GUS staining in organs from mature plant, including leaf, spikelet, stem, internode, leaf sheath, and seed. Among these organs of $P_{GLP2-1}:GUS$ plants, light signal was observed in the leaf sheath and strong blue signal displayed specifically in the scutellum. C, GUS staining of developing seeds of $P_{GLP2-1}:GUS$ transformant. Seeds were sampled at different growth stages (17 and 35 DAF). Scutellum-specific expression pattern was exhibited by all developing seeds. EV seed was used as negative control, and P_{35S} showing constitutive GUS expression was used as positive control.

Fig. S3A; Supplemental Table S1). Contrary to the dormancy-breaking phenotype of amiR-*GLP2-1* seeds, the sign of deepening dormancy was exhibited in OE-*GLP2-1* seeds. Given that *OsGLP2-1* is specifically expressed in scutellum, we further created the *OsGLP2-1* overexpression transgenic rice lines driven by its native promoter ($P_{GLP2-1}:HA-GLP2-1-eGFP$). The enhanced dormancy of $P_{GLP2-1}:HA-GLP2-1-eGFP$ seeds was consistently observed. The germination percentages of $P_{GLP2-1}:HA-GLP2-1-eGFP$ independent lines were significantly lower than that of empty vector control (Table 1). The contradictory dormancy phenotypes of amiR-*GLP2-1* and $P_{GLP2-1}:HA-GLP2-1-eGFP$

seeds suggested that *OsGLP2-1* played an important role in the maintenance of primary dormancy of the developing seed and that changed expression levels of *OsGLP2-1* led to alteration of seed dormancy state.

To further demonstrate the loss-of-function of *OsGLP2-1* in rice, two knock-out lines (*osglp2-1* 323 and 513) were created by CRISPR-CAS9 system (Fig. 2A). The 20 DAF dehusked seeds of transfer DNA (T-DNA)-free homozygous *osglp2-1* mutant were used for comparison of seed dormancy. When compared with the wild-type seed, seeds of both *osglp2-1* lines 323 and 513 exhibited dormancy-breaking phenotype, with significantly high germination percentage (Fig. 2, B and C). Together with the enhanced dormancy of P_{GLP2-1} :HA-GLP2-1-eGFP seeds, these results proved the positively regulation of seed dormancy by *OsGLP2-1*.

Altered Expression of *OsGLP2-1* in Response to ABA and GA

It is well known that seed dormancy is mainly associated with ABA and GA. Our results showed that *OsGLP2-1* functions in control of seed dormancy, suggesting if there is a regulatory relationship between *OsGLP2-1* and ABA and GA. To verify this, *OsGLP2-1* expression in response to exogenous ABA and GA treatment was examined and compared in seed halves of P_{GLP2-1} :*GUS* rice containing the embryo and scutellum collected 30 DAF. The expression level of *GUS* reporter gene was measured by reverse transcription-quantitative PCR (RT-qPCR). When compared with the relative expression level in the untreated control, *GUS* expression driven by P_{GLP2-1} was significantly increased by 50 μ M ABA treatment, but was markedly decreased by 1 and 10 μ M GA treatments (Fig. 3, A and B). In addition to use of the *GUS* reporter (P_{GLP2-1} :*GUS*

transgenic lines), we also detected *OsGLP2-1* induction/suppression in response to ABA/GA in wild-type *japonica* cv Nipponbare seeds to see the response of *OsGLP2-1* to ABA and GA. The results were consistent with that using P_{GLP2-1} :*GUS* transgenic lines: the expression of *OsGLP2-1* was induced by ABA, while suppressed by GA in wild-type developing seed. When compared with the expression level of *OsGLP2-1* in untreated control seeds, significantly activated expression of *OsGLP2-1* was displayed in 50 μ M ABA treatment at 30 min, 1 to 3 h, whereas notable inhibited *OsGLP2-1*-expressing was shown in 1 μ M GA treatment (Fig. 3, C and D). These results showed that the expression of *OsGLP2-1* was involved in both of ABA and GA signaling pathways.

The roles of ABA and GA in seed dormancy and germination are well known. To further explore the interactions of *OsGLP2-1* with these hormones in their controlled physiological processes, T₃ seeds were collected at 45 DAF and cultured on one-half strength Murashige and Skoog (MS) medium supplemented with 50 μ M ABA or 10 μ M GA3. The germination of *amiR-GLP2-1* seeds was repressed by 50 μ M ABA treatment (Supplemental Figs. S3 and S4A; Supplemental Table S2). This result showed that exogenous ABA can effectively weaken the dormancy-breaking phenotype of *amiR-GLP2-1*. In the presence of 10 μ M GA, germination of OE-*GLP2-1* seeds was stimulated but did not fully complemented the low germination potential of the seeds (Supplemental Fig. S4B). Another seed dormancy comparison assay performed on T₄ immature seeds of OE-*GLP2-1* and *amiR-GLP2-1* produced similar results (data not shown). These results showed that exogenous ABA and GA can effectively weaken and almost offset the dormancy phenotype of *amiR-GLP2-1* and OE-*GLP2-1*, respectively, and further confirmed that the regulation of seed

Table 1. Comparison of the transgenic seeds (30 DAF) germination rate

Empty vector seeds were used as a negative control. Values shown are means \pm SE of triplicate measurements. Asterisks indicate significant difference at * $P = 0.05$ and ** $P = 0.01$, respectively, in SPSS one-way ANOVA analysis with post hoc test. N, Number of seeds.

Line No.	N	Germination Percentage		
		9 d	11 d	13 d
Empty vector	30, 30, 30	34.97 \pm 3.40	38.86 \pm 2.51	38.86 \pm 2.51
P_{GLP2-1} :HA-GLP2-1-eGFP			%	
PGLP-17	11, 10, 10	13.03 \pm 11.88*	13.03 \pm 11.89**	13.03 \pm 11.89**
PGLP-22	12, 10, 10	0.00 \pm 0.00**	0.00 \pm 0.00**	0.00 \pm 0.00**
PGLP-26	14, 12, 11	3.03 \pm 3.71**	3.03 \pm 3.71**	3.03 \pm 3.71**
PGLP-29	8, 10, 10	10.83 \pm 1.02**	10.83 \pm 1.02**	10.83 \pm 1.02**
PGLP-31	12, 12, 12	16.67 \pm 5.89*	16.67 \pm 5.89*	16.67 \pm 5.89*
P_{35S} :ABI5				
AB5-44	10, 12, 10	5.56 \pm 6.80**	5.56 \pm 6.80**	5.56 \pm 6.80**
AB5-6	12, 10, 12	0.00 \pm 0.00**	2.78 \pm 3.40**	2.78 \pm 3.40**
AB5-13	14, 14, 16	0.00 \pm 0.00**	0.00 \pm 0.00**	0.00 \pm 0.00**
P_{35S} :GAMYB				
GAB6-5	10, 10, 10	96.67 \pm 4.08**	96.67 \pm 4.08**	96.67 \pm 4.08**
GAB7-2	12, 10, 10	83.89 \pm 25.97**	83.89 \pm 25.97**	87.22 \pm 26.29**
GAB8-11	12, 10, 10	93.89 \pm 3.79**	97.22 \pm 3.40**	97.22 \pm 3.40**

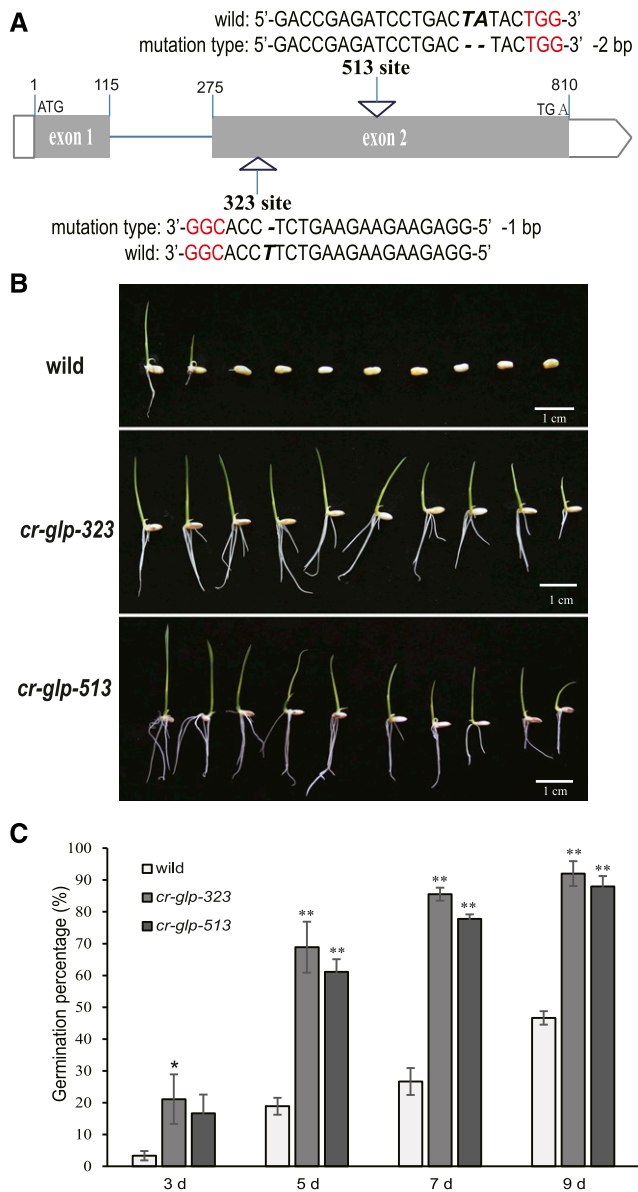


Figure 2. Dormancy-breaking phenotype exhibited on the rice CRISPR-*osgfp2-1*-seeds. **A**, Schematic diagram of CRISPR/CAS9-mediated targeted mutagenesis in *OsGLP2-1*. The ORF consists of two exons indicated as black boxes. The mutated sites (323 and 513) in 20-bp single guide RNA targeting sequences and protospacer adjacent motif are shown in bold italic letter and in red letter, respectively. **B**, and **C**, Comparisons of wild type and CRISPR-*osgfp2-1* developing seeds dormancy state. **B**, Dormancy-breaking phenotype exhibited on the CRISPR-*osgfp2-1*-seeds (7 d after sowing). **C**, Compared with the low germination rate of wild-type seeds at 3, 5, 7, and 9 d after sowing, two lines of T-DNA-free homozygous CRISPR-*osgfp2-1*-seeds, *cr-glp-323* and *cr-glp-513*, displayed significantly higher germination percent, respectively. The surface sterilized 20 DAF-dehulling seeds were sown in MS medium, 15 seeds for each sample, and 6 replicates were performed. Error bars = SE. Asterisks indicate significant difference at * $P = 0.05$ and ** $P = 0.01$ in SPSS one-way ANOVA analysis with post hoc test.

dormancy by *OsGLP2-1* was closely related to ABA and GA pathways.

Expression of *OsGLP2-1* Was Promoted by ABI5 But Suppressed by GAMYB

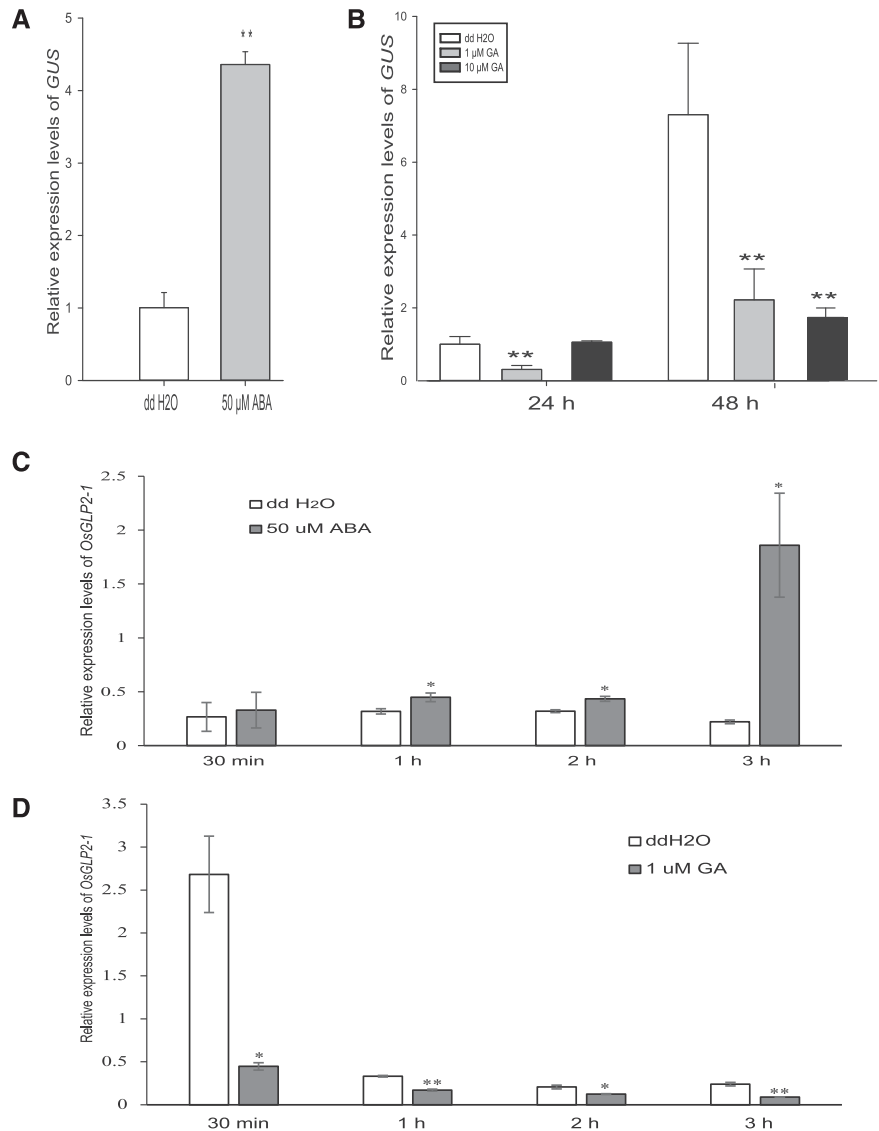
To explore the regulatory mechanism of *OsGLP2-1* expression, the 2,086-bp *OsGLP2-1* promoter sequence was analyzed using the plant cis-acting regulatory DNA elements database (Higo et al., 1999). The *OsGLP2-1* promoter contained four potential ABRE cis-elements located at sites -1865 to -1861, -792 to -788, and -296 to -292 for 5'-ACGTG-3' and -472 to -466 for 5'-MACGYGB-3' (M: C/A, Y:T/C, B:T/C/G) upstream of the translation start site for *OsGLP2-1*. The GA response complex usually includes three sequence motifs: a GA-responsive element (GARE; TAACAAA-box) for binding to GAMYB; a pyrimidine box (5'-CCTTTT-3') for binding with a DOF (DNA-binding One Zinc Finger) family transcription factor; and a 5'-TATCCAC-3' element for an unknown transcription factor. The *OsGLP2-1* promoter sequence contained the above three elements located respectively at -941 to -935, -490 to -484, and -62 to -57 bp upstream of the translation start site. The presence of these cis-element motifs suggested that *OsGLP2-1* might be regulated by ABA- and GA-response-related transcription factors.

To determine whether transcription of *OsGLP2-1* was directly regulated by transcription factors involved in the ABA and GA signaling pathways, the main transcription factors that mediate seed dormancy regulation in the ABA/GA signal pathways, i.e. ABI5 and ABL1, the DOF family transcription factors, and GAMYB, were tested in a preliminary agroinfiltration experiment (Fig. 4; Supplemental Fig. S5). Overexpression vectors for each transcription factor were constructed, and each was coexpressed with the P_{GLP2-1} :GUS expression vector in *Nicotiana benthamiana* leaves. The relative activity of GUS was measured. Among the transcription factors tested, ABI5 significantly increased the expression of GUS controlled by P_{GLP2-1} , whereas GAMYB exerted a negative impact (Fig. 4). These results suggested that the response of *OsGLP2-1* to ABA and GA was directly mediated by ABI5 and GAMYB, respectively.

ABI5 and GAMYB Coordinately Regulate *OsGLP2-1* Expression through Binding to the Respective ABRE and GARE Elements

The agroinfiltration results implied that ABI5 and GAMYB may directly regulate *OsGLP2-1* expression. To verify the physical interaction of the P_{GLP2-1} promoter with the ABI5 and GAMYB transcription factors, a yeast one-hybrid assay was performed (Fig. 5). When compared with the negative control (pLacZi+GAD), among the tested P_{GLP2-1} F1, F2, F3, and F4 promoter fragments (Fig. 5A), ABI5 directly bound to the F1

Figure 3. Response of the expression of *OsGLP2-1* to ABA and GA. A and B, Expression of GUS driven by P_{GLP2-1} was stimulated by exogenous ABA and suppressed by GA. C and D, Altered expression of *OsGLP2-1* in response to ABA and GA in wild-type seeds. The induction/suppression of *OsGLP2-1* expression was detected after ABA/GA treated within 3 h, respectively. The P_{GLP2-1} :GUS or wild-type rice embryo including the scutellum was freshly cut from dehusked 30 DAF seeds, and immediately treated with exogenous 50 μM ABA (A and C), 1 or 10 μM GA (B and D) for different time points in a growth chamber (25°C, 12-h light/12-h dark). The samples were ground in liquid nitrogen for further monitoring of GUS or *OsGLP2-1* expression by RT-qPCR. Collection of 15 embryos for each sample and three replicates were performed. Error bars = SE ($n = 3$). Asterisks indicate significant difference at $*P = 0.05$ and $**P = 0.01$ in SPSS independent-sample t test (A, C, and D) and one-way ANOVA analysis with post hoc test (B), respectively.



segment with an ABRE core cis-element (ACGT) and induced expression of *LacZ*. The GAMYB transcription factor bound to the F2 fragment and activated expression of the reporter gene, and the interaction was enhanced with multiple copies of F2 (Fig. 5B). These results are compatible with the presence of a GAMYB-binding GARE in the F2 fragment. Thus, ABI5 and GAMYB were indicated to function as transacting factors for the ABRE and GARE cis-elements, respectively, to regulate *OsGLP2-1* expression.

An electrophoresis mobility shift assay (EMSA) was conducted to confirm the results of the yeast one-hybrid assay. The assay demonstrated the DNA-binding specificity of the MBP-ABI5 fusion protein to the biotin-labeled ABRE cis-element in the *OsGLP2-1* promoter (Fig. 6, A and B). The Trx-His-GAMYB fusion protein specifically bound to the GARE cis-element in the *OsGLP2-1* promoter (Fig. 6, A and C). The shift bands displayed gradients of intensity from low to high amounts of fusion proteins (Supplemental Fig. S6), and

the binding signals were gradually suppressed by the addition of unlabeled competitive probe (Fig. 6C). With mutated probes, no competitive ability was observed (Fig. 6, B and C). It was worth noting that a more complicated pattern displayed in the binding of ABI5 with its cis-element maybe caused by the protein stability or other factors. Moreover, the freshly purified Trx-His-ABI5 or Trx-His-GAMYB fusion protein expressed in *Escherichia coli*, together with their corresponding fragments (A5-1, A5-2 and Ga-1) in *GLP2-1* promoter, respectively, were further served to a microscale thermophoresis (MST) assay, the direct bonding characteristic was found between Trx-His-ABI5 fusion protein with A5-1 or A5-2 fragment and the Trx-His-GAMYB with Ga-1 fragment. The dissociation constant (K_d) between Trx-His-ABI5 and A5-1, A5-2 was 39.86 nM and 87.74 nM, respectively; and the K_d between Trx-His-GAMYB and Ga-1 was 835.16 nM. The negative control protein, Trx-His, did not interact with

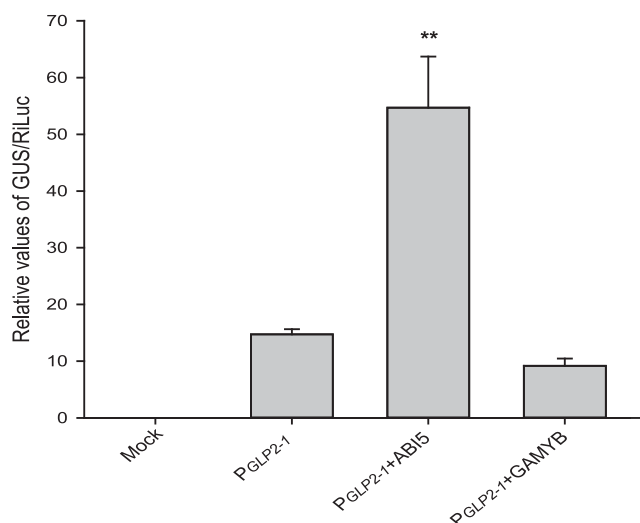


Figure 4. Effect of ABI5 and GAMYB on activity of GUS driven by P_{GLP2-1} . Effector construct: ABI5 ($P_{35S}:ABI5$) and GAMYB ($P_{35S}:GAMYB$); Reporter construct ($P_{GLP2-1}:GUS$); Internal control ($P_{NOS}:RiLuc$). The means of GUS/RiLuc ratios were obtained from three replicates of five pooled *N. benthamiana* leaves. Error bars = SE ($n = 3$). Mock, leaf sample infiltrated with MMA solution. RiLuc was used as an internal control in the agroinfiltration system. Asterisks indicates significant difference at $**P = 0.01$ in SPSS independent-sample *t* test. ABI5: LOC_Os01g64000; GAMYB: LOC_Os01g59660.

these fragments (Fig. 6, D–G). These results together with the yeast one-hybrid and EMSA results proved that the *OsGLP2-1* promoter was directly targeted by the ABI5 and GAMYB transcription factors through binding to the corresponding ABRE and GARE cis-elements, respectively, in the P_{GLP2-1} promoter.

Dynamic Subcellular Localization of OsGLP2-1

Knowledge of the subcellular localization of OsGLP2-1 may aid in understanding its biological function in rice. Thus the OsGLP2-1-eGFP fusion protein constructs, $P_{35S}:HA-GLP_{2-1}-eGFP$ and $P_{GLP2-1}:HA-GLP_{2-1}-eGFP$, were generated, and transient expression of the constructs in the leaf of *N. benthamiana* was performed. The control ($P_{35S}:eGFP$) showed constitutively expressed green fluorescent signal both in the cytosol and nucleus. The HA-GLP₂₋₁-eGFP fusion protein driven by the P_{GLP2-1} native promoter or CaMV 35S (P_{35S}) promoter exhibited single or bundles of mobile needle-shaped green fluorescent signal in the cytosol, and no signal was observed in the nucleus (Supplemental Fig. S7A). This result differed from previous reports that GLPs are usually associated with the cell wall (Li et al., 2016; Liu et al., 2016). To confirm this result, stable $P_{35S}:HA-GLP_{2-1}-eGFP$ and $P_{GLP2-1}:HA-GLP_{2-1}-eGFP$ transgenic Arabidopsis ecotype Columbia (Col-0) plants were generated, for which identical results were observed (Supplemental Fig. S7B). To verify this dynamic subcellular localization feature,

homozygous lines of $P_{35S}:HA-GLP_{2-1}-eGFP$ transgenic rice plant were further generated, and the 7-d-old seedling was used for subcellular localization observation. The result showed that, in addition to the mobile needle-shaped green fluorescence signal as shown in Arabidopsis and *N. benthamiana*, the moving round signal was also observed in the cytosol (Fig. 7; Supplemental Movies S1 and S2). These results demonstrated that OsGLP2-1 is a highly dynamic protein, and the specificity of OsGLP₂₋₁-eGFP subcellular localization will help to reveal the molecular mechanism of OsGLP2-1 in rice.

DISCUSSION

It is generally accepted that dormancy is induced by ABA during seed development on the mother plant. After maturation of the seed, germination is preceded by a decline in ABA content and the hormonal balance between ABA and GAs acts as an integrator of environmental cues to maintain dormancy or activate germination. ABI5 and GAMYB are the key positive transcription regulators of ABA and GA signaling, respectively. The expression patterns of ABI5 and GAMYB in rice show some similarities, for example, a low level of expression in organs during vegetative growth, but a relatively high level in the reproductive organs (Kaneko et al., 2004; Zou et al., 2008). In rice seeds, GAMYB is expressed specifically in the aleurone layer and scutellum. In the aleurone layer, GAMYB positively regulates α -amylase activity (Kaneko et al., 2004), but its function in the scutellum is unclear. *MFT* regulates seed germination via the ABA and GA signaling pathways in Arabidopsis, and its expression is directly regulated by ABI3, ABI5, and DELLA (Xi et al., 2010). A wheat homolog of MFT is also associated with seed dormancy and is exclusively expressed in the scutellum and coleorhiza (Nakamura et al., 2011). The overlapping scutellum expression pattern of GAMYB and ABI5-targeting genes (such as *MFT*) indicates that the possible cross-regulation of GA and ABA signals via GAMYB/ABI5 in the scutellum may be important for control of seed dormancy and germination.

GLPs are a group of proteins wide-spreading among plants. In rice, the GLP protein family contains 43 members. Although GLPs show a similar sequence to that of wheat germin, which is highly expressed during seed germination, GLPs show a variety of expression patterns and functions. For example, GLPs play important roles in plant disease resistance, including in rice (Manosalva et al., 2009; Davidson et al., 2010). Besides the similarity in protein structure, an additional common feature of germin and GLPs is their extracellular localization (Li et al., 2016; Liu et al., 2016). *OsGLP2-1* is specifically expressed in the seed scutellum and minor veins of the leaf, together with the presence of ABRE and GARE binding motifs in the promoter; these results imply that interaction between ABI5 and GAMYB may be involved in the regulation of *OsGLP2-1*.

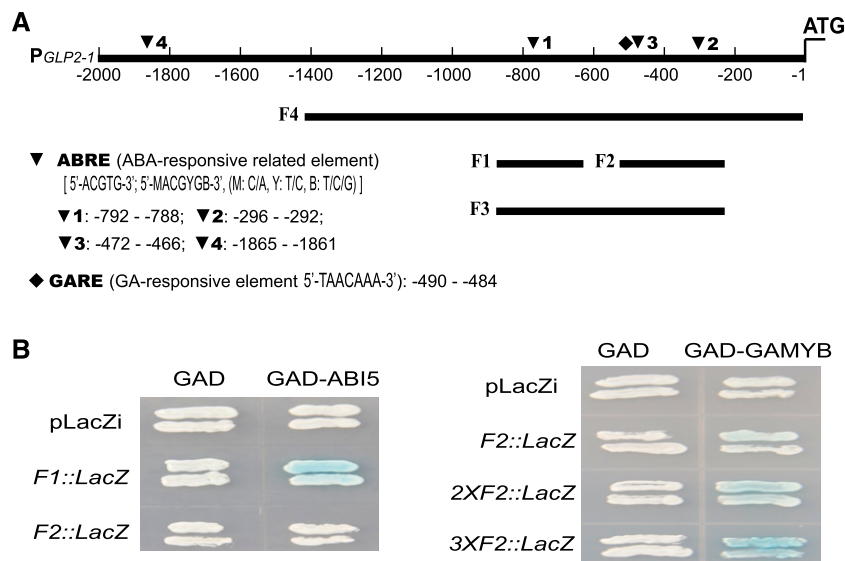


Figure 5. Binding activity of ABI5 and GAMYB to P_{GLP2-1} detected by yeast one-hybrid assay. A, Schematic diagram of the potential cis-elements in P_{GLP2-1} and promoter fragments. F1: -932 to -637 (295 bp); F2: -507 to -237 (270 bp); F3: -932 to -237 (696 bp); F4: -1419 to -1 (1419 bp); P_{GLP2-1} : -2086 to -1 (2086 bp). B, Compared with the negative controls (empty vector GAD with pLacZi; F1::LacZ, or F2::LacZ, respectively, and GAD-ABI5 with pLacZi), ABI5 directly bound to the P_{GLP2-1} promoter F1 fragment and activated expression of the *LacZ* reporter gene. GAMYB directly bound to the F2 fragment and induced *LacZ* expression; moreover, the signal increased progressively with 2 or 3 GARE tandemly repeated in 2×F2::LacZ and 3×F2::LacZ, respectively. The combinations of GAD with pLacZi, F2::LacZ, 2×F2::LacZ, or 3×F2::LacZ; and GAD-GAMYB with pLacZi were used as negative controls. These binding interactions of P_{GLP2-1} with GAMYB and ABI5 were confirmed in at least three repeated experiments.

The present results indicated that *OsGLP2-1* is involved in the ABA and GA signaling regulation of seed primary dormancy, and is directly regulated by the ABI5 and GAMYB transcription factors. Genetic analysis confirmed that the expression level of *OsGLP2-1* in rice was positively correlated with premature seed dormancy. The expression of *OsGLP2-1* is induced by ABI5 and suppressed by GAMYB, which is in accord with the process of seed development. During seed development, high quantities of ABA are synthesized to induce and maintain seed dormancy. With maturation of the seeds, ABA is degraded and dormancy is released by GAs. The *OsGLP2-1* promoter contains both ABI5 and GAMYB binding motifs (ABRE and GARE). Furthermore, in a comparison of the binding activity of GAMYB and ABI5 to the P_{GLP2-1} promoter GAMYB showed relatively stronger specific binding activity in the *in vitro* assay. In the transient expression assay ABI5 displayed high transcription activity with the complete P_{GLP2-1} promoter, whereas in the yeast one-hybrid and EMSA assays ABI5 showed relatively weak binding activity. In yeast one-hybrid assay, ABI5 directly bound to the F1 segment with an ABRE core cis-element (ACGT), but the F2 fragment, which contained an ABRE and an ABRE-like element, failed to be activated by ABI5, and the ABRE flanking sequence, i.e. coupling elements previously reported to affect the binding activity in ABA responsive pathway (Hobo et al., 1999; Niu et al., 2002). Based on these results, we speculate there is competition balance between ABI5 and GAMYB

in the regulation of *OsGLP2-1* expression. Further investigation will help to explain how the two antagonistic signals ABA and GA, in a tissue-specific manner, coregulate the target genes to determine seed dormancy and germination during seed developing.

The signal for seed dormancy (or germination inhibitors) generally originates from the seed coat, endosperm, and embryo. It is necessary to investigate on which particular seed components *OsGLP2-1* acts. Given that the dormancy phenotype of dehulled immature seeds is altered, a function for *OsGLP2-1* in the seed coat is excluded. Comparison of the dormancy of isolated immature embryos and intact seeds showed that when compared with the vector control, only the intact *OsGLP2-1* transgenic seeds exhibited significantly changed seed dormancy, whereas no significant variation was observed for the isolated embryos and the embryo-containing half-seed (Supplemental Fig. S8). These findings indicated that the control of dormancy by *OsGLP2-1* may be associated with inhibition by the endosperm, but the mechanism requires further investigation.

Germin-like proteins are usually expressed during seed germination. To visualize the expression pattern of P_{GLP2-1} :GUS on germinating seeds and seedlings, dry or germinating seeds and 7-d-old seedling were subjected to GUS staining. An identical scutellum-specific pattern of GUS expression was detected in the dry seeds and germinating seeds (Supplemental Figs. S1, A and B, S9B). After germination, the GUS signal changed to the

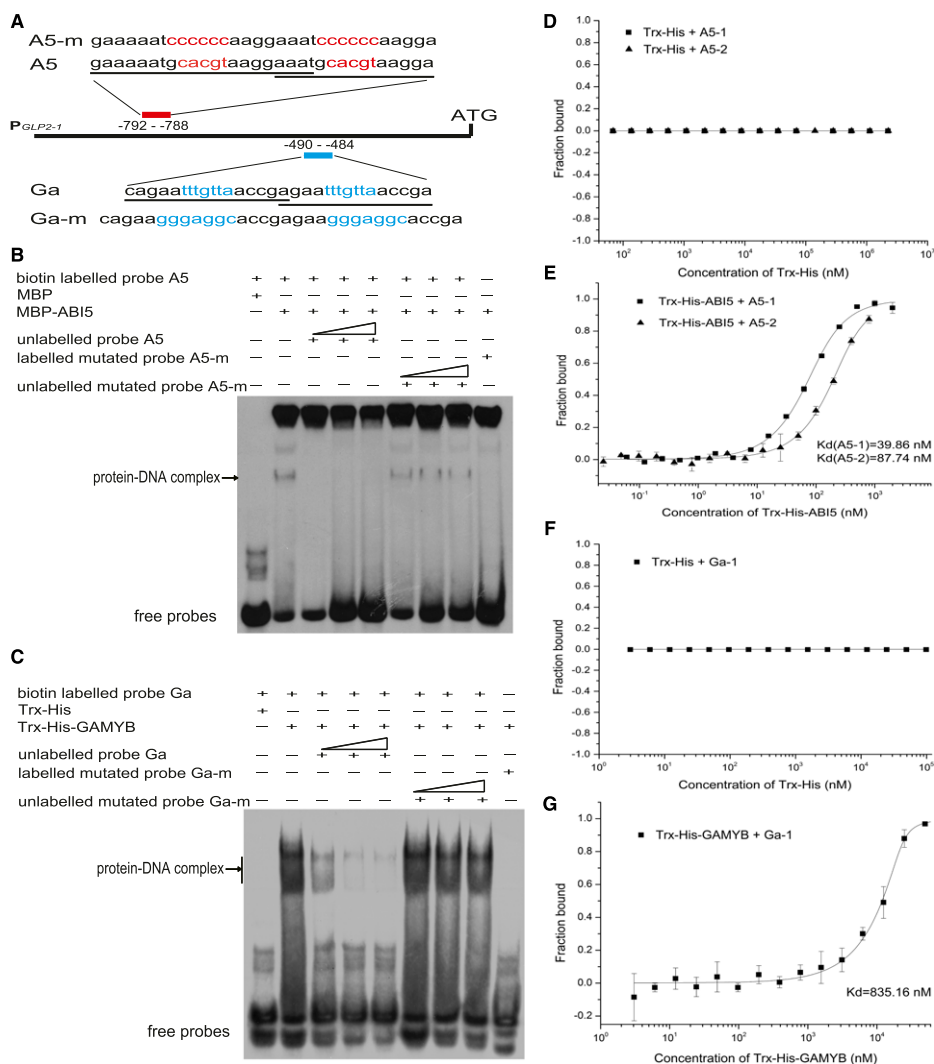
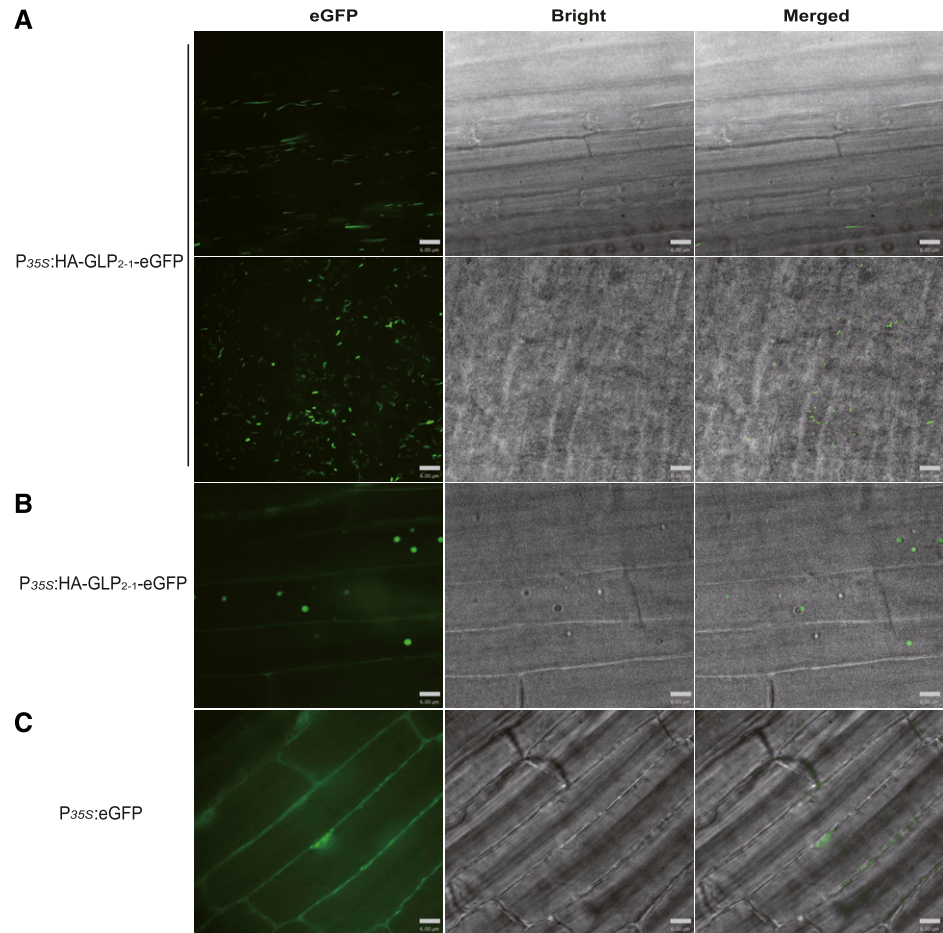


Figure 6. Physical interaction of ABI5 and GAMBY to the ABRE and GARE in *P_{GLP2-1}*. A to C, EMSA results displayed that the transcription factors (ABI5 and GAMBY) could directly recognize and bind to *P_{GLP2-1}* fragments containing the ABRE or GARE, respectively. A, The probes used in EMSA were designed based on the predicted binding sequence of ABRE (red letters) and GARE (blue letters) in the F1 and F2 fragments, respectively, together with their flanking nucleotides (underlined), two overlapped binding sites were synthesized. B and C, Binding activity of ABI5 and GAMBY with their corresponding probes. The fusion proteins MBP-ABI5 and Trx-His-GAMYB specifically bound to the corresponding probes and resulted in the retardant band, whereas no binding activity was observed with the corresponding negative controls of MBP and Trx-His. The unlabeled cold probes (10, 50, 100×) gradually competed for the corresponding binding activity, whereas the mutated biotin-labeled probes (10, 50, 100×) completely abolished the protein-DNA interaction, and the mutated cold-probes showed no competitive activities. D to G, MST analysis showing that the specially binding activity of Trx-His-ABI5 with *OsGLP2-1* promoter fragments of A5-1, A5-2 (E), and Trx-His-GAMYB with Ga-1 (G), respectively. The negative control, Trx-His, did not interact with these fragments (D and F). D and E, MST measurements of the interaction between ABI5 and A5-1, A5-2 fragments. F and G, MST assay testing the interaction between GAMBY and Ga-1 fragment. D to G data are average ± *sd*, *n* = 3. The data were analyzed with the software of MO. Affinity Analysis v2.2.4.

vascular tissue of the seedling (Supplemental Figs. S1C and S9C). It is important to note that when compared with altered dormancy phenotypes of *P_{GLP2-1}:HA-GLP2-1-eGFP*, *amiR-GLP2-1*, and *osglp2-1* knock-out seeds displayed during developing-seed-stage, there was no obvious germination variation observed in after-ripened seeds. These results suggested the main function of *OsGLP2-1* in dormancy-maintenance for developing seed. The *P_{GLP2-1}:GUS* staining in

germination seed indicated the expression of *OsGLP2-1*, but its potential role played in seed germination may be relatively weak. As previously reported, enhanced ABA content in imbibition seed failed to inhibit seed germination (Holdsworth et al., 2008b). Alternative possibility for the remaining expression of *OsGLP2-1* during germination may act like seed-stored mRNAs. Some RNAs stored from seed maturation still existed in germinated seed. It is also found that posttranscriptional

Figure 7. Subcellular localization of OsGLP2-1-eGFP in transgenic rice seedling. In 7-d-old rice seedling, the control $P_{35S}::eGFP$ leaves showed green fluorescence throughout the cytoplasm and nucleus (C); in contrast, green fluorescence from $P_{35S}::HA-GLP2-1-eGFP$ leaves exhibited moving needle-shaped signals in the cytosol, and no signal was observed in the nucleus (A). Moreover, the mobile round green fluorescent signal was also observed in $P_{35S}::HA-GLP2-1-eGFP$ seedling root (B). These dynamic green fluorescent signals of OsGLP2-1-eGFP in rice were confirmed in at least three independent experiments. Bars = 6 μm .



modification and posttranslational regulation mechanisms are involved in after-ripened seed germination (Holdsworth et al., 2008b; Gao et al., 2013). Collectively, seed development and maturation were dominated by the action of ABA, whereas seed germination was associated with multiple mechanisms.

Besides its function in seed dormancy, OsGLP2-1 might have additional functions in the plant. As we described above, during seedling and vegetative growth, *OsGLP2-1* is expressed preferentially in minor veins of the leaf, and the subcellular localization differs from that of germins and all other investigated GLPs. OsGLP2-1 is localized within the cell, and the OsGLP2-1-eGFP fusion protein resembles a short needle or round object and shows rapid movement in the cytosol. Based on these results, we speculate that OsGLP2-1 might be a component of a transporter in the cell for nutrient uptake and transport in seed development, germination, seedling development, and plant growth, but much work is required to test this hypothesis.

Taken together, the present findings show that OsGLP2-1, a previously uncharacterized protein, is responsible for maintaining primary dormancy during seed developing stage, specifically in the scutellum. The transcription factors ABI5 and GAMYB, components of the ABA and GA signaling pathways, coregulate the

expression of *OsGLP2-1*, and thereby maintain the dormancy of the developing seed. Further investigations, in particular on the subcellular localization of OsGLP2-1 and its interacting proteins, are needed to elucidate the molecular mechanism underlying its biological function in plants.

MATERIALS AND METHODS

Seed Germination and Treatment

Rice (*Oryza sativa*) ssp. *japonica* 'Nipponbare' seeds were used. The dehusked immature seeds were first surface-sterilized with 75% (v/v) ethanol for 2 min and then 30% (v/v) NaClO for 30 min, rinsed with double-distilled water, and sowed on MS salt mixture (*phyto* Technology Laboratories, catalog [cat.] no. M524) solid media, supplemented with either 50 μM ABA (Sigma, cat. no. A1049), or 1 or 10 μM GA (Sigma, cat. no. G7645). Germination was assessed at 3, 5, and 7 d after sowing. For comparison of embryo germination, two treatments were applied: (1) the embryo was isolated from 25 DAF dehusked seeds and cultured on one-half strength MS medium; and (2) immature seeds lacking the testa were cut into two halves either containing or lacking the embryo; only the embryo-containing half was used for germination. The seeds were cultured in a growth chamber at 25°C with a 12-h-light/12-hr-dark photoperiod.

Constructs

Rice genomic DNA was extracted from rice leaf with cetyltrimethylammonium bromide method (Murray and Thompson, 1980). The *OsGLP2-1* promoter (P_{GLP2-1})

2,086 bp) was amplified using the promoter-specific primers C2-Pro-5-H3 and C2-pro3-Bgl (Supplemental Table S3). The P_{GLP2-1} PCR fragment was excised with *HindIII* and *BglII*, then cloned into the binary plasmid pCAMBIA-1300-SGN (digested with *HindIII/BamHI*) to generate the recombinant plasmid P_{GLP2-1} :GUS containing the GUS report gene driven by P_{GLP2-1} .

To generate the construct for overexpression of *OsGLP2-1*, the primer pair cup25xba and cup2RSac was used to amplify the cDNA containing the *OsGLP2-1* ORF (*OsGLP2-1*-ORF). The *OsGLP2-1*-suppression construct was produced using an artificial microRNA method with the backbone sequence of OsmiR159a (Sunkar et al., 2005; Zhang et al., 2012). Based on the precursor sequence of OsmiR159a, the replacement target sequence of 5'-CTC AGTTCGAAGTTCTAA-3', spanning the second exon and 3' untranslated region of the *OsGLP2-1*-cDNA, was amplified with the primer pair C2-5a5-Xba and C2-5aR-Sac. This fragment (*amiR-GLP2-1*) and the *OsGLP2-1*-ORF fragment were separately cloned into the plant expression vector pCAMBIA-1300-D4S with *XbaI/SacI* digestion to yield *OsGLP2-1* overexpression or suppression vectors driven by the CaMV 35S promoter. The resulting constructs were designated OE-*GLP2-1* and *amiR-GLP2-1*, respectively.

To generate the constructs for subcellular localization of *OsGLP2-1*-eGFP, the *GLP2-1*-ORF fragment was amplified with the primer pair HA-C2-SBam and C2-R-Sma to yield a 680-bp product, in which the HA-tag was fused at the N terminus of *OsGLP2-1*. The product was first digested with *SmaI/BamHI*, cloned into the 130-GFP plasmid to create the plasmid P_{35S} :HA-*GLP2-1*-eGFP, then further digested with *BamHI/HindIII*; the P_{GLP2-1} promoter fragment (*BglII/HindIII*) was then inserted. Subsequently, the CaMV 35S promoter was substituted with P_{GLP2-1} . The new construct was designated P_{GLP2-1} :HA-*GLP2-1*-eGFP, in which the HA-*GLP2-1*-eGFP fusion protein was driven by the P_{GLP2-1} native promoter. Finally, the recombinant constructs P_{35S} :HA-*GLP2-1*-eGFP and P_{GLP2-1} :HA-*GLP2-1*-eGFP were used for observation of subcellular localization via transient and stable expression in the plant.

CRISPR-CAS9-Induced Mutation in the *OsGLP2-1* Gene

The *osglp2-1* mutant was developed using CRISPR/CAS9-based editing of *OsGLP2-1* in rice by Biogle in 2016. Two targets in the second exon of *OsGLP2-1* (*cr-glp-323*: 5'-GGAGAAGAAGAAGTCTCCA-3'; *cr-glp-513*: 5'-GACCGA GATCCTGACTATAC-3'; Fig. 2A) were selected and the targeting specificity of target sequences (including protospacer adjacent motif) was confirmed using a BLAST search against the rice genome (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Hsu et al., 2013). The designed targeting sequences were synthesized and annealed to form the oligo adaptors, which were further inserted into pBGK032 vector digested by *Bsa* I, to produce CRISPR/CAS9 plasmids. Transformation of rice was carried out via *Agrobacterium tumefaciens* strain EHA105. The mutated *osglp2-1* plant was identified by sequencing of PCR product with primers of C2-Cri-S/C2-Cri-R flanking the *OsGLP2-1* genome. In order to obtain the T-DNA free *osglp2-1* mutant, the crosses between wild-type plant and the T_1 homozygous mutated plant were further performed. From F_2 population plants, T-DNA-free *osglp2-1* homozygous mutant was selected based on the absence of hygromycin band, while the presence of the *osglp2-1* mutated site in a genomic DNA PCR detection.

Plant Stable Transformation

The plant expression constructs were introduced into *A. tumefaciens* strain EHA105 and transformed into rice via *A.*-mediated transformation (Hiei et al., 1994), and stable *Arabidopsis thaliana* (ecotype Col-0) transgenic plants were obtained by floral-dip transformation (Clough and Bent, 1998). For each construct, about 30 independent T_0 transformants were obtained, and further screened by germinating seeds from T_2 lines with 50 μ g/mL hygromycin. The hygromycin-resistant homozygous seeds were used for further experiments. Seeds from homozygous lines transformed with the empty vector pCAMBIA 1300, or the construct harboring the *GUS* (or *eGFP*) gene driven by the CaMV 35S promoter, were used as a negative and positive control, respectively.

RT-qPCR

Freshly harvested immature rice seeds were ground to powder with the SPEX SamplePrep Geno/Grinder in liquid nitrogen. Total RNA was extracted following the method previously reported (Wang et al., 2012), and further treated with TURBO DNase according to the TURBO DNA-free Kit (Applied

Biosystems, AM1907). cDNAs were produced using the Invitrogen superscript III First Strand Synthesis System (cat. no. 18080-051). RT-qPCR was performed with the SYBR Green Real-time PCR Master Mix (TOYOBO, QPK-201) to determine the relative expression level of *OsGLP2-1* or *GUS*, using the primers listed in Supplemental Table S3. Rice *ACTIN* was used as an internal control.

GUS Staining

Staining of GUS activity was performed with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide as described previously (Gallagher, 1992). The GUS assay was first carried out with leaves from 14-d-old P_{GLP2-1} :GUS transgenic rice seedlings, and with various organs (leaf, spikelet, internode, stem, leaf sheath, and seed) at the reproductive stage (25 DAF). Subsequently, the tissue-specific expression of GUS in minor veins of the leaf and in the scutellum with T_3 plant were intensively examined. The assay was conducted on leaves at various growth stages (42, 52, 64, and 86 d after sowing), and on freshly harvested seeds at different development stages (17 and 35 DAF). To visualize the expression pattern of P_{GLP2-1} :GUS during seed germination, dry or germinating seeds and 7-d-old seedling were also subjected to GUS staining. Briefly, dry seeds were imbibed in double-distilled water and incubated in a growth chamber at 25°C with a 12-h-light/12-hr-dark photoperiod. The germinated seeds (after 3 d of imbibition) stripped of the glume and 7-d-old seedlings were collected separately for GUS staining.

Transient Expression of *OsGLP2-1* in *Nicotiana benthamiana*

To investigate the effect of transcription factors on the expression of *OsGLP2-1*, a transient expression assay was conducted in *N. benthamiana*. According to the potential cis-elements predicted by the plant cis-acting regulatory DNA elements database, the candidate genes of upstream of *OsGLP2-1* were cloned. The cDNAs containing the ORF of different transcription factors were amplified from rice with appropriate primers (Supplemental Table S3), and cloned into the plant expression vector 1300-DAS downstream of the CaMV 35S promoter. For the reporter construct, the *OsGLP2-1* promoter fragment (*HindIII/BglII*) was cloned into the plant vector 1300-intGUS (*HindIII/BamHI*) to generate the plasmid 130- P_{GLP2-1} :intGUS in which P_{GLP2-1} drives the GUS report gene (intGUS). The construct 121- P_{NOS} -*Renilla* luciferase (RiLuc) was used as an internal control. To avoid contamination of the reporter protein expressed from *Agrobacterium*, the reporter genes of both intGUS and RiLuc ORFs contained an intron. The plasmids were introduced into *A. tumefaciens* strain GV3101 for agroinfiltration.

Agroinfiltration of *N. benthamiana* was performed in accordance with a previous procedure with some modifications (Li et al., 2013). The freshly cultured single clones of GV3101 carrying the different plasmids were independently grown at 28°C overnight and then diluted by 1% (v/v) to continuous cultured until $OD_{600} = 1.0$, resuspended in MMA buffer (10 mM MgCl₂, 10 mM MES, and 100 μ M acetosyringone) to wash one time, resuspended in MMA, and then adjusted to $OD_{600} = 1.5$. The reporter, effector, and 121- P_{NOS} -RiLuc constructs were mixed (2:3:1). Mixture of 130- P_{GLP2-1} :intGUS and 121- P_{NOS} -RiLuc (2:1) without the effector construct was used as a negative control, and the MMA buffer was used as a mock control. After agroinfiltration, the *N. benthamiana* plants were cultured in a greenhouse for 3 d; then leaves were sampled for RiLuc and GUS detection.

Yeast One-Hybrid Assay

Binding of the *OsGLP2-1* promoter to the transcription factors ABI5 and GAMYB was detected with a yeast one-hybrid assay. P_{GLP2-1} fragments (F1: -932 to -637 [295 bp]; F2: -507 to -237 [270 bp]; F3: -932 to -237 [696 bp]; and F4: -1419 to -1 [1419 bp]) with predicted cis-elements were amplified by PCR with specific primers (Supplemental Table S3). The fragments were cloned into the pLacZi vector (Clontech) to generate the constructs pLacZi- P_{GLP2-1} -F1, -F2, -F3, and -F4, respectively. To confirm binding activity, duplicated fragments of 2 \times F1, 3 \times F1, and 2 \times F2, 3 \times F2 were constructed. The ORF fragments of the ABI5 and GAMYB effectors were separately cloned into the pGAD424 vector (Clontech). The pLacZi-Fn::LacZ series reporter gene plasmids and pGAD424-ABI5 or pGAD424-GAMYB constructs were cotransformed into *Saccharomyces cerevisiae* strain EGY48. The transformants were selected on synthetic complete medium lacking Ura and Leu.

EMSA

The MBP-ABI5 fusion protein was expressed in the pMAL-p2X vector (BioLabs, cat. no. E8000S) and purified in accordance with the manufacturer's instructions. The specific primer pair AB5-S-Bam and AB5-R-H3 was used to amplify the ABI5-ORF fragment, which was cloned into the pMAL-p2X vector (*Bam*HI/*Hind*III). The Trx-His-GAMYB fusion protein was constructed in the pET32a (+) expression vector via *Bam*HI/*Hind*III digestion. *Escherichia coli* strain BL21 (DE3) pLysS cell harboring the fusion constructs was induced by 0.2 mM isopropylthio- β -galactoside at 16°C overnight (about 18 h) at 150 rpm. Cells expressing the ABI5 or GAMYB fusion protein were collected and lysed with a cell disrupter (JNBIO, JN-mini) at 1000 bar on ice. The recombinant proteins MBP-ABI5 and Trx-His-GAMYB were purified with Amylose Resin (BioLabs, cat. no. E8021L) and Ni Sepharose excel (GE Healthcare, cat. no. 17-3712-02), respectively, and then applied to the EMSA using the Chemiluminescent Nucleic Acid Detection Module Kit (Prod. no. 89880). The GARE- or ABRE-containing probes were separately labeled with biotin by Thermo Fisher Scientific (Fig. 6). The unlabeled probe was used as a competitive probe, and in the mutated probe the corresponding cis-element was changed. To confirm the binding activity of ABI5 and GAMYB to the corresponding cis-elements of GARE and ABRE in the P_{GLP2-1} promoter, different amounts of MBP-ABI5 (0, 0.3, 0.5, 1.0, and 1.5 μ g) and Trx-His-GAMYB (0.05, 0.1, 0.3, 0.5, and 1.0 μ g) fusion proteins were separately subjected to the EMSA. The expressed protein of MBP and Trx-His from the corresponding empty vector of pMAL-p2X and pET-32a (+), respectively, was used as a negative control.

Microscale Thermophoresis Assay

Protein-DNA binding experiments were performed with a Monolith NT.115 instrument (NanoTemper Technologies; www.nanotemper-technologies.com). The Trx-His-ABI5 fusion protein expressed from pET32a (+) in *E. coli* was further induced as described above. The recombinant proteins of Trx-His-ABI5 and Trx-His-GAMYB were purified using imidazole-gradient purification in Ni-NTA agarose (Qiagen). The *OsGLP2-1* promoter fragments (A5-1: 5'-CCG GAGAAAAATGCACGTAAGGAAATAATAAT-3' located at -805 to -774; A5-2: 5'-GCAGGCAGGCCACAACACGTTTATTCAGACTT-3' at -311 to -280; and Ga-1: 5'-ACAGAAATTTGTTAACCGAACGACGACGACGCGTAAT GCATA-3' at -496 to -458, the underlined bases indicate ABRE or GARE element) were, respectively, synthesized and labeled with 5-carboxyfluorescein (FAM) at the 5' end (Thermo Fisher Scientific). The labeled FAM-A5-1, FAM-A5-2, or FAM-Ga-1 fragment with 16 titrations of Trx-His, Trx-His-ABI5, or Trx-His-GAMYB proteins were loaded onto standard treated silicon capillaries (MO-K002 Monolith NT.115; NanoTemper Technologies), and fluorescence was measured. The experiment was accompanied by a step-by-step gradient dilution process. The optimized measuring parameters of instrument were as follows: light emitting diode power 20%; MST power 20% for Trx-His-ABI5 and 80% for Trx-His-GAMYB; fluorescence before time, 5 s; MST on time, 30 s; fluorescence after time, 5 s; delay time, 25 s. Data were analyzed using software MO. Affinity Analysis v2.2.4 (Nano Temper Technologies).

Confocal Microscopy

Leaf samples from the transient *N. benthamiana*, seedling of stable transgenic Arabidopsis, and rice plants were observed with confocal microscope (Leica TCS SP8 and UltraView Vox).

Statistical Analyses

Statistical analysis was performed using independent-sample *t* test in Statistical Package for Social Science (SPSS) for two groups comparison and one-way ANOVA post hoc tests for more than two groups comparison.

Accession Numbers

Rice genomic sequence data from this article can be found in the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu>) under the following accession numbers: *OsGLP2-1* (LOC_Os02g29000), *ABI5* (LOC_Os01g64000), *GAMYB* (LOC_Os01g59660), *ABL1* (LOC_Os06g10880), *DOF2* (LOC_Os02g15350), *DOF7* (LOC_Os07g32510), and *DOF9* (LOC_Os09g29960).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. GUS staining of P_{GLP2-1} :GUS transformant during seed germination.

Supplemental Figure S2. Transcript levels of *OsGLP2-1* in the leaf of OE-*GLP2-1* and amiR-*GLP2-1* rice detected by RT-qPCR.

Supplemental Figure S3. Dormancy phenotype of immature seeds of amiR-*GLP2-1* rice at 9 d after sowing.

Supplemental Figure S4. Germination rate of OE-*GLP2-1* and amiR-*GLP2-1* seeds with hormone treatment.

Supplemental Figure S5. Influence of the transcription factors on the relative GUS activities (GUS/RiLuc) controlled by P_{GLP2-1} .

Supplemental Figure S6. Protein concentration gradient assay in EMSA for MBP-ABI5 and Trx-His-GAMYB recombinant proteins.

Supplemental Figure S7. Subcellular localization of *OsGLP2-1*-eGFP.

Supplemental Figure S8. Percentage germination of OE-*GLP2-1* and amiR-*GLP2-1* rice seeds on one-half strength MS solid medium.

Supplemental Figure S9. GUS staining of developing seed, embryo and etiolated seedling of P_{GLP2-1} :GUS transformant.

Supplemental Table S1. Percentage germination of amiR-*GLP2-1* transgenic seeds (35 DAF) on MS medium.

Supplemental Table S2. Percentage germination of amiR-*GLP2-1* transgenic seeds (35 DAF) on MS medium supplemented with 50 μ M ABA.

Supplemental Table S3. Primers used in this study.

Supplemental Movie S1. The dynamic movement of the needle-shape green fluorescence in P_{35S} :HA-*GLP2-1*-eGFP rice seedling leaf.

Supplemental Movie S2. The mobile round green fluorescent signal of *GLP2-1*-eGFP in 3-d-old seedling root.

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