The rice **GERMINATION DEFECTIVE 1**, encoding a B3 domain transcriptional repressor, regulates seed germination and seedling development by integrating GA and carbohydrate metabolism

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**SUMMARY**

It has been shown that seed development is regulated by a network of transcription factors in Arabidopsis including LEC1 (LEAFY COTYLEDON1), L1L (LEC1-like) and the B3 domain factors LEC2, FUS3 (FUSCA3) and ABI3 (ABA-INSENSITIVE3); however, molecular and genetic regulation of seed development in cereals is poorly understood. To understand seed development and seed germination in cereals, a large-scale screen was performed using our T-DNA mutant population, and a mutant germination-defective1 (gd1) was identified. In addition to the severe germination defect, the gd1 mutant also shows a dwarf phenotype and abnormal flower development. Molecular and biochemical analyses revealed that GD1 encodes a B3 domain-containing transcription factor with repression activity. Consistent with the dwarf phenotype of gd1, expression of the gibberellic acid (GA) inactivation gene OsGA2ox3 is increased dramatically, accompanied by reduced expression of GA biosynthetic genes including OsGA20ox1, OsGA20ox2 and OsGA3ox2 in gd1, resulting in a decreased endogenous GA4 level. Exogenous application of GA not only induced GD1 expression, but also partially rescued the dwarf phenotype of gd1. Furthermore, GD1 binds to the promoter of OsLFL1, a LEC2/FUS3-like gene of rice, via an RY element, leading to significant up-regulation of OsLFL1 and a large subset of seed maturation genes in the gd1 mutant. Plants over-expressing OsLFL1 partly mimic the gd1 mutant. In addition, expression of GD1 was induced under sugar treatment, and the contents of starch and soluble sugar are altered in the gd1 mutant. These data indicate that GD1 participates directly or indirectly in regulating GA and carbohydrate homeostasis, and further regulates rice seed germination and seedling development.

**Keywords:** B3 domain transcription factor, EAR motif, germination-defective1, gibberellin, rice, seed germination.

**INTRODUCTION**

Seed development is an important phase in the life cycle of land plants, offering plants an indispensable opportunity to maintain the species and to survive in a hostile environment. In flowering plants, seed development may be divided into two major stages: embryo/endosperm morphogenesis and seed maturation. A large body of evidence obtained over recent years has shown that many genetic loci control the developmental regulation from embryogenesis to germination (McCarty, 1995; Holdsworth *et al.*, 1999). It has been extensively demonstrated that LEAFY COTYLEDON 1 (LEC1), LEC2, FUSCA3 (FUS3) and ABSCISIC ACID INSENSITIVE 3 (ABI3) play a central role in this process. LEC1 encodes a conserved eukaryotic CCAAT-box binding HAP3 subunit, while LEC2, FUS3 and ABI3 all encode proteins with a plant-specific B3 DNA-binding domain (Giraudat *et al.*, 1992; Lotan *et al.*, 1998; Luerssen *et al.*, 2001).
et al. (2001). The four mutants lec1, lec2, fus3 and abi3 severely block the seed development and share some common phenotypes, such as reduced expression of seed storage proteins (Kroj et al., 2003). By contrast, enforced expression of LEC1 or LEC2 is sufficient to induce embryo development in vegetative cells (Lotan et al., 1998; Stone et al., 2001). LEC2 represses expression of GA3ox2 and may regulate embryogenesis partly by stimulating expression of auxin biosynthesis genes (Stone et al., 2001, 2008; Curaba et al., 2004; Braybrook et al., 2006). Restriction of expression of FUS3 to the epidermis produces cotyledon-like leaves, and FUS3 negatively modulates GA biosynthesis and positively regulates abscisic acid (ABA) biosynthesis (Gazzarrini et al., 2004). GA3ox2 is also a direct downstream target of FUS3 (Curaba et al., 2004). These loci may act synergistically to adjust the optimal hormone balance to maintain seed development (To et al., 2006).

Recently, a striking functional symmetry within the B3 domain-containing transcription factors was revealed by identification of a novel B3 domain transcriptional repressor named VP1/ABI3-LIKE (VAL; Suzuki and McCarty, 2008). In Arabidopsis, three genes (VAL1, VAL2 and VAL3) are categorized within the VAL sub-group. In addition to a B3 domain, the VAL proteins contain three other domains that may be involved in chromatin remodeling: a PHD (plant homeodomain)-like domain, a CW-type zinc finger, and an EAR (ethylene response factor-associated amphiphilic repression) motif. The val1 mutant was first identified by screening for mutants with altered expression of sugar response genes (Tsukagoshi et al., 2005). The val1, val2 and val3 single mutants have no obvious phenotype compared to the wild-type under normal growth conditions, while the val1 mutant exhibits strong embryonic traits when germinated on medium containing paclobutrazol, an inhibitor of GA biosynthesis (Suzuki et al., 2007). However, the val1 val2 double mutant and val1 val2 val3 triple mutant seedlings show growth arrest and develop a variety of embryonic phenotypes during their growth and development (Suzuki et al., 2007). Expression of LEC1, LEC2, FUS3, ABI3 and other seed maturation genes was up-regulated in the val1 val2 double mutant, suggesting that VAL genes play a role in repressing the embryonic pathway and are essential for the transition from embryo development to seed germination and vegetative development (Suzuki et al., 2007). These findings in Arabidopsis reveal that two sub-families of B3 domain transcription factors serve as activators and repressors of seed development, respectively, but the mechanism of how those regulators are involved in the seed development is still unclear. In contrast to ABI3, FUS3 and LEC2, which have well-defined cis-elements in their downstream target genes, cis-elements specific to VAL transcription factors remain to be elucidated (Suzuki et al., 1997; Ezcurra et al., 2000; Reidt et al., 2000; Monke et al., 2004; Braybrook et al., 2006). VAL transcription factors may target RY elements (typical sequence CATG-CATG) for repression through recruitment of chromatin modification and remodeling factors (Suzuki et al., 2007; Guerrero et al., 2009; Kagale and Rozwadowski, 2011).

In rice (Oryza sativa), it was found that 91 B3 domain-containing genes exist (Swaminathan et al., 2008), but OsLFL1 (LEC2/FUS3-LIKE), which is most close to FUS3 in Arabidopsis, is the only rice B3 gene with a phenotype reported to date (Swaminathan et al., 2008). It has been shown that OsLFL1 binds directly to the RY motif in the Early heading date 1 (Ehd1) promoter, and over-expression of OsLFL1 delays flowering by repressing Ehd1 expression (Peng et al., 2007, 2008). In addition to late-flowering, additional phenotypes such as dwarf stature, small leaves and leaf-like petals were also observed on over-expression of OsLFL1 in rice and Arabidopsis, suggesting that OsLFL1 may have a similar function to LEC2 and FUS3 of Arabidopsis (Peng et al., 2007, 2008). In cereals, the correct transition from embryo development to germination is an important agronomic trait, but little is known about the molecular and genetic regulation of this process (Holdsworth et al., 1999). Here, we report the identification and characterization of a rice mutant germination-defective1 (gd1), which is defective in seed germination and seedling development. When imbibed in water, some of the mutants arrested during germination period. Once germinated, the growth rate of gd1 is also much slower than that of wild-type. Sequence alignment showed that GD1 has high similarity to VALs in Arabidopsis. Further molecular and biochemical analyses revealed that GD1 has transcriptional repressor activity and ability to bind the RY element. In addition, plants over-expressing OsLFL1 partly mimic the gd1 mutant. The data clearly indicate that GD1 participates directly or indirectly in GA homeostasis by suppressing OsLFL1 expression. These results extend our understanding on the role of B3 genes in regulating rice seed germination and seedling development.

RESULTS

Phenotype of germination-defective1

To identify seed development-related mutants, a large-scale screening was performed using a previously described T-DNA mutant population (Ma et al., 2009), and the mutant germination-defective1 (gd1), which is defective in seed germination and seedling development, was obtained. When cultured on MS medium, wild-type seeds germinate within approximately 2 days and grow normally, but gd1 germinates and grows much more slowly than wild-type (Figure 1a). At 11 days after germination, the shoots and roots of gd1 are much shorter than those of wild-type, with approximately one-third of the mutants arrested at the germination stage (Figure 1b,c).
After germination, $gd_1$ showed distinct dwarf and small leaf phenotypes (Figure 1d). Most of the $gd_1$ plants died during vegetative growth, and only <10% flowered. The panicle of $gd_1$ was smaller than that of wild-type, and the flowers also exhibited abnormality (Figure 1e). Rice flowers contain six stamens, one pistil and two stigmas (Figure S1a); however, both the number and identity of the floral organs were altered in $gd_1$ mutants. $gd_1$ mutant plants often exhibited seven or eight stamens (Figure S1b,c), and abnormalities were also found in stigmas, with three stigmas often fused together (Figure S1b). In many flowers, an abnormal additional organ appeared (Figure S1d,e), and a stamen-like organ was fused with an imperfect stigma (Figure S1f). Iodine staining showed that approximately 50% of the pollen grains aborted in the $gd_1$ mutants (Figure 1f,g).

GD1 encodes a B3 domain-containing transcription factor

The flanking region of T–DNA was amplified by SiteFinding PCR (Wang. et al., 2011), and the amplified fragment was sequenced. BLAST alignment (Altschul et al., 1990) revealed that the T–DNA was inserted into the 11th exon of a gene (LOC_Os07 g37610) located on chromosome 7 (Figure S2a,b). RT-PCR analysis showed that the T–DNA insertion led to knockout of full-length gene expression (Figure S2c). However, the gene portions both upstream and downstream of the T–DNA insertion site were still expressed (Figure S2c), as the T–DNA also contains an activation tag (actin1 promoter) at its left border (Ma et al., 2009), which activated expression of the downstream portion of GD1. However, we were unable to amplify any products using primers flanking the T–DNA insertion site, indicating that $gd_1$ is a knockout mutant. GD1 shares high similarity with the VAL genes in Arabidopsis (Figure S2b). The coding sequence of GD1 contains 2868 bp, which encodes a polypeptide of 956 amino acids. By searching the rice genome (http://rice.plantbiology.msu.edu/), another homolog, OsVAL2 (LOC_Os07 g48200), was also found. These proteins share five conserved domains: a PHD domain at the N–terminus, a B3 domain for DNA binding, a CW-type zinc finger, a nuclear localization signal, and an EAR motif at the C–terminus (Figure S2b). It has been shown that EAR motif-containing transcriptional repressors play an important role in modulating plant defense and stress responses (Kazan, 2006). Recently, the PHD domain of HSI2 (HIGH-LEVEL EXPRESSION OF SUGAR INDUCIBLE GENE2)/VAL1 has been implicated in H3K27 trimethylation.
to repress seed-specific genes in Arabidopsis (Veerappan et al., 2012). In addition, a proline- and glutamine-rich extension was found at the N-terminus of GD1, which is absent in all other VAL proteins, implying that GD1 may have distinct functions.

To examine whether the T-DNA insertion was responsible for the mutant phenotype, co-segregation analysis between the T-DNA insertion and mutant phenotype in the T1 generation was performed. The 666 mutants with a defective phenotype were confirmed to be homozygous for the T-DNA insertion using primers P1, P2 and P4 (Figure S2a), whereas the 692 normal plants were either wild-type (235) or heterozygous (457), indicating that the gd1 mutant phenotype is due to the T-DNA interruption of GD1. For further confirmation, a 13 kb genomic fragment containing the entire 7246 bp GD1 coding region, a 4614 bp upstream region and a 1092 bp downstream sequence was cloned into binary vector pCAMBIA1300 and transformed into calli derived from the heterozygous seeds (Figure 2a). The individual transformants were confirmed by PCR using primers P1, P2 and P3 shown in Figure 2(a), and transgenic plants with a homozygous gd1 mutant background were obtained and found to be restored to a wild-type phenotype (Figure 2b,c), showing that loss of function of GD1 led to the gd1 mutant phenotype.

**Expression pattern of GD1**

Quantitative real-time PCR analysis revealed that GD1 was ubiquitously expressed in all tissues, including roots, flowers, stems, leaves and calli (Figure 3a). The highest expression level of GD1 was detected in leaf blades and flowers, whereas the expression level was relatively low in young shoots, leaf sheaths, roots and stems. We next examined the expression patterns of GD1 under ABA and GA treatments (Figure 3b). The transcript of GD1 in leaves showed a slight increase upon ABA treatment, but the expression of GD1 was increased twofold 6 h after GA treatment. We also investigated the effect of sucrose on GD1 expression, as shown in Figure 3(b), and its expression was strongly up-regulated by sucrose after 1 and 6 h.

**GD1 is a nuclear-localized transcriptional repressor**

To study the subcellular localization of GD1, GD1 was fused with GFP and expressed in onion epidermal cells. As shown in Figure 4(a), fluorescence was detected in both cytoplasm and nucleus for the negative control GFP. In contrast, for the GD1-GFP fusion protein, fluorescence was only detected in the nucleus, suggesting that GD1 is a nuclear-localized protein.

To test whether the EAR motif of GD1 has transcriptional repressor activity, we used a protoplast assay system to detect the transcriptional activity of GD1. In this system, various truncated versions of GD1 were fused with GAL4DB as effector plasmids (Figure 4b), and the firefly luciferase (LUC) gene under the control of five copies of GAL4 was used as the reporter. After introducing both plasmids into protoplasts, the LUC activity was measured to determine the GD1 transcriptional activity. As shown in Figure 4(b), the LUC activity was strongly repressed when the full-length GD1 effector was co-expressed in the protoplasts. However, the repressed LUC activity was released when various truncated GD1 constructs without the EAR

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**Figure 2.** Complementation of the gd1 mutant. (a) Genomic DNA fragment used for complementation. (b) Complementation restored the gd1 mutant to a normal wild-type phenotype. (c) Complementation was confirmed by PCR using the primers indicated in (a). Heter, heterozygous gd1 mutant; Homo, homozygous gd1 mutant; Homo+fragment, homozygous gd1 mutant with complementary fragment; Heter+fragment, heterozygous gd1 mutant with complementary fragment.
motif (amino acids 1-872, amino acids 1-767, amino acids 1-613 and amino acids 1-394) were co-transformed. Among them, the fragment without the EAR motif and the nuclear localization signal (amino acids 1-767) completely lost its ability to inhibit LUC activity. The LUC activity decreased significantly when various C-terminal truncations of the EAR motif (amino acids 606-956, amino acids 757-956 and amino acids 867-956) were used as the effectors (Figure 4b). All these data indicate that the C-terminus plays an important role in the repression process, and the EAR motif serves as an active repression domain. Similar results were also obtained from a yeast one-hybrid system (Figure 4c). Both the full-length and C-terminal fragment strongly inhibited expression of the reporter gene compared with the empty vector, while transformants containing the N-terminal fragment without the EAR motif retained normal gene expression (Figure 4c).

The B3 domain of GD1 specifically binds to the RY motif

Previously it was shown that B3 transcription activators, such as ABI3, FUS3 and LEC2, specifically bind the RY element to positively regulate the expression of downstream target genes (Reidt et al., 2000; Monke et al., 2004; Braybrook et al., 2006). The protoplast expression assay was used to detect whether the B3 domain of GD1 uses a similar binding site. Two LUC reporter plasmids containing 4 x RY DNA-binding elements and 5 x GAL4 binding elements were used (Figure 5a). For the effector plasmids, full-length GD1 (amino acids 1-956) and three truncated versions of GD1 including a C-terminal fragment containing the B3 domain (amino acids 410-956), the B3 domain (amino acids 410-612) fused to a VP16 activation domain, and an N-terminal fragment (amino acids 1-394) fused to VP16 were constructed (Figure 5a). As shown in Figure 5a, full-length GD1 and the C-terminal fragment containing the B3 domain (amino acids 410-956) greatly inhibited the LUC activity of the RY reporter. When the B3 domain was fused to the VP16 activation domain, the LUC activity of RY reporter was enhanced compared with the GAL4 control. When a fragment without the B3 domain was fused to the VP16 domain, the LUC activity was almost the same in the various reporters. Therefore, the RY motif is a candidate to serve as GD1 binding site, and the B3 domain is responsible for recognition of the target DNA sequence. To further investigate the binding activity of the B3 domain to the RY motif, an in vitro gel-shift assay was performed. Due to unsuccessful expression of full-length GD1 in Escherichia coli, we used a truncated GD1 containing the B3 domain in the binding experiment. As shown in Figure 5(b), the B3 domain of GD1 directly bound to RY motif tetramer. A competition experiment showed that unlabeled RY oligonucleotide competed for this binding. OsLFL1, which exhibited the same specific binding activity to the RY motif, was used as a positive control. These results suggested that the B3 domain of GD1 bound specifically to the RY motif. Identification of the RY motif as a downstream binding site of GD1 is critical for understanding of the repression mechanism of GD1.

GD1 negatively regulates GA metabolism

B3 transcription factors have been reported to regulate seed germination and seedling development by participating in the ABA and GA pathways. We investigated whether the dwarf phenotype of gd1 is due to GA deficiency. Four-week-old wild-type and mutant plants were treated with GA3, and the response was analyzed 10 days after treatment. As shown in Figure 6(a), GA partially rescued the dwarf phenotype of the gd1 mutant, indicating that GD1 may regulate GA homeostasis. In accordance with this result, the endogenous GA3 level in the gd1 mutant was down-regulated compared with wild-type (Figure 6b). Quantitative real-time PCR analysis of several genes involved in GA biosynthesis and catabolism revealed that expression of the GA inactivation gene OsGA2ox3 was increased dramatically, while expression of GA biosynthetic genes, including OsGA20ox1, OsGA20ox2 and OsGA3ox2, was decreased in the gd1 mutant (Figure 6c and Figure S3).
In addition, OsCPS1 and OsKO1 were found to be down-regulated in the leaves of 2-month-old gd1 mutant plants (Figure 6c). Interestingly, OsCPS1 and OsKO1 were significantly up-regulated in the leaves of 4-month-old mutant plants (Figure S3). Moreover, expression of OsGA2ox3 was significantly induced in gd1 after exogenous GA treatment for 2 days (Figure 6d). These results indicate that GD1 is involved in the regulation of GA metabolism, either directly or more likely through other components.

**Expression of OsLFL1 is upregulated in the gd1 mutant**

It has been reported that expression of VAL genes in Arabidopsis represses the expression of LEC1 and the B3 network in developing seeds, which is essential for repression of embryonic pathways (Suzuki et al., 2007; Suzuki and McCarty, 2008). Due to the defect in seed germination, we speculate that GD1 may be involved in transition between the seed and vegetative phases. We first compared the expression level of OsLFL1 in gd1 mutant and the wild-type. Quantitative real-time PCR results showed that gd1 had a higher level of the OsLFL1 transcript (Figure 7a). OsLFL1 is the only B3 domain-containing protein characterized in rice so far, and probably has similar functions to LEC2 and FUS3 (Peng et al., 2007, 2008). The increased expression of OsLFL1 in gd1 suggested that the negative effect of GD1 on the embryonic pathway may be
similar to the results found in Arabidopsis. Furthermore, we identified RY elements within the 1.5 kb promoter regions of OsLFL1 and GD1. As expected, this DNA-binding study provided additional evidence that the B3 domain of GD1 is directly involved in regulation of OsLFL1 and GD1 itself (Figure 7b,c). In addition, genes including one encoding a protease inhibitor/seed storage/LTP family protein, non-specific LTP2 and OLEO2 were also expressed at higher levels in the gd1 mutant compared to wild-type (Figure 7a). Although we do not know their exact roles in seed development, the accumulation patterns suggest that they are downstream targets of B3 proteins. During germination, failure to repress OsLFL1 and many seed development-associated genes is probably responsible for the developmental retardation phenotypes in gd1. Interestingly, expression of OsVAL2 was found to be down-regulated in gd1 (Figure 7a).

Plants over-expressing OsLFL1 partly phenocopy the gd1 mutant

As very few homozygous gd1 seeds were obtained, making it difficult to generate OsLFL1 knockdown transgenic plants in the gd1 background, OsLFL1 over-expression lines were generated to further determine the role of OsLFL1 in mediating the GD1-regulated GA homeostasis. OsLFL1 transgenic lines showed a similar dwarf phenotype to gd1 during seedling and adult plant growth periods (Figure 8a). In addition, ectopic expression of OsLFL1 in rice also causes dwarf stature and small leaves, which resemble the gd1 phenotypes except for the delay in flowering. We used the OsLFL1–2 and OsLFL1–14 lines for further expression analysis (Figure 8b). As expected, expression of OsGA2ox3 and non-specific LTP2 was induced in OsLFL1 over-expression plants (Figure 8c), mimicking the expression patterns
in the gd1 mutant (Figures 6c and 7a). These results suggest that OsLFL1 is the downstream target of GD1.

Carbohydrate metabolism is affected in the gd1 mutant

In Arabidopsis, the val1 mutant showed altered expression of sugar response genes. Interestingly, we found that the number of starch granules was significantly reduced in the stem of the gd1 mutant compared to the wild-type (Figure 9a). Measurement of the carbohydrate content in the leaves also revealed that the starch content decreased significantly in both flag leaves and the top second leaves of the gd1 mutant (Figure 9b,d); however, soluble sugars including glucose, fructose and sucrose accumulated to high levels in gd1 (Figure 9c,e), suggesting that GD1 participates in regulation of carbohydrate metabolism. To study the potential involvement of GD1 in carbohydrate metabolism, the expression patterns of some related genes were further investigated. As shown in Figure 9(f), expression of the α-amylase genes RAMy1A and RAMy3D was induced, whereas expression of the transcript of SSIIIa (encoding starch synthase) was inhibited in the gd1 mutant (Figure 9f), consistent with the carbohydrate alteration observed in the mutants. One exception is ADP-glucose pyrophosphorylase large subunit 1 (encoded by AGPL1), which catalyzes a rate-limiting step of starch biosynthesis in plants. AGPase family genes are known to accumulate in response to sugars and ABA (Akihiro et al., 2005; Ohdan et al., 2005). Expression of AGPL1 was elevated in the gd1 mutant, which may be explained via feedback regulation by the increased endogenous sugar content.

DISCUSSION

Seed development and germination are highly complex and fine-tuned developmental processes (Finkelstein et al., 2008; Santos-Mendoza et al., 2008). During recent years, huge amounts of work have been performed in Arabidopsis and maize (Zea mays) to identify components associated with seed maturation and germination. Many of these are involved in GA and ABA signaling, biosynthesis or catabolism (White et al., 2000; Finkelstein et al., 2002; Olszewski et al., 2002; Peng and Harberd, 2002; Nambara and Marion-Poll, 2003, 2005; Gubler et al., 2005; Fang et al., 2008; Suzuki et al., 2008; Nakashima et al., 2009). The key roles played by ABA and GA in regulating seed development and germination are well-established (Piskurewicz et al., 2008, 2009), and reduction of ABA or increase of GA in seeds results in a switch from the seed
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maturation program to the germination program (Koornneef et al., 2002; Finch-Savage and Leubner-Metzger, 2006). In addition, roles of sugar and other hormones in regulation of seed germination have also been proposed (Gibson, 2004; Rolland et al., 2006; Yuan and Wysocka-Diller, 2006); however, the molecular mechanism is still poorly understood. Recent studies indicate that cross-talk between ABA- and auxin-dependent responses occurs during seed germination and early seedling development in which ABA-dependent repression of growth is potentiated by auxin (Belin et al., 2009).

Rice is one of the most important crops in the world. The correct transition from seed maturation to germination is an important agronomic trait. Although several loci have been identified that regulate rice seed maturation and germination processes, the complete pathway and the underlying mechanism remain unclear (Agrawal et al., 2001; Fang et al., 2008). Here, we identified a gd1 mutant from a T-DNA mutant population that exhibits defect in seed germination and seedling development, including dwarf and sterile phenotypes. GD1 is expressed ubiquitously and is regulated by hormone and sugar treatment, indicating an essential role in plant development.

GD1 encodes a B3 domain-containing protein that shares high similarity with VAL proteins in Arabidopsis. Previous reports in Arabidopsis have shown that two sub-families of B3 domain transcription factors, including AB3/FUS3/LEC2 and VAL, are critical determinants in seed and vegetative development (Suzuki and McCarty, 2008). AtVAL1 functions as a transcriptional repressor for sugar-inducible genes and plays an important role during the transition from seed maturation to germination (Tsukagoshi et al., 2005, 2007; Suzuki et al., 2007). The VAL family in Arabidopsis is required for repression of the LEC1/B3 transcription factor network in germinating seedlings (Suzuki et al., 2007). Except for the B3 domain, VAL sub-family proteins do not have any other similarities to AB3/FUS3/LEC2 proteins. AtVAL1 and related proteins have been reported to be B3 domain–EAR motif active transcription repressors (Tsukagoshi et al., 2005). A number of reports have indicated that EAR motif-containing proteins play a key role in the transcriptional repression of plant defense- and stress-associated gene expression (Ohta et al., 2001; Sakamoto et al., 2004; Song et al., 2005; Yang et al., 2005; Kazan, 2006; Kagale and Rozwadowski, 2010). Our transient expression analysis in both Arabidopsis protoplasts and yeast indicated that the EAR motif in the C-terminus of GD1 functions as an active repression domain, suggesting that GD1 is involved in seed germination and seedling development by negatively regulating target gene expression.

Currently, our knowledge about the function of B3 genes in grass species is still poor and fragmentary. In rice, there are several proteins that are homologous to the products of the Arabidopsis genes, but only one of them, OsLFL1, which is homologous to FUS3, has been studied in depth (Peng et al., 2007). Expression of OsLFL1 was enhanced in the gd1 mutant, consistent with the results in Arabidopsis. In combination with the evidence in Arabidopsis, which demonstrates the positive regulatory role of the AB3/...
FUS3/LEC2 genes in the embryonic pathway, de-repression of OsLFL1 in the gd1 mutant partially explains gd1 defective phenotype in germination. These results suggest that GD1 has a similar function to AtVAL proteins. The molecular mechanisms controlling the embryo pathway are probably conserved between monocot and dicot species, at least to some extent. Like other B3 factors, GD1 binds to the RY element through the B3 domain. Interestingly, more than 10 RY elements were found within the 1.5 kb genomic sequence upstream of GD1. In addition, one RY element was also found in the upstream sequence of OsLFL1. Gel-shift experiments revealed that GD1 binds to the promoters of both GD1 and OsLFL1, indicating that GD1 regulates the expression of itself and other B3 factors. However, in OsLFL1 over-expression lines, the transcript levels of GD1 and its homolog remain the same as in wild-type. All the data above suggest that OsLFL1 functions downstream of GD1. The data presented also demonstrate the complex regulation network within B3 transcription activators and repressors. In order to further elaborate how GD1 regulates seed germination and seedling development, several ChIP experiments for testing the interaction between GD1 and OsLFL1 in vivo were performed, but the unstable nature of the GD1 protein made all our attempts failed.

The phenotypes of most adult gd1 mutants are reminiscent of plants that are defective in GA synthesis or action. Expression of GD1 may be induced by GA, the endogenous GA level was significantly reduced in gd1, and the dwarf phenotype of the gd1 mutant may be partially rescued by exogenous application of GA. Expression analysis clearly showed that expression of the GA deactivation gene OsGA2ox3 was strongly induced in gd1 under GA treatment compared with wild-type; however, the expression levels of the GA biosynthesis genes OsGA20ox1, OsGA20ox2 and OsGA3ox2, were decreased in the gd1 mutant. These data suggest that GD1 participates in maintaining GA homeostasis by positively regulating GA biosynthesis and negatively regulating GA deactivation. Inconsistent expression changes for OsCPS1 and OsKO1, which are also involved in GA biosynthesis, were probably due to the differentially developmental manner (Silverstone et al., 1997; Smith et al., 1998; Hedden and Phillips, 2000). The oscillation of GA concentration is critical for regulating seed germination and seedling development. The levels of bioactive GAs are maintained via feedback and feed-forward regulation of GA metabolism, and several factors that influence GA metabolism have been identified (Yamaguchi, 2008). Microarray data analysis in atval mutants suggested that VAL genes may be involved in the regulation of GA synthesis, because AtGA3ox1, one of the key GA biosynthesis genes expressed during seed germination, is down-regulated more than 10-fold in the val1/val2 double mutant compared to wild-type (Suzuki et al., 2007). GA biosynthesis was also shown to be regulated by the LEC2 and FUS3 pathways (Curaba et al., 2004; Gazzarrini et al., 2004). Lines over-expressing OsLFL1 showed dwarf stature, small leaves and leaf-like petals, and GA metabolism genes showed a similar expression pattern to the gd1 mutant (Peng et al., 2007). Therefore, we can derive a scenario for the possible role of GD1 in the regulation of B3 transcription factors and GA metabolism: GA induces GD1 expression, and GD1 negatively regulates B3 transcription factors such as OsLFL1 and GD1 itself, then directly or indirectly modulates the GA level, regulating seed germination and seedling development (Figure 10).

Sugars, as energy sources and structural components, have important functions in plant development. Many genetic approaches have been used to identify the

**Figure 8.** Over-expression of OsLFL1 partly phenocopies gd1 mutant. (a) Phenotypes of OsLFL1 over-expression lines. Top: 2-week-old stage; scale bars = 2 cm. Bottom: grain-filling stage; scale bars = 10 cm. (b) RT-PCR confirmation of over-expression of OsLFL1 in the two OsLFL1 over-expression lines. (c) Determination of the transcript level of OsGA2ox3 and nsLTP2 in OsLFL1 transgenic lines by quantitative real-time PCR. The data were referenced to OsActin1 as an internal control and normalized against wild-type. Values are means ± SD of three biological replicates. Student’s t test was used for statistical analysis. Asterisks indicate statistically significant differences compared with wild-type (***P < 0.05, **P < 0.01).
regulatory pathways that control sugar signaling in Arabidopsis. Various alleles of \textit{aba1}, \textit{aba2}, \textit{aba3}, \textit{abi4} and \textit{abi5} were isolated that have altered sugar responses, indicating a close correlation between the sugar and ABA pathways (Fedoroff, 2002; Rook et al., 2006). Consistent with the results obtained for \textit{VAL1}, \textit{GD1} showed increased expression under sugar treatment. The contents of starch and soluble sugars are altered in \textit{gd1}, suggesting that \textit{GD1} is involved in regulation of the metabolism of starch and sugar. The expression of genes involved in carbohydrate metabolism also varied between \textit{gd1} and wild-type. The enhanced expression of \textit{AGPL1} is indicative of a high endogenous sugar level and ABA level. However, the molecular basis of how \textit{GD1} is involved in the carbohydrate metabolism has not yet been determined.

In conclusion, \textit{GD1} plays a critical role in the regulation of B3 transcription factors, and also contributes to GA and carbohydrate catabolism regulation. Correct expression of \textit{GD1} is crucial for proper seed and plant development. To better understand the regulatory role of \textit{GD1}, future work will focus on identification of its interaction partners and downstream targets.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

All rice lines used in this study were derived from the \textit{japonica} cultivar Nipponbare. The plants were grown in paddy fields under natural conditions except where specifically indicated. Germination of \textit{gd1} was assessed on plates containing MS0 medium (Sripaoraya et al., 2003), using seeds harvested from heterozygous \textit{gd1} plants.

For various treatments, the rice seeds were pre-soaked for imbibition in water at 37°C for 2 days. Then the soaked seeds were sown and cultivated in a growth chamber at 28°C with constant light. Two-week-old seedlings were subjected to treatment with 100 \(\mu\)M ABA, 10 \(\mu\)M GA3 or 6% sucrose, respectively. The aerial parts of treated or control seedlings were harvested at the 0, 1, 6 and 12 h time points. For the rescue of the \textit{gd1} phenotype by GA plants grown in the soil for 2 weeks were sprayed with 10 \(\mu\)M GA3.

![Figure 9. Regulation of carbohydrate metabolism in the \textit{gd1} mutant.](image) (a) Reduced number of starch granules in cross-sections of stems of wild-type and \textit{gd1}. (b, c) Content of starch and soluble sugars including glucose, fructose and sucrose in the flag leaves of wild-type and \textit{gd1}. Values are means ± SE (\(n=10\)). Similar results were obtained for three independent experiments. Asterisks indicate statistically significant differences compared with wild-type (*\(P \leq 0.05\), **\(P \leq 0.01\)).

(d, e) Content of starch and soluble sugars including glucose, fructose and sucrose in the top second leaves of wild-type and \textit{gd1}. Values are means ± SE (\(n=10\)). Similar results were obtained for three independent experiments. Asterisks indicate statistically significant differences compared with wild-type (*\(P \leq 0.05\), **\(P \leq 0.01\)).

(f) Expression pattern of carbohydrate metabolism-associated genes in wild-type and \textit{gd1}. Values are means ± SD of three biological replicates. Student’s t test was used for statistical analysis. Asterisks indicate statistically significant differences compared with wild-type (**\(P \leq 0.01\)).

![Figure 10. Proposed model for \textit{GD1}-regulated GA homeostasis.](image)
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for 10 days. For gene expression analysis, seedlings were sampled after spraying 10 μM GA3 treatment for 2 days.

Vector construction and rice transformation

A 13 kb genomic DNA fragment containing the 7246 bp entire GDI1 coding region, a 4614 bp upstream region and a 1092 bp downstream sequence was inserted into the binary vector pCAMBIA1300 (CAMBIA, Canberra, Australia) by digesting with XhoI and SalI to generate a transformation plasmid for the complementation test. The plasmid was introduced into Agrobacterium tumefaciens AGL1 by electroporation, and rice transformation was performed as previously described (Liu et al., 2007). Calli derived from seeds of heterozygous plants were used. The genotype of each transformant was determined by PCR. For PCR, the P1 (5′-GAGCTTGCCGAAATGATTCCTA-3′), P2 (5′-CTAGGGTTGCTGCTCGAGG-3′), and P3 (5′-GGCAACATGTCTGT CTTGCTGT-3′) primers were used.

To generate the OsLFL1 over-expression vector, the full-length cDNA fragment of OsLFL1 was amplified using primers 5′-CCCCGAGGAATCCCGATG-3′ and 5′-GCCAGGGAGTGCCTTG-3′. Then the fragment was cut using Xbal and SalI, and inserted into the pCAMBIA2300 (CAMBIA, Canberra, Australia) vector.

Identification of the T-DNA insertion site

The flanking sequence of the inserted T-DNA fragment in the mutant was determined using SiteFinding PCR as described previously (Wang et al., 2011). The products of PCR were cloned into the pGEM-T-easy (Promega, Madison, WI, USA) and then sequenced. A total of 200 ng RNA was used for RT-PCR reactions (30 cycles) in a total volume of 20 μL using an RT-PCR kit (Toyobo, Osaka, Japan). The primers used for RT-PCR were P5 (5′-GCTTGGCCAA TGGTGTACTACCC-3′), P6 (5′-GCTCCTCCCATGATTAGTGGG-3′), P7 (5′-TCTGA TGAATGCATGCGACC-3′), OsActin1F (5′-TGAGACC TTCAC ACCCTTGTG-3′) and OsActin1R (5′-TCTTGGGAGTCTCCATTT CC-3′).

RNA preparation and real-time quantitative PCR

Total RNAs were isolated using the guanidinium isocyanate/acidic phenol method (Chomczynski and Sacchi, 1987). Synthesis of first-strand cDNA and real-time PCR analysis were performed as described previously (Yang et al., 2009). OsActin1 was used as an internal control. Gene-specific primers were designed and are listed in Table S1. Three biological repeats were performed for each gene.

Subcellular localization of GDI1–GFP fusion proteins

The GDI1 cDNA was amplified using primers 5′-CTTAGGGTTCT GAGGCCCA-3′ and 5′-GAGCTTGGCTTGTGCTCGAGG-3′, and cloned into the Nhel and SacI sites of the transient expression vector pGFP2(GA)/II. Transient expression assays of GFP localization in onion epidermal cells were performed by particle bombardment (model PDS–1000, Bio-Rad, Hercules, CA, USA). After overnight incubation in the dark, confocal images were collected using a Zeiss LSM 510 Meta confocal laser scanning microscope (Jena, Germany).

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Transient expression assay in protoplasts and LUC activity determination

For the transcriptional repression activity assay, the reporter plasmid GAL4–LUC and effector plasmids with various lengths of GDI1 were used. The GAL4–LUC plasmid includes five repeats of the yeast GAL4 protein binding site, and the minimal TATA region of the CaMV 35S promoter located upstream of the firefly luciferase gene (Fujimoto et al., 2000; Ohta et al., 2000). The effector plasmids were constructed by fusing various lengths of the GDI1 coding region (amino acids 1–956, 1–872, 1–767, 1–613, 1–394, 606–956, 757–956 and 867–956) with GAL4DB. Arabidopsis mesophyll protoplasts were isolated and transformed by the polyethylene glycol-mediated method (Yoo et al., 2007). To normalize values, plasmid pPTRL (Ohta et al., 2001), which includes a luciferase gene from Renilla reniformis under the control of the CaMV 35S promoter, was used as an internal control. Luciferase activity was quantified using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA). The results are reported as the ratio of firefly relative light units (RLUs) versus Renilla RLUs as obtained using a luminometer (Promega). Experiments were performed in triplicate.

In order to explore the relationship between GDI1 and the RY element, a reporter plasmid with 4× RY DNA-binding element (CATGCATGCATGCATGCATG) was constructed. Four effectors (amino acids 1–956, the fragment comprising amino acids 410–956 fragment, a fusion of amino acids 410–612 with the VP16 activation domain, and a fusion of amino acids 1–394 with VP16) were co-transformed with GAL4–LUC and RY–LUC reporters, respectively. The RY–LUC plasmid includes four repeats of RY motif. Luciferase activity was then assayed.

Electrophoretic mobility shift assays

Gel-shift assays were performed as described by the manufacturer of the equipment used (Promega). Because of failure to obtain full-length GDI1 protein after many attempts, we prepared only the B3 domain of GDI1 protein containing amino acids 387–613 using E. coli strain BL21 (DE3). OsLFL1 was used as a positive control. For GDI1, the oligonucleotides 5′-GAATTCGCTTCCCTCCT CATTGCAGAA-3′ and 5′-TCTGCGACAGCCAGTGCTGTCC AC-3′ were used, and for OsLFL1 the oligonucleotides 5′-CGCGA TCGCGCGTCCCGAACGATC-3′ and 5′-AAATGCACAGGAGAAG GGTTTCTAGCTTC-3′ were used. Fragments were sub-cloned into expression vector pGEXP–1 (Amersham Pharmacia Biotech, Tokyo, Japan). The glutathione S-transferases (GST) fusion protein was extracted from bacteria utilizing glutathione-tagged Sepharose (GE Healthcare, Piscataway, NJ, USA). The eluted GST fusion protein was assayed by SDS-PAGE.

To test whether GDI1 bind RY elements, complementary 59 bp single-stranded oligonucleotides containing the RY motif tetramer TGATCATGCATGCATGCATGCGTAGTGCTACATGCATGCAGC GCATGCATGACC were synthesized. For downstream gene promoter binding experiments, complementary single-stranded oligonucleotides derived from their promoters were synthesized as DNA probes. To obtain double-stranded RY motif-containing fragments, two complementary oligonucleotides were mixed and heated in a water bath at 95°C for 5 min, and cooled to room temperature for annealing.

GA4 quantification

Quantification of endogenous GAs was performed as described previously (Li et al., 2011). Rice panicles (approximately 3 g fresh
weight) of gd1 and wild-type were harvested 100 days after sowing.

Carbohydrate analysis

Carbohydrate analysis was performed as described previously (Stitt et al., 1989). For extraction of soluble sugars and starch, two leaf discs (8 mm diameter) were taken from the fully expanded flag leaves or top second leaves of field-grown plants using a cork borer, and frozen immediately in liquid nitrogen.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Morphology of flowers in wild-type and the gd1 mutant.

Figure S2. Cloning of the GD1 gene.

Figure S3. Expression analysis of GA synthesis genes and inactivation genes in 4-month-old wild-type and gd1 plants by quantitative real-time PCR.

Table S1. Primers used for quantitative real-time PCR.

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