Comprehensive Gene Expression Analysis of Rice Aleurone Cells: Probing the Existence of an Alternative Gibberellin Receptor(s)

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One Sentence Summary of Most Important Findings

GID1-DELLA is the sole mechanism for GA perception for gene expression in rice aleurone cells.
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Abstract

Current gibberellin (GA) research indicates that GA must be perceived in plant nuclei by its cognate receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1). Recognition of GA by GID1 relieves the repression mediated by the DELLA protein, a model known as the GID1-DELLA GA perception system. There have been reports of potential GA-binding proteins in the plasma membrane that perceive GA and induce α-amylase expression in cereal aleurone cells, which is mechanistically different from the GID1-DELLA system. We therefore examined the expression of the rice α-amylase genes (RAmys) in rice (Oryza sativa) mutants impaired in the GA receptor (gid1) and DELLA repressor (slr1), and confirmed their lack of response to GA in gid1 mutants and constitutive expression in slr1 mutants. We also examined the expression of GA-regulated genes by genome-wide microarray and quantitative reverse-transcription PCR analyses, and confirmed that all GA-regulated genes are modulated by the GID1-DELLA system. Furthermore, we studied the regulatory network involved in GA signaling by using a set of mutants defective in genes involved in GA perception and gene expression, namely gid1, slr1, gid2 (GA-related F-box protein mutant), and gamyb (GA-related transacting factor mutant). Almost all GA up-regulated genes were regulated by the four named GA-signaling components. On the other hand, GA down-regulated genes showed different expression patterns with respect to GID2 and GAMYB; e.g. a considerable number of genes are not controlled by GAMYB or GID2 and GAMYB. Based on these observations, we present a comprehensive discussion of the intricate network of GA-regulated genes in rice aleurone cells.
Introduction

Gibberellins (GAs) comprise a large family of tetracyclic diterpenoid plant hormones involved in a wide range of plant growth responses, including seed germination, stem elongation, leaf expansion, flowering, and pollen maturation (Richards et al., 2001; Thomas et al., 2005). In the past decade, genetic studies of GA-signaling mutants of rice and Arabidopsis thaliana have revealed factors essential for GA perception including the GA receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1, Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006), the GA signaling repressor protein, DELLA (Peng et al., 1997; Itoh et al., 2002), and an F-box protein for DELLA degradation, GIBBERELLIN INSENSITIVE DWARF2/ SLEEPY 1 (GID2/SLY1, Mcginnis et al., 2003; Sasaki et al., 2003). Functional analyses of these proteins have enabled us to construct a molecular model for GA signaling (Ueguchi-Tanaka et al., 2007; Davière and Achard, 2013; Locascio et al., 2013). In this model, DELLA protein represses GA action in the absence of GA. When GA is present, the GID1 receptor binds with GA and develops the ability to interact with DELLA protein. Interaction triggers the degradation of DELLA via the SCF$^{GID2/SLY1}$ proteasome pathway. The SCF complex is an E3 ligase, which consists of Skp1, Cullin, F-box protein and a RING-H2 motif, which add a polyubiquitin chain to DELLA, thus inducing degradation via the 26S proteasome complex. The ensuing degradation of DELLA triggers GA action.

Prior to establishment of the GID1-DELLA GA perception system, researchers speculated about the existence of a GA-binding protein (GBP) in the aleurone cells of
cereal species. Jelsema et al. (1977) first reported GBP activity in aleurone homogenates derived from wheat (*Triticum aestivum*) seed. Hooley and colleagues demonstrated that α-amylase can be induced in aleurone protoplasts by the application of a GA derivative that was impermeable with respect to plasma membranes (Beale et al., 1992; Hooley et al., 1991). When GA was microinjected into the cytoplasm of barley (*Hordeum vulgare*) aleurone protoplasts, Gilroy and Jones (1994) reported the absence of α-amylase induction. Later, Hooley and colleagues examined GBPs in the plasma membrane of oat (*Avena fatua*) aleurone cells using photoaffinity-labeled GA and discovered two GBPs. These were a 60 kDa protein localized in the microsomal fraction (Hooley et al., 1993) and a 50 kDa protein in the cytosolic fraction (Walker et al., 1994). Two other GBPs of 68 and 18 kDa were detected in the plasma membrane fraction of oat (*Avena sativa*) aleurones using the same photoaffinity-labeling method (Lovegrove et al., 1998). These observations strongly suggest that GA perception occurs in the plasma membrane, which induces α-amylase expression. Thus, there should be a GA receptor in the plasma membrane of cereal aleurone cells. The above-mentioned characteristics of GBPs cannot be explained with respect to GID1; for example, rice GID1 is a 40 kDa soluble protein that localizes to the nucleus (not the plasma membrane). Furthermore, the GID1-DELLA interaction is thought to occur primarily in the nucleus because of the nuclear localization of DELLA proteins (Ueguchi-Tanaka et al., 2005). Although the identity of GID1 as the GA receptor has been established for many years, the potential existence of GBP in aleurone cells has persisted. Several reviews and textbooks contain information about an alternative GA perception
mechanism that is mediated by GBPs (Hartweck and Olszewski, 2006; Ueguchi-Tanaka et al., 2007; Taiz and Zeiger, 2010). To investigate this alternative mechanism, we examined the expression of GA-regulated genes in half-seeds of rice (Oryza sativa) lacking embryos. This research utilized four GA signaling mutants containing defects in GID1, DELLA (SLR1 in rice), GA-related F-box (GID2), and GA-related MYB (GAMYB) proteins (see Supplemental Fig. S1 for the presumed function of these components). These proteins are encoded by single genes in rice, which facilitated our study of the GA perception system. The comprehensive microarray analysis using these mutants revealed that the genes responsive to GA in the wild-type (WT) aleurone cells did not respond to GA in the gid1 and slr1 mutants. This observation clearly demonstrates that GA perception is solely undertaken by the GID1-DELLA system and negates the idea of an alternative system at the level of gene expression. Furthermore, based on transcriptome and quantitative reverse-transcription PCR (qRT-PCR) analyses, we present a comprehensive discussion of GA signaling that begins with the perception of GA by GID1 and ends with the expression of downstream genes.

RESULTS

Expression of Rice α-amylase (RAmy) Genes in Different GA Mutants

We used halved seeds lacking embryos; these were obtained from rice GA-signaling mutants, namely gid1-4, slr1-1, and gid2-5 (null mutants for GID1, DELLA, and GID2, respectively). We also included gamyb-2, a null mutant for GAMYB, which is an important transcription factor for GA signaling in cereals (Gubler et al., 1995; Kaneko et
al., 2004). We first examined the expression pattern of RAmy genes in the mutant half-seeds by qRT-PCR, because it has been reported that RAmy genes might be induced in cereal aleurone cells though the GBP activity as previously mentioned. There are nine genes of RAmy in the rice genome (RAP DB; http://rapdb.dna.affrc.go.jp/). Among them, RAmy1A, 1B, 1C, 3B, 3C, and 3E were greatly up-regulated by GA in wild-type (WT) seeds (Fig. 1); up-regulation of these RAmy genes did not occur in the gid1 mutant background. In slr1 seeds, regardless of GA treatment, the RAmy genes were expressed at levels similar to the GA-treated WT seeds. These results indicate that the induction of RAmy genes completely depends on the GID1-DELLA system. In gid2 seeds, gene expression was generally not induced; exceptions included RAmy3B, 3C, and 3E, which showed a slight but significant up-regulation by GA (see arrows in Fig. 1). This indicates that degradation of SLR1 by GID2 is a prerequisite to fully induce the expression of these genes, but some RAmys are slightly induced without SLR1 degradation. In the case of gamyb seeds, expression of RAmy1A, 1B, 1C and 3B was not induced by GA, whereas RAmy3C and 3E were up-regulated by GA (arrowheads in Fig. 1). These findings indicate that RAmy induction depends on GAMYB; however, there may be a bifurcation for RAmy3C and 3E expression that does not require GAMYB (see discussion). For the RAmy2A gene, although it was also up-regulated in WT by GA, but only very slightly and its expression pattern was essentially same as that of RAmy1 genes (Supplemental Fig. S2).

In contrast to the above-mentioned RAmy genes, expression of RAmy3A and 3D was down-regulated by GA in the WT seeds, but not in the gid1 background (Fig. 1).
Expression of these two genes did not occur in *slr1* seeds regardless of GA treatment. These results indicate that suppression of *RAmy3A* and *3D* by GA is negatively regulated by the GID1-DELLA perception system. As for *gid2* seeds, *RAmy3A* expression was suppressed significantly (but not completely) by GA; *RAmy3D* expression was not down-regulated in the *gid2* background (see arrows, Fig. 1). In the *gamyb* background, the down-regulation of *RAmy3A* by GA was almost complete, while *RAmy3D* expression was only partially suppressed (arrowheads, Fig. 1).

**In silico Analysis of GA-regulated Transcriptomes in Signaling Mutants**

Microarray experiments were conducted to examine the change in GA-regulated expression profiles in half-seeds lacking embryos. Based on these results, 615 and 704 probes corresponding to 447 up- and 471 down-regulated genes, respectively, showed significant alterations in gene expression in WT seeds (Supplemental Table S1 and S2, respectively). Using these GA-regulated genes, enrichment analysis of Gene Ontology terms was performed using agriGO (Du et al., 2010) (Supplemental Fig. S3). With regards to the molecular function (MF) of GA up-regulated proteins, the GO term “hydrolase activity” was extremely enriched (*P* value = 1.83e^{-05}); indicating the increased predominance of hydrolases including α-amylase, glucanase, and proteinase, which hydrolyze glycosyl and peptide bonds that reside in various compounds present in germinating seeds. In terms of cellular components (CC), the extracellular region, cell wall, endoplasmic reticulum, and vacuole were significantly enriched. This trend seems
logical considering the role of hydrolases and transporters in catabolism, which includes cell wall loosening and endosperm weakening during seed germination.

In the case of GA down-regulated genes, transporter activity was enriched ($P$ value = 0.00446), and this indicates the increased MF of cation:sugar, solute:hydrogen, and sugar:hydrogen symporters involved in the uptake of sugars into cells. In terms of biological processes, the GO terms “response to endogenous stimulus” and “response to abiotic stimulus” were enriched, which relates to genes stimulated by abscisic acid, such as *ABSCISIC ACID-INSENSITIVE1* (*ABI1*), *ABI5*, and *OsSnRK6* (Gosti et al., 1999; Finkelstein and Lynch, 2000; Nakashima et al., 2009) supporting cross-talk between GA and ABA during seed germination (Ho et al., 2003).

**GA-regulated Gene Expression in Rice Aleurones Depends on GID1-DELLA**

We compared the effect of GA on the expression of GA-regulated genes in WT and *gid1* seeds to identify those that are not controlled by GID1. In Fig. 2A, the y-axis shows the log$_{10}$ ratio of the GA-induced, signal intensity change in WT seeds. Similarly, the x-axis represents the log$_{10}$ value of the change between GA-treated and untreated *gid1* seeds. In this analysis, the clustering of data points (probes) around the y-axis (x=0 line) would imply that the GID1-mediated system is the sole mechanism for GA perception. Although most probes were concentrated around the y-axis as indicated by the regression line (Fig. 2A), there were six outliers (Fig. 2A and 2B) beyond the threefold difference (±0.48 differences in log$_{10}$ ratio) in cut-off points (see dashed lines, Fig. 2A). Thus, we directly examined the change in expression of corresponding genes in WT
and *gid1* seeds by qRT-PCR (Fig. 2C). All of the genes were up- or down-regulated by GA in the WT at different levels in qRT-PCR, but their expression was not significantly altered in *gid1* seeds where expression was comparable to WT seeds not treated with GA (Fig. 2C; *RAMy3B* was presented in Fig. 1). These results clearly demonstrate that all GA-related changes in expression depend on GID1 activity.

We next examined the involvement of SLR1 in GA perception using the method described above (Fig. 3). Almost all probes were concentrated around the y-axis (Fig. 3A), suggesting that SLR1 controls expression of corresponding genes. However, similar to the results obtained for *gid1*, 18 outlying probes corresponding to 11 up- (including *RAmy1C* and *RAmy3E*) and three down-regulated genes were observed (Fig. 3A and Supplemental Table S3). We examined the change in expression for all corresponding genes by qRT-PCR (Fig. 3B). In the WT, these genes were up- or down-regulated by GA at different levels. In contrast, their expression was not significantly altered in *slr1* seeds and was comparable with the WT seeds treated with GA (Fig. 3B, for *RAmy1C* and 3E, see Fig. 1). These results demonstrate that all GA-related changes in expression depend on SLR1 activity and thus are mediated by the GID1-DELLA-GA perception system.

**Involvement of GID2 and GAMYB in GA Signaling**

We also examined the involvement of GID2 in the expression of GA-regulated genes using the same approach (Fig. 4A). The probes were concentrated around the y-axis, with 100 outliers (see Supplemental Table S4). Among these, 22 probes corresponded
to 12 GA up-regulated genes. Of these, we examined the expression of nine genes (corresponding to 16 probes, Fig. 4A); four of these were \textit{RAmy1C}, \textit{3B}, \textit{3C}, and \textit{3E} (Fig. 1). In the qRT-PCR analysis, up-regulation of all of the genes by GA was inhibited in \textit{gid2} seeds (Fig. 4B), although five genes (\textit{RAmy3B}, \textit{3C}, and \textit{3E}, \textit{Os04g0227500}, \textit{Os5g0527300}, and \textit{Os03g0279700}) were slightly up-regulated (arrows in Figs. 1 and 4B). These results demonstrate that GA-dependent up-regulation of most genes are dependent on GID2 function. For the GA down-regulated genes, 48 genes corresponding to 78 probes were identified that deviated from the untreated control by more than threefold. Among these, we examined the expression of eight genes corresponding to 18 randomly selected probes that include \textit{RAmy3A} (presented in Fig. 1). These genes were down-regulated by GA in \textit{gid2} seeds to the level observed in GA treated WT seeds (Fig. 4B), whereas the expression of several genes (\textit{Os10g0517500}, \textit{Os03g0277600}, \textit{Os08g0327700}, and \textit{Os11g0582400}) was significantly but not completely down-regulated (see arrowheads, Fig. 4B). These results demonstrate that the expression of several genes is down-regulated by GA independent of GID2, in contrast to \textit{gid1} and \textit{slr1}.

We also examined the involvement of GAMYB (Fig. 5A). The probes were concentrated around the y-axis, with a considerable number of outliers in GA down-regulated genes (Supplemental Table S5). For GA up-regulated genes, there were 27 genes corresponding to 30 probes. Among these, expression of six up-regulated genes, namely, \textit{RAmy3E} (Fig. 1) and \textit{Os02g0740400}, \textit{Os04g0227500}, \textit{Os05g0527300}, \textit{Os03g0279700}, and \textit{Os04g0364800} (Fig. 5B), were examined by
qRT-PCR. In general, induction of these genes in GA-treated gamyb was lower than that observed in GA-treated WT seeds (arrowheads). These results suggest that most of the GA up-regulated genes depend on GAMYB function, although several genes are slightly up-regulated in the absence of GAMYB. As for GA down-regulated genes, there were 65 genes corresponding to 93 probes that deviated from the untreated control by more than threefold. In contrast to the up-regulated genes, down-regulated genes were broadly dispersed around the y-axis and beyond the y=x (red) line, although the y-axis had a greater density of probes (Fig. 5A). qRT-PCR was conducted on seven genes corresponding to 13 probes (dispersed around the y=x line, purple dots), and eight genes corresponding to 10 probes were located between y=x and x=-0.48 lines (> threefold difference, green dots). All genes clustering around the y=x line (purple dots) were down-regulated by GA in gamyb to the levels observed in GA-treated WT seeds, whereas the genes lying between y=x and x=-0.48 (green dots) were significantly but not completely down-regulated in gamyb (Fig. 5B). These results demonstrate that some genes can be down-regulated by GA in the absence of GAMYB.

Hierarchical Cluster Analysis of the GA-signaling Mutants

To elucidate the relationship of the four GA signaling components, we performed a cluster analysis of GA up- and down-regulated genes by hclust hierarchical clustering (Ben-Hur et al., 2002; Smolkin and Ghosh, 2003). The first group, Up-group1, contained the majority of GA up-regulated genes (435 genes), which includes the six GA up-regulated forms of RAMy (Supplemental Table S6). The Up-group1 genes were
generally not induced in the four mutants (Fig. 6A; see expression of two representative
genes denoted with purple lines, and Supplemental Figs. S4A and B); however, some
genes deviated by more than threefold (dashed lines in Fig. 6A). Outliers in the gid1 and
slr1 mutant backgrounds were directly examined by qRT-PCR as mentioned previously,
and confirmed that their expression was controlled by the GID1 and SLR1 (Figs. 2 and
3). In gid2, change in expression of RAmY genes sometimes deviated by more than a
three-fold increase (light blue lines in Fig. 6A). The observation is consistent with
qRT-PCR (Fig. 1), namely, RAmY3B, 3C and RAmY3E expression was partially induced
in gid2. The second group of GA up-regulated genes, Up-group2, was small and
contained only 12 genes with expression patterns similar to Up-group1 (Fig. 6B and
Supplemental Table S7). The expression patterns of two representative genes from
Up-group2 are shown in Supplemental Fig. S4C and D (also see purple lines in Fig. 6B).
Os04g0364800 showed significant down-regulation in the gamyb mutant in the
microarray analysis (arrowhead in Fig. 6B), but qRT-PCR demonstrated no change in
expression (arrowhead in Supplemental Fig. S4D). Although Up-group2 has two upward
(+) outliers in slr1, qRT-PCR analysis showed that their expression was not significantly
different than the WT (Fig. 3). Taken collectively, cluster analysis confirmed that most
GA up-regulated genes fall under strict control of the four GA-signaling components,
GID1, SLR1, GID2, and GAMYB.

In the case of GA down-regulated genes, 355 genes (including RAmY3D) were
assigned to Down-group1 (Supplemental Table S8), which is comprised of genes
showing no change in expression by GA in the four mutants (Fig. 6C). Two
representative genes (purple lines in Fig. 6C; Supplemental Fig. S5A and B) showed no expression change by GA in gid1, gid2, or gamyb, and not expressed in slr1, demonstrating that they are strictly regulated by GID1, SLR1, GID2, and GAMYB. However, there were some downward outliers in the slr1, gid2, and gamyb backgrounds. qRT-PCR demonstrated no actual change in the expression of the outlier, Os04g0179100, in slr1 (Fig. 3B), while the three most down-regulated genes in gid2 (green lines in Fig. 6C) were down-regulated at a level similar to the WT upon GA treatment (arrows in Supplemental Fig. S5C to 5E). On the other hand, the expression of Down-group2, which contains 53 genes (Supplemental Table S9), shows essentially the same pattern as Down-group1 (Fig. 6D). Actually, down-regulation of two representative genes did not occur in gid1, gid2, or gamyb, but constitutively occurred in slr1 (purple lines in Fig. 6D and Supplemental Fig. S5F and G). Several genes were downward outliers in the gamyb mutant, such as Os11g0138300 (arrow in Fig. 6D), and this was confirmed by qRT-PCR (Fig. 5B, probe #120).

Down-group3 contains 43 genes (Supplemental Table S10), which did not show significant differences with respect to GA treatment in the gid1, slr1, and gid2 mutants, but were down-regulated in gamyb (Fig. 6E). Down-regulation of two representative genes (purple lines) did not occur in gid1 or gid2 but occurred in gamyb, and were constitutively down-regulated in slr1 (arrows in Supplemental Fig. S6A and B).

Down-group4 contained only two genes (Supplemental Table S11), which were down-regulated in gid1 and gamyb in microarray analysis (Fig. 6F). However, qRT-PCR confirmed that down-regulation of these two genes occurred only in gamyb but not in
gid1 (arrows and arrowheads in Supplemental Fig. S6C and D, respectively). Thus the expression of Down-group4 is essentially the same as Down-group3.

Down-group5 is a small group that contains five genes (Supplemental Table S12), including RAm3A (light blue lines, Fig. 6G). As shown by analysis of two representative genes (Fig. 6G, purple lines), expression of Down-group5 was down-regulated in gid2 and gamyb (arrows in Supplemental Fig. S7A and B). Down-group6 contains 26 genes (Supplemental Table S13), and the expression pattern was similar to Down-group5 (Fig. 6H; Supplemental Fig. S7C and D). Down-group6 contained many genes that were down-regulated by GA by less than threefold in the gid2 and gamyb backgrounds, suggesting that these genes are not fully down-regulated in gid2 and gamyb. For example, expression analysis of two outlier genes (Fig. 6H, green lines; and arrowheads in Supplemental Fig. S7E and F), indicated that many genes in this group are partially down-regulated in the absence of GID2 and GAMYB.

Taken collectively, cluster analysis allowed us to briefly categorize GA down-regulated genes into three categories with respect to their dependence on GID2 and GAMYB. The first category includes genes that are regulated by GID1, SLR1, GID2, and GAMYB, and Down-group1 and 2 include many of these genes. The second category includes genes regulated by GID1, SLR1 and GID2, but not by GAMYB, and these are primarily assigned to Down-group3 and 4. The last category includes genes regulated by GID1 and SLR1 (but not by GID2 or GAMYB); these are generally present in Down-group5 and 6.
Several GA Suppressed Genes are Positively Regulated by SLR1

The above results demonstrate that a number of genes (including *RAmy3A*) within Down-groups 3, 4, 5 and 6 are down-regulated by GA without the aid of GAMYB. Recently, a novel DELLA function has been proposed where DELLA protein interacts with transacting factor(s) containing DNA-binding domain and enhances gene expression by targeting promoter sequences (Zentella et al., 2007; Yoshida et al., 2014). Based on this, we hypothesized that elevated gene expression in the absence of GA may depend on the transactivation activity of SLR1. To examine the effect of SLR1 on the expression of *RAmy3A*, we attempted a transient gene expression experiment. As an effector, we used SLR1 fused to the transactivation domain of VP16 (SLR1-VP16, Hirano et al., 2012) to enhance the transcriptional activity of SLR1. After SLR1-VP16 bombardment, the expression of endogenous *RAmy3A* was examined by qRT-PCR. Bombardment with SLR1-VP16 consistently increased the endogenous expression of *RAmy3A* relative to the vector control (Fig. 7). We also examined the effect of SLR1-VP16 on *RAmy3D* expression, largely because *RAmy3D* was significantly down-regulated by GA in the absence of GAMYB (Fig. 1). As expected, *RAmy3D* expression was enhanced by SLR1-VP16, and similar results were observed for four other GAMYB-independent, GA down-regulated genes (Fig. 7). This suggested that SLR1 functions as a positive transacting factor for expression of these genes. We also examined the effect of SLR1-VP16 on the expression of GA up-regulated *RAmy1A*, 1C, and 3C. Expression of *RAmy1B*, 3B, and 3E were low in GA-untreated seeds and thus were not investigated (Supplemental Fig. S8). Although expression of *RAmy1A*, 1C,
and 3C differed between experiments, bombardment with SLR1-VP16 consistently diminished their expression (Fig. 7).

DISCUSSION

GID1-DELLA is the Sole Mechanism for GA Perception in Rice Aleurones

In the present work, we investigated the potential existence of an alternative GA receptor as predicted by previous observations (Hooley et al., 1991; Gilroy and Jones, 1994). The alternative GA receptor was expected to have certain biochemical characteristics such as a plasma membrane location, the ability to perceive GA outside of aleurone cells, and the ability to induce hydrolytic enzymes such as α-amylase (e.g. RAmy) after GA perception (Hartweck and Olszewski, 2006; Ueguchi-Tanaka et al., 2007; Taiz and Zeiger, 2010). We examined GA-mediated induction of RAmy in rice aleurone cells using mutants for GID1 and the rice DELLA protein, SLR1. Six RAmy genes showed enhanced expression in the aleurones of WT seeds, whereas none were induced in gid1 seeds (Fig. 1). Furthermore, all genes were expressed in GA-untreated slr1 seeds at levels similar to GA-treated WT seeds (Fig. 1). For RAmy3A and RAmy3D, expression was reduced in the presence of GA and the expression of RAmy3A and RAmy3D in gid1 and slr1 mutants contrasted with other RAmy genes. In other words, RAmy3A and RAmy3D were not down-regulated in gid1, and their expression was low in slr1 even in the absence of GA (Fig. 1). These observations clearly demonstrated that GA-mediated expression of all RAmy genes in rice aleurone cells is strictly regulated by the GID1-DELLA system. We also performed a comprehensive analysis of GA-related...
gene expression by using microarrays, and confirmed by qRT-PCR that all GA up- and
down-regulated genes are regulated by the GID1-DELLA perception system (Figs. 2
and 3). Thus, the GID1-DELLA remains the only perception system known that can
mediate GA-dependent gene expression in rice aleurone cells.

Involvement of GID2 and GAMYB in GA-dependent Expression in Rice Aleurones

We also evaluated the involvement of GID2 and GAMYB in GA-dependent gene
expression in rice aleurone cells. In the case of up-regulated genes, expression was not
significantly up-regulated by GA treatment in the four mutants (Figs. 2 to 5), indicating
that their expression depends on GID1, SLR1, GID2 and GAMYB (Fig. 8A). In this
mechanistic model, the absence of GA allows SLR1 to suppress GAMYB and thus
prevents the expression of GA-inducible genes such as \textit{RAm}y1\textsl{A, B, and C}. In the
presence of GA, SLR1 is degraded through GID2, which results in the activation of
GAMYB. However, several genes were partially up-regulated in either \textit{gid2} or \textit{gamyb}
mutants (Figs. 4 and 5) or in both mutants ("overlapping genes"), including \textit{RAm}y3\textsl{C,}
\textit{RAm}y3\textsl{E, Os04g0227500, Os05g0527300, Os03g0279700, and Os03g0131200}
(Supplemental Tables S4 and S5). The high frequency of "overlapping genes" suggests
that an unknown mechanism may be responsible for the partial dependence on GID2
and GAMYB. This might depend on alternative transcription factor(s) (TF), which can
partially restore the function of GAMYB (Fig. 8B). The partial induction of "overlapping
genes" in \textit{gid2} indicates that such TF’s expression are induced upon GID1-SLR1
interaction, and that degradation of SLR1 is not essential. This is consistent with
previous reports that the suppressive function of DELLA proteins can be partially deactivated by the GID1-DELLA interaction (Ariizumi et al., 2008; Ueguchi-Tanaka et al., 2008). Thus, it is possible that the GID1-SLR1 interaction may allow activation of certain TFs, thus resulting in the induction of some GA up-regulated genes (narrow arrow in Fig. 8B).

In the case of down-regulated genes, the situation is more complicated. The expression of down-regulated genes is generally under control of the four GA signaling factors, which are primarily in Down-group1 and 2. Since GAMYB is considered to be a trans-activating factor (Gubler et al., 1995), the down-regulation of genes by GAMYB may be an indirect effect of GAMYB. One possibility is that GAMYB influences carbon metabolites, which are released by catabolic enzymes induced by GA via a GAMYB-mediated mechanism (Fig. 8C). It is well-established that carbon metabolites function to suppress genes in cereal aleurone cells (Chen et al., 2006). An alternative explanation could be that TFs exerting repressive activity on their target genes are positively regulated by GAMYB (Fig. 8C). In this respect, it is important to mention the existence of multiple TFs that are regulated by GAMYB (Supplemental Table S1).

Hierarchical cluster analysis (Fig. 6) clearly demonstrated that there are certain number of genes that are down-regulated by GA independent of GID2 or GAMYB, which is clearly different from the GA up-regulated genes. Recent studies suggest that the DELLA protein has two possible roles, and one of these functions is an inhibitory effect on the transcription inducing activity of targets such as PIFs, (de Lucas et al., 2008, Feng et al., 2008), MYC2 (Hong et al., 2012), and SPLs (Yu et al., 2012). In the
other role, DELLA promotes transcriptional activity by collaborating with other TFs carrying DNA-binding domains (Yoshida et al., 2014, Zentella et al., 2007). Moreover, the transactivation activity of SLR1 is important for inducing dwarfism in rice (Hirano et al., 2012). The present study showed that SLR1-VP16 enhanced the expression of some genes including RAmy3A and RAmy3D (Fig. 7). These observations suggest that SLR1 functions as a positive regulator for the expression of GA down-regulated genes.

Since DELLA proteins (including SLR1) have been considered to lack DNA-binding domains (DBD) (Sun et al., 2012; Davière and Achard, 2013), they would need to interact with a TF to modulate DNA binding (Fig. 8D and E). In accordance with this model, the down-regulation of genes by GA independent of GID2 could be explained by the inhibitory effect of GID1-SLR1 complex formation on the SLR1-TF interaction (Fig. 8E). In contrast, the down-regulation of genes that depend on GID2 suggests that SLR1 degradation is essential to diminish the SLR1-TF interaction (Fig. 8D). Previously, Zentella et al. (2007) comprehensively searched genes down-regulated by GA and up-regulated by DELLA, and identified 14 GA-related genes including GA20 oxidase (GA biosynthesis), GA3 oxidase (GA biosynthesis), and GID1s; these authors discussed the functions of DELLA with respect to feedback regulation in GA signaling.

Our findings suggest that genes up-regulated by SLR1 are not limited to those involved in GA feedback regulation, but also include a diverse array of genes (Supplemental Tables S8 to S13). Thus, it is possible that DELLA functions as a transactivator not only in GA feedback regulation but also in other biological processes.
MATERIALS AND METHODS

Plant Material

Seeds of WT (Oryza sativa cv. Nipponbare), gid1-4 (Ueguchi-Tanaka et al., 2005), gid2-5 (Sasaki et al., 2003), slr1-1 (Ikeda et al., 2001) and gamyb-2 (Kaneko et al., 2004) were used. Since the mutant plants carrying homozygous alleles are either lethal or sterile, PCR was used to screen for homozygous F2 seeds, and DNA from seed embryos was utilized as template. Half seeds lacking embryos were placed in incubation medium (10 mM sodium acetate, pH 5.2, 2 mM CaCl2; with or without 10⁻⁵ M GA₃) for 36 h at 30°C and then used for RNA extraction.

RNA Isolation and qRT-PCR Analysis

Total RNA was extracted from incubated embryoless half-seeds as described previously (Sambrook et al., 1989). RNA was then treated with Amplification Grade Deoxyribonuclease I (Invitrogen) and used for qRT-PCR experiments. More than three biological replicates containing independently-isolated RNA samples were analyzed. The first strand cDNA was synthesized from 1 µg of total RNA with the Omniscript reverse transcription kit (Qiagen). The resulting cDNA was diluted 1:20 and used as template; transcripts were quantified by qRT-PCR with a C1000™ Thermal Cycler (Bio-Rad) and a SYBR Green PCR kit (Qiagen). For quantification, a linear standard curve and threshold cycle number versus log (designated transcript level) were constructed from a series of diluted DNA fragments (10⁻¹⁷, 10⁻¹⁹, 10⁻²¹, and 10⁻²₃ M). Each cDNA sample was subjected to different cycles of PCR amplification (35 to 40
cycles) to confirm the linear pattern of PCR amplification for each gene. The rice *Ubiquitin (Ubi)* gene was used as an internal standard for normalizing cDNA concentration variations. The primer sequences used in this study are listed in Supplemental Table S14.

**Microarray Hybridization and Data Analysis**

Agilent 44K rice oligoarray (Agilent Technologies) containing 44,000 probes, was used for two-color analysis. Each probe consists of a 60-mer oligonucleotide corresponding to a full-length cDNA of rice. Four biological replicates consisting of independently isolated RNA samples were analyzed. All microarray experiments were performed according to the manufacturer's manual. The Feature Extraction software (Agilent Technologies) was used to delineate and measure the Cy3 and Cy5 signal intensities of each spot in the array. The resulting data were normalized using the Variance-stabilizing normalization (VSN) algorithm (Huber et al., 2002)(45). To identify differentially expressed genes between GA-untreated and -treated WT seeds, the normalized values of log2 signal ratios were analyzed using a simple non-parametric statistical method (RankProduct method), as described by Breitling et al. (2004). The P-value cutoff was set at 0.01, and the multiple testing was taken into account using the percentage of false prediction (*pfp* < 0.05) (Breitling et al., 2004; Hong et al., 2006). Under these conditions, we finally obtained 1,319 probes that corresponded to GA-regulated genes in WT seeds (Supplemental Table S1 and S2). All microarray data from this study were deposited in the GEO repository under accession code (XXXX).
Hierarchical Clustering

Array data were analyzed using hierarchical clustering with average linkage and ‘hclust’ (included in the R software package), which is based on the agglomeration method (Ben-Hur et al., 2002; Smolkin and Ghosh, 2003). The Pearson correlation coefficient was used for measuring similarity between gene expression profiles in all microarray data. The clustered dendrogram was divided using the ‘cut-tree’ function to classify genes according to the expression pattern.

Gene Ontology Analysis

Probes representing 615 and 704 GA up- and down-regulated genes, respectively, were used for GO analysis with agriGO (Du et al., 2010) (http://bioinfo.cau.edu.cn/agriGO/index.php). Fisher’s exact test was used to identify the enriched GO term(s) with an FDR-adjusted $P$ cutoff of $<0.05$. Boxes contained gene ontology terms and descriptions, FDR-adjusted $P$-values, the total number of GO annotated and background genes from GA-regulated genes, and the total number of GO annotated and background genes from the entire array.

DNA Bombardment

DNA bombardment was performed as described by Sutoh and Yamauchi (2003) using wild type (cv. Nipponbare) embryoless grain quadrisections derived from the same seed. For the construction of effector plasmid, we inserted the maize $Ubi$ promoter and $NOS$
terminator into the *HindIII/Xba* and *SacI/EcoRI* site of pUC19, respectively, to produce pUbi/pUC19 as a control vector. DNA sequences of SLR1-VP16 (Hirano et al., 2012) was then ligated into the *SmaI* site of pUbi/pUC19. About 1.5 pmoles of plasmid DNA were delivered into aleurone cells and incubated at room temperature for 16 h. For qRT-PCR, RNA was extracted from one embryoless grain quadrisection as mentioned above.

Supplemental Material

**Supplemental Figure S1.** Simplified model of GA up-regulated gene expression.

**Supplemental Figure S2.** qRT-PCR analysis of *RAmy2A* in embryoless half-seeds of *gid1, slr1, gid2,* and *gamyb*.

**Supplemental Figure S3.** Gene Ontology analysis of GA-regulated genes.

**Supplemental Figure S4.** Expression analysis of two representative genes in Up-group1 (A) and (B), and Up-group2 (C) and (D) by RT-PCR.

**Supplemental Figure S5.** Expression analysis of two representative genes in Down-group1 (A) and (B) and Down-group2 (F) and (G) by qRT-PCR.

**Supplemental Figure S6.** Expression analysis of two representative genes in Down-group3 (A) and (B), and Down-group4 (C) and (D) by RT-PCR.

**Supplemental Figure S7.** Expression analysis of two representative genes in Down-group5 (A) and (B) and Down-group6 (C) and (D) by qRT-PCR.

**Supplemental Figure S8.** Expression analysis of endogenous *RAmy1B, 3B,* and *3E* in comparison to endogenous *Ubi* expression in embryoless half-seeds in the absence of
GA by qRT-PCR.

**Supplemental Table S1.** List of GA up-regulated probes.

**Supplemental Table S2.** List of GA down-regulated probes.

**Supplemental Table S3.** List of probes up- or down-regulated by GA by more than 3-fold in the slr1.

**Supplemental Table S4.** List of probes up- or down-regulated by GA by more than 3-fold in the gid2.

**Supplemental Table S5.** List of probes up- or down-regulated by GA by more than 3-fold in the gamyb.

**Supplemental Table S6.** List of probes categorized under Up-group1.

**Supplemental Table S7.** List of probes categorized under Up-group2.

**Supplemental Table S8.** List of probes categorized under Down-group1.

**Supplemental Table S9.** List of probes categorized under Down-group2.

**Supplemental Table S10.** List of probes categorized under Down-group3.

**Supplemental Table S11.** List of probes categorized under Down-group4.

**Supplemental Table S12.** List of probes categorized under Down-group5.

**Supplemental Table S13.** List of probes categorized under Down-group6.

**Supplemental Table S14.** List of primers used in this study.

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Figure Legends

**Figure 1.** Expression of RAmy genes in *gid1, slr1, gid2*, and *gamyb* mutant seeds. Embryoless half-seeds of WT and the four GA-related mutants were incubated with (+) or without (-) $10^{-5}$ M GA$_3$ for 36 h. RAmy expression was normalized by the Ubi expression in each sample and shown in comparison to that in GA-untreated WT seeds. Arrows and arrowheads indicate induction or suppression of RAmy genes by GA in *gid2* and *gamyb*, respectively. The lack of suppression of RAmy3D in *gid2* is also indicated by an arrow. The data represent means ± SD of four replicates.

**Figure 2.** Contribution of GID1 in GA-dependent gene expression. (A) RNAs were independently extracted from WT and *gid1* embryoless half-seeds and used for microarray analysis. The y-axis represents log$_{10}$ ratios of the signal intensities of corresponding probes in GA-treated and untreated WT seeds, whereas the x-axis shows that of *gid1* seeds. Probes that were up- or down-regulated by GA more than threefold (±0.48 differences in log$_{10}$ ratios shown by dashed lines) in *gid1* are noted as numbered, black dots. RAmy3B (probe #1) is indicated. (B) List of probes exceeding ±0.48 differences in log$_{10}$ ratios in *gid1*. A single gene is often represented by more than one probe. WT±GA and *gid1±GA* represent fold differences in log$_{10}$ values between GA-treated and untreated seeds of WT and *gid1*. The annotation for each gene is derived from RAP-DB (http://rapdb.dna.affrc.go.jp/). (C) qRT-PCR analysis of the genes listed in panel B is shown as the same manner in Fig. 1. The numbers at the upper left side of each graph correspond to the same probes presented in panel A. The qRT-PCR
result for *RAmy3B* is shown in Figure 1. The data presented are means ± SD of four replicates.

**Figure 3.** Contribution of SLR1 to GA-related gene expression. (A) Data are presented essentially as shown in Fig. 2A, except the x-axis shows the signal intensity change in *slr1* seeds. Table indicating *RAmy*s and their corresponding probes is superimposed on (A). (B) qRT-PCR analysis of the genes corresponding to numbered probes in (A) is shown as the same presentation in Fig. 2C. The qRT-PCR results for *RAmy1C* and *3B* were presented in Fig. 1. The data represent means ± SD of four replicates.

**Figure 4.** Contribution of GID2 to GA-related gene expression. (A) The data are presented as shown in Fig. 2A, except the x-axis shows the signal intensity change in *gid2* seeds. Probes examined by qRT-PCR (16 of 22 probes representing up-regulated genes; 18 of 78 probes for down-regulated) are demarcated with numbered, black dots. (B) qRT-PCR expression analysis of genes corresponding to numbered probes in (A) is shown as the same presentation in Fig. 2C. Arrows and arrowheads indicate partial induction or incomplete suppression by GA in *gid2*, respectively. The data presented are means ± SD of four replicates.

**Figure 5.** Contribution of GAMYB in GA-related gene expression. (A) The presentation is essentially the same as Fig. 2A, except the x-axis shows the signal intensity change in *gamyb* seeds. Probes examined by qRT-PCR are presented as black (up-regulated),
purple (down-regulated, dispersed around y=x line), and green (down-regulated, dispersed between y=x and x=-0.48) numbered dots. (B) qRT-PCR expression analysis of genes corresponding to numbered probes in (A) is shown as the same presentation in Fig. 2C. Arrowheads indicate partial induction in gamyb. The data presented are means ± SD of four replicates.

Figure 6. Expression profiles classified by hierarchical cluster analysis. The y-axis represents log_{10} ratios of the signal intensities of corresponding probes of GA-treated and untreated seeds of plants indicated in the x-axis, namely, WT, gid1, slr1, gid2, and gamyb. Threefold differences in intensity (±0.48 differences in log_{10} ratio) are indicated as dashed lines. Purple lines represent the expression profiles of two representative genes for each group, which were confirmed by qRT-PCR (Supplemental Figs. 4-7). The expression profiles of RAmy genes are shown as light blue lines in panel (A), RAmy1A, 1B, 1C, 3B, 3C, and 3E and RAmy3D in panel (C), and RAmy3A in panel (G). Green lines in panels (C) and (H) represent the expression profiles of genes having obvious downward outliers in gid2 and/or gamyb, which were confirmed by qRT-PCR (Supplemental Figs. 5 and 7). The arrowhead in panel (B) shows the obvious downward outlier Os04g0364800, which was confirmed by qRT-PCR (see Supplemental Figure 4D). The arrow in (D) shows Os11g0138300, an obvious downward outlier that was confirmed by qRT-PCR as presented in Fig. 5B (probe #120).
Figure 7. Bombardment with SLR1-VP16 enhances the expression of *RAmy3A*, *RAmy3D*, and other GAMYB-independent GA-down regulated genes in aleurone cells. SLR1-VP16 (black bars) and a control plasmid (white bars) were independently bombarded into aleurone cells and the expression of endogenous genes as revealed by qRT-PCR are shown in comparison to endogenous *Ubi* expression. The effect of SLR1-VP16 on expression of *RAmy1A*, *RAmy1C*, and *RAmy3C*, which are up-regulated by GA, was also observed.

Figure 8. Proposed models for GA-mediated gene expression involving GID1, SLR1, GID2 and GAMYB in rice aleurone cells. (A) Up-regulation by GA as mediated by GID1, SLR1, GID2 and GAMYB. (B) Up-regulation by GA partially controlled by GID2 and GAMYB. (C) Down-regulation by GA under the control of GID1, SLR1, GID2 and GAMYB. (D) Down-regulation by GA under the control of GID1, SLR1, and GID2 but not GAMYB (GID2 is essential for down-regulation). (E) Down-regulation by GA under the control of GID1 and SLR1, but not GID2 nor GAMYB. The typical expression pattern for each group is presented below each model.

Supplemental Figure Legends

Supplemental Figure S1. Simplified model of GA-regulated gene expression in rice aleurone cells.
Supplemental Figure S2. qRT-PCR analysis of RAmy2A in embryoless half-seeds of gid1, slr1, gid2, and gamyb. Experimental conditions are described in Fig. 1. The data presented are means ± SD of four replicates.

Supplemental Figure S3. Gene Ontology analysis of GA-regulated genes. GO analysis by agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) with respect to MF, CC, and biological process (BP) for GA up- or down-regulated genes. The enrichment level of the term is indicated by the color intensity of boxes. Solid, dashed, and dotted lines represent two-, one- and zero- enriched terms between connected boxes, respectively.

Supplemental Figure S4. Expression analysis of two representative genes in Up-group1 (A) and (B), and Up-group2 (C) and (D) by RT-PCR. The arrowheads in panel (D) show no GA-mediated changes in the expression of Os04g0364800 by GA, although the corresponding array data indicated significant down-regulation (arrowhead in Fig. 6B). The data presented are means ± SD of four replicates.

Supplemental Figure S5. Expression analysis of two representative genes in Down-group1 (A) and (B), and Down-group2 (F) and (G) by qRT-PCR. The expression of three genes having obvious downward outliers in gid2 (green lines in Fig. 6C) was also analyzed (arrows in panels C-E). The data presented are means ± SD of four replicates.
Supplemental Figure S6. Expression analysis of two representative genes in Down-group3 (A) and (B), and Down-group4 (C) and (D) by RT-PCR. The down-regulation of genes in gamyb is indicated by arrows. Although array data showed downward outliers in Down-group4 in gid1 (Fig. 6F), down-regulation in gid1 was not detected by qRT-PCR (arrowheads in panels C and D). The data presented are means ± SD of four replicates.

Supplemental Figure S7. Expression analysis of two representative genes in Down-group5 (A) and (B) and Down-group6 (C) and (D) by qRT-PCR. Down-regulation of genes in gid2 and gamyb are indicated by arrows. The expression of genes not fully down-regulated in gid2 or gamyb (green lines in Fig. 6H) is indicated by arrowheads in panels (E) and (F). The data presented are means ± SD of four replicates.

Supplemental Figure S8. Expression analysis of endogenous RAm1B, 3B, and 3E in comparison to endogenous Ubi expression in embryoless half-seeds in the absence of GA by qRT-PCR.
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Figure 4. Contribution of GID2 to GA-related gene expression. (A) The data are presented as shown in Fig. 2A, except the x-axis shows the signal intensity change in gid2 seeds. Probes examined by qRT-PCR (16 of 22 probes representing up-regulated genes; 18 of 78 probes for down-regulated) are demarcated with numbered, black dots. (B) qRT-PCR expression analysis of genes corresponding to numbered probes in (A) is shown as the same presentation in Fig. 2C. Arrows and arrowheads indicate partial induction or incomplete suppression by GA in gid2, respectively. The data presented are means ± SD of four replicates.
Figure 5. Contribution of GAMYB in GA-related gene expression. (A) The presentation is essentially the same as Fig. 2A, except the x-axis shows the signal intensity change in *gamyb* seeds. Probes examined by qRT-PCR are presented as black (up-regulated), purple (down-regulated, dispersed around y=x line), and green (down-regulated, dispersed between y=x and x=-0.48) numbered dots. (B) qRT-PCR expression analysis of genes corresponding to numbered probes in (A) is shown as the same presentation in Fig. 2C. Arrowheads indicate partial induction in *gamyb*. The data presented are means ± SD of four replicates.
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