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Running Title:

Gene regulation by gibberellin in rice aleurone

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Common mechanisms regulating expression of rice aleurone genes that contribute to the primary response for gibberellin

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Abstract

During germination of cereal seeds, aleurone cells respond to gibberellins (GA) by synthesizing and secreting hydrolytic enzymes that mobilize the reserved nutrients. It has been shown that products of early GA response genes, like a transcription factor GAMyb, act as key molecules leading to this regulation. Pivotal roles of GAMyb on expression of hydrolase genes have been well documented, whereas regulation of *GAMyb* expression itself remains obscure. In order to understand virtual mechanisms of the GA-mediated expression of genes, it is important to know how GA control expression of early GA response genes. Using an aleurone transient expression system of rice (*Oryza sativa* L.), we examined GA responsive domains of early GA response genes in the aleurone, such as *GAMyb* and *OsDof3*. The 5' upstream region could not confer GA response. Extensive analyses revealed the presence of enhancer-like activities in a large first intron. In Arabidopsis, intron enhancers have been identified in MADS-box homeotic genes, *AGAMOUS* (*AG*) and *FLOWERING LOCUS C* (*FLC*), in which large introns should not only confer proper gene expressions, but also associate with gene silencing by covalent modifications of both DNA and histone. These evidences prompt us to assign that chromatin-based control might be important for initial GA action. Based on this assumption, we have identified DNA methylation of the *GAMyb* locus in germinated rice seeds.

1. Introduction

Gibberellins (GA) are tetracyclic diterpenoid hormones essential for many aspects of plant growth and development, such as seed germination, stem elongation, leaf expansion, flower initiation and anther development [1, 2]. Molecular genetic analyses of the GA-related mutants have identified crucial components of GA signaling [3, 4]. The DELLA proteins, a subgroup of the GRAS family of putative transcription factors [5], are located in the nucleus and rapidly disappeared after GA application [6, 7, 8]. Loss-of-function mutations in the *DELLA* gene produce a constitutive GA response phenotype in some cases [9, 10]. Expression of the DELLA protein lacking the DELLA domain, which is a constitutive repressor form, shows a dominant GA-insensitive phenotype [9, 10, 11, 12]. These results provide a reliable concept that the DELLA proteins are robust repressors of GA signaling and the functions of GA are established by removing the repressions of the DELLA proteins [6, 13].

Other GA signaling components illustrate molecular events that involve the degradation of the DELLA proteins. The F-box protein is subunit of a SCF E3 ubiquitin ligase complex that plays a key role for substrate specificities in the 26S proteasome pathway [14]. Mutations in both Arabidopsis *SLY1* [15] and rice *GID2* [16], encoding GA-related F-box

proteins, result in a GA-insensitive dwarfism. In these mutants, DELLA proteins accumulate at high-levels in nuclei even in the presence of GA. These findings indicate that this F-box protein class is positive regulators of GA signaling and capable of destabilizing the DELLA proteins via proteolysis. Recently, an initial factor of GA signaling has been proposed. Ueguchi-Tanaka *et al.* identified a recessive rice mutant *gid1* that showed a strong GA-insensitive dwarfism [17]. Mutant phenotypes suggest that GID1 is able to destabilize the DELLA protein and acts as a positive regulator of GA signaling. The GID protein directly binds to bioactive GA and interacts with rice DELLA protein in a GA-dependent manner. Detailed mechanisms of GID1 that recruit the DELLA protein into proteolysis pathway are still elusive; however, several lines of evidence indicate that GID1 is a functional receptor for GA.

The observations with several plants suggest that the DELLA proteins have a highly conserved role in GA signaling across higher plants [18], whereas organizations of the *DELLA* genes are various in different species. Arabidopsis has five distinct genes in the DELLA subfamily (*GAI*, *RGA*, *RGL1*, -2 and -3), from which encoded negative regulators of GA signaling appear to have partially redundant and overlapping functions [19, 20]. For example, *GAI*, *RGA* and *RGL1* are negative regulators of stem elongation [11, 19, 21, 22]. *RGL2* mainly controls seed germination [20, 23]. Set of *RGA*, *RGL1* and *RGL2* seem to function as major repressors of floral development

[23, 24]. In contrast to Arabidopsis, GA response system of small cereal plants is simple. Each of rice and barley has a single DELLA gene, *SLR1* and *SLN1*, so that recessive mutations of each gene exhibit solo deficiencies in the GA-regulated growth [9, 10]. Upon application of GA, these DELLA proteins disappear in aleurone [8], young leaf [7] and anther [25], but visible physiological responses are differing in each tissue. These observations raise a fundamental question of why unique SLR1 and SLN1 could generate various processes of such plant growth that are comparable to Arabidopsis.

The response of cereal aleurone cells to GA is one of the most understood hormone responses in plants [26]. In the germinated cereal seeds, GA, which are occurred in embryo, penetrate into the aleurone layer and induce the production of hydrolytic enzymes, such as α -amylase and protease. Induced enzymes are secreted into the starchy endosperm, where they degrade and mobilize the stored nutrients to support seedling growth. R2R3-type Myb transcription factor GAMyb is proposed to be an important regulator of GA action in cereal aleurones [27]. GAMyb specifically binds to the GA-responsive element of hydrolase genes and *trans*-activate their expressions. Loss-of-function mutations in rice *GAMyb* have recently shown to abolish the GA-mediated induction of α -amylase gene in the aleurone and impair in floral development, particularly, in pollen development [28]. Overexpression of barley *GAMyb* perturbs normal anther development [25]. These genetic proofs demonstrate that GAMyb is a true GA signaling

component essential for seed germination and floral organ development.

Although the processes of hydrolase induction in the aleurone have been well understood, many questions remain as to how GA control expression of *GAMyb*. Previous analyses in barley aleurone reveal that *GAMyb* expression is stimulated by GA at early stage of germination [27]. After the treatment of GA, SLN1 degradation occurs rapidly prior to *GAMyb* expression. The level of *GAMyb* expression is decreased in a dominant dwarf allele of *SLN1* [8]. Temporal and reciprocal appearances observed between *GAMyb* and SLN1 suggest that *GAMyb* is likely a candidate target for the DELLA protein. If functional linkage between GA signaling and gene functions that trigger a specific GA action could be resolved, it would be expected to extend our understanding for other processes of the GA-regulated plant growth. In this regard, we preliminary employed a transient expression system in rice aleurone and made an attempt to identify regulatory elements required for the GA responsibility of rice *GAMyb*.

2. Materials and methods

2.1. Plant material and growth conditions

Dehusked and embryo-less one-half-seeds of mature rice seeds (*Oryza sativa* cv Yukihihikari) were sterilized with 2.5% (w/v) sodium hypochloride for 20

min, rinsed extensively with sterilized water, and immersed in incubation solution composed of 20 mM CaCl₂, 10 µg mL⁻¹ chloramphenicol, 10 µg mL⁻¹ ampicillin and 25 units mL⁻¹ nystatin. After incubation in the dark at 30 °C, germinated seeds were dissected into seedling, root, scutellum and endosperm containing the aleurone layer at d 2.5. Embryo-less half-seeds were incubated for different times in 10⁻⁵ M GA₃ or at various hormonal conditions for 2.5 d. Samples were immediately frozen in liquid nitrogen.

2.2. RT-PCR analysis

Total RNA was extracted from frozen samples by the standard SDS-phenol method [29]. One microgram of DNase-treated RNA from different tissues was used to synthesis first-strand cDNA using random primer and reverse transcription system (Promega, Madison, USA). After completion of RT, 1 µl of RT products was amplified using each cDNA specific primer (see Supplementary Data online). PCR amplifications were performed in a final volume of 25 µl using Ex Taq DNA polymerase (Takara Bio, Shiga, Japan). The PCR condition was 5 min at 94 °C, followed by 28 cycles (*18S rRNA* and *RAmy1A*) or 35 cycles (*GAMyb*, *RPBF* and *SLR1*) of 30 sec at 94 °C, 30 sec at 55 °C and 90 sec at 72 °C, followed by 5 min at 72 °C. The PCR products were run on 3% agarose gel in TBE (89 mM Tris-borate, 2 mM EDTA) and visualized by ethidium staining. *18S rRNA* was used as an internal control

to normalize each of templates. The linear range of accuracy for the detection of each transcript was determined by comparing samples at different number of cycles.

2.3. Transient gene expression in germinated rice aleurone

Rice genomic DNA was isolated according to the method by Rogers and Bendrich [30]. Each fragment of the *GAMyb* and *RPBF* regions (-1095 to +953, -966 to +2019) was generated from the genomic DNA by PCR amplification. The amplified fragment was ligated with a gene cassette that carried a firefly luciferase gene (*F-luc+*; Promega) and the 3' terminator of a nopaline synthetase gene (*Nos-t*). The ligated fragments were cloned between restriction sites for *Hind* III and *Eco* RI in the plasmid pUC18 and used as original *GAMyb* and *RPBF* reporter constructs. A series of deletion mutants of the *GAMyb* and *RPBF* promoters were generated by PCR reactions from parental constructs. After the amplified fragments had been ligated with the *F-luc+::Nos-t* gene cassette, the ligated fragments were cloned into pUC18.

To construct reporter plasmids for gain- or loss-of-function analyses (Figure 3, 4), each requisite fragment from the upstream promoter (-482 to -91 in *GAMyb*, -335 to -91 in *RPBF*), the core promoter (-90 to +1, -90 to +1), exon 1 (+2 to +290, +2 to +128) and intron 1 (+291 to +953, +129 to +2019)

regions was amplified by PCR from parental constructs as the template. PCR fragments were replaced with or inserted into the corresponding sites of the cauliflower mosaic virus 35S (*35S*) reporter construct, using the 5'-terminal (*Hind* III), the internal (*Eco* RV) and the 3'-terminal (*Bam* HI) restriction sites for the *35S* promoter. Nucleotide substitutions on the *GAMyb*, *RPBF* and *35S* promoters shown in Fig. 5 were generated by a PCR-based oligonucleotide-directed mutagenesis [31].

Particle bombardment was carried out with a biolistic helium gun device (IDERA GIE-III; Tanaka Co., Ltd, Sapporo, Japan) as described previously [32, 33]. After bombardment, embryo-less one-half-seeds were grown in the dark at 30 °C in the absence or the presence of 10⁻⁵ M GA₃ for 2.5 d. F-Luc+ activity was determined by total light units emitted from each reporter reaction following the manufacturer's instructions (Dual-luciferase reporter assay system; Promega). Values of F-Luc+ activity were normalized with respect to *Renilla* luciferase activity due to the internal control [32]. Each reporter activity was assayed at least three times and averaged.

2.4. Bisulfite genomic sequencing

Bisulfite treatment was performed as described [34] with some modifications. Genomic DNA (30 µg) from each tissue of 2.5-d-germinated rice seeds was digested completely with restriction enzymes that cut outside of the *GAMyb*

region (*Pst* I, -2259 to +7; *Dra* I, -406 to +870). Digested products were separated on 1% agarose gel and purify DNA of objective sizes using QIAquick gel extraction kit (Qiagen Sci., Maryland, USA). DNA (0.5 μ g) in 19 μ l of water was denatured at 97 °C for 5 min. After quenched on ice, 6 N NaOH (1 μ l) was added and incubated at 37 °C for 15 min. Pre-warmed bisulfite solution (55°C; 107 μ l of 4.04 N sodium bisulfite (Sigma, S-9000), 7 μ l of 10 mM hydroquinone and 6 μ l of 6 N NaOH) was added to the DNA solution, mix gently, and overlaid with mineral oil. The bisulfite conversion was conducted using 5 cycles of 95 °C for 5 min, 55 °C for 3 hr. After bisulfite treatment, DNA was desalted with Wizard DNA clean-up system (Promega) following the manufacturer's instructions and recovered in 47.5 μ l of water. 6 N NaOH (2.5 μ l) was added to the DNA solution and incubated at 37 °C for 15 min. DNA was then recovered by ethanol precipitation with glycogen carriers and 3 M NH₄OAc and dissolved in 25 μ l TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

A 2 μ l aliquot of the bisulfite treated DNA was used for each 50 μ l volume of PCR reaction with a specific set of primers for the *GAMyb* region of interest (see Supplementary Data online). PCR products were cloned and 16 individual clones from each tissue were sequenced using Big Dye terminator cycle sequencing on an ABI sequencer according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems).

3. Results

3.1. Expression of the GA-regulated genes in germinated rice seeds.

Transcription factor GAMyb is a central regulator of hydrolase genes in rice aleurone [28, 35]. Before a detailed promoter analysis of *GAMyb* was taken place, we obtained an overview of the expression pattern of genes involved in GA action in rice aleurone. The expression level of genes in different tissues of germinated seeds or at various conditions of hormonal treatments in the aleurone was compared using semiquantitative RT-PCR (Fig. 1). *RAmy1A*, encoding an α -amylase, is one of the downstream targets of GA signaling in rice aleurone [36]. The mRNA accumulation of *RAmy1A* was markedly abundant in scutellum and aleurone tissues in 2.5-d-germinated seeds. After treating the aleurone with GA, the *RAmy1A* mRNA was visible at 12 h and reached to a high-level at d 1. The stimulating effect by GA decreased by antagonistic hormone, abscisic acid (ABA). *GAMyb* showed a spatial pattern of expression similar to *RAmy1A*, although it temporarily preceded *RAmy1A* expression in the GA-treated aleurones. This observation further confirms that *GAMyb* is an early gene conferring primary responses in the GA-treated cells.

GA response in the aleurone seems not to be controlled by GAMyb alone, but also involve the combinatorial interactions with other regulatory

proteins. We previously showed that Dof-type transcription factor OsDof3, referred to RPBF (rice prolamine- or pyrimidine-box binding factor) in this study, had an accessory role as a transcription cofactor with GAMyb [33]. The *RPBF* transcript was preferentially expressed in the GA-treated aleurones and appeared at early stage of germination. High-level expressions of *RAmy1A* were consistent with the sites where *GAMyb* and *RPBF* were coordinately expressed. This indicates that these factors function as a unit of initial regulators in GA action. We also examined the pattern of expression of the *SLR1* gene, encoding a rice DELLA protein. *SLR1* mRNA levels were ubiquitous in almost all tissues tested and seemed to be slightly changed in response to GA in the aleurone. The expression pattern of *SLR1* supports an idea that GA signaling cascade is working in the seedling as well as in the aleurone and high-response of aleurone cells to GA is not controlled by *SLR1* transcription or steady-state amounts of *SLR1* mRNA [37].

3.2. Gene structure of rice *GAMyb* and *RPBF* genes

We searched for genes encoding *GAMyb* and *RPBF* in the rice genome database. Each of *GAMyb* and *RPBF* is found as a single gene per haploid genome (Fig. 2). The basis for gene structure of rice *GAMyb* has already been known [38]. *GAMyb* is mapped at the middle of the long arm of chromosome 1 and contains four exons and three introns with sizes of 653, 1257 and 70

base pair (bp). Three kinds of *GAMyb* transcript are found in the database, due to splicing variants at intron 3 or different transcription initiations. *RPBF* is located on chromosome 2. Comparison with the *RPBF* cDNA [32] and genomic DNA sequences showed the transcribed region interrupted by a single intron. The site of transcription initiation was determined by PCR-based primer extension analysis using total RNA from germinated seeds (data not shown). The initiation site for *GAMyb* transcription was mapped at distal and proximal positions (-953 and -1051 from the ATG start codon). *RPBF* had a major transcription start site at 2019 bp upstream from the ATG start codon. It is noteworthy that both of *GAMyb* and *RPBF* have a relatively large size intron (653 bp in *GAMyb*, 1881 bp in *RPBF*) in the 5'-untranslated region (5'-UTR) that is just prior to the ATG start codon.

3.3. The regulatory regions from GAMyb and RPBF showed GA-dependency in transfected rice aleurone cells

The GA-regulated promoter of hydrolase genes has been studied extensively using a transient expression system in the aleurone. Gomez-Cadenas *et al.* previously proposed a moderate but reliable GA induction from the reporter plasmid that contained 1031 bp promoter and the first intron of barley *GAMyb* [39]. To test the abilities of the *GAMyb* and *RPBF* promoters, helium gas-mediated transfection of a reporter gene into rice aleurone was carried

out. The 5' regulatory region from *GAMyb* and *RPBF* extending from -1095 to +953 and -966 to +2019, respectively, was fused to the firefly luciferase coding region. The constructs were cobombarded into embryo-less half-seeds along with an internal control construct, *Renilla* luciferase (Fig. 2). Each of the *GAMyb* and *RPBF* constructs generated 3.3- and 9.3-fold higher level of the reporter activity in the GA-treated aleurones than those of the untreated controls. The transient expression results are consistent with our own RNA gel blot findings [33], which showed moderate and tight regulation in each expression of *GAMyb* and *RPBF* in response to GA. The genomic regions used in the study are likely to contain most *cis*-acting elements required for correct GA responses of *GAMyb* and *RPBF*.

To determine the 5' boundary of the *GAMyb* and *RPBF* promoters, a series of 5'-terminal deletions was made. The relative increase in the GA-induced reporter activity was similar in all *GAMyb* deletions, except for one construct. Only the removal of the region between -117 and -1 caused a loss of GA response. Truncated versions of the *RPBF* promoter with deletions to positions -966, -682 and -335 directed strong GA inductions. Deletion to position +1 almost abolished promoter activity. These observations reveal the presence of GA-responsive domains downstream of -117 of *GAMyb* and -335 of *RPBF*. The -117 region of *GAMyb* is very close to a position -98, which is the 5' end of the longest *GAMyb* cDNA. To cover the dual mode of *GAMyb* transcription, the 5' deletion constructs with -482 of *GAMyb* and -335 of

RPBF were chosen for subsequent experiments.

3.4. The GAMyb and RPBF promoters have independent domains for the GA-mediated expression in the aleurone

To dissect control regions downstream of the *GAMyb* and *RPBF* promoters, we employed comparative studies with a general *35S* promoter for GA response (Fig. 3). The 5' regulatory region was divided into functional domains; namely, the upstream promoter (upstream of -90), the core promoter (downstream of -90), the first exon (exon 1) and the first intron (intron 1), and combinations of each domain were replaced with or introduced into two functional domains of the *35S* promoter that had been proposed by Benfey *et al.* (domain B, -343 to -91; domain A, -90 to +8) [40]. When the upstream promoters from *GAMyb* and *RPBF* were placed upstream of domain A of the *35S* promoter, the absolute levels of the reporter activity decreased by roughly 20-30% compared with the wild-type *35S* construct, whereas they showed no response to GA (GMD-1, RPD-1 in Fig. 3). The constructs by replacing domain A with the core promoter plus exon 1 regions reduced the reporter activity to 12% levels of reporter expression driven by the parental *35S* promoter in the absence of GA (GMD-2, RPD-2). The GA treatment overcame these reductions in some extents, for which weak responses to GA were found. We observed

dramatically higher levels of the reporter expression from the constructs that contained the exon 1 plus intron 1 regions (GMD-3, RPD-3). The exon 1 plus intron 1 region from *GAMyb* and *RPBF* elevated the reporter activity 6.8- and 12.3-fold, respectively, and application of GA further stimulated both reporter expressions by 3.2-fold. Taken together, these observations imply that the upstream promoter of *GAMyb* and *RPBF* should not confer GA response. Two or more dispersed elements are probably required for proper GA response of *GAMyb* and *RPBF*. In addition, the location of the control region within a large intron 1 raises the possibility that it functions either as a transcriptional enhancer or as a post-transcriptional determinant of RNA abundance.

For the purpose of comparing the relevance among dispersed control regions, a single domain of the *GAMyb* and *RPBF* promoters were replaced with that of the *35S* promoter (Fig. 4). When domain B from *35S* was fused upstream of -90 in *GAMyb* and *RPBF*, overall levels of the reporter activity increased ca. 4- to 6-fold in both control and GA treatments (GMD-2, 3, RPD-2, 3). These constructs therefore showed normal patterns of hormonal control expected of native promoters. The result demonstrates that regulatory elements downstream of -90 in *GAMyb* and *RPBF* are essential for GA response. The constructs replacing the core promoter with domain A of *35S* significantly elevated the reporter activity, but poorly responsive to GA (GMD-1, 3, RPD-1, 3). It could be concerned several reasons for these

results that domain A contained positive elements and acted as enhancer, or that negative effects present in intact promoters were moved away by replacement. Although the reporter construct containing domain A only did not produce high-level of reporter expression in our previous study [32]. Benfey *et al.* proposed a tandem repeat of the sequence, TGACG, in domain A that had been shown to interact synergistically with other enhancer sequences to activate transcription [40]. The possibility that domain A from the *35S* promoter significantly affects *GAMyb* and *RPBF* promoter activities could not be ruled out.

Lack of both intron 1 regions of *GAMyb* and *RPBF* significantly decreased absolute levels of reporter expression (GMD-1, 2, RPD-1, 2). In case of *RPBF*, not only the basal level of reporter expression but also GA-responsibility was reduced. The lines of experiments performed in this study depicted similar properties in the regulatory regions of *GAMyb* and *RPBF* besides intron 1. The intron 1 from *RPBF* possessed higher abilities to stimulate reporter expression and therefore *RPBF* constructs with intron 1 often proposed better responsibilities to GA than those of *GAMyb* (Fig. 2-4). These observations may measure a way of regulation by multiple and different control regions that together influence *GAMyb* and *RPBF* expressions. Core promoter configurations act as negative domains and restrict the expression, whereas intron 1 is not simply *cis*-acting enhancers but also confers high-responsiveness to GA. GA-responsibilities of *GAMyb* and *RPBF* are

likely achieved by synergistic interactions with all or subset of these regulatory regions.

3.5. Normal expressions of GAMyb and RPBF are under negative regulation by the core promoters

We divided 90-bp of the *GAMyb* and *RPBF* core promoters into six parts by 15-bp and created constructs that had sequential mutations, by converting each part to a *35S* version (Fig. 5). The most profound effects were caused by mutations upstream of TATA box-like sequences, regardless of whether no pivotal effects were found with other mutations (data not shown). The *GAMyb* construct carrying a mutation from -60 to -46 showed strong increase in basal reporter activities (GMS-1), but no effect was detected in a mutation from -45 to -31 (GMS-2). The negative domain of the *RPBF* promoter is overlapping but not identical to that contained in *GAMyb*. The reporter activity conferred by a mutation from -60 to -46 increased the *RPBF* promoter activity to 6- and 2-fold without or with GA treatment (RPS-1). The magnitude of the reporter increase became remarkable in a mutation from -45 to -31 (RPS-2). The observations indicate that a set of sequences from -60 to -31 was required for effective suppressions in *RPBF*, whereas sequences from -45 to -31 are sufficient to repress the *GAMyb* promoter. None of the *35S* fragments introduced into the *GAMyb* and *RPBF* promoters contained

TGACG sequences that had been defined as pivotal *cis*-acting enhancer elements [40]. This evidence confirms an earlier assumption that positive elements are not acquired from the *35S* promoter, but the segment removed contains *cis*-elements that confer negative effects.

We identified cognate sequences matching canonical CCAAT box in identified regulatory regions. To test whether the CCAAT box may also serves as a negative element, each sequence from *GAMyb* and *RPBF* was introduced into a position in order that the original distance from the TATA box had been restored and tested its effect on the *35S* promoter (CMS-1, CMS-2). In the absence of GA, each construct exchanging the CCAAT box to *GAMyb* and *RPBF* versions exhibited 39.3% and 77.9% levels of reporter expression driven by the original *35S* promoter. Application of GA increased both reporter expressions by roughly 3-fold. The effects of replacement were qualitatively similar but quantitatively dissimilar between the *GAMyb* and *RPBF* CCAAT boxes. The CMS-2 construct possesses binary CCAAT box sequences from the *35S* and *RPBF* promoters. Chimaeric and/or synergic effects should be concerned for the CMS-2 construct. The CCAAT box sequences are shown to play a part in GA-response of *GAMyb* and *RPBF* by negative effects, particularly without GA, but unlikely to be sufficient for full suppressions, because declines in reporter expression are remarkable for whole changes of -90 region (GMD-2, RPD-2). In addition to the CCAAT box, perhaps other elements along the core promoter and exon 1 are required for

significant suppressions of *GAMyb* and *RPBF*.

3.6. DNA methylation of the *GAMyb* locus in germinated seeds

We assumed that regulatory sequences present in a large intron 1 were commonly required for high-level and GA response of *GAMyb* and *RPBF* expressions. Although the fact that there are significant differences among transient and endogenous expressions of genes complicates the interpretation, similar regulations by intron sequences have been demonstrated in plant MADS box genes, such as *PLENA* [*PLE*; 41], *AG* [42] and *FLC* [43]. *FLC* and *AG* are regulated epigenetically [44]. Both *FLC* intron 1 and *AG* intron 2 serve as *cis*-elements for proper gene expressions and are also required for maintenance of their repressions by complex chromatin modifications. These evidences provide clues for further delineation in the chromatin control of early GA response genes.

Chromatin structure is modulated by numerous covalent modifications of both DNA and histone. The interplay between DNA methylation and histone modification in plant is proving to be complex and might be specific to the type of genes examined [45]. We studied the level of DNA methylation of the *GAMyb* locus to investigate whether GA responsibilities might be linked with chromatin configuration. Genomic fragments containing the *GAMyb* region were enriched from three distinct tissues; namely, young seedlings

excised from 2.5-d-germinated seed, embryo-less half-seeds incubated without or with 10^{-5} M GA₃ for 2.5 d. Each pool of genomic fragments was modified by the bisulfite method, amplified by PCR, cloned and sequenced. The methylation frequencies were estimated for 16 PCR clones from each tissue and expressed as histogram, assigned to each cytosine (Fig. 6).

The *GAMyb* segments spanning the core promoter, exon 1, intron 1 and exon 2 upstream of the ATG start codon (-21 to +976) were completely unmethylated in all tested tissues (data not shown), whereas the upstream promoters were frequently methylated. Histograms displayed binary pattern of DNA methylation in the *GAMyb* promoter, associated with heavy methylation ranging from -1054 to -724 (distal cluster) and lower methylation from -512 to -252 (proximal cluster). Notable changes among examined samples were observed in the proximal cluster. The young seedling showed approximately the same overall degree of methylation (12.3%), as revealed by the GA-untreated aleurone (11.1%). In the seedling, the proximal cluster composed of 72.1% CpG, 6.6% CpNpG and 21.3% asymmetric methylation. CpG and CpNpG methylation in the GA-untreated aleurone reduced to 43.6% and 3.7%, respectively, whereas asymmetric type increased to 52.7%. After treating the aleurone with GA, it retained the majority of CpG methylation (55.3%) and showed a subtle reduction in CpNpG (2.6%) and asymmetric methylation (42.1%). Similar pattern in tissue specificities was observed in the distal cluster, but had little effects on

the level of methylation. Given the general proposal that DNA methylation plays a major role in gene silencing and the symmetry of the CpG site is important for stable maintenance of methylation pattern in vegetative tissues, methylation profile of the *GAMyb* locus is likely to be rigid in the seedling and rather plastic in the aleurone.

4. Discussion

The DELLA proteins restrain GA action by repressing the set of genes that is targeted by GA signaling. In order to clarify functional relevance among the DELLA protein and specific target genes, we identified regulatory domains of early GA response genes that were involved in GA action in rice aleurone. Our analyses defined negative regulations by a core promoter region and enhancer-like activities present in a large intron 1 of *GAMyb* and *RPBF* that are putative targets of the DELLA protein. Promoter analyses suggested that crucial sequences for proper expression of *GAMyb* and *RPBF* appeared to be located within a segment, which might be involved in the assembly of the CCAAT-box binding proteins.

The CCAAT box is one of the most general promoter elements, being present in 25% of eukaryotic promoters [46]. These motifs are recognized by different classes of the CCAAT-binding complex, a trimer composed of distinct subunits; NF-YA (CBF-B/HAP-2), NF-YB (CBF-A/HAP-3) and

NF-YC (CBF-C/HAP-5) [47]. In plants, all the subunits are encoded by multiple and distinct genes and show different patterns in their expressions [48, 49]. For example, five NF-YA, 10 NF-YB and 10 NF-YC subunit genes have been identified in the rice genome [50]. This implies that NF-Ys have highly divergent roles in plant development. It is noteworthy that Arabidopsis NF-YC can deliver nuclear regulators without DNA-binding motifs to a target promoter via protein-protein interaction [51]. It has yet been known of which set of NF-Ys virtually modulate *GAMYb* and *RPBF* expressions in rice aleurone; however, nucleotide changes in the CCAAT box of *GAMYb* and *RPBF* resulted in a high-basal expression of the reporter gene and uncoupled the reporter expression from the regulation by GA. The data are consistent with an idea that the assembly of NF-Ys at the CCAAT box controls the level of *GAMYb* and *RPBF* expressions by an inhibitory effect.

When introduced into the *35S* promoter, the CCAAT box alone is not sufficient to establish suppression of the reporter expression in the absence of GA (Fig. 5). This suggests that additional sequences act as negative elements in combination with a pivotal role of the CCAAT box. Analogies of the promoter sequences prompt us to address for which transcription factor(s) lead to significant repressions. From a comparison of the *GAMYb* and *RPBF* promoters, we could define that both fragments contained an aberrant GA/TC-rich sequence, including dinucleotide GA/TC-repeats (GA₅, GA₄ and TC₅ from -108 to +75 in *GAMYb*; TC₃, TC₄ and TC₃ from -139 to -56

in *RPBF*). GA-repeat sequences have been found in the promoters of many genes in animals. In *Drosophila*, GAGA elements are known to be natural targets for GAF proteins [52]. GAF protein is a member of the trithorax group (trxG) proteins [53]. The Polycomb group (PcG) and trxG proteins are required for determining the state of expression of developmental regulators, such as homeotic genes [54]. It has been believed that the PcG proteins participate in gene silencing of homeotic loci by condensing chromatin, whereas trxG maintain transcriptional activity by keeping an open chromatin structure [55]. PcG response elements (PREs) are thought to act as negative elements that recruit the PcG proteins. GAF protein also binds to PREs that contain GA-repeat sequences, suggesting a shared role for GAGA elements that contributes to induce conformational changes in chromatin structure [52].

Sequence homologies with GAF and its related proteins have not been reported in the plant genomes [56]. A novel family of DNA binding proteins, BASIC PENTACYSTEINE (BPC), has been proposed to be a plant substitute of GAFs [57]. BPC bind to GA-repeat sequences and influence expression of genes in different plant species and processes [57, 58]. Arabidopsis BPC1 functions as an active repressor of the homeotic gene *SEEDSTICK* (*STK*) [59]. Similar to the case of *GAMyb* and *RPBF*, the *STK* regulatory region is defined at a 5' region flanking the transcription start site and the 5'-UTR with a large intron 1, where multiple sites for BPC1 binding are predicted.

Biochemical studies have revealed that BPC1 could induce conformational changes in the *STK* regulatory region by cooperative binding to multiple binding sites. In our study, distinct 15-bp mutation in GA-rich elements of *GAMyb* and *RPBF* showed no detectable changes on reporter expressions (Fig. 5, not shown data), whereas potential sites for BPC1 binding (RGARAGRRA, R is G plus A) [59] were often observed throughout the regulatory regions. This observation, aligned with the similarity to the *STK* regulation by BPC1, suggests that GA/TC-rich elements of *GAMyb* and *RPBF* are putatively working as sub- and multiple elements by recruiting rice BPC-like proteins [57].

Another intriguing finding has come from the observation of enhancer-like activities present in a large intron 1. In general, introns are thought to increase the protein production by elevating both mRNA accumulation and translational efficiency [60]. Potential mechanisms of the intron-mediated gene expression have been explored by Arabidopsis introns inserted into different positions of the reporter gene [61]. This analysis revealed position-dependent and -independent effects from introns on the reporter expression. The ability to enhance mRNA abundance clearly depends on the position proximity to the promoter, whereas effects on translation appear less dependent on intron location. These findings support an idea that introns control gene expression by two distinct mechanisms and stimulating effects on mRNA abundance are coupled with transcription. In

our reporter analysis, reporter fusions containing intron 1 from *GAMyb* and *RPBF* showed significantly increased both the absolute levels and GA-dependent expressions (Fig. 3). It remains unclear whether the *GAMyb* and *RPBF* introns influence the reporter expression by increasing transcription or by promoting translation. Nonetheless, nuclear run-on assays using nuclei from barley aleurone cells revealed that GA caused an increase in the rate of *GAMyb* transcription [8]. This implies that intron 1, positioned nearest to the transcription start site, may stimulate *GAMyb* and *RPBF* transcriptions, at least in part, under the control of GA.

Arabidopsis homeotic genes, such as *AG*, *FLC* and also *STK*, have shown to be regulated by intragenic sequences [42, 43, 59, 62]. Most of *cis*-elements essential for transcriptional control have been identified within a 3.0 kbp *AG* intron 2 and a 3.5 kbp *FLC* intron 1. These large introns do not house *cis*-elements that recruit transcription factors, but also serve as the main target of gene silencing. *AG* intron 2 is hypermethylated in plants that have been altering in DNA methylation [34]. Environmental cues cause changes in histone modifications, particularly in specific regions of the *FLC* locus, at the 5' end of the gene and within intron 1 [63, 64]. These evidences provide a concept that larger size of intron have a more open space and propose the accessibility of DNA for complex chromatin modifications. In addition, GA-repeat sequences are found in the regulatory regions of *AG* and *FLC* [65]. The intron regulation linked with GA-repeat sequences is reminiscent of the

control of *GAMyb* expression in the aleurone.

By the analogy with the *AG* and *FLC* regulations, we examined DNA methylation of the *GAMyb* locus. The 5' region flanking the transcription start site, exon 1 and leader of intron 1 were completely excluded from DNA methylation. Our analysis showed different patterns of DNA methylation in the upstream promoter (Fig. 6). Methylation patterns in the aleurone appeared to be distinguished from that of the young seedling regardless whether the aleurone was treated by GA or not. In the seedling, the 5' region nearest to the transcription start site was masked by dense CpG methylations, whereas methylations in the aleurone were shifted upstream and substituted by asymmetric types. Plant DNA methylation occurs at CpG, CpNpG and asymmetric contexts, mediated by complex network of distinct cytosine methyltransferases [66]. The Arabidopsis DRM and CMT3 are required for non-CpG methylation at specific gene loci [67]. DRMs are supposed to act as *de novo* methyltransferases [68]. CMT3 is a plant specific chromomethylase that has a chromodomain at the C-terminal catalytic part [69]. The chromodomain of CMT3 was shown to interact, *in vitro*, with specific types of methylated histone, indicating a direct link between DNA methylation and histone modification [70]. If CMT3-like machineries were involved in establishment of asymmetric methylations of the *GAMyb* promoter, histone modification around the *GAMyb* locus would be expected. Priority of DNA methylation and histone modification on gene silencing

remains obscure, but histone modification precedes and emphasizes the effect of DNA methylation in some cases [71]. In this regard, further analyses of histone modification, particularly on an open space of large intron 1, are needed to clarify chromatin configurations of the *GAMyb* locus that are likely influenced by GA.

SLR1 is a single DELLA protein in rice and thus appears to regulate all aspects of the GA-regulated rice growth [9]. Several explanations for how a unique SLR1 controls different developmental processes have been suspected. Nature of SLR1 might be changed by post-translational modifications [72]. Other control factors, such as ABA [73] and components related to SLR1 (SLR1-like1 and 2) [74], should be involved in influencing GA response. Although very low level of gene expression is detected in the seedling, the induction of α -amylase gene by GA occurs limited in the aleurone [75]. This suggests that specific linkage may occur between GA signaling and early GA response genes that are specialized in the aleurone. We proposed that presets of DNA methylation of the *GAMyb* promoter were possibly differing in the tissues or at hormonal conditions. At this stage, it seems difficult to speculate a relationship between diverse methylation contexts and expression of *GAMyb*; however, DNA methylation of promoter regions usually inhibits transcription. This general concept allows us to speculate that the rice body plan is capable of changing the configuration of *GAMyb* chromatin and determine the capacity to respond to GA. In the

aleurone, *GAMyb* chromatin is presumably potentiated and susceptible for GA signaling. This idea is strengthened by the fact that expression of *BPBF*, which is a barley counterpart of *RPBF* [76, 77], is also occurred by decreasing CpG methylation during endosperm development [78].

In summary, there is a parallel regulation between early GA response genes and plant homeotic genes. Homeotic gene plays an important role for determining a crucial fate of growth and development. Higher chromatin structures are necessary for temporal and spatial activation or silencing of these types of genes and provide an additional level of gene control beyond transcription. The precise connection with homeotic genes suggests that expression of early GA response genes may be controlled by chromatin modulation. The DELLA proteins are believed to regulate transcription, and as far as we know, they do not show any DNA-binding activities. This raises the possibility that unknown DNA-binding proteins or chromatin factors are needed to recruit the DELLA proteins to specific target loci. Soluble GA receptor GID1 [17] and F-box protein GID2 [16] have shown to interact with SLR1 and putatively stimulate its proteolysis, but these are GA signaling components acting post GA application. Therefore, further studies are needed to identify functional partners of SLR1 that coordinately access to the regulatory regions of early GA response genes that have been dedicated in this study.

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Figure legends

Fig. 1. RT-PCR analysis of genes involved in gibberellin action in germinated

rice seeds. (a) Organ-specific expression patterns of *GAMyb*, *RPBF*, *SLR1* and *RAmy1A*. Total RNA was isolated from the indicated tissues of 2.5-d-germinated rice seeds. (b) Embryo-less half-seeds were incubated in the presence of 10^{-5} M GA₃ for different times as listed on the top of the panel. (c) Embryo-less half-seeds were treated by different hormonal conditions (no hormone, 10^{-5} M GA₃ and 10^{-5} M GA₃ plus 10^{-4} M abscisic acid) for 2.5 d. *18S rRNAs* are indicated to ensure equal loading on the lane.

Fig. 2. Transient expression analysis of the *GAMyb* and *RPBF* promoters to direct expression of the luciferase reporter gene in rice aleurone. (a) Schematic representation of genomic organizations of *GAMyb* and *RPBF*. Shaded boxes represent exons (coding regions), open boxes indicate non-coding exons, and the lines connecting them denote introns. Numbers present nucleotide positions (base pairs) from the major transcription start site (+1). The ATG start and stop codons are indicated. Rice genomic location number is presented in the parenthesis. (b) Deletion analysis of the *GAMyb* and *RPBF* promoters. The promoter region, the 5'-untranslated region (1st exon and 1st intron) and the firefly luciferase (*F-luc+*) coding region are schematically represented. The numbers to the left of the construct diagrams indicate the 5' end of each deletion. At the right panel, results of the transient expression assays with the different constructs are shown. Transfected embryo-less half-seeds were incubated for 2.5 d in the absence or

the presence of 10^{-5} M GA₃. The induction fold by GA is indicated on the top of graphs. For the estimation of transfection efficiency, the *Renilla* luciferase (*R-luc*) reporter construct was cobombarded with each construct. The bars in the right of graphs indicate relative F-Luc+ activities (normalized with respect to R-Luc activity) \pm standard errors of the mean.

Fig. 3. Effects of the *GAMyb* and *RPBF* promoter fragments on the cauliflower mosaic virus 35S (*35S*) promoter activities in the aleurone. (a) Diagrams of the forefather constructs are shown at the top (*CaMV35S* and *GAMyb*). Various combinations of domain B (-343 to -91) and A (-90 to +8) from the *35S* promoter [40] and *GAMyb* fragments containing the upstream promoter (-482 to -91), the core promoter (-90 to +1), exon 1 (+2 to +290) and intron 1 (+291 to +953) regions were linked to the *F-luc+* reporter as indicated at the left of the panel. Experimental details are the same as in Fig. 2. (b) Diagrams of the forefather constructs are shown at the top (*CaMV35S* and *RPBF*). Various combinations of domain B and A from the *35S* and *RPBF* promoter fragments containing the upstream promoter (-335 to -91), the core promoter (-90 to +1), exon 1 (+2 to +128) and intron 1 (+129 to +2019) regions were linked to the *F-luc+* reporter construct as indicated in (a). The bars indicate normalized F-Luc+ activities \pm standard errors of the mean.

Fig. 4. Effects of functional domains from the cauliflower mosaic virus 35S (*35S*) promoter on the *GAMyb* and *RPBF* promoter activities in the aleurone. (a) Diagrams of the forefather constructs are shown at the top (*CaMV35S* and *GAMyb*). Various combinations of domain B (-343 to -91) and A (-90 to +8) from the *35S* promoter and *GAMyb* fragments containing the upstream promoter (-482 to -91), the core promoter (-90 to +1), exon 1 (+2 to +290) and intron 1 (+291 to +953) regions were linked to the *F-luc+* reporter construct as schematically indicated at the left of the panel. Experimental details are the same as in Fig. 2. (b) Diagrams of the forefather constructs are shown at the top (*CaMV35S* and *RPBF*). Various combinations of domain B and A from the *35S* promoter and *RPBF* fragments containing the upstream promoter (-335 to -91), the core promoter (-90 to +1), exon 1 (+2 to +128) and intron 1 (+129 to +2019) regions were linked to the *F-luc+* reporter construct as indicated in (a). The bars indicate normalized F-Luc+ activities \pm standard errors of the mean.

Fig. 5. Effects of mutations in the regulatory regions of the *GAMyb* and *RPBF* promoters for gibberellin response in the aleurone. (a) Sequences of the *GAMyb*, *RPBF* and *35S* promoter regions from -120 to +15 and their mutations used in the experiment. The transcription start site and the TATA box are shown in bold capital letters. The CCAAT box-like sequences indicated by capital letters and GA-repeat sequences are underlined.

Tandem TGACG repeat sequences of the *35S* promoter are boxed. Nucleotide sequences under the lines mark the mutations used in the experiment. (b) Mutation analysis of the *GAMyb*, *RPBF* and *35S* promoters. The diagrams at the left of the panel indicate schematic representations of the promoter regions used in the experiment. Open box represents the region of a 15-bp mutation. GMD-2 and RPD-2 are the same constructs used in Fig. 3. Position of the TATA box is indicated by eclipse. At the right panel, results of the transient expression assays with the different constructs are shown. Experimental details are the same as in Fig. 2. The bars indicate normalized F-Luc+ activities \pm standard errors of the mean.

Fig. 6. Methylation pattern found at the *GAMyb* locus in different tissues of germinated rice seeds. Distribution and frequency of 5-methyl cytosine (m^5C) were estimated for 16 cloned PCR products of bisulfite-treated genomic DNA from each tissue of 2.5-d-germinated rice seeds (young seedling, embryo-less half seeds incubated in the absence or the presence of 10^{-5} M GA_3). Histograms represent the frequency of m^5C over the total cytosines at positions containing CpG, CpNpG and asymmetric sequences in the top strand. Potential sites for each type of cytosine methylation are indicated on the top of the panel by vertical bars. Numbers under the panels present relative nucleotide positions (base pairs) from the major transcription start site (+1).

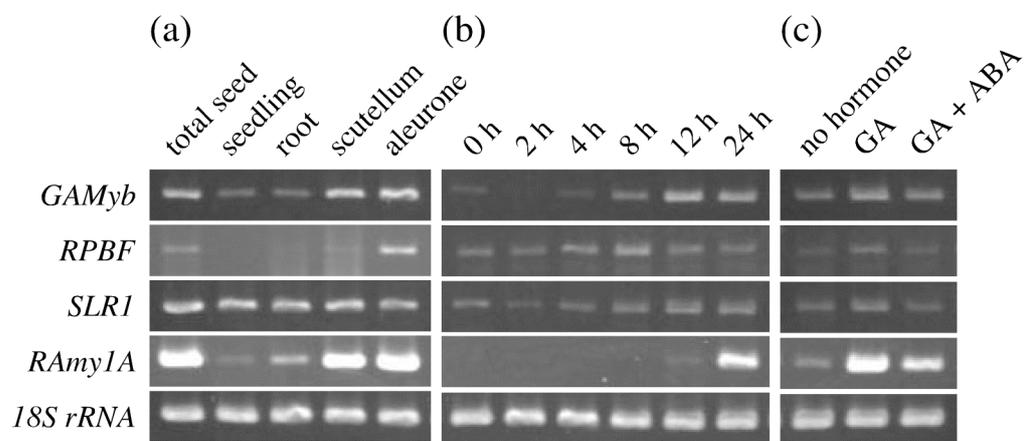
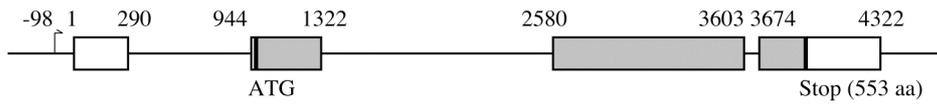


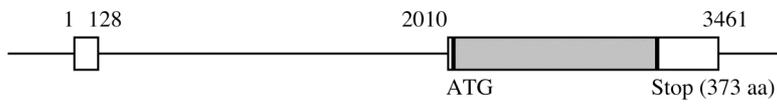
Figure 1

(a)

GAMyb gene (Os01g59660)



RPBF gene (Os02g15350)



(b)

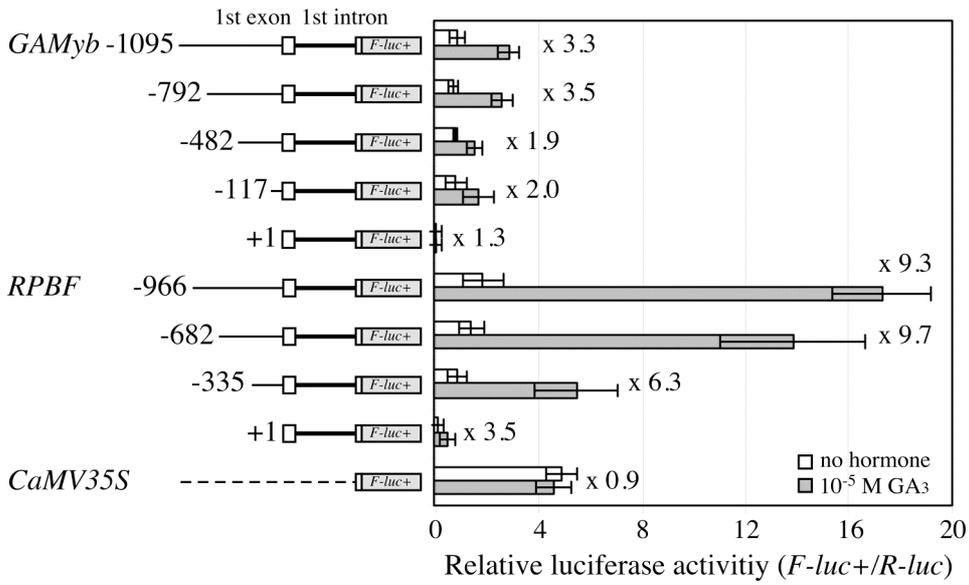
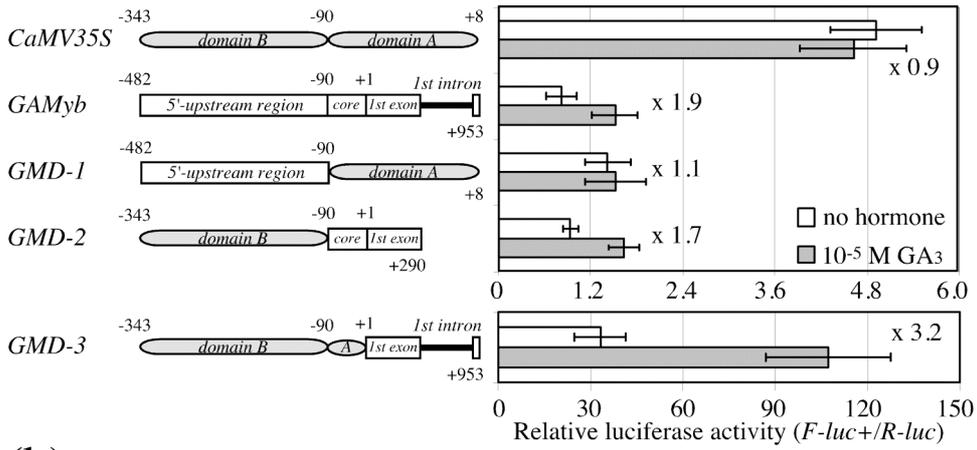


Figure 2

(a)



(b)

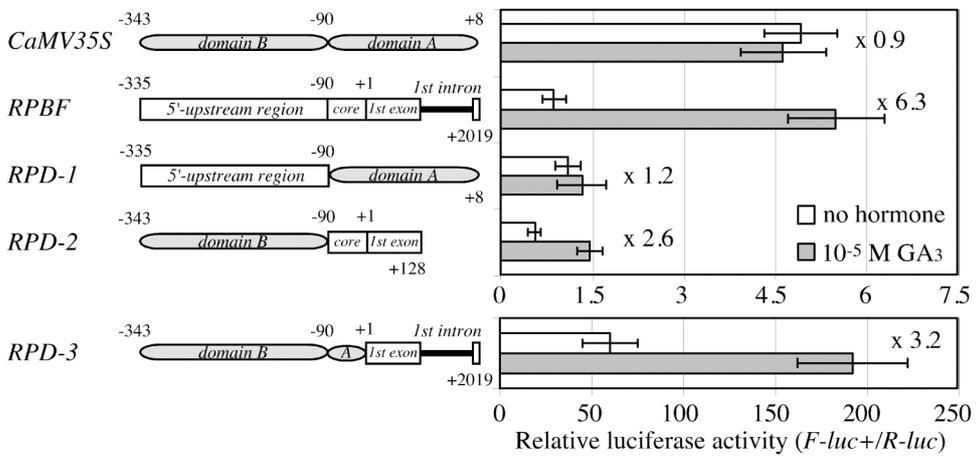
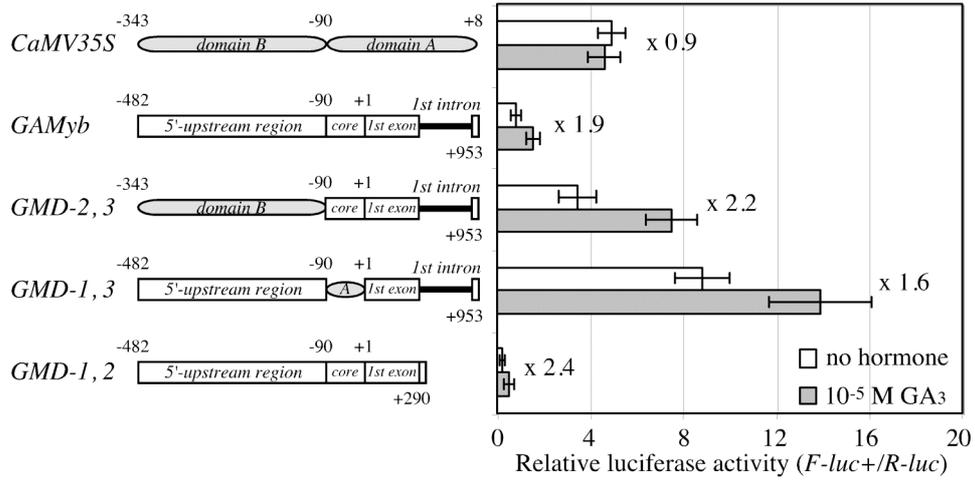


Figure 3

(a)



(b)

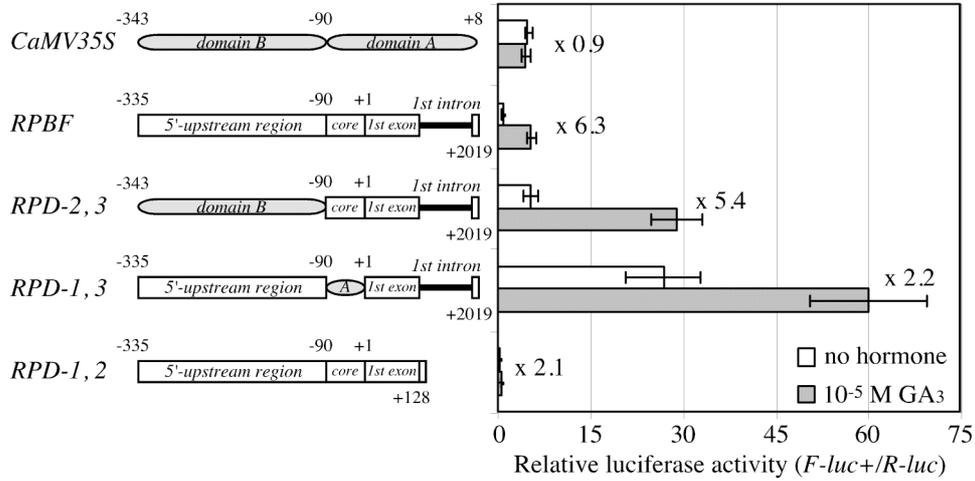


Figure 4

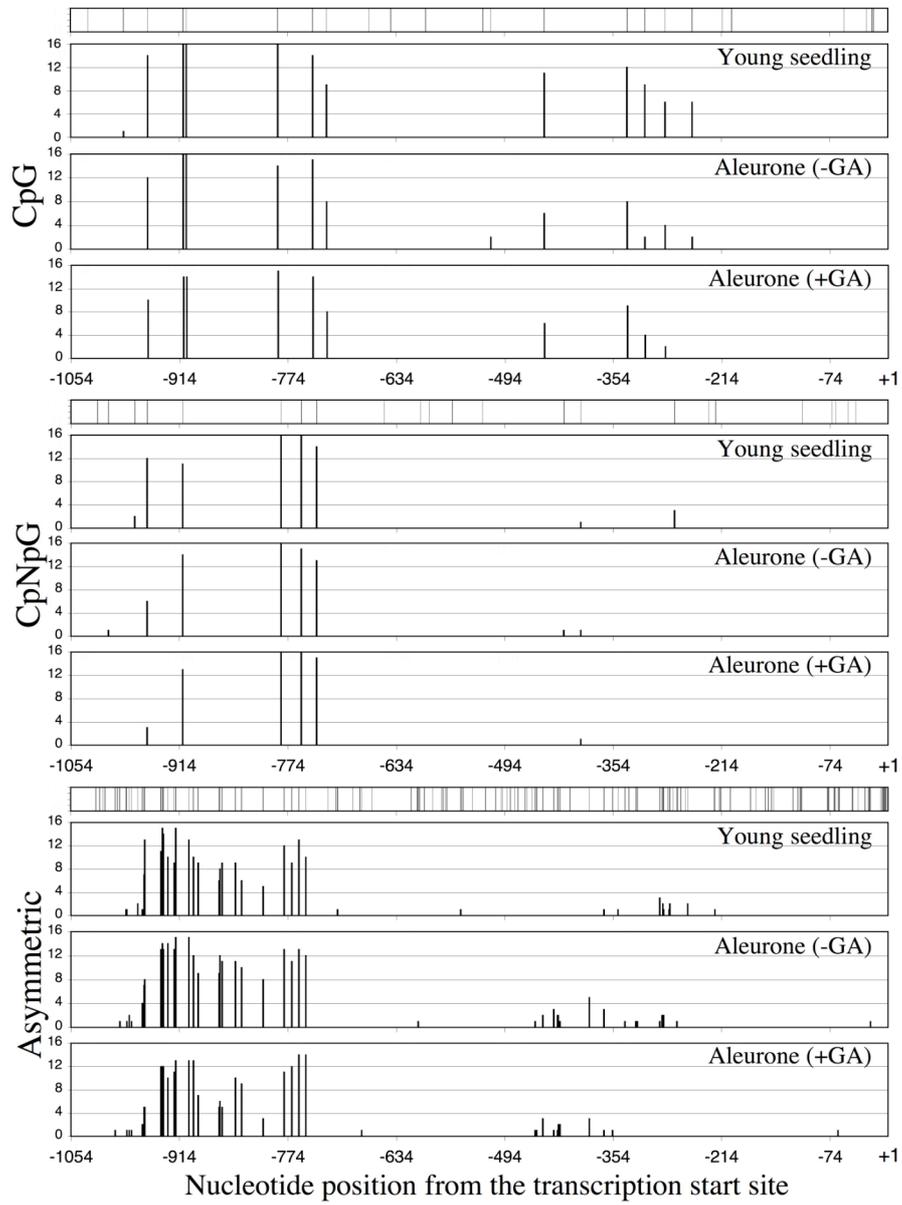


Figure 6

Oligonucleotide primers for RT-PCR analysys		
Gene	Forward primer	Reverse primer
<i>GAMyb</i>	GCAATTGGAGCTCCGCTCGG	AGCCCGGTGTTCTTCTG
<i>RPBF</i>	CAACAACACTACAGCATGGCGC	TTGCTGATCGCCGACGA
<i>SLR1</i>	CATGAAGCGCGAGTACCAAGAA	GGTTGTAGTGCACGGTGTCCGT
<i>RAmy1A</i>	ATGGTGAACAAACACTTCTTGT	CGGCGATGTCGTCCACCTTGC
<i>18S rRNA</i>	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

Oligonucleotide primers for bisulfite genomic sequence of the <i>GAMyb</i> locus		
Gene region	Forward primer	Reverse primer
-1054 to -691	TGTGTGTAGAATGAGGAG	CCTCCACTTATACACTCTA
-709 to -292	TAGAGTGTATAAGTGGAGG	TTRAAAACCATTTTATCACTC
-316 to -79	TTAYGAGTGATAAAATGGTTTT	CACCCCATTTTTCTCTCC
-97 to +217	GGAGAGGAAAAATGGGGTG	TCCTTTRCTCCTCCCARCTC
+101 to +509	GGTGGGYTTAAGYGGTGGYGG	TRTCTTCCCCCATAATTCACA
+410 to +760	TGGTTTTGTTYGTGGGGATTGAATT	ACAAACCAATTARACATRACTACAT
+643 to +975	TGYGAATTTGGAATYAAGAGGTT	TCTCRCTCTTACCCRATACAT

Y, C + T; R, G + A

Supplementary Data