Functional Dissections between GAMYB and Dof Transcription Factors Suggest a Role for Protein-Protein Associations in the Gibberellin-Mediated Expression of the *RAmy1A* Gene in the Rice Aleurone

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In the germinated cereal aleurone layer, gibberellic acids (GA) induce expression of a number of genes encoding hydrolytic enzymes that participate in the mobilization of stored molecules. Previous analyses suggest that the key events controlling the GA-regulated gene expression in the aleurone are formation of active transcription machinery referred to as the GA responsive complex, followed by recruiting GAMYB. In general, bipartite promoter contexts composed of the GA-responsive element and the pyrimidine box are observed within the regulatory regions of cereal GA-responsive genes. Protein factors that recognize each promoter sequence were identified and distinct effects on the GA-mediated activation of gene expression have been also investigated; however, the connection and intercalation between two promoter motifs remain obscure. In this study, I have evaluated cooperative function of GAMYB and a pyrimidine box-binding protein OsDOF3 that influenced the promoter activity of the most predominant GA-responsive gene (*RAmy1A*) of rice (*Oryza sativa*). Transient expression of OsDOF3 in the germinated aleurone prolonged GAMYB function on the reporter expression in the absence of GA. The synergistic effect required a set of DNA bindings of two proteins on the *RAmy1A* promoter region. The yeast two-hybrid assay showed the physical interaction of GAMYB and OsDOF3 in yeast cells, indicating that the association of GAMYB and OsDOF3 may be a functional unit in transcription regulation. The results showed the accessory function of OsDOF3 responsible for a dosage-dependent mediation of GA signaling that leads to high-level expression of physiological target genes.

Gibberellins (GAs) are diterpenoid hormones that play crucial roles in plant growth and development, including seed germination, leaf expansion, stem elongation, and flower and fruit development (Hooley, 1994). During the germination of cereal seeds, the de novo-synthesized GAs are transported from the embryo to the aleurone layer where GAs trigger the expression of hydrolytic enzymes through transcription activation of corresponding genes (Jacobsen et al., 1985). The induced enzyme activities are secreted into the starchy endosperm and mobilize the reserved nutrients to support the seedling growth.

Detailed analyses of the GA-regulated promoters have provided the basis for the discovery of transcription factors involved in GA actions in the aleurone. The research has culminated in determining the function of a R2R3-type Myb transcription factor GAMYB (Gubler et al., 1995). GAMYB induces the expression of genes such as high- and low-pI α-amylases, proteinase, and β-glucanase, through direct binding to GA-responsive element (GARE) that seems to be essential for the GA induction of these genes (Gubler and Jacobsen, 1992; Gubler et al., 1999). Recent evidence indicate that the GAMYB function has not only connected with GA signaling, but also made it a target of antagonistic effects by abscisic acid (Gómez-Cadenas et al., 2001; Zentella et al., 2002). The involvement of GAMYB as a transactivator of GA signaling may not to be restricted in the cereal aleurone system. Given the timing and pattern of expressions of cereal *GAMyb* and its related genes in Arabidopsis that precede or overlap with the site of GA actions, such as flower development (Gocal et al., 1999; Murray et al., 2003), seed maturation (Diaz et al., 2002), and stem elongation (Gocal et al., 2001), it is tempting to speculate that GAMYB plays ubiquitous roles in the GA signaling cascade throughout the plant growth.

These diverse functions appear not to be controlled by GAMYB alone, but also may involve the combinatorial interaction with additional regulatory proteins. For instance, the GA induction of the *GAMyb* gene expression in the aleurone is moderate mostly due to a high-level expression in the absence of GA (Gómez-Cadenas et al., 2001; Gubler et al., 2002). This accounts for a discrepancy between tight regulations of target gene expressions and suggests the necessity of posttranscriptional control of GAMYB or the presence of cofactors responsible for effective responses to GA. Antagonistic effects against the GAMYB func-

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tion have been reported. Raventós et al. (1998) identified a barley (*Hordeum vulgare*) transcription repressor (HRT) capable of binding the GARE region of an α-amylase gene and repressing its activity (Raventós et al., 1998). A Mak-like kinase KGM seems to abolish the GAMYB function by direct physical interaction in the barley aleurone cells (Woodger et al., 2003).

The pyrimidine box is another promoter element that is observed in cereal GA-responsive promoters examined thus far (Huang et al., 1990). Mutations on the pyrimidine box caused the reduction of the GA induction with a lesser magnitude than those of GARE in the GA-treated aleurone (Gubler and Jacobsen, 1992; Rogers and Rogers, 1992). The pyrimidine box alone could not confer the hormone responsiveness to the minimal promoter of the cauliflower mosaic virus 35S (*CaMV35S*), suggesting accessory roles of the pyrimidine box on the transcription response to GA (Skriver et al., 1991). Another group and I successfully identified aleurone proteins that recognized the pyrimidine box sequence from barley (BPBF; Mena et al., 2002) and rice (*Oryza sativa*; OsDOF3; Washio, 2001). A member of Dof proteins, prolamine box-binding factors PBF that were originally identified to be transcription factors regulating the expression of genes for stored proteins in developing seeds (Vicente-Carbajosa et al., 1997; Mena et al., 1998), BPBF and OsDOF3 are likely to be a pyrimidine box-binding protein in the germinated aleurone.

The analysis of the *BPbf* and *OsDof3* expressions in germinated seeds confirmed that the temporal and spatial appearances of the transcripts were consistent with those of high-level expressions of potential target genes. In contrast, another group and I failed to detect any positive roles on the reporter expressions influenced by the expression of the Dof proteins in the aleurone cells (Washio, 2001; Mena et al., 2002). Recently, Isabel-LaMoneda et al. (2003) have identified a new barley Dof protein (scutellum and aleurone-expressed DOF [SAD]) that plays a positive role on the expression of the GA-regulated gene targeted by BPBF. This suggests that multiple interactions between distinct classes of the Dof protein on the pyrimidine box motif might lead to proper levels of GA-regulated gene expression.

In this report, I have evaluated the role of a pyrimidine box-binding protein of rice in the germinated aleurone. The recombinant OsDOF3 was able to bind in vitro the pyrimidine box motifs from the GA-controlled α-amylase gene (*RAmy1A*; Huang et al., 1990). In transient expression experiments, OsDOF3 promoted the activity of the *RAmy1A* promoter through the trans-activation mediated by GAMYB. I identified that OsDOF3, binding the pyrimidine box, affected the DNA binding of GAMYB to GARE, suggesting that intercalation of the OsDOF3 function is important for combinatorial regulation of the transcription response to GA.

RESULTS

Promoter Analysis of the *RAmy1A* Gene

The transcript of a rice gene for α-amylase *RAmy1A* is known to be abundant in the aleurone during germination (O'Neil et al., 1990; Ranjhan et al., 1992). Functional promoter analysis using transgenic rice seeds has confirmed that the 5′-regulatory region extending from −232 (nucleotide position relative to the site for transcription initiation) to +31 is sufficient for hormonal regulation by GA (Itoh et al., 1995), along which are found the distal (Pyr-1, −312) and proximal pyrimidine boxes (Pyr-2, −214), GARE (−148), and three potential sites for the Dof binding (D-1, −191; D-2, −109; D-3, −78; Fig. 1A; Huang et al., 1990). To test the abilities of identified promoter motifs, transient expression of reporter genes influenced by mutations on a distinct site for predicted motifs was analyzed. Nucleotide substitutions previously reported to inactivate the abilities of each promoter motif were introduced in GARE (Gubler et al., 1995) and in the sites for the Dof binding (Kang and Singh, 2000). Expression analyses of the reporter constructs are shown in Figure 1B. The upstream portion from the *RAmy1A* promoter (−380 to +27) had a stimulatory effect on the GA-induced expression of the reporter gene in transfected aleurone cells. The drastic loss of the GA induction associated with a mutation on GARE (M4) further verifies the importance of this motif in the GA-regulated expression of genes. Other mutations of the proximal pyrimidine box (M2) and one site for the Dof binding (M5) also reduced the GA-induced activities to 48% and 54% of the wild-type sequence, respectively, but the effects were not as marked as seen when GARE was mutated. The results demonstrate that a complex of multiple promoter elements contribute to the *RAmy1A* expression in the GA-treated aleurone cells. The pivotal role of GARE and accessory functions of the pyrimidine box are in agreement with the presumed functions of those reported in the GA-responsive promoters (for review, see Jacobsen et al., 1995).

OsDOF3 Binds the Pyrimidine Box from the *RAmy1A* Promoter

The pyrimidine box binding protein of rice, OsDOF3, was initially identified by the Southern/western-blot method using the promoter fragment of a rice gene for type III carboxypeptidase (Washio, 2001). Sequence analysis shows that the *OsDof3* cDNA encodes a 371-amino acid polypeptide related to the PBF factors of cereal plants. The predicted amino acid sequence of OsDOF3 aligns well with those of maize (*Zea mays*; Vicente-Carbajosa et al., 1997), wheat (*Triticum aestivum*), and barley PBF proteins (Mena et al., 1998; Fig. 2). The N-terminal sequence of OsDOF3 contains four Cys residues remi-

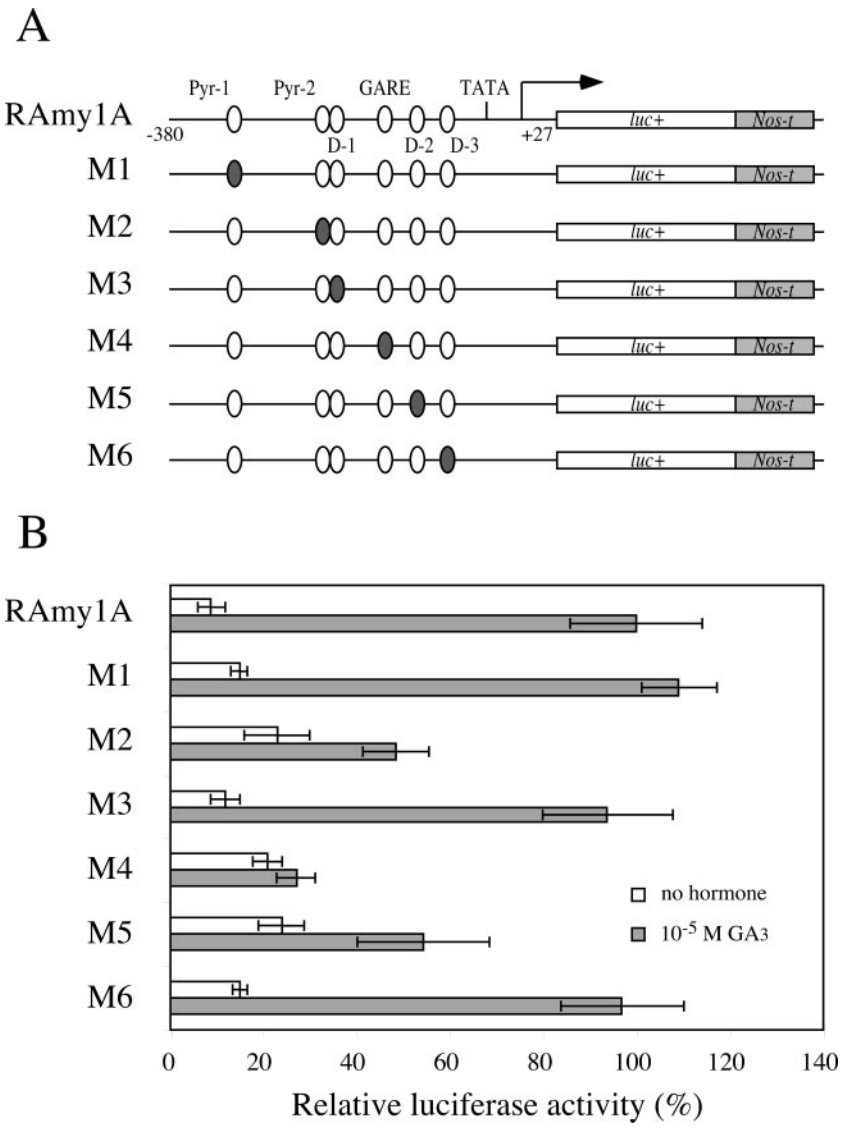

Figure 1. Transient expression analysis of the relative abilities of the *RAmy1A* promoters containing mutations to direct expression of the luciferase reporter gene in the rice aleurone. A, Schematic representation of genomic organizations of the *RAmy1A* promoter (Huang et al., 1990). A, 5′-upstream regulatory sequence (407 bp long; −380 to +27) from the *RAmy1A* gene was fused to a reporter gene cassette containing the firefly luciferase coding region (*luc+*) and the 3′ terminator from the nopaline synthase gene (*Nos-t*). The positions of predicted promoter motifs are indicated by open ellipses. Their sequences and positions in the *RAmy1A* promoter (referred to the transcription initiation site) are indicated. Nucleotide substitutions in the mutated versions are indicated by shaded eclipses.B, Relative luciferase reporter activities in rice aleurone transfected with the *RAmy1A* reporter constructs. Tissues were incubated in the absence (no hormone) or in the presence of 10^{-5} M GA_3 for 3.5 d. All values have been shown relative to the reporter activity of the wild-type *RAmy1A* reporter construct that was given a value of 100% in the GA treatment. The bars represent SE of the mean.

niscent of the Dof zinc finger and shows around 80% sequence identities with PBFs, whereas the C-terminal parts are divergent showing several insertions and deletions. An Asn-rich stretch at the C terminus that is characteristics of cereal PBFs (Mena et al., 1998) is also lacking in OsDOF3. I could not find any sequences encoding a polypeptide similar to cereal PBF except for OsDOF3 in the current database from rice nuclear genome. Hence, OsDOF3 is likely to be a unique rice protein compatible with cereal PBFs.

To address whether OsODF3 binds the pyrimidine box elements, the His-tagged recombinant proteins were expressed in *Escherichia coli* and were purified through Ni affinity chromatography. Interaction between the recombinant OsDOF3 and DNA fragments derived from the *RAmy1A* promoter was assayed by electrophoretic mobility shift assay (EMSA; Fig. 3). The OsDOF3 bound the probe fragments derived from the distal (RAM1, −380 to −85) and proximal parts (RAM2, −84 to +27) of the *RAmy1A* promoter, resulting in formation of high-mobility complexes. The specificity of the binding was examined following nucleotide substitutions in the probes. This showed that mutations on two pyrimidine boxes (M1 and M2) and one site for the Dof binding (M5) effectively abolished the interactions. Pentanucleotide sequence from the pyrimidine box (CTTTT; Huang et al., 1990) and D-3 (AAAAG) is capable of matching a favored substrate selected by in vitro DNA binding of maize Dof proteins (CTTTT or AAAAG; Yanagisawa and Schmidt, 1999). These results indicate preferential binding of OsDOF3 to two pyrimidine box and D-3 motifs in the *RAmy1A* promoter context. When the results of the transient expression in Figure 1 are in consideration, the distal pyrimidine box and D-3 motifs that did not have a remarkable influence on the *RAmy1A* promoter activity are thought to exclude from following analyses.

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ZmPBF    MDMISGSTAA TSTPHNNQQA VMLSSPIIKE EARDPKQTRA MPQIGGSGER   50
TaPBF    MEEVFSSNSK SKAGQMAGEA I--------- ---------- -----AGAEK   26
HvPBF    MEEVFSSNSK SKAGQMAGEA A--------- ---------- -----AAAEK   26
OsDOF3   M--------- ASGGALS--P VEEKPTVVKT TKAEQHEEEA AVAVKSAAEM   39

ZmPBF    KPRPQLPEAL K@PR@DSNNT KFCYYNNYSM SQPRYF@KA@ RRYWTHGGTL  100
TaPBF    KPRPKPEQKV E@PR@KSGNT KFCYYNNYSM SQPRYF@KA@ RRYWTHGGSL   76
HvPBF    KSRPKPEQKV E@PR@KSGNT KFCYYNNYSM SQPRYF@KA@ RRYWTHGGSL   76
OsDOF3   MKKSSPC--- -@PR@NSIKT KFCYYNNYSM AQPRYF@RE@ RRYWTQGGSL   85

ZmPBF    RNVPIGGGCR KNKHASRFVL GSHTSSSSSA TYAPLSPSTN ASSSNMS---  147
TaPBF    RNVPIGGGCR KPKRSGTSDA HKLGVASSPE PTTVVPPST- --CTGMN---  120
HvPBF    RNVPIGGGCR KPKRPGTSDA HKLGMASSSE PTGVVPPSN- --CTGMN---  120
OsDOF3   RNVPVGGGCR KSKRSSASSA SASAASPPAP AVGAAPPVVP ALSSAISKLL  135

ZmPBF    ---------- -INKHM--MM VPNMTMPTP- ---TTMGLF- ---PNVLPTL  176
TaPBF    ---------- -FANVLPTFM SVGFEIPSS- ---LSLTAFG SSSSSNTAAM  155
HvPBF    ---------- -FANVLPTFM SGGFDIQSS- ---LSLTTFG SSSSSNPTAL  155
OsDOF3   QSEPMAAPCA DFPNVLPTFV STGFELPAAA GDRLSLGSFG AFGNLSAAVA  185

ZmPBF    MPTGGGGG-- ---F------ ------DFTM DNQH------ ----------  193
TaPBF    MSPGGTTS-- ---FLDVLRG GAGGLLDGSL SQNN------ --GYYYGGPA  192
HvPBF    MSPGGTTS-- ---FLDVLRG GAGGLLDGSL GPNN------ --GYYYGGHA  192
OsDOF3   APGGGGGSST TTSFMDMLRG -VGGLFDGVG NSHQMGGNGG GGGSYYAPLI  234

ZmPBF    ----RSLSFT PMSLPSQGPV PMLAAGGSEA -TPSFLEMLR GGIFHGSSSY  238
TaPBF    IGSGNGMLMT PPAVSFGIPV PMQQHGDLVV -GGNGIGAAT ASIFQGATS-  240
HvPBF    NGSSIGMLMT PPTVSFGIPS PMQQHGGLVV -GGNGIGGTT SSTFQGSAG-  240
OsDOF3   TGAGNGMLMP PPPLP-PFSG SLMQHGMQGL FANHAMGGGG GGV-------  276

ZmPBF    NTSLTMSGGN NG--MDKPFS LPSYGAMC-- ---TNGLSGS TTNDARQL--  279
TaPBF    ------EEGD DG--MGGVMG LQWQPQVG-- ---NGGGGGG VSGGVHHLGT  277
HvPBF    ------EEGD DG--TGSIMG LQWQPHVG-- ---NGGGGVV GLGGAHHLGT  277
OsDOF3   -----MNAGE DGSVMAGLGG GQWPPALGGA DEQQGGGDGG EAVMTKDTGG  321

ZmPBF    ---------- VGPQQDNKAI MKSSNNNGV SLLNLYWNKH NNNNNNNNNN  319
TaPBF    GNNVTMGNSN IHNNNNNDSG G---DDNNGG SSRDCYWINN GGSNPWQSLL  324
HvPBF    GNNVTMGNNN NNNNQNNNNG GGAGDDDDGG SSRDCYWINN GGSNPWQSLL  327
OsDOF3   GASSSASRPD YFYGWNTPPA ASSLEAASAA TP-------P LPPEQRHGRA  364

ZmPBF    NNNNNNKGQ                                              328
TaPBF    NSTSLM                                                 330
HvPBF    NSTSLM                                                 333
OsDOF3   SSTAARP                                                371
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Figure 2. Alignment of the amino acid sequence predicted from the *OsDof3* cDNA with cereal PBF proteins. OsDOF3 shares a Dof DNA-binding domain very closely related to those of maize (ZmPBF; Vicente-Carbajosa et al., 1997), wheat (TaPBF; Mena et al., 1998), and barley PBF proteins (HvPBF; Mena et al., 1998), although there is no significant homology outside the Dof domain. Amino acid residues identical to those among PBF proteins are in bold. The four Cys residues putatively involved in the formation of a zinc finger are indicated by the open letters. Dashes indicate gaps introduced in the maximized alignment. The *OsDof3* cDNA accession number is AB028131.

The *OsDof3* and *GAMyb* Gene Expressions in Germinated Rice Seeds

The expression pattern of the *OsDof3* gene in different tissues of germinated seeds or at various conditions of hormonal treatments in the aleurone was compared with that of the *GAMyb* gene to understand how they were regulated. The *GAMyb* transcripts ubiquitously appeared in all tissues, with the endosperms showing the highest levels of mRNA accumulation (Fig. 4A), where it temporarily preceded those of the GA-regulated gene for α-amylase (*RAmy1A*; Fig. 4B). This further exemplifies that *GAMyb* is an immediate early gene conferring primary responses in the GA-treated cells. In germinated seeds, the *OsDof3* transcripts were present at a relatively low, but detectable, level. The mRNA levels of *OsDof3*, raised onset of imbibition, were clearly visible at d 1, and reached a high level at d 3. The temporal appearance of the *OsDof3* transcripts was in a parallel manner to high-level expressions of a potential target gene (Fig. 4B). The lower levels of the *OsDof3* mRNA render clear impression on RNA images with respect to tissue specificity in the scutellum and endosperms (Fig. 4A), and also to hormonal regulations (Fig. 4C). The expression analysis of *OsDof3* suggests secondary roles on the expression of target genes, followed by penetration of the *GAMyb* functions in the GA-treated aleurone cells.

OsDOF3 Has No Transcription Activation Ability in Yeast and Plant Cells

Gubler et al. (1999) determined functional domains of barley GAMYB responsible for transcriptional control using yeast one-hybrid studies. The results showed the presence of two transcriptional activation (TAD) and two negative regulation domains (NRD) for transcription control of this protein. Sequence comparison between barley and rice GAMYB showed high sequence identity through the predicted functional domains, indicating that both proteins are likely to have very similar functions (Gubler et al., 1997).

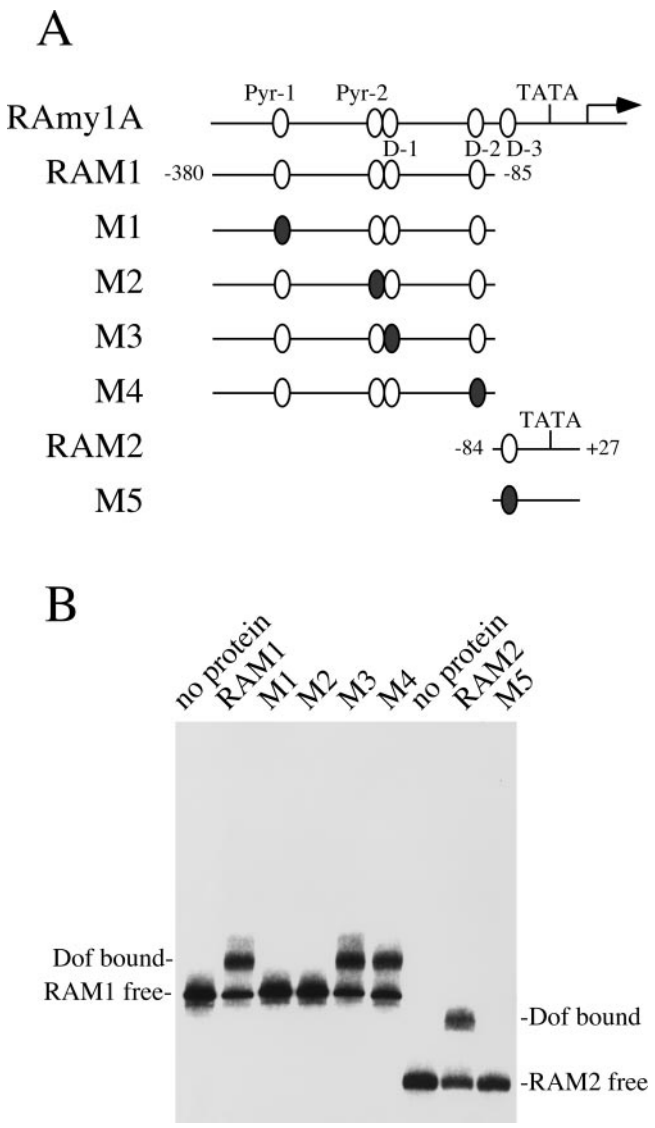

Figure 3. EMSA of the recombinant OsDOF3 protein and the *RAmy1A* promoter fragments. A, Schematic representation of the promoter fragments from the *RAmy1A* gene. A 407-bp-long 5′-upstream sequence was divided into two parts (RAM1 and RAM2) to ensure a clear resolving of the DNA-protein complexes in EMSA. Nucleotide changes were introduced in five putative sites for the Dof binding (Yanagizawa and Schmidt, 1999), including two pyrimidine boxes (Pyr-1 and Pyr-2, and D-1 to D-3). The positions of nucleotide changes in mutation promoters (M1–M5) are indicated by shaded ellipses. B, EMSA with the ^{32}P-labeled probes derived from the *RAmy1A* promoter and its mutated versions affected in the Dof binding. Each probe listed on the top of the panel was incubated with the recombinant OSDOF3 protein (5.0 ng reaction^{-1}). DNA-protein complexes (Dof bound) were separated from free probes (RAM1 free and RAM2 free) by PAGE.

To identify functional domains in rice GAMYB as well as in OsDOF3, I constructed fusion genes that consisted of the DNA-binding domain of the yeast transcription factor GAL4 (GAL4-BD) with full-length or various deletions of two rice proteins (Fig. 5A). Resultant plasmids were transformed in the yeast cell SFY526 that contained the *lacZ* reporter gene targeted by the DNA binding of GAL4-BD; transcription activation was monitored by reporter expression (Fig. 5B). I found only a low level of trans-activation of the reporter expression with any of the full-length or C-terminal deletion of GAMYB because the production of fusion proteins possessed by the Myb domain caused moderate growth defects in transfected yeast cells. GAL4-BD fused to the C-terminal part of GAMYB beside the Myb domain successfully trans-activated the reporter expression, and the level of activation was enough to compare with that seen in GAL4 protein used as a positive control. Other N-terminal deletions with predicted TADs resulted in activation of the reporter to some extent. In contrast, I could not see a significant trans-activation with any of the OsDOF3 effectors analyzed. The evidences obtained from the yeast trans-activation experiments demonstrate potential pivotal roles on transcription activation of GAMYB and the absence of TAD in OsDOF3.

To assess in planta functions of the proteins encoded by the *GAMyb* and *OsDof3* cDNAs, intact aleurones were transfected by the biolistic method with the effector plasmid designated to generate each protein and incubated in the absence of GA (Fig. 6A). The effect in the cells was monitored by activation of *luc+* placed downstream of the *RAmy1A* promoter (Fig. 6B). With the *OsDof3* expression alone, no or only a slight reporter activation was observed in the absence of GA in comparison with that of noneffector control. Transfection of the *GAMyb* effector resulted in a 4-fold induction in the GA-untreated cells, indi-

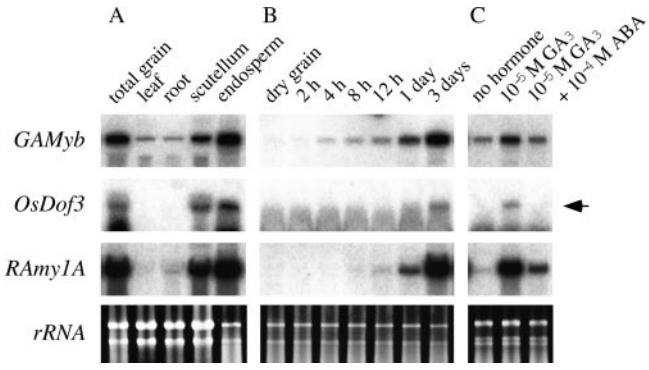

Figure 4. GA-mediated accumulation of mRNA for *GAMyb* and *OsDof3* in germinated rice seeds. A, Leaves, roots, scutellum, and endosperms were excised from 2.5-d-germinated seeds. B, De-embryonated one-half-seeds were incubated in the presence of 10^{-5} M GA_3 for different times as listed on the top of the panel. C, One-half-seeds were treated by different hormonal conditions (no hormone, 10^{-5} M GA_3 and 10^{-5} M GA_3 plus 10^{-4} M abscisic acid) for 2.5 d. Total RNA (10 μg lane^{-1} for *OsDof3* and *GAMyb*, or 2.5 μg lane^{-1} for *RAmy1A* and *rRNA*) from each type of tissue was subjected to electrophoresis. Gels were blotted, and blots were hybridized to the probe indicated to the left of each panel. The band corresponding to the size of the *OsDof3* transcripts is indicated by an arrow. Ethidium bromide-stained RNAs are indicated to ensure equal loading on the lane.

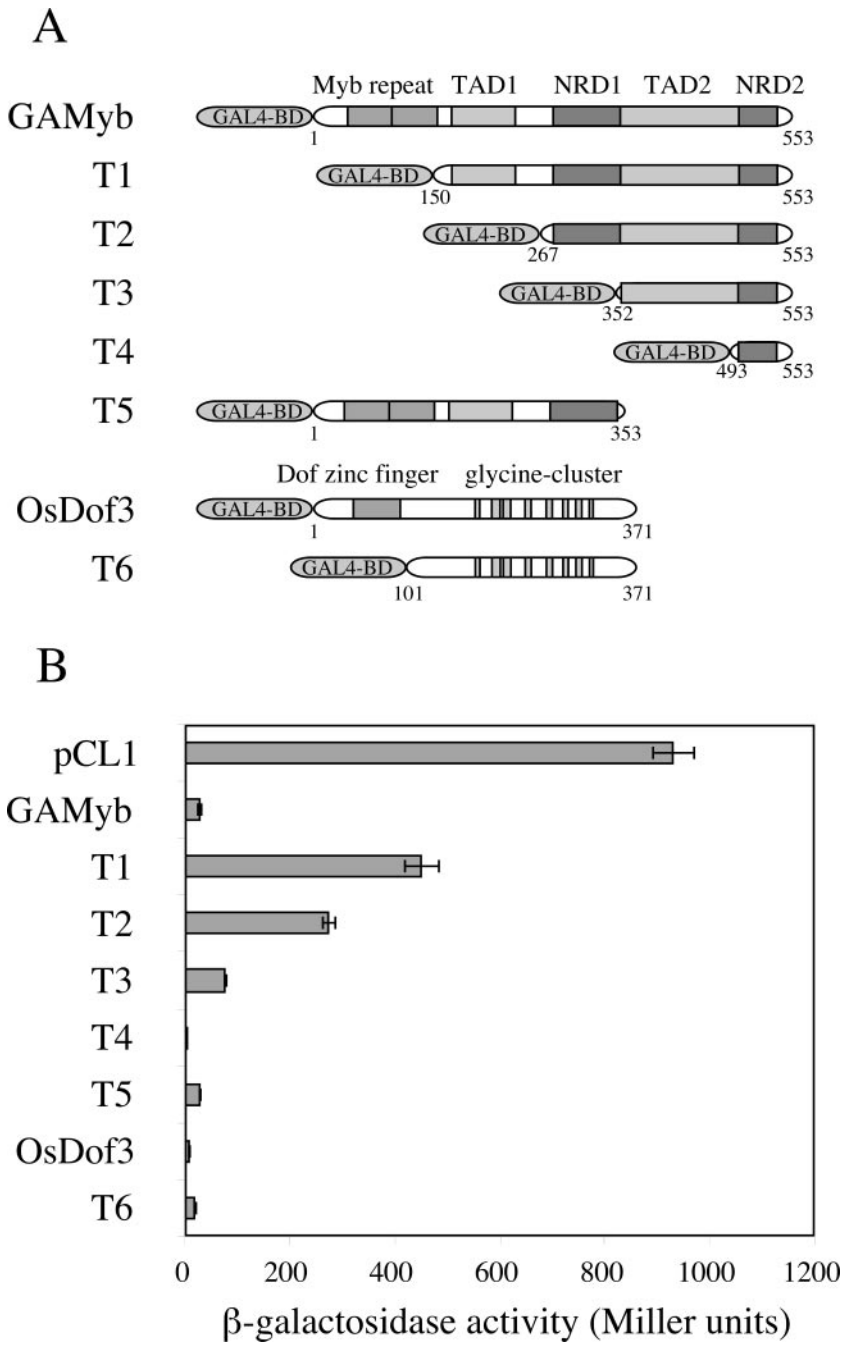

Figure 5. Transcription activation of the *lacZ* reporter gene by OsDOF3 and GAMYB in yeast cells. A, Schematic representation of the effector constructs. OsDOF3, GAMYB, and their truncated derivatives (T1–T6) fused to GAL4-BD for expression in yeast. The localization of the Myb DNA-binding domain (Myb repeat), TADs (1 and 2), and putative NRDs (1 and 2) relative to those in barley GAMYB, which have been deduced from trans-activation experiments in yeast (Gubler et al., 1999), are shown by shaded boxes. The Dof DNA-binding domain (Dof zinc finger) and Gly-rich regions (Gly cluster) of OsDOF3 are also indicated. The numbers refer to the amino acid position in rice GAMYB and OsDOF3. B, β-Galactosidase activity resulting from the transfection of the effector constructs as indicated in A. Measurement of β-galactosidase activity in liquid assays were made from at least three independent replicates and are expressed as Miller's units (Miller, 1992). The bars represent SE of the mean.

cating that in the aleurone, GAMYB is a transactivator of transcription. The activation of the reporter expression was evidenced further by cotransfection with the *GAMyb* and *OsDof3* effector plasmids by which activation of the reporter expression accounted for over a 9-fold induction, but the level was not a match to that observed in the GA-treated sample.

When the proximal pyrimidine box was lacking (M-2), entire levels of the reporter expressions were reduced to 60% of the wild-type reporter. The synergistic effect that was revealed among the *GAMyb* and *OsDof3* effectors especially disappeared. The deficiency of GARE caused drastic decreases of the reporter expressions with any samples (M-4), but the synergy of the GAMyb and OsDof3 effectors was observed minuteness. The results confirm that the functional interaction between GAMYB and OsDOF3 depends on the join of DNA bindings to the *RAmy1A* promoter. It also suggests that OsDOF3 activates a target gene by recruiting GAMYB that possesses a TAD function in the aleurone cells.

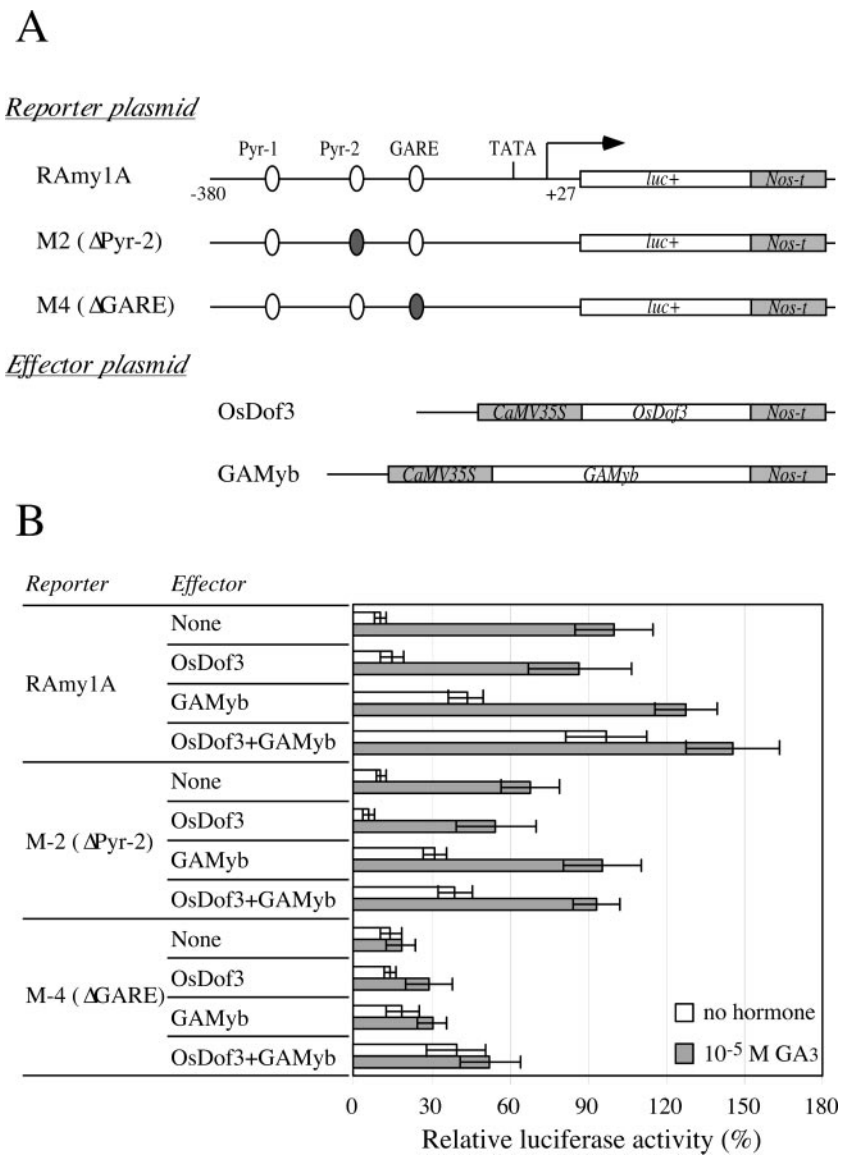

Figure 6. Transient expression of the *RAmy1A* promoter influenced by the expression of OsDOF3 and GAMYB in the rice aleurone. A, Schematic representation of the reporter and effector constructs used in the transient expression assays. The effector constructs contain the *GAMyb* and *OsDof3* cDNAs under the control of the *CaMV35S* promoter. The reporter constructs are identical to those used in Figure 1. Nucleotide changes introduced in mutation promoters (M2 and M4) are indicated by shaded ellipses. B, Relative luciferase reporter activities in the rice aleurone, transfected with indicated combinations of the reporter and effector constructs. Tissues were incubated in the absence (no hormone) or in the presence of GA (10^{-5} M GA_3) for 3.5 d. The effects of the *OsDof3* and *GAMyb* cDNA expressions on the reporter expression are represented relative to the reporter activity from the wild-type *RAmy1A* reporter only that was given a value of 100% in the GA treatment. The bars represent the SE of the mean.

Physical and Functional Interactions between GAMYB and OsDOF3

Given the general observations that a couple of the promoter motifs, GARE and the pyrimidine box, always exist in a close distance in the reported GA-responsive promoters (Huang et al., 1990), the synergistic effect on *RAmy1A* promoter activation are likely to be mediated through protein-protein interaction. That hypothesis has been supported by the experimental evidence providing a direct interaction between barley GAMYB and BPBF, which is a homologous protein of OsDOF3, in the yeast two-hybrid studies (Y2H; Diaz et al., 2002). Y2H analysis was used to test whether OsDOF3 could interact with GAMYB (Fig. 7). Full-length OsDOF3 was expressed as a bait with a C-terminal fusion to GAL4-BD. Interactions were monitored pair-wise with C-terminal fusion proteins to the GAL4 activation domain (GAL4-AD) for the *lacZ* reporter activation. The OsDOF3 fusion protein did not affect the activation of *lacZ* in combination with the GAL4-AD construct alone, indicating that OsDOF3 does not interact with GAL4-AD and it does not activate transcription autonomously. Combination with OsDOF3 and GAMYB affected the *lacZ* activation enough to compare with that observed in combination with p53 and T-antigen, that is, a known pair demonstrated previously to interact via Y2H. Activation of semiquantitative *lacZ* assays allows a discrimination of strong interaction between GAMYB and OsDOF3. Deletions of the N- or C-terminal segments of GAMYB significantly reduced its ability to interact with OsDOF3. In summary, the entire polypeptide of GAMYB should be essential to ensure a strong interaction with OsDOF3.

To demonstrate that Y2H results were not dependent on additional yeast-specific factors, I next tested the pattern of complex formation when GAMYB and

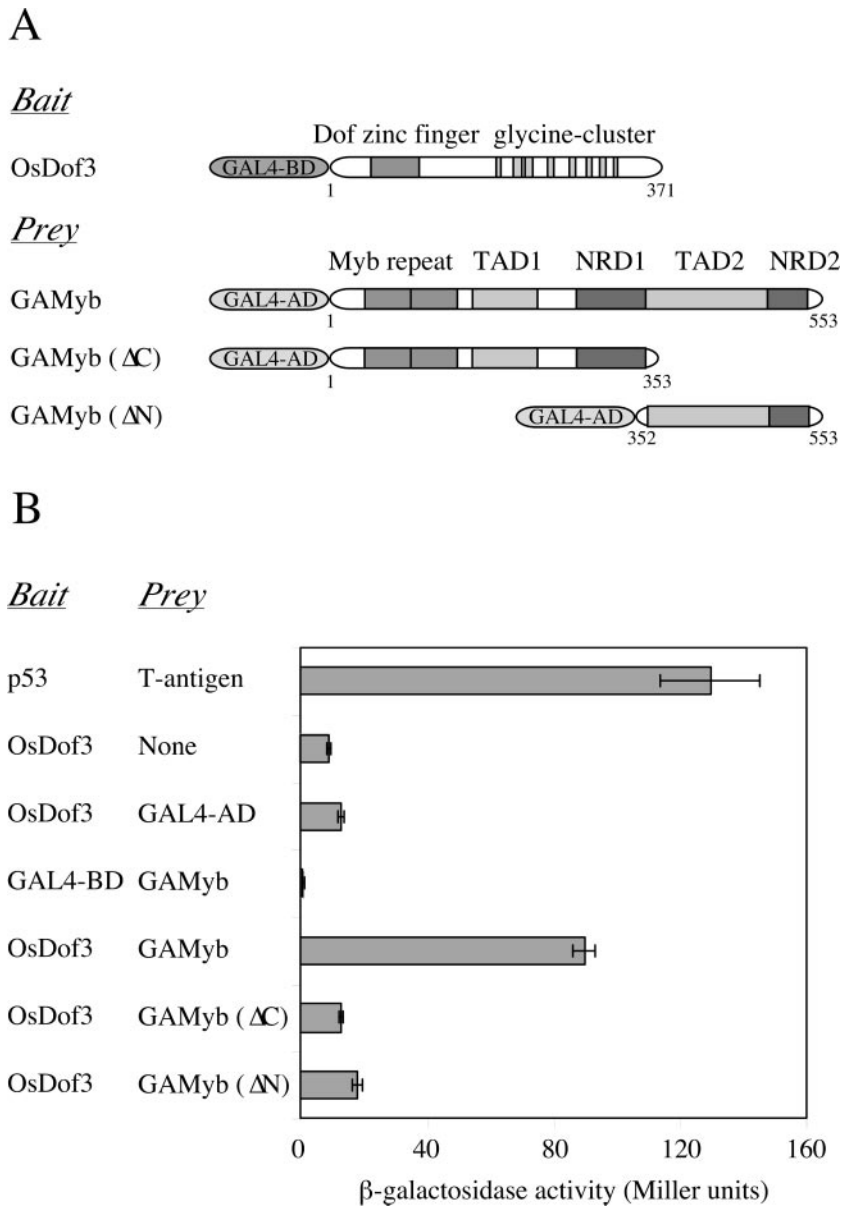

Figure 7. Interaction between OsDOF3 and GAMYB in the yeast two-hybrid system. A, Schematic representation of the effector constructs. OsDOF3 was fused to GAL-BD for expression as a bait in yeast cells. GAMYB and its deletion derivatives fused to GAL4-AD were used as preys in Y2H. Each location of functional domains of OsDOF3 and GAMYB is indicated relative to those present in Figure 5. B, β-Galactosidase activity resulting from the transduction of various combinations of the bait and prey constructs, as indicated to the left of the histogram. Enzyme activity from the combination of p53 and T-antigen clones was included as a positive control for interacting proteins. β-Galactosidase activity obtained from each combination is expressed as Miller's units. The bar represents SE of the mean.

OsDOF3 proteins were incubated together with the *RAmy1A* promoter fragments in vitro. Arabidopsis Dof protein OBP1 has been shown to interact with specific bZIP proteins and stimulate the DNA binding of a partner to the recognition element in the target promoters (Zhang et al., 1995). It raises a possibility that OsDOF3 has a similar effect on the DNA binding of GAMYB to GARE. His-tagged recombinant proteins containing the entire polypeptide for OsDOF3 and GAMYB were produced in *E. coli* and were subjected to combinatorial EMSA (Fig. 8). Distinct incubation of the recombinant OsDOF3 and GAMYB proteins with the probe fragment from the *RAmy1A* promoter (A 148-bp *Eco*RI/*Aat*I fragment containing Pyr-2, D-1, GARE, and D-2) resulted in formation of high- and low-mobility complexes (Fig. 8, lanes 2 and 4). Mutation on an objective site for each binding of two proteins significantly reduced the formation of complexes (Fig. 8, lanes 3 and 5), indicating that the DNA bindings of recombinant proteins are specific. The band intensity could be correlated directly with the amount of OsDOF3 present (Fig. 8, lanes 6 and 7). In the incubation of a fixed amount of GAMYB, I found that the addition of OsDOF3 yielded a new lower mobility band above the complex with GAMYB binding alone (indicated by asterisk, Fig. 8, lanes 8–10). The slowest mobility complex observed with simultaneous incubation of GAMYB and OsDOF3 appeared to be formed with cobinding of both proteins to the probe because the band intensities obtained with GAMYB or OsDOF3 alone also decreased (Fig. 8, lane 7, 10 and lane 8, 10). The modulation of complex formation affected by coincubation of GAMYB and OsDOF3 was not ob-

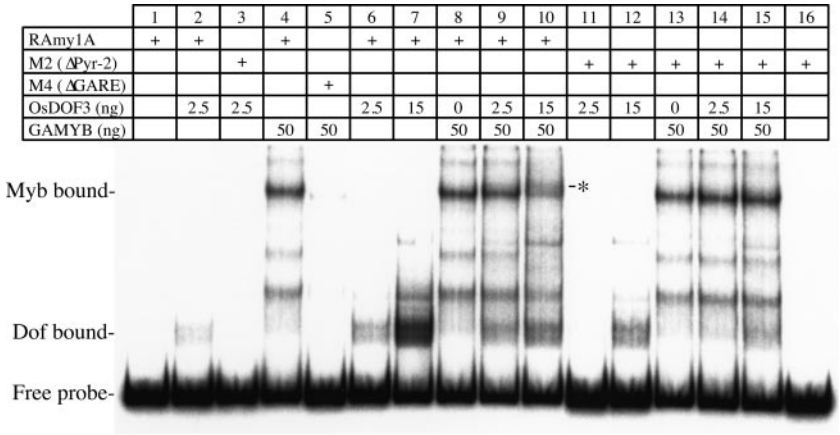

Figure 8. The effect of OsDOF3 on GAMYB binding to the GARE sequence from the *RAmy1A* promoter. Detail components added to the incubations, namely the presence (+) of the probe (RAmy1A, M2, and M4) and added amounts (nanograms per reaction) of the recombinant proteins (OsDOF3 and GAMYB), are listed in parenthesis on the top of the panel. The bindings of equal amounts of GAMYB (50 ng reaction^{-1}) to GARE from the wild-type (RAmy1A; lanes 8–10) or mutated promoters (M2; lanes 13–15) were analyzed in differing amounts of OsDOF3 (0, 2.5, and 15 ng reaction^{-1}). Lanes 1 and 16 contain the probe fragment only. The positions of the free probes (Free probe), and DNA-protein complexes resulting from the interactions with OsDOF3 (Dof bound) and GAMYB (Myb bound) are indicated.

served when the pyrimidine box element (Pyr-2) in the probe had been mutated to the one to which OsDOF3 could not bind (Fig. 8, lanes 11–15).

The results show that OsDOF3 included in the incubation does not affect the affinity of GAMYB binding to GARE in vitro, that is, the contrary the case for Arabidopsis OBP1 protein (Zhang et al., 1995), but can bind together with GAMYB to the *RAmy1A* promoter region. In transient expression experiments in Figure 6, the addition of *GAMyb* and *OsDof3* effectors, as well as the promoter sequences requirements for both factors' binding, appeared to act synergistically, giving rise to substantial levels of the *RAmy1A* promoter activity. The possibility exists that the interaction between GAMYB and OsDOF3 may be critical in facilitating cooperative binding to the target promoters.

GA Dose Dependency of the *GAMyb* and *OsOof3* Gene Expressions in the Germinated Aleurone

To delineate how cooperative interaction of the *GAMyb* and *OsDof3* genes occurs in the aleurone, I explored this question by northern-blot analysis using RNA samples prepared from de-embryonated one-half-seeds that had been incubated in various concentrations of GA (Fig. 9). Relative levels of mRNAs for an α-amylase increased linearly according to the applied concentrations of GA (*RAmy1A*). The maximum induction in band intensities occurred at 10^{-4} M GA_3, accounting for over a 10-fold induction, whereas an adequate level of the *GAMyb* transcripts had already been presenting in the absence of GA and moderately increased up to 10^{-6} M GA_3. Higher concentrations of GA did not give rise to the expression of *GAMyb* well, showing an inconsistency with a tight regulation of target gene expressions across the GA concentrations tested. The induction of *OsDof3* began at 10^{-8} M GA_3 and was maintained up until 10^{-3} M GA_3. These data are interpreted as indicating that higher concentrations of GA are required for simultaneous expression of the *GAMyb* and *OsDof3* genes, whereas lower concentrations induce only *GAMyb*. High dosages of GA are likely to be necessary for a full response to induce target gene expressions in the aleurone, in which the *GAMyb* and *OsDof3* functions could contact each other.

DISCUSSION

In this study, I have found some biochemical evidence for the GA-controlled expression of genes. The DNA binding of a rice Dof protein OsDOF3 is capable of stimulating GA response of an α-amylase promoter by mediating the trans-action of GAMYB, possibly through protein-protein interaction. The Dof proteins are a group of transcription factors specific to the plant kingdom (Yanagisawa, 2002). These proteins compose of relatively small size of polypeptides and share a conserved 52-amino acid sequence, including a variant sequence of the zinc fingers, designated the Dof domain. In Arabidopsis, completion of the genome analysis indicates the presence of 37 members of the *Dof* gene family (Riechmann et al., 2000), whereas there are as many as 21 genes present in a current rice database (Goff et al., 2002). The Dof proteins all recognize similar target sequences with a CTTT (or an AAAG) core (Yanagisawa and Schmidt, 1999); however, these proteins do not share significant sequence homology outside the Dof domain. This suggests that each action of a distinct Dof protein should rest on specific interaction with other regulatory protein(s).

Substantial effects in plants have been convincingly demonstrated by the genetic evidence for two genes in Arabidopsis, *DAG1* and *DAG2*. The lesion of *DAG1* by T-DNA insertion increased sensitivity to red light and raised germination potentials of mutated seeds (Papi et al., 2002). The phenotype of *DAG1* mutation has been reverted by the knockout of *DAG2* (Gualberti et al., 2002), implying an opposite effect on germination between *DAG1* and *DAG2*. This supports a scenario that combinatorial regulations are important in the Dof functions.

Reliable reports on cooperative actions between the Dof protein and a specific partner have been made. The maize Dof1 and Dof2 proteins control the expression of genes participating in carbon metabolism (Yanagisawa, 2000). Previously described maize PBF binds the prolamine box in zein gene promoters and interacts with the bZIP transcription activator Opaque2 (Vicente-Carbajosa et al., 1997). Similarly, Arabidopsis OBP1 influences the DNA binding of bZIP proteins via protein-protein interactions and regulates the expression of stress-related genes (Chen et al., 1996). A barley counterpart of the PBF protein, BPBF, should especially be noted. During development of barley seed, barley GAMYB and BPBF transactivate *Hor2* and *Itr1* promoters through binding to each objective promoter element (Diaz et al., 2002).

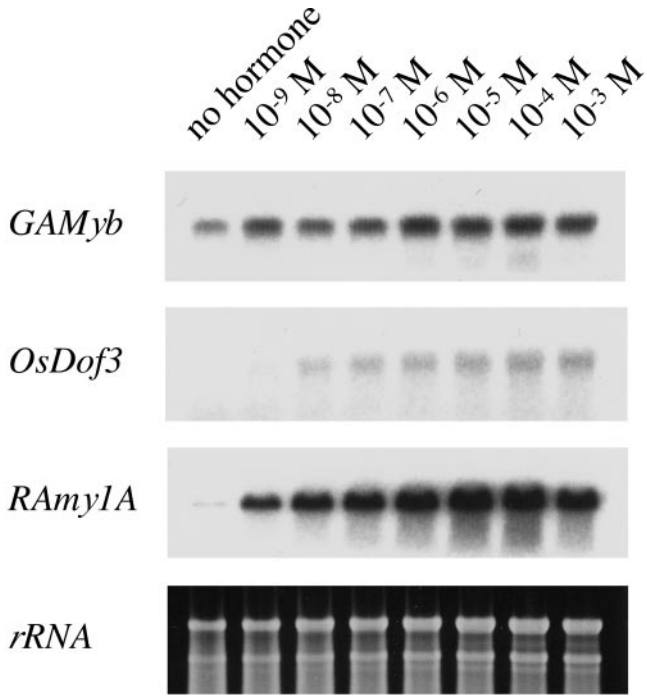

Figure 9. Northern-blot analysis showing the effect of GA_3 concentrations on the mRNA accumulation for rice GA-responsive genes in the aleurone. De-embryonated one-half-seeds were incubated for 2.5 d in the different molarities of GA_3 as listed above each lane. Total RNA (10 μg lane^{-1} for *OsDOF3* and *GAMyb* or 2.5 μg lane^{-1} for *RAmy1A* and *rRNA*) from each tissue was subjected to northern-blot analysis as described in the text. The blots were hybridized to the probes indicated at the left of the panel.

The mutation of the Dof-binding sequence influences not only the trans-activation of BPBF, but also that of GAMYB. Moreover, BPBF is capable of interacting with GAMYB in Y2H. These results strongly suggest a virtual combination of the Dof and GAMYB factors in vivo.

Using Y2H analysis, I have confirmed reported interactions found in the Dof and GAMYB proteins. The observation previously reported for BPBF suggested that an interaction between the C-terminal part of BPBF and barley GAMYB was taking place (Diaz et al., 2002). Expression of the GAL4-BD and C-terminal part of OsDOF3 fusion conferred detectable background levels of the *lacZ* reporter expression (Fig. 5). Similarities of the amino acid sequences between OsDOF3 and PBFs are scarcely detectable outside the Dof domain (34% similarities between OsDOF3 and BPBF; Fig. 2); regardless, these proteins belong to a phylogenetically related class (Yanagisawa, 2002). These conditions make it difficult to identify basic polypeptide domains necessary for the interaction between the Dof and GAMYB proteins. The C-terminal part of OsDOF3 contains a number of Gly residues where complete matching of the amino acid sequences is observed occasionally among the PBF proteins (Fig. 2). High flexibility of a Gly-rich peptide is thought to make it a good candidate for interaction with other proteins (Sachetto-Martins et al., 2000). In that context, OsDOF3, binding the pyrimidine box, may potentiate GAMYB action through protein-protein association of the C-terminal part.

This speculation is also supported by the result of combinatorial EMSA (Fig. 8). Concerted binding to the *RAmy1A* promoter, together with GAMYB, depends on the amounts of OsDOF3 binding to the pyrimidine box rather than that included in the incubation. This result proposes that not only the physical interaction and also mutual binding to the target promoter are necessary for a productive running of both factor's contributions. As referred earlier, GAMYB plays a role in GA response throughout the life cycle of the plant, as well as in the germinated seeds. It means that GAMYB manages to control the expressions of diverse set of genes that mediate GA actions in different biological aspects, putatively with specific partner(s). Potential target for GAMYB action has been postulated in the promoters of the floral identity *LEAFY* (Gocal et al., 2001) and cell wall-loosening expansin genes (Lee et al., 2001). OsDOF3 might approach to the target promoter, as the marker of the pyrimidine box along with GAMYB binding to GARE, and then make a decoy for assembling high-order complexes of the transcription machinery leading to high-level expression of gene. It is possible that a number of genes for hydrolytic enzymes receive a benefit of cooperative action of GAMYB and Dof proteins in the germinated aleurone because each promoter possesses a set of motifs targeted by both

factors, namely GARE and the pyrimidine box, in the juxtaposition (Huang et al., 1990).

The stimulating effect of OsDOF3 on the GAMYB function is puzzling because my result is in conflict with the inhibitory effect of BPBF on the GA-regulated expression of the *Al21* promoter in the aleurone (Mena et al., 2002). In transient expression experiments, BPBF could largely revert the GAMYB-mediated trans-activation, even if the *BPBF* gene expression had been up-regulated by GA. The authors presented a possible scenario that the repression activity of BPBF would compete with the function of another Dof transcription activator(s) in such a way that *DAG1* and *DAG2* have opposite effects on germination of Arabidopsis seeds (Gualberti et al., 2002). Recently, a barley gene encoding a new Dof protein, highly abundant in the germinated scutellum and aleurones, has been identified (SAD; Isabel-LaMoneda et al., 2003). The SAD protein, expressed in *E. coli*, binds the pyrimidine box sequences present in the *AL21* promoter and transiently trans-activates a reporter expression from this promoter in transfected aleurone cells. Synergistic effect on the *AL21* promoter activation and physical interaction in Y2H have been confirmed between the SAD and GAMYB proteins, suggesting the possibility of SAD competing with BPBF in barley aleurone cells. In the same report, the presence of rice counterparts for SAD has been pointed out. Previously identified OsDOF2 is likely to be a SAD-homologous protein (Washio, 2001). For the present, the function of OsDOF2 on the GA-regulated gene expression is unclear, but the distribution of the *OsDof2* transcripts in the germinated seeds somewhat differs from that of *SAD*. The *OsDof2* expression is biased in the seedling tissues containing the scutella and not abundant in the aleurones (Washio, 2001). The cooperative action of OsDOF2 and OsDOF3 in the germinated rice seeds is possibly due to their being different from that of SAD and BPBF.

The belief of the stimulating effect of OsDOF3 is strengthened by the fact that mutations of the pyrimidine box in several GA-responsive promoters resulted in decreased expression in response to GA (for review, see Jacobsen et al., 1995). This evidence argues for the pyrimidine box ultimately being recognized by a trans-factor possessing positive roles on the GA-regulated gene expression. Previous experiments with BPBF in the analogous yeast one-hybrid system showed trans-activation ability in the C-terminal part, which was 10 times higher than that of OsDOF3, in the same yeast strain SFY526 (Diaz et al., 2002). This shows the fact that the functions of the C-terminal part are substantially differing between BPBF and OsDOF3. Two proteins are presumed to evolve in a different manner, while keeping a function that interacts with GAMYB.

The genes for hydrolytic enzymes are not all subjected to the same regulation by GA. There are temporal and quantitative differences in their expression among plant species and varieties. As for the tissue-specific expressions of α-amylase genes in the germinated seeds, barley is less specific than rice (Karrer et al., 1991). In the aleurone layers, the progress of barley α-amylase gene expression is slower than that of rice *RAmy1A* gene by nearly one-half (Sugimoto et al., 1998). The placement, order, and orientation of the promoter sequences, including the pyrimidine box and GARE, also appear to be different in each promoter (Cercós et al., 1999). Multiple interactions between distinct classes of the Dof protein might be playing diverse roles in providing a fine-tuning of gene expression, varying interaction between GAMYB binding to cognate GAREs.

Expression kinetics of the *GAMyb* and *OsDof3* genes showed the necessity of high-level and long-time treatments of GA for simultaneous expressions in the aleurone cells (Figs. 4 and 9). The importance of active GA levels for germination is best shown by studies of GA biosynthesis. The rice genes for GA 3β-hydroxylase, which catalyzes the final step of active GA synthesis, are expressed differing with a temporal and spatial pattern of mRNA accumulation in the germinated seeds in which only the synthesis in the scutellum epithelium is essential for α-amylase expression in the aleurone (Kaneko et al., 2002). The evidence supports an idea that local change of active GA levels affected by additional endogenous and environmental cues is important for plant processes (Hay et al., 2002). The GAs, being continuously supplied from the epithelium, ensure the specificity of the aleurone cells to high-dose responses of GA and produce numerous amounts of hydrolytic activities through possible cooperation between the *GAMyb* and *OsDof3* gene functions.

Recent evidence provides a reliable concept that the GA signal cascade causes derepression of the repressible function of the DELLA proteins of the GRAS family (Olszewski et al., 2002). Arabidopsis has five genes in the DELLA subfamily, from which encoded negative regulators of GA responses appear to have partially redundant or overlapping functions (Lee et al., 2002; Wen and Chang, 2002). By contrast, rice and barley seem to have just one *DELLA* gene so that only one DELLA protein exerts pleiotropic responses of GA in these plants (Ikeda et al., 2001; Chandler et al., 2002). The working of the DELLA proteins in cereal aleurones has been identified (Ikeda et al., 2001; Gubler et al., 2002), but little is known about detailed mechanisms as to how simple behaviors of the DELLA protein contribute to generate various processes of such plant growth. One explanation is that rice and barley DELLA proteins govern a complex battery of components that cause GA responses, and release them from their own repressible functions according to the relevant body plan. From this point of view, it will be crucial to determine whether the *OsDof3* gene expression is a

secondary response directed by the trans-action of GAMYB or a primary response that is behind with tight regulations of the repressible functions of the DELLA protein. These various pieces of evidence allow us to speculate concerning a better understanding of plant cellular responses to GA.

MATERALS AND METHODS

Plant Materials

The rice (*Oryza sativa* cv Yukihikari) used in this study was kindly supplied by the Hokkaido Central Agricultural Experiment Station (Iwamizawa, Japan). Dehusked seeds and de-embryonated one-half-seeds were surface sterilized in 2.5% (w/v) NaClO for 20 min, rinsed extensively with sterile water, and immersed in water containing 20 mM $CaCl_2$, 10 μg mL^{-1} chloramphenicol, 10 μg mL^{-1} ampicillin, and 25 units mL^{-1} nystatin. After the incubation in the dark at 30°C, germinated seeds were dissected into leaves, roots, scutella, and endosperms containing the aleurone layer at d 2.5. Samples were immediately frozen in liquid nitrogen.

Northern-Blot Analysis

Total RNA was extracted from frozen samples according to Washio and Ishikawa (1994). DNA fragments for probes were prepared from each cDNA or a genomic clone as follows. The cDNA clone of rice *GAMyb* was isolated from a rice aleurone library with reference to the sequence information from the published cDNA sequence (Gubler et al., 1997). The *RAmy1A* gene was obtained by PCR (Huang et al., 1990). To avoid cross-hybridizations among the *Dof* gene family, regions of nucleotide sequences from the *OsDof3* cDNA (42–207) were amplified by PCR (Washio, 2001). The *Pst*I/*Eco*RI (1,452–2,046) fragments were prepared from the cDNA clone for rice *GAMyb* (Gubler et al., 1997). The *Pst*I genomic fragments containing the first exon of the *RAmy1A* gene (+27–+325) were also prepared (Huang et al., 1990). Each probe fragment was labeled by the random priming procedure. Northern hybridizations were performed as described previously by Sambrook et al. (1989). Hybridization results were viewed using an image analyzer (BAS2000; Fuji Film, Tokyo).

Transient Gene Expression in the Germinated Rice Aleurone

Nucleotide substitutions on the *RAmy1A* promoter shown in Figure 1 were generated by a PCR-based in vitro mutagenesis according to the manufacturer's instructions (Takara Shuzo, Kyoto). The −380 to +27 fragment, containing the GA-responsive region from the *RAmy1A* promoter, and its mutation versions were ligated with a gene cassette that carried a firefly luciferase gene (*luc+*; Promega, Madison, WI), and the 3′ terminator of a gene for *Nos-t*. The ligated fragments were cloned between restriction sites for *Hind*III and *Eco*RI in the plasmid pUC18 and were used as the reporter constructs. Effector plasmids were created by insertion of the *Bgl*II fragments (100–1,282) from the *OsDof3* cDNA, and the *Kpn*I/*Sph*I fragments (109–2,185) from the *GAMyb* cDNA, between the *CaMV35S* promoter and *Nos-t* of a plant expression vector pBI221 (Clontech, Palo Alto, CA) in a correct orientation.

Particle bombardment was carried out with a biolistic helium gun device (IDERA GIE-III; Tanaka, Sapporo, Japan) as described previously with some modifications (Washio, 2001). The weight-ratio of the reporter and effector constructs was set to 5:1, by which 20 μL of gold suspension (50 ng mL^{-1}, 1.5–3.0 μm in size) was mixed with 30 μL of plasmid solution containing 1.0 μg of the reporter, 2.0 μg of the reference plasmid, and if needed, 0.2 μg of the effector plasmids or an empty vector. After bombardment, de-embryonated one-half-seeds were grown in the dark at 30°C in the absence or in the presence of 10^{-5} M GA_3 for 3.5 d. *Luc+* expression was determined by total light units emitted from each reporter reaction, following the manufacturer's instructions (Dual-luciferase reporter assay system; Promega). The relative *luc+* activities from each reaction, normalized with reference to *Renilla* luciferase activity due to the internal control (Washio, 2001), are shown. Each reporter was assayed at least three times and averaged.

Production of Recombinant Proteins in *Escherichia coli*

A 1,152-bp cDNA fragment, spanning from nucleotide positions 126 to 1,277 in the *OsDof3* cDNA, was amplified by PCR using oligonucleotide primers as follows; the forward primer was 5′-GGGGCATATGGCGAG-CGGCGGCGCATTATCC-3′, which incorporated a *Nde*I site (underlined) on a first initiation codon, and the reverse primer was 5′-TCTGGATCCAAC-CTTGGCATGGTTGATTTG-3′, which added a new *Bam*HI site at the 3′ end of the PCR product. Similarly, a cDNA fragment (396–2,126) of the *GAMyb* cDNA was obtained using a set of primers (forward; 5′-GGGGCATATGTA-TCGGGTGAAGAGCGAGAGC-3′, reverse; 5′-GTTAGGATCCAAACAA-TGAGCAAGAACGA-3′). The PCR fragments were cloned into the *Nde*I/*Bam*HI-digested pET15b vector (Novagen, Madison, WI) to express His-tagged recombinant proteins.

E. coli strain Rosetta DE3 (Novagen) was transformed with the expression plasmids. A fresh colony was inoculated in 2× yeast tryptone (YT) liquid medium containing 150 μg mL^{-1} carbenicillin and was grown for the middle log phase at 37°C. Expression of recombinant proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactoside for 3.5 h at 25°C. The insoluble fraction, containing recombinant proteins, from each transformant was solubilized by 6 M urea. Purification was achieved through Ni-agarose affinity chromatography under denaturing conditions including 6 M urea, following the manufacturer's instructions (Novagen).

EMSA

The *Dra*I/*Aat*I fragment (−380 to −85) and the *Aat*I/*Pst*I fragment (−84 to +27) from the *RAmy1A* promoter, and the mutation derivatives were endolabeled by a fill-in reaction with a [α-^{32}P] dNTP mixture (3,000 Ci mM^{-1}; ICN, Costa Mesa, CA) and Klenow enzyme, and were gel purified. Reactions were performed in 20 μL of a mixture containing 20 mM HEPES-KOH (pH 7.9), 100 mM KCl, 1 mM dithiothreitol, 0.02% (w/v) Nonidet P-40, 4% (w/v) Ficoll 400, 0.5 μg of poly(dI-dC), 0.5 μg of poly(dA-dT), 0.2 ng of radiolabeled probe (approximately 20,000 cpm), and 5.0 ng of the recombinant OsDOF3 protein. After incubation for 30 min at 25°C, the DNA-protein complexes were resolved on a 4% (w/v) polyacrylamide gel with 0.5× 89 mM Tris-HCl and 2 mM EDTA at 100 V for 3.5 h at 4°C. The gel was dried and subjected to autoradiography.

For combinatorial EMSA, a combination of recombinant proteins (OsDOF3 and GAMYB) was added to the reaction composed of 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM dithiothreitol, 0.05% (w/v) Nonidet P-40, 10% (w/v) glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of poly(dA-dT), and 0.2 ng of radiolabeled probe as described in Figure 8. The DNA-protein complexes were resolved on a 6% (w/v) PAGE.

Yeast Transcription Activation Assay

The rice *GAMyb* cDNA was prepared for cloning into the pGBT9 vector (Promega) by PCR amplification with the forward primer; 5′-TGAGGATC-CATGTATCGGGTGAAGAGCGAG-3′ to introduce a *Bam*HI site before the start codon, and the reverse primer; 5′-GTTAGGATCCAAACA-ATGAGCAAGAACGA-3′ to create a new *Bam*HI site at the 3′ end of the PCR product. Similarly, the *OsDof3* cDNA was amplified by the PCR reaction with a set of primers, 5′-AGGGAATTCATGGCGAGCGGC-GGCGCATTA-3′ (forward) and 5′-TCTGGATCCAACCTTGGCATGGTTGATTTG-3′ (reverse). N- or C-terminal deletions of the *GAMyb* and *OsDof3* cDNAs were prepared by PCR amplification of the corresponding regions of each cDNA. The PCR products were digested with *Bam*HI or *Eco*RI/*Bam*HI and were cloned into objective sites in the GBT9 vector for generating fusion proteins with GAL4-BD. pCL1, encoding the full length of GAL4 protein, was used as a positive control of transcription activation in yeast.

Yeast expression constructs were transformed into yeast strain SFY526 (Promega) that had a β-galactosidase gene under the control of the *GAL1* promoter. Transcription activation of the *lacZ* reporter gene influenced by GAL4-BD fusion proteins was measured by β-galactosidase assay as described previously (Miller, 1992). Values represent the average of three repeat assays for each treatment.

Yeast Two-Hybrid Assay

The *OsDof3* cDNA in the GBT9 plasmid was used as a bait to create a fusion protein with the GAL4-BD and OsDOF3. The full-length and truncated fragments from the *GAMyb* cDNA with additional *Eco*RI and *Bam*HI flanking sites were amplified by PCR and ligated with *Eco*RI/*Bam*HI-digested GAD424 plasmids as described above. Obtained GAD424 constructs, expressed as fusion proteins with GAL4-AD, were used as preys. Yeast transformations were performed with various combinations of bait and prey constructs as shown in Figure 7. Transformants were screened for growth in Trp- and Leu-depleted agar medium. Interactions between a bait and a prey protein were evaluated by quantification of β-galactosidase activity in liquid cultures (Miller, 1992). Values represent the average of three repeat assays for each treatment.

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