

Two *cis*-acting elements necessary and sufficient for gibberellin-upregulated proteinase expression in rice seeds

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Received 3 October 2002; revised 4 February 2003; accepted 21 February 2003.

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Summary

In germinating rice seeds, a cysteine proteinase (REP-1), synthesized in aleurone-layer cells, is a key enzyme in the degradation of the major storage protein, glutelin. The expression of the gene for REP-1 (*Rep1*) is induced by gibberellins (GAs) and repressed by abscisic acid (ABA). To identify GA-responsive elements in the *Rep1* promoter, we developed a transient expression system in rice aleurone cells. Deletion and point-mutation analyses indicated that the GA-response complex was composed of TAACAGA, TAACGTA, and two copies of CAACTC. The two former sequences were identical to GAREs conserved in the promoter of genes for α -amylase and proteinases in cereals. The latter, termed as CAACTC regulatory elements (CAREs), were novel GAREs. Gain-of-function experiments revealed that two pairs of GARE and CARE were necessary and sufficient to confer GA inducibility. The sequences were also required for effective transactivation by the transcription factor OsGAMYb. Four copies of either GARE or CARE showed transactivation neither by OsGAMYb nor by GA induction. CARE and GARE were also found in the promoters of a rice α -amylase gene, *RAmy1A*, and a barley proteinase gene, *EPB1*, which are expressed in germinating seeds. Mutations of CARE in their promoters caused a loss of GA inducibility and GAMyb transactivation, suggesting that CARE is the regulatory element for GA-inducible expression of hydrolase genes in the germinating seeds.

Keywords: aleurone, GARE, gibberellin, Proteinase, Rice.

Introduction

During cereal seed germination, gibberellins (GAs) which are synthesized in the embryo, diffuse to the aleurone-layer cells and induce the expression of hydrolytic enzymes such as α -amylases and proteinases in the cells. The hydrolases are secreted into the endosperm, where they degrade and mobilize the starch and storage proteins, which, in turn, fuel the embryo growth. GA-inducible expression of the gene in the aleurone layer is antagonized by abscisic acid (ABA; Fincher, 1989; Jacobsen and Beach, 1985).

Cis-regulatory elements involved in the GA induction of α -amylase genes have been studied extensively in a transient expression system, using barley aleurone cells. GA-inducible expression of the high-pI α -amylase gene, *Amy6-4*, depended on the presence of three regulatory elements, GARE (TAACAG/AA), amylase box (TATCCAT), and pyrimidine box (C/TCTTTT) in the promoter region (Gubler and Jacobsen, 1992). The GA-dependent expression of the low-pI α -amylase gene, *Amy32b*, required one additional regulatory region, the Opaque-2 binding sequence (Lanahan

et al., 1992). Moreover, GARE and pyrimidine box were shown to be essential for GA-induced expression of the cysteine proteinase gene *EPB1* (Cercós *et al.*, 1999). Gubler *et al.* (1995) obtained a cDNA clone for a GA-induced Myb-like transcription factor, HvGAMYb, which bound specifically to GARE in the α -amylase gene promoter and which transactivated the expression of the gene. HvGAMYb was also shown to transactivate the expression of the genes for proteinases and glucanase (Gubler *et al.*, 1999). In barley, GA-inducible gene expression is repressed by the zinc finger protein, HRT, which binds to GARE (Raventós *et al.*, 1998).

In contrast to barley, no transient expression system has been established so far using rice aleurone cells. Therefore, information on the mechanisms of GA-regulated gene expression in rice is derived mainly from analyses of the mutants. The rice dwarf mutant, *d1*, is defective in the α -subunit of the heterotrimeric G protein (Ashikari *et al.*, 1999; Ueguchi-Tanaka *et al.*, 2000). GA induction of

α -amylase activity in aleurone cells of *d1* is greatly reduced (Ueguchi-Tanaka *et al.*, 2000). In addition, GA applied to the aleurone layer induced a lower expression of the genes for α -amylase, *RAmy1A*, and a rice GAMyb homolog, *OsGAMyb*, in *d1* than in the wild type. The GARE element is present in the *RAmy1A* promoter. These facts suggest that a heterotrimeric G protein is involved in the GA-dependent expression of *OsGAMyb*, and that the *OsGAMyb* protein activates the GA-inducible gene expression through GARE in the *RAmy1A* promoter. However, direct evidence for such a pathway is lacking to date.

A cysteine proteinase, REP-1, was identified as the key enzyme in the degradation of glutelin in the endosperm of germinating rice, and its cDNA clone, pRP60, was isolated and sequenced (Kato and Minamikawa, 1996). The deduced amino acid sequence indicated that REP-1 belongs to the papain family. GA induced the expression of the REP-1 gene, *Rep1*, in the aleurone-layer cells, and its induction was antagonized by ABA (Shintani *et al.*, 1997). The genomic clone was isolated, and its sequence revealed that *Rep1* is an intronless gene (Kato *et al.*, 1999). Ho *et al.*, (2000) isolated a cysteine proteinase gene, *OsCP3A*, which corresponded to *Rep1*. *OsCP3A* expression was induced in cultured suspension cells by nitrogen starvation, and in seeds by GA. The *OsCP3A* promoter region contained GA-response elements as well as nitrogen-regulatory elements (Ho *et al.*, 2000).

In the present study, we developed a transient expression system in the rice aleurone layers. We carried out deletion- and point-mutation analyses as well as gain-of-function experiments to define the regulatory elements required for the GA-induced gene expression and transactivation by *OsGAMyb*. We identified a novel GA-responsive sequence, the CAACTC regulatory element (CARE), and demonstrated that the two pairs of CARE and GARE are necessary and sufficient for GA-inducible expression and effective transactivation by *OsGAMyb*. Furthermore, we found CARE in the promoters of *RAmy1A* and *EPB1*, and showed that it was involved in the GA-dependent expression of these genes in the germinating seeds.

Results

GA-inducible expression of Rep1 and RAmy1A follows that of OsGAMyb

We examined the occurrence of mRNAs of *Rep1*, *RAmy1A*, and *OsGAMyb* in the embryo-less half-seeds of rice, which were incubated with or without GA (Figure 1). None of the three mRNAs was detectable before the start of the incubation. In the absence of GA₃, small amounts of the mRNAs of *Rep1* and *OsGAMyb* were detected at 12 h, and the levels were maintained until 72 h. *RAmy1A* mRNA appeared at 72 h. In the presence of GA₃, *Rep1* and *RAmy1A* mRNAs

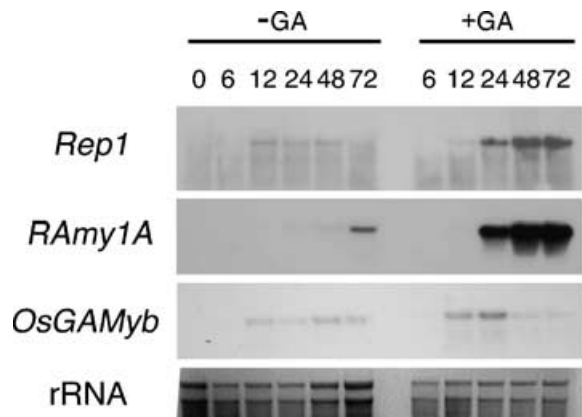


Figure 1. RNA gel blot analyses of mRNAs for *Rep1*, *RAmy1A*, and *OsGAMyb* in embryo-less half-seeds of rice.

Embryo-less half-seeds were incubated at 27°C for 0, 6, 12, 24, 48, and 72 h with gibberellin (+GA) or without GA (–GA) 0.5 μM GA₃. Total RNA was extracted from the seeds, and 10 μg (*Rep1* and *RAmy1A*) or 20 μg (*OsGAMyb*), respectively, of total RNA was run on a 1.4% agarose gel. Preparations of probes for *Rep1*, *RAmy1A*, and *OsGAMyb* are described in Experimental procedures. For detection of rRNA, which was used as a loading control, the gel was stained with 0.04% methylene blue.

accumulated between 12 and 48 h, and remained at a high level until 72 h. In contrast, *OsGAMyb* mRNA was also detected at 12 h, but its level decreased after it had reached a peak at 24 h. Thus, *OsGAMyb* mRNA reached peak levels 24 h before the *Rep1* and *RAmy1A* mRNAs.

5'-terminal deletion analyses indicate that the region downstream of –184 is required for GA induction of Rep1

Previously, the rice aleurone cells could not be prepared for transient gene expression as readily as the aleurone layers of barley. We developed a protocol including the incubation of embryo-less half-seeds of rice for 3 days, and the dissection of the half-seeds prior to the pericarp removal (see Experimental procedures for details), which allowed us to establish a transient expression system in rice aleurone.

To identify the GA-response elements of the *Rep1* promoter, a series of 5'-terminal deletions of the promoter were constructed and fused to the β -glucuronidase (GUS) coding region (Figure 2). When we transfected the rice seeds with the construct RX-808 containing the longest promoter region, the exogenous application of GA₃ increased the GUS activity sixfold as compared to the untreated control. The relative GA₃-dependent increase in the GUS activity was similarly high in all deletion mutants but one. Only the removal of the region between –184 and –112 caused a loss of GA inducibility, indicating the presence of GA-response elements downstream of –184. Exogenous ABA reduced the GA₃-induced GUS activity of RX-184-transfected tissue to a quarter (data not shown). This is in line with the reported ABA-triggered reduction of *Rep1* mRNA levels in rice seeds (Shintani *et al.*, 1997).

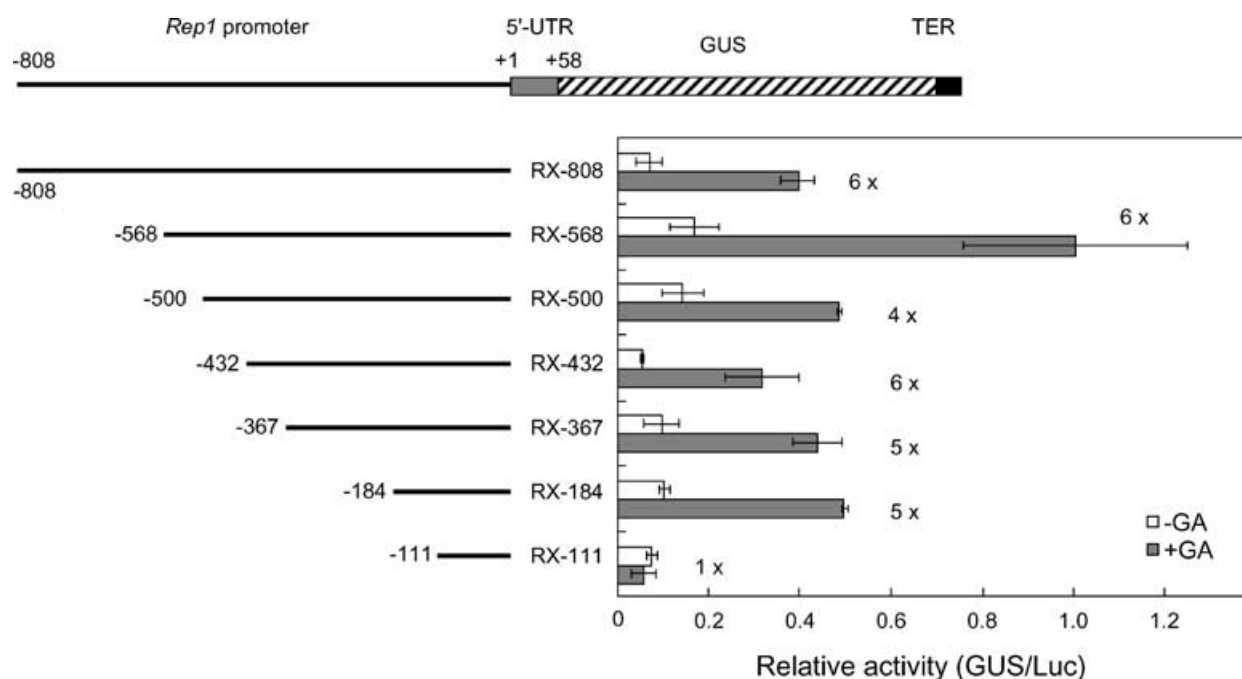


Figure 2. Deletion analysis of the *Rep1* promoter with the transient expression system.

A diagram of the forefather construct, RX-808, is shown at the top. The promoter region of *Rep1*, the 5'-untranslated region (UTR) of *Rep1* from +1 to +58, the β -glucuronidase (GUS) coding region, and the terminator (TER) of the nopaline synthase gene are indicated. The numbers to the left of the construct diagrams indicate the 5' end of each deletion. At the right, results of the transient expression experiments with the different constructs are shown. Transfected embryo-less seeds were incubated for 20 h with or without 0.5 μ M GA₃. For the estimation of transformation efficiency, the luciferase (Luc) reporter construct pAHC18 was co-bombarded with each deletion construct. Bars shown in the graph on the right indicate relative GUS activities (normalized with respect to luciferase activity) \pm standard errors (SEs; $n = 4$). Deletion of bases downstream of -184 abolished the gibberellin (GA) response, indicating that GA-responsive elements are located in this region.

Four putative Myb-binding sequences exist in the *Rep1* promoter downstream of -184

GARE located in the promoter regions of the genes for α -amylase and proteinases interacted with the Myb-type *trans*-acting factor, HvGAMyB (Gubler *et al.*, 1995, 1999). In a previous study, the consensus sequence that binds to c-Myb, YAACKGHH, was derived by a site-selection protocol (Weston, 1992). Comparing this sequence with the -184 to -1 region of the *Rep1* promoter, we found four putative Myb-binding sites, CAACTCAC, CAACTCCC, TAACGTAG, and TAACAGAA (Figure 3). The motif CAACTC was identical in the former two sites, and these sequences were named CAREs. The latter two sites corresponded to GARE.

Mutational analyses indicate that CARE and GARE are essential for GA-inducible expression of *Rep1*

To investigate whether CARE and GARE were associated with the GA-inducible expression of *Rep1*, we carried out mutational analyses of the four sites. A single *Xho*I site (CTCGAG) was introduced into each of CARE or GARE of the construct RX-184. The four constructs were termed mCAAC-1, mCAAC-2, mGARE-1, and mGARE-2, depending on the mutated site. In cells that were transfected with RX-

184, the GUS activity could be increased by more than fourfold by exogenous GA (Figure 4). In comparison, the relative GA-dependent increase in GUS activity was reduced to about twofold in the CARE mutants. In addition, these mutants showed the decrease of the GUS activities in the absence of GA₃ compared to that of RX-184. No statistically significant GA induction of GUS activity was detected in GARE mutants (Figure 4). We conclude that two GAREs are necessary for GA induction, and that CARE modulates the absolute level of expression and enhances the level of GA-induced expression.

A minimal promoter acquired GA inducibility by addition of two pairs of CARE and GARE

To examine the functional significance of the arrangement in pairs of CAREs and GAREs in the GA-responsive promoters, different fragments of the *Rep1* promoter were fused to the minimal promoter region of the cauliflower mosaic virus (CaMV) 35S RNA gene coupled to the GUS reporter gene. The minimal promoter alone (pNoins) did not react to the exogenous GA₃, whereas GUS activity increased about sixfold in response to GA₃ in cells transfected with a construct that included both CARE and GARE couples from *Rep1* (Figure 5, p(184-66)). Only one CARE/

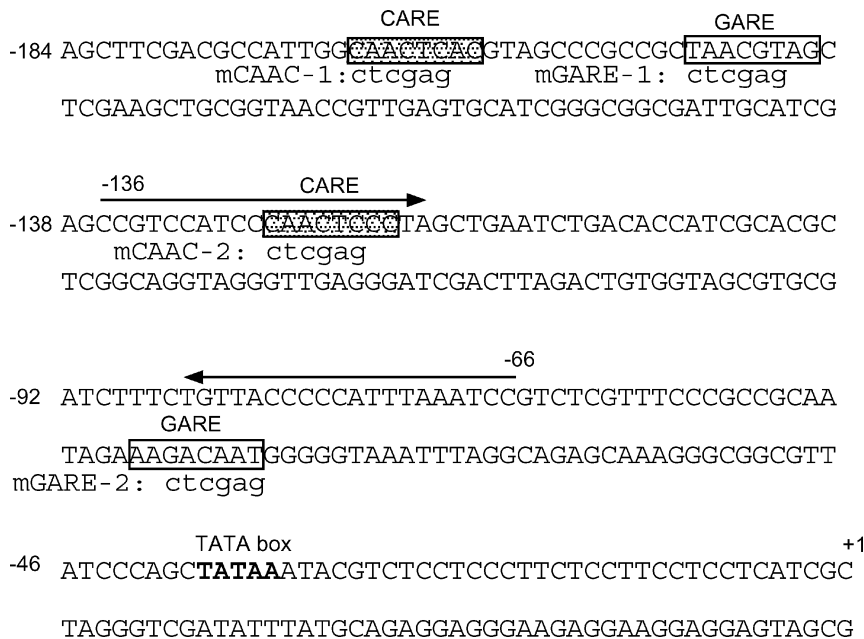


Figure 3. Sequences of both DNA strands in the *Rep1* promoter region from -184 to +1. Putative c-Myb binding sequences, YAACKGHH (Weston, 1992), are boxed. Meshed and open boxes indicate the CAACTC regulatory element (CARE) and GARE, as indicated. Small letters under the boxes mark the point mutations used in the experiments shown in Figure 4. The primers for construction of pGC and pGC² are represented by arrows. The TATA box is shown in bold.

GARE couple (region -136 to -66 of the *Rep1* promoter; pGC) did not confer GA responsiveness, whereas two copies of this couple (pGC²) did. This indicates that first, one pair each of CAREs and GAREs are required for GA inducibility, and secondly, the CARE/GARE couples located

in the *Rep1* regions -170 to -138 and -130 to -70, respectively, are functionally interchangeable. GA responsiveness induced by the three CARE/GARE couples (pGC³) did not significantly differ from that induced by two of them (pGC²), whereas the presence of four couples (pGC⁴) appeared to

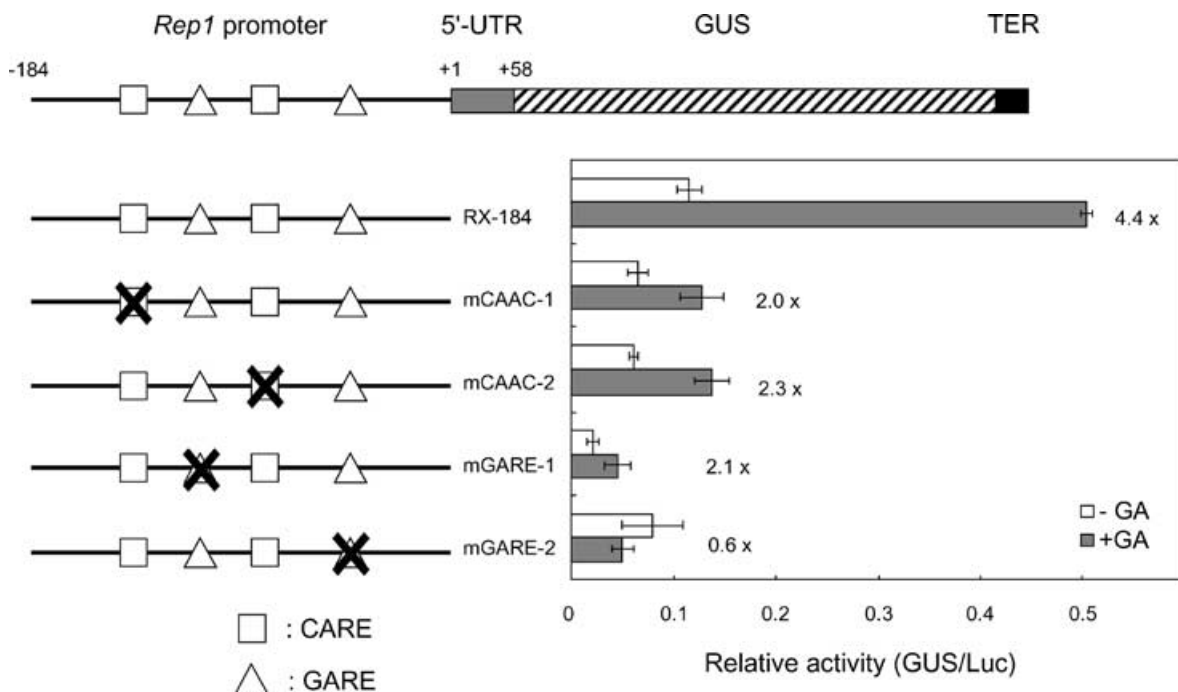


Figure 4. Point mutagenesis analysis of the CAACTC regulatory element (CARE) and GARE. A diagram of the forefather construct, RX-184, is shown at the top. The positions of CAREs and GAREs are indicated; crossed-out symbols stand for point mutations at that site. Experimental details are the same as in Figure 2. Results from the transient expression of the constructs are shown at the right. Bars indicate relative β-glucuronidase (GUS) activities (normalized with respect to luciferase activity) ± standard errors (SEs; n = 4). Both GAREs were needed for gibberellin (GA) responsiveness, and both CAREs were necessary to achieve the full response.

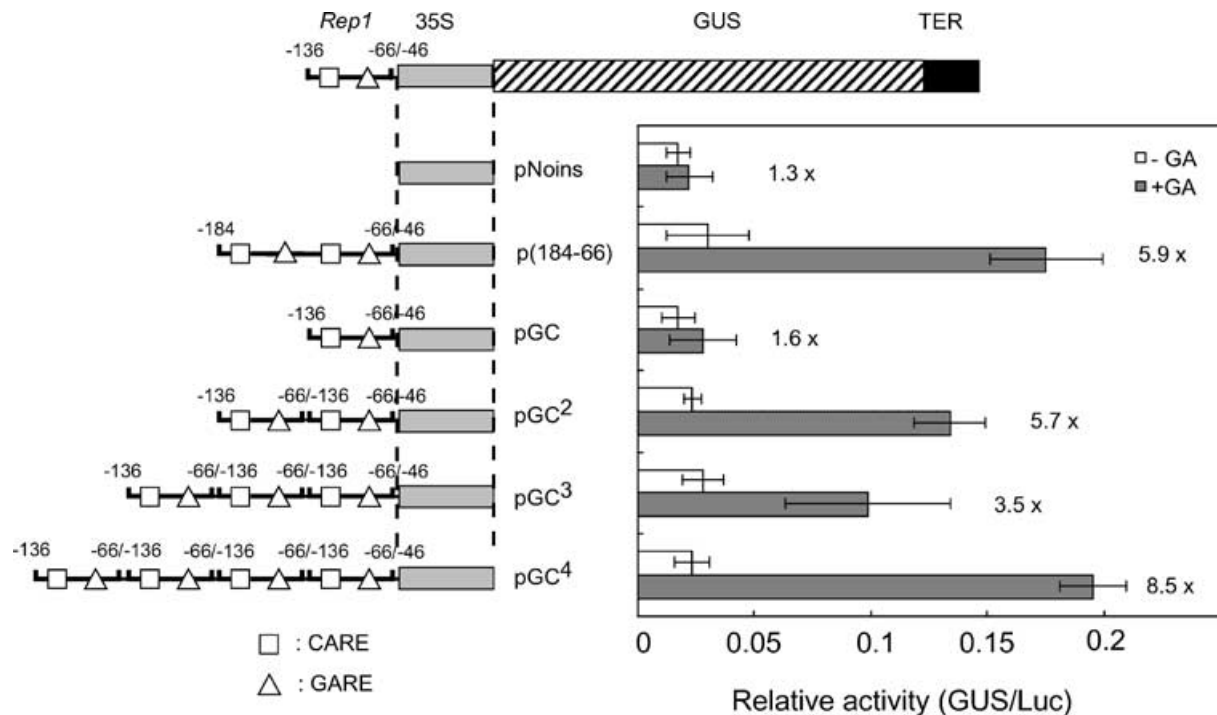


Figure 5. Multiple copies of the CAACTC regulatory element (CARE) and GARE are needed for gibberellin (GA) responsiveness.

A diagram of the forefather construct, pGC, is shown at the top. Various combinations of fragments of the *Rep1* promoter region containing pairs of CARE and GARE were linked to the β -glucuronidase (GUS) reporter, as indicated. Experimental details are the same as in Figure 2. Results from the transient expression of the constructs are given at the right. Bars indicate relative GUS activities (normalized with respect to luciferase activity) \pm standard errors (SEs; $n = 4$). At least two CARE/GARE couples were required to induce GA responsiveness.

further enhance GA-dependent GUS activity (Figure 5). Thus, it seems that GA-responsiveness is amplified stepwise with every two pairs of CAREs and GAREs.

Cooperation of CARE and GARE conferred GA inducibility

As one CARE/GARE couple could replace the other in *Rep1* without the loss of function as shown above, we tested whether single CARE and GARE sequences could also functionally replace each other. In analogy to the experiments shown in Figure 5, we created constructs of the CaMV 35S minimal promoter coupled to the GUS reporter gene, equipped either with two or four CAREs (pCC and pCC², respectively), or with two or four GAREs (pGG and pGG², respectively). None of these constructs exhibited GA responsiveness (Figure 6). Thus, CARE and GARE act cooperatively as GA-response complexes.

Transactivation by OsGAMYb depends on both CARE and GARE

In barley, HvGAMYb binds to GARE and activates the transcription of the α -amylase gene (Gubler *et al.*, 1995). Because the mRNA of *OsGAMYb* appears prior to that of *Rep1* (Figure 1), *OsGAMYb* may transactivate the expres-

sion of *Rep1*. We overexpressed *OsGAMYb* in rice aleurone cells using a construct with the CaMV 35S promoter (pBIOsGAMYb), and determined the GUS activity as a result of concomitant expression of pGC², pCC², or pGG², respectively. The GUS activity of pGC² was dramatically increased by pBIOsGAMYb even in the absence of GA₃ (Figure 7). The effect was much weaker in pGG² and was absent in pCC². This suggests that *OsGAMYb* transactivation of *Rep1* expression requires the interaction of *OsGAMYb* with a complex of both CAREs and GAREs.

CARE in the promoters of hydrolase genes involved in GA induction and transactivation by GAMYb

As in *Rep1*, CARE is also present in the promoters of the rice α -amylase gene, *RAmy1A*, and a barley proteinase gene, *EPB1* (Figure 3). To test whether CARE is involved in GA induction and transactivation by GAMYb in these genes, the CAACTC sequences in all the three promoters were changed into *Xho*I sites (CTCGAG). The constructs containing the original or mutated promoters, respectively, and the GUS reporter genes were expressed in rice (*Rep1*, *RAmy1A*) or barley (*EPB1*). In all cases, the GA-mediated enhancement of the GUS activity was dramatically decreased in cells that contained the mutated promoter as compared to the original one, but these mutations did not completely

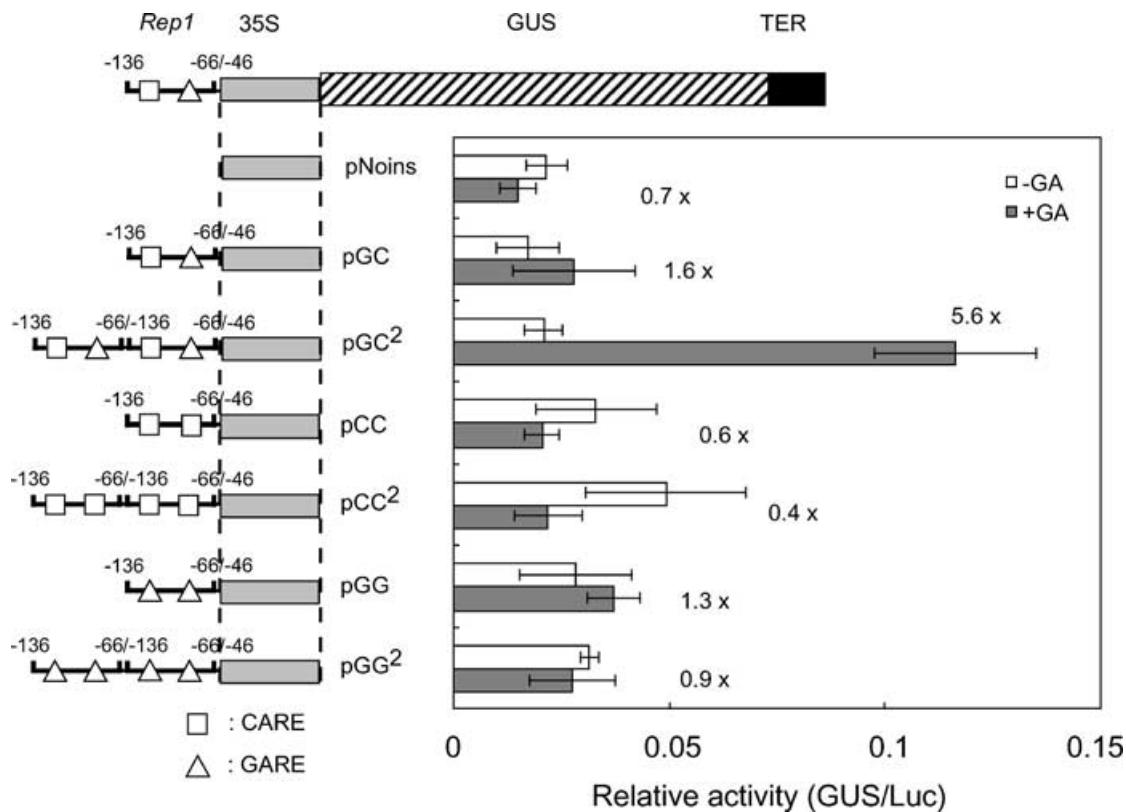


Figure 6. Effect of multiple copies of the CAACTC regulatory element (CARE) or GARE on gibberellin (GA) inducibility.

A diagram of the forefather construct, pGC, is shown at the top. Various combinations of fragments of the *Rep1* promoter containing CAREs, GAREs, or both, were linked to the β -glucuronidase (GUS) reporter as indicated. Experimental details are the same as in Figure 2. Bars in the graph at the right indicate relative GUS activities (normalized with respect to luciferase activity) \pm standard errors (SEs; $n = 4$). Pairs of CAREs or GAREs, respectively, could not replace CARE/GARE couples in inducing GA responsiveness.

lose GA inducibility (Figure 8). The transactivation of the gene by the overexpressed GAMyb was lost in the CARE mutations in the promoters of *RAmy1A* and *EPB1*. These results suggest that CARE amplifies GA responsiveness through transactivation by GAMyb. Thus, CARE is involved in the GA-inducible gene expression of *RAmy1A* and *EPB1* as well as of *Rep1*.

Discussion

We developed a transient expression system using the rice aleurone tissue to elucidate the mechanisms of GA-dependent gene induction. Similar approaches have been successful in barley (Cercós *et al.*, 1999; Gubler and Jacobsen, 1992; Gubler *et al.*, 1995; Lanahan *et al.*, 1992). Our system enabled us to directly analyze the *cis*-elements and *trans*-factors of the cysteine proteinase gene *Rep1* in rice. We demonstrated that GA activated the *Rep1* promoter. However, the induction fold by which the activity of a GUS reporter linked to the *Rep1* promoter was amplified by GA, as compared to the GA-free control, seemed lower than that in the barley α -amylase and *EPB1* genes (Cercós *et al.*, 1999; Lanahan *et al.*, 1992). In this study, we focused

on the *cis*-acting elements. It is possible, although, that the 3'-untranslated region (UTR) was also involved in the GA-dependent regulation of *Rep1*, as it is in barley *EPB1* (Cercós *et al.*, 1999). Fortunately, *Rep1* does not possess introns (Kato *et al.*, 1999), so that the analysis of its GA-dependent regulation can be expected to be less complicated than that of the α -amylase genes, in which introns are known to have a regulatory function (Gallie and Young, 1994).

Six tandem-repeats of GARE conferred GA-inducible expression to the CaMV 35S minimal promoter, while a single copy of GARE did not (Skriver *et al.*, 1991). There is a variety of GARE-associated elements, such as the amylase box, pyrimidine box, and the Opaque-2 binding sequence (Gubler and Jacobsen, 1992; Lanahan *et al.*, 1992). Rogers and Rogers (1992) suggested that the GA-responsive gene expression requires GARE plus a coupling element, which may be a general or a constitutive element. A coupling element has been well characterized in the ABA-response promoter by Shen and Ho (1995). They showed that a *cis*-acting element named coupling element 1 (CE1), together with a single copy of the G-box-like ABA-responsive element, constitute a minimal unit for ABA responsiveness. In this case, a single regulatory element on the promoter was

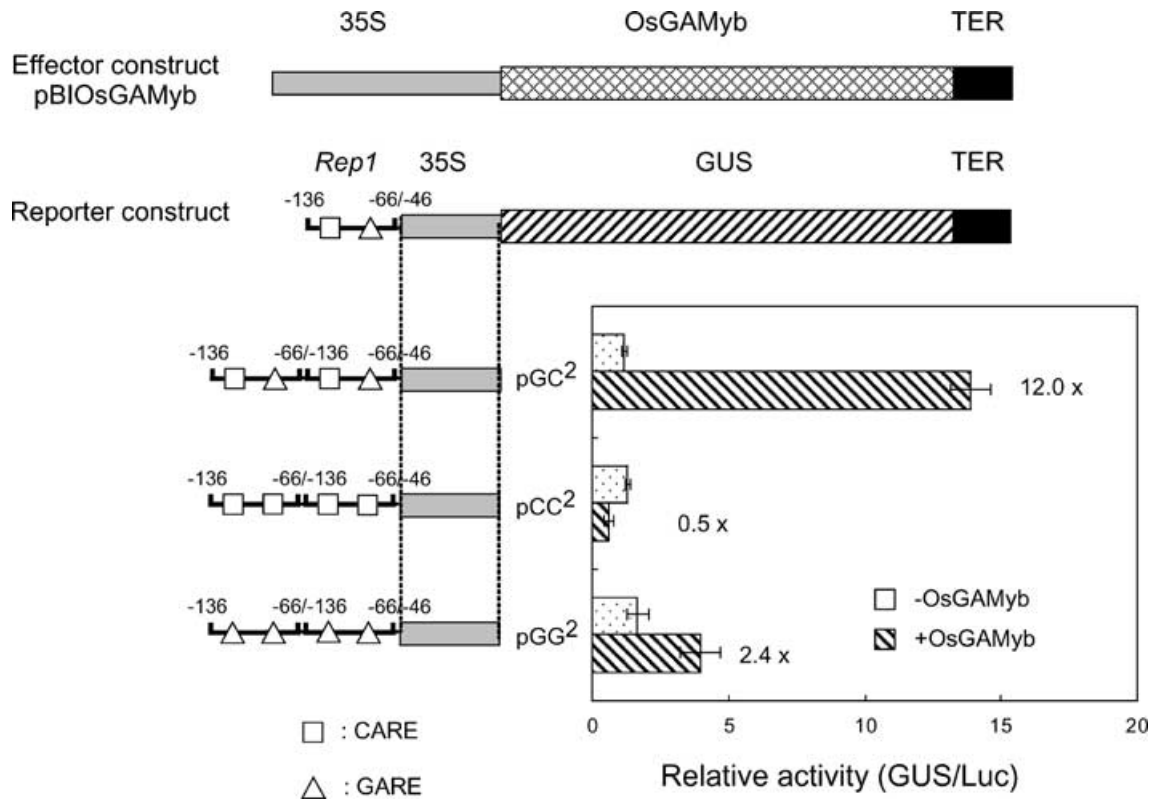


Figure 7. Transactivation by OsGAMyB.

The effector construct, pBIOsGAMyB, containing the cauliflower mosaic virus (CaMV) 35S promoter fused to *OsGAMyB* cDNA, is shown on the top. A diagram of the forefather reporter construct, pGC, is shown under the effector construct. Various combinations of fragments of the *Rep1* promoter containing CAACTC regulatory elements (CAREs) and/or GAREs were fused to the reporter construct, as indicated. Reporter construct, effector construct, and the internal control pAHC18 were expressed concomitantly. The relative activity of the β -glucuronidase (GUS) reporter (normalized with respect to luciferase (Luc) activity) in each construct is shown by the bars in the graph at the right; values are means \pm standard errors (SEs; $n = 4$). The full reaction was mediated only by two CARE/GARE couples (pGC²).

not sufficient; a second element was needed to initiate the transcription. We found that two couples of GARE/CARE were required in the *Rep1* promoter for GA-inducible expression. The CAREs could not be functionally replaced by additional copies of GARE. The complex of two CAREs and GAREs each, which is sufficient for efficient gene induction by GA, seems suitable for the analysis of the interaction of transcription factors involved in GA-inducible gene expression.

OsGAMyB was previously shown to transactivate the expression of *Amy32b* in the barley aleurone cells (Gubler *et al.*, 1997). We demonstrated here that it transactivated the expression of *Rep1* in rice as well (Figures 7 and 8). Gubler *et al.* (1995) showed that HvGAMyB bound to GARE. Thus, GARE in the *Rep1* promoter might interact with OsGAMyB. However, four copies of GARE (pGG²) showed a lower induction by OsGAMyB than two combined pairs of CAREs and GAREs (Figure 7, pGC²). Moreover, multiple copies of CARE were insufficient to induce GA responsiveness. Therefore, we hypothesized that CARE-binding proteins may stabilize the binding of GAMyB to GARE. Proteins

binding to CARE may be Myb-like transcription factors because CARE is a putative Myb-binding sequence (Weston, 1992). However, OsGAMyB itself is unlikely to interact with CARE because CARE is not identified as a HvGAMyB-binding sequence as determined by random-binding site-selection experiments (Gubler *et al.*, 1999).

CAREs are involved in GA responsiveness and transactivation by GAMyB also in *RAmy1A* and *EPB1* (Figure 8). In contrast to *Rep1*, *RAmy1A* and *EPB1* possess only single copies of both CARE and GARE. We suppose that the single CARE/GARE couples interacted with other regulatory elements present, i.e. the amylase box in *RAmy1A* and the pyrimidine box in *EPB1*, to produce GA responsiveness.

A database search for GARE and CARE in the promoter sequences of GA-inducible genes in vegetative tissues revealed the presence of GARE in two rice expansin genes, *Os-EXP2* and *Os-EXP4* (Cho and Kende, 1997), and in the *LEAFY* gene, which is involved in flowering in *Arabidopsis thaliana* (Blázquez *et al.*, 1998). CARE was found exclusively in the promoter regions of hydrolase genes that were expressed in germinating cereal seeds, suggesting that

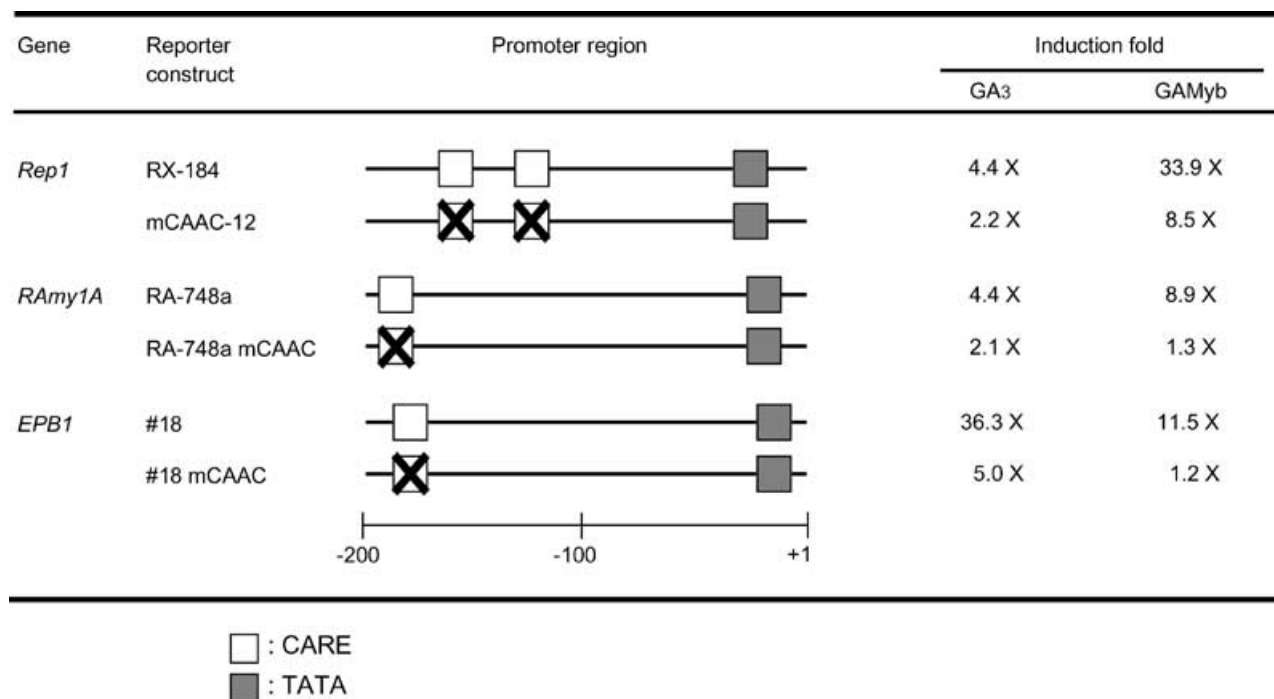


Figure 8. Effects of mutations in the CAACTC regulatory elements (CAREs) on gibberellin (GA) induction and GAMyB transactivation of hydrolase gene expression.

Point mutations were induced in CAREs in the promoter regions of a rice α -amylase gene, *RAmy1A*, a barley proteinase gene, *EPB1*, and *Rep1*. The diagrams show the structure of the promoter regions of the reporter genes. Point mutations of CARE (CAACTC) to a *Xho*I site (CTCGAG) are indicated by crossed-out symbols. *Rep1* and *RAmy1A* promoters were analyzed in the rice aleurone transient expression system, whereas *EPB1* promoter constructs were introduced into the barley aleurone cells. For examination of the GA₃ effects on the β -glucuronidase (GUS) activity, the aleurone layers were incubated in 0.5 μ M GA₃ for 20 h after bombardment. For examination of transactivation of these promoters by GAMyB, the effector constructs pBIOsGAMyB and Ubi-HvGAMyB were co-bombarded with each reporter construct into rice and barley aleurone cells, respectively, and each aleurone tissue sample was incubated without GA₃ for 20 h after bombardment. The induction fold represents the average from four independent bombardments. In all cases, point-mutating CAREs resulted in dramatic decreases of activity.

CARE is involved in the seed-specific gene expression. However, we did not detect CARE in the promoter regions of the barley α -amylase genes, *Amy32b* and *Amy6-4*.

In summary, our findings suggest that in the *Rep1* promoter, two pairs of CAREs and GAREs cooperate with at least two different transcription factors to form a GA-response complex. Future studies will focus on the identification of the binding proteins and their functions, and on the significance of the alternating arrangement of CAREs and GAREs in the promoter for the stability of the response complex.

Experimental procedures

Plant material

Embryo-less half-seeds of rice (*Oryza sativa* L. cv. Nipponbare) were sterilized in 1% sodium hypochlorite and 0.05% Tween 20 for 25 min, and then washed five times with sterile water. For the analysis of GA-inducible gene expression, half-seeds were incubated at 27°C in shooting buffer (20 mM sodium succinate (pH 5.5), 20 mM CaCl₂, 10 μ g ml⁻¹ chloramphenicol) in the pre-

sence or absence of 0.5 μ M GA₃, respectively. After incubation, the seeds were frozen in liquid nitrogen and stored at -80°C until use. For particle bombardment, sterilized half-seeds were incubated for 3 days at 22°C in shooting buffer. Upper and lower end of the seeds were cut off. The seeds were cut longitudinally and divided into two flat pieces with a razor blade. After removal of the pericarps, the seed tissue samples were subjected to particle bombardment. Embryo-less half-seeds of barley (*Hordeum vulgare* L. cv. Himalaya) were prepared as described by Belanger *et al.* (1986). The half-seeds were incubated for 3 days at 22°C on vermiculite soaked with shooting buffer, and were then subjected to particle bombardment after removal of the pericarps.

RNA isolation and RNA blot analysis

Total RNA was prepared as described by Karrer *et al.* (1991) with modifications. Three grams of embryo-less half-seeds were homogenized in an Ultra-disperser (Janke & Kunkel, Staufen, Germany) for 3 min in 15 ml TLE buffer (0.2 M Tris-HCl (pH 8.2), 0.1 M LiCl, 5 mM EDTA, 1% SDS) containing 2 mM aurintricarboxylic acid, 50 mM 2-mercaptoethanol, and 15 ml 85% phenol. The homogenate was centrifuged at 3000 g for 10 min at 4°C. The aqueous phase was mixed with an equal volume of 85% phenol. After this procedure had been repeated thrice, the aqueous phase was mixed with a 1% volume of 10 M LiCl and two volumes of ethanol, and was then kept at -80°C for

1 h. After centrifugation at 3000 *g* for 10 min at 4°C, the precipitate was dissolved in 4 ml sterile water, mixed with 1 ml of 10 M LiCl, and kept at 4°C overnight. After centrifugation, as described above, the precipitate was dissolved in 1 ml sterile water, and RNA in the solution was precipitated by the addition of 2 ml ethanol. The mixture was placed at –80°C for 1 h, and then centrifuged again as described above. The precipitate was dried and dissolved in 100 µl sterile water.

For RNA blot analysis, the partial *OsGAMyb* cDNA and *RAmy1A* cDNA were amplified by reverse transcription-polymerase chain reaction (RT-PCR; Huang *et al.*, 1990a) with total RNA from rice embryo-less half-seeds incubated for 24 h in the presence of GA₃. The amplified cDNA fragments of *OsGAMyb* and *RAmy1A* contained the reported sequences 1008–1743 (Gubler *et al.*, 1997) and 549–1268 (Huang *et al.*, 1990b), respectively. The cDNA clone pRP60 was used to detect *Rep1* mRNA (Shintani *et al.*, 1997). These three cDNA probes were labeled with α³²P-dCTP using a random primer DNA labeling kit (TaKaRa Shuzo, Kyoto, Japan). RNA blot analysis and hybridization were carried out as described by Shintani *et al.* (1997). After hybridization, the nylon membrane (Hybond-N; Amersham Bioscience Corp. Piscataway, NJ, USA) was washed twice with 2 × SSC (1 × SSC, 0.15 M NaCl, and 15 mM sodium citrate (pH 7.0)) containing 0.1% SDS at 65°C for 1 h. For detection of rRNA, the gels were stained with 0.04% methylene blue in 0.5 M sodium acetate buffer (pH 5.2) after electrophoresis.

Construction of deletion mutants

A series of deletion mutations of the *Rep1* promoter were generated by PCR using the λgRP60 clone (Kato *et al.*, 1999) as a template. To ligate the amplified fragments into vectors, *HindIII* and *SpeI* sites were created at the 5'- and 3'-ends of the fragments, respectively. After the amplified fragments had been cloned into the vector pCR 2.1 (Invitrogen, Carlsbad, CA, USA), fragments cut by *HindIII* and *SpeI* were inserted into the GUS vector, pBI221 (Jefferson *et al.*, 1987), replacing the CaMV 35S promoter. The resultant plasmids were named RX-111, RX-184, RX-367, RX-432, RX-500, RX-568, and RX-808, according to the position at the 5'-end.

Construction of point mutants from RX-184

The *Rep1* promoter region from –184 to +58 was point-mutated at five locations. The point mutations were generated by PCR-based oligonucleotide-directed mutagenesis (Picard *et al.*, 1994) with 6-point changes to *XhoI* sites. The amplified fragments digested with *HindIII* and *SpeI* were inserted into pBI221 as described above. The resultant plasmids were named mGARE-1, mGARE-2, mCAAC-1, mCAAC-2, and mCAAC-12, indicating the element in which the point mutation was located.

Construction of plasmids for gain-of-function analysis

To construct plasmids for gain-of-function analysis, the regions –184 to –66 and –136 to –66 of the *Rep1* promoter were amplified by PCR using RX-184 as the template. PCR fragments were cloned to pGEM-T Easy Vector (Promega, Madison, WI, USA). The cloned fragments were cut at *EcoRI* sites in the multicloning site of the pGEM-T Easy Vector. GUS constructs were made using p101RZ, including 68 bp of the ABA-response complex 3 (ABRC3), the CaMV 35S minimal promoter region from –46 to +100, the GUS coding region, and the terminator of the nopaline synthase gene. The fragments of the *Rep1* promoter were inserted into p101RZ in replacement of ABRC3. We confirmed

copy numbers and direction of the fragments inserted into p101RZ by sequencing. The construct carrying the *Rep1* promoter region –184 to –66 was designated p(184–66). The plasmids containing one, two, three, and four copies of the region –136 to –66 were named pGC, pGC², pGC³, and pGC⁴, respectively. The sequences of GARE and CARE in the region –136 to –66 were mutated to CARE and GARE, respectively, by PCR, using RX-184 as the template. The resultant PCR fragments were cloned to pGEM-T Easy Vector, and one or two copies of the fragments were inserted into p101RZ as described above. The constructs carrying two and four CARE and two and four GARE were named pCC, pCC², pGG and pGG², respectively.

Construction of the effector plasmid carrying *OsGAMyb* cDNA

The fragment of the *OsGAMyb* cDNA from 389 to 2064 (Gubler *et al.*, 1997) was generated by the RT-PCR procedure as described above, except that *Bam*HI and *Sac*I sites were created at the 5'- and 3'-ends of the fragment, respectively. The amplified fragment was cloned into pGEM-T Easy Vector. The cDNA cut by *Bam*HI and *Sac*I was inserted into pBI221 in replacement of the GUS coding region. This construct, named pBIOsGAMyb, was co-bombarded with the GUS reporter constructs as described below.

Construction of the GUS reporter including the *RAmy1A* promoter and the *Act1* intron

Rice genomic DNA was isolated as described by Rogers and Bendrich (1985). A fragment of the *RAmy1A* promoter region (–748 to +31) was generated from the genomic DNA by the RT-PCR procedure described by Itoh *et al.* (1995). *HindIII* and *NheI* sites were created at 5'- and 3'-ends of the fragment, respectively. The amplified fragment cut with *HindIII* and *NheI* was cloned into *HindIII* and *XbaI* sites of the GUS vector pBI221. The resultant construct was named RA-748. To enhance the GUS activity under the *RAmy1A* promoter, the *Act1* first intron (McElroy *et al.*, 1991) was inserted between the *RAmy1A* promoter and the GUS coding region. The fragment of the *Act1* first intron from +65 to +549 was generated from the genomic DNA by PCR. To join the amplified fragment to RA-748, *Bam*HI sites were created at the 5'- and 3'-ends of the fragment, and the fragment cut with *Bam*HI was inserted into the *Bam*HI sites of RA-748. The resultant plasmid was designated as RA-748a. CARE in the *RAmy1A* promoter was mutated to a *XhoI* site as described above. The mutated fragment digested with *HindIII* and *NheI* was cloned into *HindIII* and *XbaI* sites of pBI221, and the plasmid obtained was joined to the *Act1* first intron as described above. The resultant construct was named RA-748amCAAC.

Construction of the GUS reporter gene fused to the *EPB1* promoter

The GUS reporter gene was fused to the *EPB1* promoter #18 (Cercós *et al.*, 1999). CARE in the *EPB1* promoter was point-mutated to a *XhoI* site as described above. The mutated fragment of the *EPB1* promoter was cloned into the *KpnI* sites of the construct #18. The resultant construct was named #18mCAAC.

Transient expression

Gold particles (1 µm diameter; Tokuriki-Honten Co., Ltd, Tokyo, Japan) were coated with DNA by ethanol precipitation. The luciferase (Luc) reporter plasmid pAHC18 (Christensen and Quail,

1996) was always used together with the DNA constructs, to serve as an internal control. In experiments without effector plasmids, pAHC18 was co-bombarded with each GUS reporter plasmid at a weight ratio of 2 : 5, and 7 µg of DNA was conjugated to 1 mg of gold particles. In tests with an effector construct, 6 µg of DNA was conjugated to 1 mg of gold particles, and each GUS reporter construct, effector construct, and the internal control (pAHC18) were co-bombarded at a weight ratio of 5 : 5 : 2. Each seed tissue sample was bombarded with 50 µg of gold particles using a biolistic device (IDERA GIE-III, Tanaka Co., Ltd, Sapporo, Japan), according to the manufacturer's instructions. After bombardment, batches of three seed tissue samples were incubated in 5 ml shooting buffer on a shaker for 20 h at room temperature. The tissue was then homogenized in a mortar in 200 µl extraction buffer (100 mM potassium phosphate (pH 7.8), 2 mM DTT, 2 mM EDTA, 5% glycerol). The homogenate was centrifuged at 15 000 g for 30 min at 4°C, and the GUS and Luc activities were assayed in the supernatant. Luc activity was determined in 20 µl extract using a commercial kit (Pica Gene, Toyooki, Tokyo, Japan) and a luminometer (NU-600; Nichion, Funabashi, Japan). GUS activity was measured spectrofluorometrically (RF-5000; Shimadzu, Kyoto, Japan) in 50 µl extract that had reacted with 4-methylumbelliferyl-β-D-glucuronide as a substrate, according to Kosugi *et al.* (1990). Values of GUS activity were normalized with respect to Luc internal activity.

Acknowledgements

We thank Mr Seiichiro Kiyota, National Institute of Agrobiological Sciences, for his gift of rice seeds, Dr Peter Quail, University of California, Berkeley, for his gift of pAHC18, and Dr Tuan-hua David Ho and Dr Rodolfo Zentella, Washington University, for their gifts of plasmids, p101RZ, the EPB1:GUS construct #18, and Ubi-HvGA-Myb, and for critically reading the manuscript. This work was partly supported by the Itoh Science Foundation to D.Y. and by the SASAKAWA Scientific Research Grant from the Japan Science Society to K.S.

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