Expression of the REB transcriptional activator in rice grains improves the yield of recombinant proteins whose genes are controlled by a *Reb*-responsive promoter

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The gene encoding the rice transcription factor, REB (rice endosperm bZIP) was cloned from a bacterial artificial chromosome library of rice. The cloned 6,227-bp-long *Reb* gene is composed of six exons and five introns and is flanked by a 1.2-kb 5′ promoter and a 1.2-kb 3′ terminator region. The function of the *Reb* gene was explored by a transient assay by using a rice immature endosperm system. The effector constructs containing the native gene or fusion genes linking *Reb* to the rice actin (*Act*) or globulin (*Glb*) gene promoters and the reporter gene construct Glb-β-glucuronidase (GUS) were used in this study. When these effector constructs were cotransferred with the reporter *uid*A gene encoding GUS under the control of the *Glb* promoter into immature rice endosperm cells, the *Glb* promoter was activated. The transient GUS expression was 2.0 to 2.5-fold higher with the effector construct than without. When the upstream activation sequence containing the GCCACGT(A/C)AG motifs of the *Glb* promoter was deleted, the activation by REB was abolished. On the other hand, a gain-of-function experiment showed that inserting the upstream activation sequence into the glutelin-1 (*Gt1*) promoter made it responsive to activation by REB. When cotransformed with *Reb* gene, mature transgenic rice grains containing the human lysozyme gene driven by the *Glb* promoter produced 3.7-fold more lysozyme. Accumulation of recombinant lysozyme in mature seed ranged from 30.57 to 279.61 μg·mg^{-1} total soluble protein in individual transformants from 30 independent transformation events. Thus, our results show that REB is not only a transcriptional activator, it can also be used to increase the expression of recombinant protein in transgenic rice grains.

Reb gene | lysozyme expression | transgenic plant | gain-of-function | loss-of-function

The developing endosperm of cereal grains and the developing cotyledons of legume grains are highly suitable as bioreactors for the production of recombinant proteins (1–3). Among the tested recombinant proteins are a protein-engineered enzyme (1), recombinant antibodies in the form of single chain antibody fragments (scFvs) (2, 3), the human milk protein beta casein (4), the antimicrobial protein lactoferrin (5), and thioredoxin (6). Although expression levels of 53 ± 2.8 μg·mg^{-1} total soluble protein (TSP) (range 37–77 μg·mg^{-1}) have been obtained with recombinant (1,3-1,4)-β-glucanase in the barley endosperm (1), the production level of single chain antibody fragments reached only 32 ng·mg^{-1} TSP in rice endosperm and 1.4 ng·mg^{-1} TSP in wheat endosperm (3). A high level of expression in transgenic grains becomes an important issue when the commercial production, application, or purification requires a high concentration of recombinant protein in the grain. Successful increases of recombinant protein synthesis and storage have been achieved by codon optimization of the transgene to a C + G content of more than 60% (1), by selection of a stronger promoter (7), and by using a signal peptide to target the protein into the endo-

plasmic reticulum for deposition into protein bodies (1, 2). A further or alternative way of increasing the amount of recombinant protein might be the transgenic expression of transcription factors that have the ability to transactivate the target genes by binding to nucleotide sequence motifs in the gene promoter (8–11). This possibility has been investigated with the experiments reported in the present communication.

In cereals, bZIP proteins have been identified as transcriptional factors for genes encoding storage proteins in the endosperm (10–20). The basic amino acids containing domain of these proteins bind to a recognition nucleotide sequence in the promoter, whereas their leucine repeat domain interacts with the transcriptional machinery, leading to a dramatic increase of transcription initiation of the gene in the endosperm of the cereal grain (11, 15). Nakase *et al.* (21) have cloned a bZIP protein gene from rice. This protein, named REB for rice endosperm bZIP protein, binds specifically to the sequences GCCACGTAAG and GCCACGTCAG in the distal part of the rice globulin (*Glb*) gene promoter (21). Its function as a transcriptional activator or suppressor, however, remains to be elucidated.

We have isolated a rice *Reb* gene from a bacterial artificial chromosome (BAC) library and shown with transient expression analyses that the REB protein can specifically enhance the expression of the β-glucuronidase (GUS) reporter gene under the control of the *Glb* gene promoter. The enhancement requires the presence of the REB upstream activation sequence (UAS) in the *Glb* promoter. It is further demonstrated that REB can increase the expression of human lysozyme in developing transgenic rice grains.

Materials and Methods

Cloning of *Reb* Gene. *Screening of BAC library.* With reference to the *Reb* gene sequence (21), PCR primers were designed and used to screen a rice BAC library (22). The forward primer had the sequence 5′-CTGATATGTGCCCATGTTCCAAAC-3′, and the reverse primer was 5′-CCTTGCTGAATGCAGAT-GTTTCAC-3′. The screening strategy of three-dimensional DNA pools of the BAC library has been described (23). PCR was carried out with 100 ng of pooled BAC DNA/10 mM Tris (pH 8.3)/1.5 mM MgCl$_2$/50 mM KCl/0.5 μM dNTP and used a program of denaturing at 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min. The plasmid
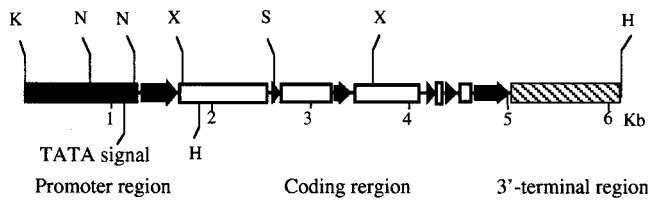
Fig. 1. Restriction map of the rice Reb gene isolated from BAC clone 42B9. The gene sequence of 6.227 kb consists of five introns (open bar) and six exons (solid arrow) flanked by 1.2 kb of the 5′ promoter (solid bar) and 1.2 kb of the 3′ region (striped bar). The restriction enzymes indicate K (*KpnI*), N (*NruI*), X (*XhoI*), S (*StuI*), and H (*HindIII*).

DNA of a positive BAC clone was prepared as described (22), the BAC DNA was digested with *HindIII*, and the presence of the *Reb* gene was confirmed by Southern analysis (24).

Subcloning and sequencing. The *Reb* gene was retrieved from the BAC by subcloning two fragments into the pBluescript KS+ vector (Stratagene). First, the promoter and partial coding region was obtained as a *KpnI–HindIII* fragment (Fig. 1). In a second step, a *HindIII* fragment containing the remaining coding region and the 3′ terminator region was obtained by shotgun cloning (Fig. 1). The two fragments were ligated at the internal *HindIII* site, generating an intact *Reb* gene. The DNA was sequenced with an automatic ABI 371 instrument, and alignment of the sequence to the cDNA (21) established the structure of the gene (Fig. 1). The plasmid containing the gene is designated as pAPI266 (*Native-Reb*).

Construction of expression plasmids. Plasmids API212 (*Glb-GUS*), carrying the rice globulin gene promoter fused to the GUS reporter gene, and API142 (*Gt1-GUS*), containing the rice glutelin 1 gene promoter fused to the GUS reporter gene, were used for transient expression assays. The globulin promoter (GenBank accession no. X63990) and glutelin 1 promoter (GenBank accession no. Y00687) were obtained from M202 *(Oryza sativa Joponica* subsp.) by PCR amplification. Plasmid API267 (*Glb-Reb*) was prepared by cloning the *Reb* coding region (*NruI–SacI* fragment, Fig. 1) into the *Glb-GUS* plasmid after removal of the GUS gene by digestion with *SmaI–SacI*, thus replacing the GUS gene with the *Reb* gene. Using the same strategy, plasmid API277 (*Actin-Reb*) was made by replacing the GUS gene of plasmid *Act1-D-GUS* (7) with the complete *Reb* gene (*NruI–SacI* fragment). A gene encoding the mature polypeptide of human lysozyme (EC 3.2.1.17) with a G + C content of 68.4% was synthesized (Operon Technologies, Alameda, CA) based on the sequence of GenBank accession no. J03801. The DNA was digested with *DraI–XhoI* and ligated into the *NaeI–XhoI* sites of the expression cassette in plasmid API241, which contains the rice globulin gene promoter and signal peptide (GenBank accession no. X63990). The resulting plasmid was named pAPI264.

Transient Expression Assays. Details of the transient expression assays developed by Y. S. Hwang will be presented elsewhere. Briefly, rice spikelets with immature endosperm (7–9 days after pollination) of M202 were collected from plants grown in the greenhouse at 30°C. The spikelets were sterilized with 70% ethanol for 10 min. After evaporation of residual ethanol, the endosperm was dissected, and 10 immature endosperms were placed on a filter paper in a Petri dish containing AA medium (25) supplemented with 20 mM ammonium nitrate. Fifty microliters of gold particles (60 mg·ml^{-1} at 1:1 ratio of 1.0 and 1.5–3.0 μm diameter gold particle) were coated with 5 μg of DNA consisting of a mixture of the reporter gene, the effector gene, and the internal control gene at a molar ratio of 1:1:1. DNA coating was carried out as described in the instruction manual of

the Biolistic PDS system (Bio-Rad). As internal control, pAHC18 containing the luciferase gene driven by an ubiquitin promoter (26) was used. In tests without the effector gene, effector DNA was replaced by pBluescript DNA. Particle bombardment was carried out with a Biolistic helium gun device at 1100 psi (Biolistic PDS 1000/He system, Bio-Rad). After bombardment, the immature endosperms were incubated at 25°C for 24 h in 5 ml of AA medium supplemented with 20 mM ammonium nitrate, 50 μg·ml^{-1} cefotaxin, and 50 μg·ml^{-1} timentin (SmithKline Beecham) to prevent bacterial growth. The endosperms were then harvested and ground with 55 μl of extraction buffer (0.1 M potassium phosphate, pH 8.0/1 mM EDTA/10 mM DDT/5% glycerol/0.2 mM leupeptin/0.2 μM phenylmethylsulfonyl fluoride). The extract was centrifuged at 25,000 × g for 5 min at 4°C. From the resulting supernatant, a 20-μl aliquot was added to 180 μl of luciferase assay buffer (0.25 M Tricine, pH 7.8/150 mM magnesium chloride/10 mM ATP/1 mM DDT/100 μg·ml^{-1} BSA). Another 20-μl aliquot was added to 200 μl of GUS assay buffer (Tropix, Bedford, MA). Luciferase activity was measured after incubation at 25°C for 20 min and GUS activity at 37°C for 1 h with a Monolight 2010 chemi-illuminometer according to the manufacturer's instructions (Analytical Luminescence Laboratory, San Diego). GUS activity was normalized to the luciferase activity and expressed relative to the activity of *Glb-GUS*. All data are averaged from at least six assays of two independent experiments.

Transformation and Plant Regeneration. Microprojectile-mediated transformation of rice was carried out according to the procedure described in ref. 25. Calli were derived from the hypocotyls of germinating mature seeds of the cultivar TP309 (*Oryza sativa*, subsp. *Japonica*). Calli with a diameter of 2–4 mm were selected and placed on an N6 medium (Sigma) supplemented with 0.3 M mannitol and 0.3 M sorbitol for about 20 h before bombardment. Bombardment was carried out with the Biolistic PDC-1000/He instrument (Bio-Rad). Fifty microliters of gold particles (60 mg·ml^{-1} at 1:1 ratio of 1.0 and 1.5–3.0 μm diameter gold particle) were coated with effector DNA, target DNA, and selection marker DNA in a ratio of 3:3:1 (wt/wt) and accelerated with a helium pressure of 1,100 psi. After a 2-day incubation, calli were transferred to N6 selection media containing 35 mg·l^{-1} hygromycin B and allowed to grow in dark at 26°C for 45 days. Calli resistant to hygromycin B were transferred to regeneration media to generate plantlets as described in ref. 25. After shoots had reached a height of 1–3 cm, the plantlets were transferred to rooting media (MS plus 0.05 mg·l^{-1} α-naphthaleneactic acid, Sigma). After 2 weeks, the plantlets were transferred to soil and grown in a greenhouse to maturity.

PCR Analysis of Transgenic Plants. Genomic DNA samples were prepared from transgenic plant leaves as described in ref. 27 and used as template for amplification with two pairs of primers for identification of the transgenes. For the rice *Reb* gene, the forward primer 5′-CCATCCAATCCAATCCACTCCAAC-3′ is based on a 3′ untranslated terminal sequence of the gene, and the reverse primer covers the vector sequence 5′-AGGCGATTAAGTTGGGTAACG-3′ (see Fig. 5). For the human lysozyme transgene, the 5′-CCTAGCCAAAGTCTTCGAGCG-GTG-3′ forward primer hybridizes to the 5′ end of the gene's ORF and the reverse primer 5′-GCGATGTTGTCTTG-CAGC-3′ to its 3′ end (Fig. 5). The PCR mixture contained 100 ng of genomic DNA/10 mM Tris (pH 8.3)/1.5 mM MgCl$_2$/50 mM KCl/0.5 μM dNTP. Amplification used a program of denaturing at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. The PCR products were resolved by electrophoresis in a 1.2% agarose gel.

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Antibody Preparation and Western Blotting. A peptide with 15 aa, PPPQSAAAAQQQGGG, from the position 19–23 of rice *Reb* gene was synthesized, and antiserum were prepared from rabbit by Alpha Diagnostic (San Antonio, TX). The total protein was extracted from immature seeds of transgenic lines at 14 days after pollination by using the extraction buffer (66 mM Tris, pH 6.8/2% SDS/2% β-mercaptoethanol). The resulting extract of total protein was run on a 12% polyacrylamide gel and blotted onto a nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad). The blot was blocked with blocking solution (5% nonfat milk powder/0.02% sodium azide in PBS, pH 7.4) at 4°C overnight. Then, the blot was incubated with anti-REB antibody with 1:2,000 dilution of reaction solution (5% nonfat milk powder/0.05% Tween 20 in PBS, pH 7.4) at room temperature for 2 h. After washing with PBS for three times, 5 min each time, the blots were incubated with mice anti-rabbit IgG-conjugated alkaline phosphatase (1:2,500) in incubation solution (5% nonfat milk powder in PBS, pH 7.4) at room temperature for 2 h. After washing three times with TBS (pH 7.4), the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (Sigma).

Lysozyme Activity Assay. The lysozyme assay used a procedure developed by Jianmin Huang (Applied Phytologics, Sacramento, CA). Briefly, 20 individual seeds from each T1 transgenic plant were ground in 1 ml of chilled extraction buffer (PBS plus 0.35 M NaCl). After centrifugation at $25,000 \times g$ at 4°C for 5 min, the resulting supernatant was recovered. A series of dilutions was placed in a 96-well microtiter plate containing 250 μl of 0.015% *Micrococcus letus* cells in each well (Sigma). Human lysozyme (EC 3.2.1.17, Sigma) was used as standard. Lysozyme activity was measured as a decrease in turbidity by using Microplate Reader 3550 (Bio-Rad). The lysozyme concentration in the samples was read from a standard curve for different concentrations of human lysozyme. The lysozyme expression level in a given transgenic plant was computed as the average for the lysozyme content of all seeds of that plant. Total soluble protein in seed extracts was estimated with the Bradford protein assay (Bio-Rad).

Results

Cloning of the *Reb* Gene from a Rice BAC Library.
A rice BAC library was screened with the three-dimensional pool PCR strategy (23) by using PCR primers based on the cDNA sequence of the *Reb* gene (21). One positive clone, 42B9, was obtained. Restriction site mapping of 42B9 indicated that there was no convenient restriction sites for subcloning the entire *Reb* gene in a single fragment. The complete *Reb* gene was therefore generated by ligating a 1,775-bp fragment containing the promoter and the 5′ coding region to a 4,452-bp fragment containing the 3′ coding and terminator region (Fig. 1). DNA sequencing of the *Reb* gene revealed five introns, six exons, 1.16 kb of the 5′ promoter sequence, and 1.2 kb of the 3′ region totaling 6,227 bp (Fig. 1). Comparison of the ORF of our gene with the *Reb* cDNA sequence (Accession no. AB021737) revealed 99.97% DNA sequence identity and 99.99% amino acid identity resulting from two amino acid changes: $Ile_{165} \rightarrow Asn$ and $Glu_{215} \rightarrow Lys$. These differences are likely to be because of polymorphisms among rice varieties.

Transcriptional Activation of the Globulin Promoter by REB.
To determine whether binding of the REB protein to the motif [GCCACGT(A/C)AG] in the globulin (*Glb*) gene promoter activates transcription of this promoter, plasmids containing fusions of the *Reb* coding region with the *Glb* promoter and the rice actin (*Act*) gene promoter were prepared (Fig. 2A). These plasmids as well as the expression plasmid containing the native *Reb* gene (pAPI266) were cobombarded into the rice endosperm
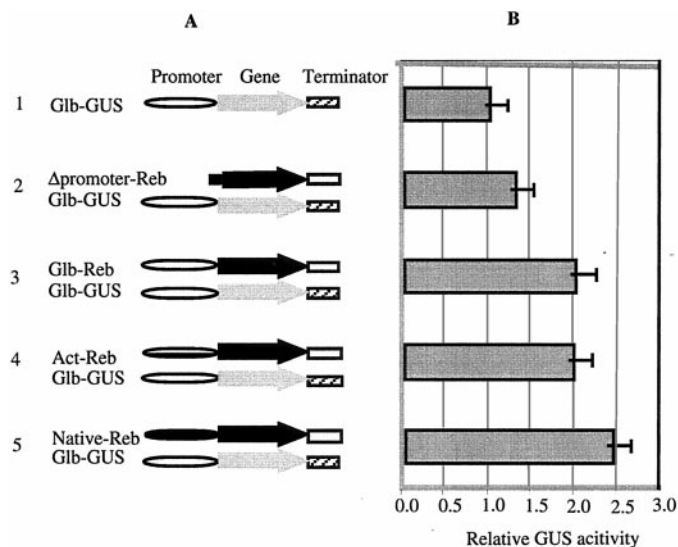

Fig. 2. The *trans*-activation function of the *Reb* gene toward the *Glb* promoter as analyzed with the GUS transient expression assay. (*A*) Schematic diagram of the constructs used for the transient assays. 1, GUS reporter construct with the *Glb* promoter and the *Nos* terminator; 2, Δpromoter-*Reb*-Term cobombarded with *Glb-GUS-Nos* reporter gene construct; 3, *Glb-Reb-native-Term* cobombarded with *Glb-GUS-Nos* reporter gene construct; 4, *Act-Reb-Term* cobombarded with *Glb-GUS-Nos* reporter gene construct; and 5, *Native-Reb-Term* cobombarded with *Glb-GUS-Nos* reporter gene construct. (*B*) Relative GUS activity corresponding to effector/reporter combinations shown in *A*. The error bar indicates the standard variation. The symbols indicate the *Glb* promoter (open bar), the *Act* promoter (horizontal striped bar), the *Reb* native promoter (black bar), the *Reb* gene (black arrow), the *GUS* gene (gray arrow), the *Reb* terminator (gray bar), and the *Nos* (striped bar).

with the plasmid containing the GUS reporter gene driven by the *Glb* promoter and the internal control plasmid containing the luciferase gene driven by the ubiquitin promoter.

Setting the level of GUS expression by the *Glb* promoter as 1, the codelivery of the plasmids containing the *Reb* gene increased GUS expression irrespective of whether the gene was driven by its own promoter, the *Glb* promoter, or the *Act* promoter (Fig. 2B). The increases were 2.43-, 2.01-, and 1.98-fold, respectively. The activation of GUS expression was abolished when a promoterless *Reb* construct was cobombarded with *Glb-GUS* (Fig. 2B). These results suggested that the REB protein functions as a transcriptional activator of the *Glb* promoter.

Identification of the UAS for REB.
Using a band-shift assay, Nakase *et al.* (21) have shown that the binding motifs of REB are GCCACGTAAG or GCCACGTCAG. An analysis of the *Glb* promoter sequences revealed two copies of GCCACGTAAG and one copy of GCCACGTCAG clustered around −700 bp distal to the TATA box of the *Glb* promoter (Fig. 3A). If the binding motifs for REB signify a UAS, deletion of this sequence would abolish the transactivation by REB (experiment 3 in Fig. 3A). On the other hand, insertion of the binding motif into a promoter that cannot be activated by REB might introduce transactivation responsiveness. Accordingly, the 200 bp with the three motifs located at positions −642 to −842 from the TATA box were deleted from the *Glb* promoter (Fig. 3A). The deletion has no effect on the expression of GUS, as both *Glb-GUS* and *GlbΔUAS-GUS* show the same level of background GUS expression (experiments 1 and 3 in Fig. 3). When *Native-Reb* was cobombarded with *GlbΔUAS-GUS* (experiment 4), transcriptional activation by REB was lost (experiment 4 in Fig. 3). These data indicate that REB activation of *Glb-GUS* occurs through this 200-bp fragment containing REB binding motifs.
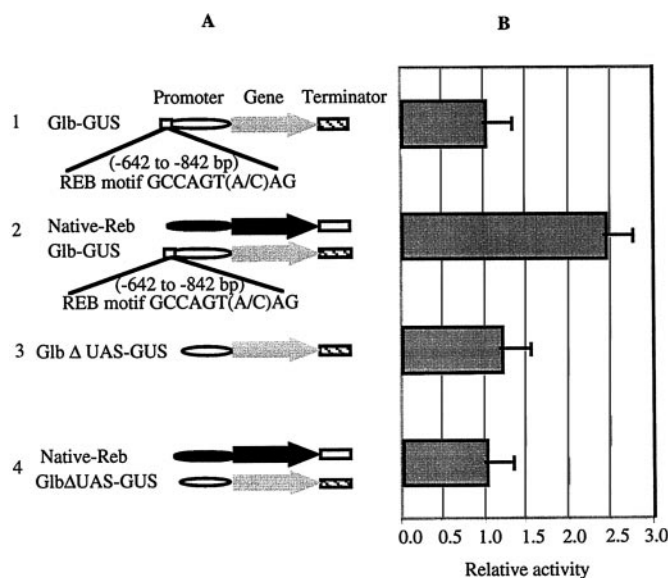
Fig. 3. The loss-of-function analysis of the REB *trans*-activation by transient assay. (*A*) Schematic diagram of constructs used for loss-of-function analysis. 1, the GUS reporter construct with the *Glb* promoter and the *Nos* terminator; 2, *Native-Reb-native terminator* construct cobombarded with *Glb-GUS-Nos* reporter construct; 3, *GlbΔUAS-GUS-Nos* construct; 4, *Native-Reb-Term* cobombarded with the *Glb* reporter construct in which REB UAS motifs of the *Glb* promoter are deleted. (*B*) Relative GUS activity corresponding to effector/reporter combinations shown in *A*. The error bar indicates the standard variation. The symbols indicate the *Glb* promoter (open bar), the *Reb* native promoter (black bar), the *Reb* gene (black arrow), the *GUS* gene (gray arrow), the *Reb* terminator (gray bar), and the *Nos* (striped bar).

Fig. 4. The gain-of-function analysis of the REB *trans*-activation by transient assay. (*A*) Schematic diagram of plasmid constructs used for the gain-of-function analysis. 1, *Gt1-GUS-Nos*; 2, *Gt1-GUS-Nos* with effector *Native-Reb-Term*; 3, modified *Gt1-UAS-GUS-Nos*; 4, modified *Gt1+UAS-GUS-Nos* with effector *Native-Reb-Term*. (*B*) Relative GUS activity corresponding to reporter constructs, *Gt1-GUS-Nos*, *Gt1-UAS-GUS-Nos*, and REB effector constructs shown in *A*. The error bar indicates the standard variation. The symbols indicate the *Gt1* promoter (box bar), the *Reb* native promoter (black bar), the *Reb* gene (solid bar), the *GUS* gene (gray arrow), the *Reb* terminator (gray bar), the *Nos* terminator (striped bar), and the *Reb* UAS (open box).

Scanning the rice glutelin1 (*Gt1*) promoter sequence does not reveal the presence of REB binding motifs. This suggested that the *Gt1* promoter could serve as a candidate for constructing a gene that gains the REB response function through addition of the UAS from the *Glb* promoter. A construct containing the *Gt1* promoter linked with the *GUS* gene was tested, and the results established that cobombardment of developing endosperm with the native *Reb* gene and the *Gt1-GUS* does not activate the *Gt1* promoter (experiments 1 and 2 in Fig. 4). The 98-bp *Glb* fragment (from −626 to −724 distal to TATA box of the *Glb* promoter) containing three copies of GCCACGT(C/A)AG (Fig. 3) was inserted into the *Gt1* promoter at a position −630 bp distal to TATA box of the *Gt1* promoter to generate *Gt1+UAS-GUS* (Fig. 4*A*). Addition of the 98-bp fragment to the *Gt1* promoter does not significantly increase its capacity for GUS expression (experiment 3 in Fig. 4). The small increase of GUS activity observed could be because of the presence of some endogenous REB protein in immature rice endosperm. However, when *Gt1+UAS-GUS* construct was cotransformed into the endosperm with the native *Reb* gene, a 2.5-fold increase in GUS activity was obtained (experiment 4 in Fig. 4).

The loss of activation function, when the 200-bp fragment (from −642 to −842 distal to TATA box of the *Glb* promoter) was removed from the *Glb* gene promoter, and the gain of this function, when the 98-bp fragment (from −626 to −724 distal to TATA box of the *Glb* promoter) was added to the *Gt1* promoter, establishes the 98 bp of the fragment as a UAS.

The *Reb* Gene Increases Human Lysozyme Expression in Transgenic Rice Seed. Because transient gene expression of REB in rice immature endosperm *trans*-activates the *Glb* promoter, transgenic plants expressing a target gene driven by the *Glb* promoter might be expected to produce more of the target protein when additional REB protein is present. A synthetic human lysozyme
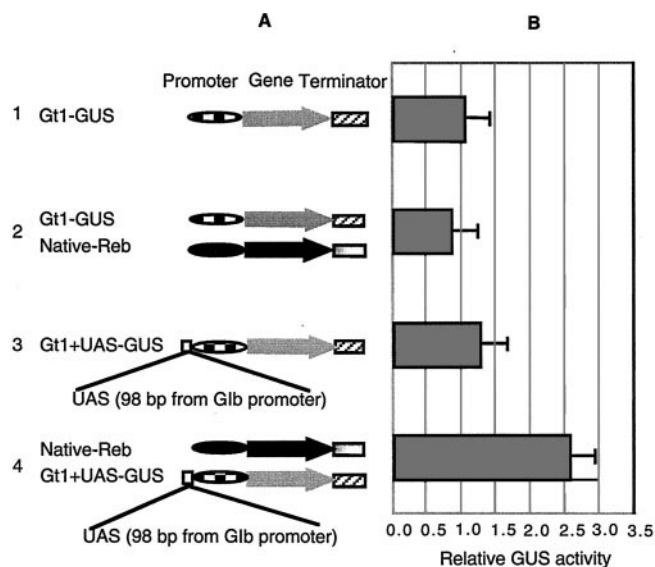
gene was linked with the *Glb* promoter to generate *Glb-Lys*, which was used to transform rice with or without *Native-Reb*. Additional transformants were made by cobombardment of *Glb-Lys* with the *Act-Reb* construct as well as with the *Act-Reb* construct alone. Eight individual transgenic rice plants were obtained from independent transformation events with *Act-Reb* construct. All of them were late flowering and displayed high sterility and broad leaves (data not shown). Analysis of these plants was not pursued. In contrast, normal plant phenotypes were obtained among transformants containing *Glb-Lys* alone or both *Glb-Lys* and native *Reb*. To determine whether the *Reb* gene and *Glb-Lys* are present in the transgenic rice genome, one primer designed from vector sequence and another designed from the *Reb* gene 3′ terminator were used to identify these lines. In this case, only the recombinant *Reb* gene could be amplified (Fig. 5*A*). PCR analysis confirmed the presence of transgenes into the rice genome. As shown in Fig. 5*C*, 10 of 11 plants from independent transformation events contained both *Reb* and the lysozyme transgenes. The REB protein of immature seeds from five randomly selected transgenic lines was detected by Western blotting. The expression level of the REB protein in transgenic lines ranged from 25% to 71% higher than that in untransformed TP309 (Fig. 6). This demonstrated that the transgenic *Reb* gene was active in transgenic plants. Increased REB expression provides the potential in enhancing target gene expression in transgenic seeds.

Seeds of confirmed transgenic rice plants were harvested at maturity, and the lysozyme activity was analyzed. Lysozyme expression in the seeds from 30 independent transformation events containing both the *Native-Reb* and the *Glb-Lys* ranged from 30.57 to 279.61 μg·mg^{-1} TSP with an average of 125.75 ± 68.65 μg·mg^{-1} TSP (Fig. 7). Seeds of 17 transgenic events containing the *Glb-Lys* gene alone expressed lysozyme in amounts ranging from 7 to 76 μg·mg^{-1} TSP with an average of 33.95 ± 20.55 μg·mg^{-1} TSP (Fig. 7). No lysozyme activity was
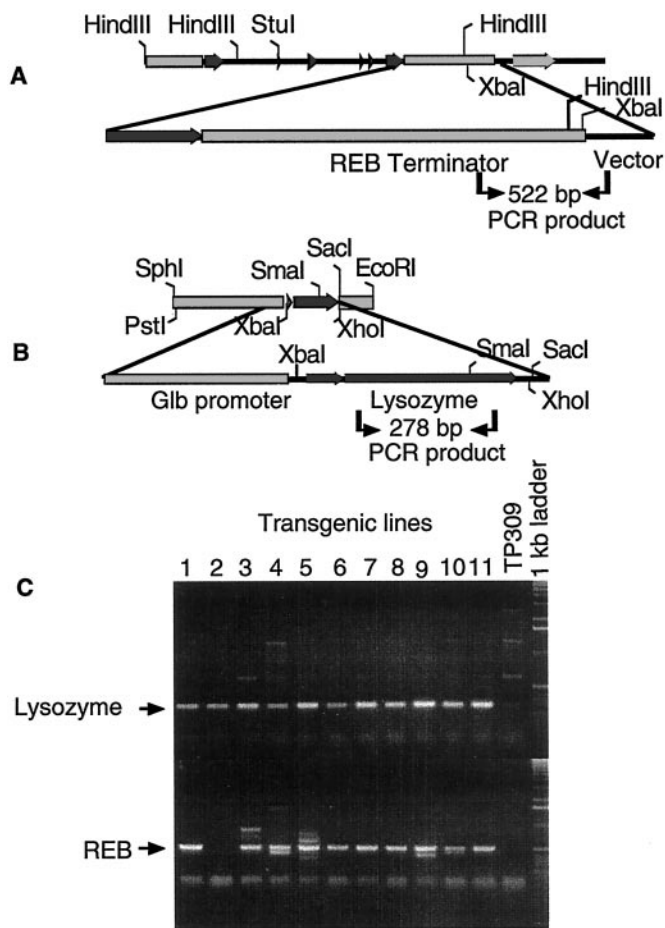
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Fig. 5. PCR analysis of T_0 transgenic plants containing the *Reb* and the human lysozyme gene. (*A*) The plasmid construct, API266 (*Native-Reb*), the primer positions (indicated by arrows), and amplified fragment size. One primer was designed from the vector region and the other from the *Reb* terminator. Only transformants can be amplified. (*B*) The diagram shows construct API264 (*Glb-Lys*), the primer positions, and the amplified fragment size. The primers hybridize to an internal sequence of the human lysozyme gene. (*C*) PCR analysis of *Native-Reb/Glb-Lys* cotransformed plants. Arrows mark the 522-bp fragment of the *Reb*/vector region and the 278-bp fragment from internal sequence of the human lysozyme gene.

detected in untransformed rice seeds (Fig. 7). The results showed that the expression level of the lysozyme increased an average of 3.7-fold when the seeds were transgenic for both the *Reb* gene and *Glb-Lys*. Statistical analysis (*t* test) showed that the amount of lysozyme in seeds from the plants transgenic for the *Reb* gene and *Glb-Lys* is significantly higher than in the plants transgenic for the *Glb-Lys* alone ($P < 0.001$).

Discussion

Several lines of evidence indicate that REB is a transcriptional activator that can increase the expression of recombinant protein as demonstrated here with β-glucuronidase in the developing endosperm and human lysozyme in the transgenic rice seeds. With transient gene expression analysis, we showed that expression of the *Reb* gene driven by either its own promoter or by the promoters of the actin or the globulin genes can double the expression of the GUS protein (Fig. 2). Transcriptional activation by REB is through an interaction with the UAS located in the 5′-distal region of the *Glb* promoter (Fig. 3). Addition of the UAS to a nonresponsive promoter provided the transactivation function of the REB (Fig. 4). Production of human lysozyme
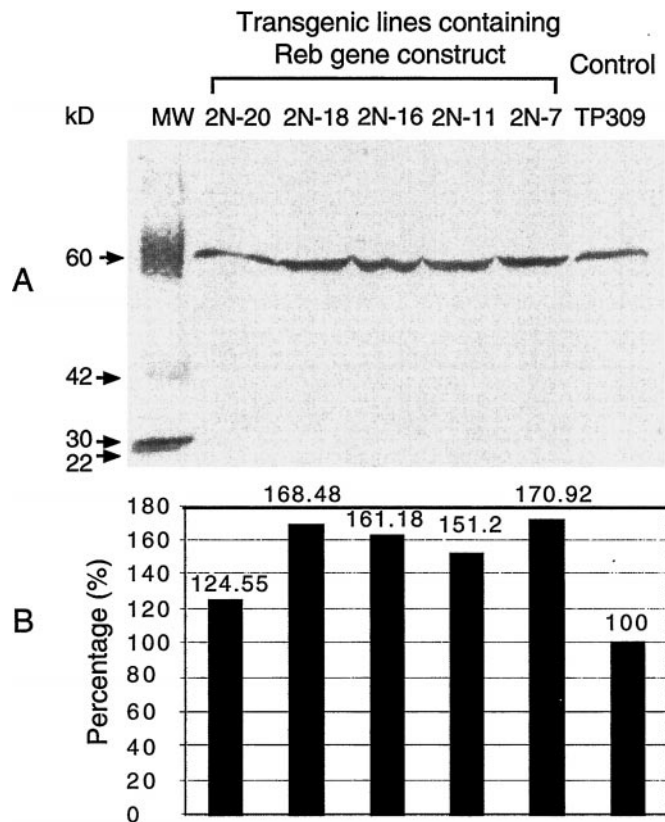

Fig. 6. The detection of the REB protein expression in immature seed of the transgenic plants by Western blotting. (*A*) Western blot of five transgenic lines containing *Glb-lys/Native-Reb*. TP309 is used as base-line control. (*B*) The quantitative analysis of the REB protein based on *A* by the Kodak 1D Program.

from *Glb-Lys* in developing transgenic rice seeds was 3.7-fold higher when coexpressed with REB. The increasing dosage of REB protein with the lysozyme expression level is consistent in the detected transgenic plants.
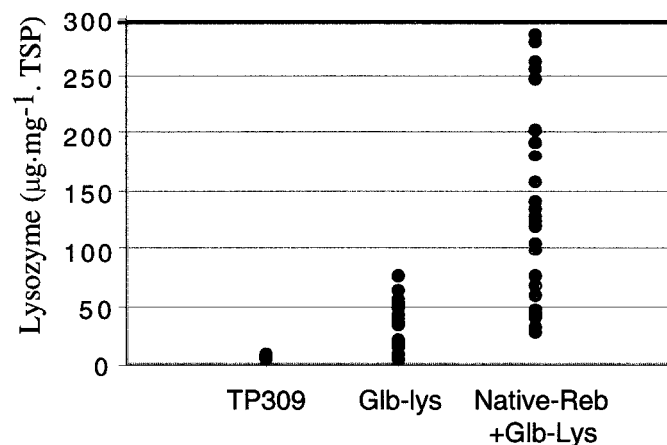

Fig. 7. The expression of human lysozyme in mature seed of T_0 transgenic plants. Seeds of 30 plants containing the *Reb* and lysozyme genes and seeds from 17 plants containing only the lysozyme gene were analyzed for lysozyme activity. The lysozyme gene was under the control of the *Glb* promoter, and the *Reb* gene was under the control of its own promoter. Individual seeds from each plant were analyzed. Transgenic seeds lacking detectable amounts of lysozyme were excluded. The activities of 20 lysozyme-positive seeds per plant, including both hemizygous and homozygous seeds, were averaged and plotted on the graph.

The increased expression of human lysozyme in transgenic plants carrying the *Native-Reb* gene is unlikely to be because of other factors, such as the position of integration. All transgenic plants with and without the *Native-Reb* gene were obtained with the same procedure and at the same time. The majority of both types of transgenic plants grew normally in the greenhouse. Thus, they are assumed to share the same random variation, and any difference in expression between the means of the two groups should be attributed to the presence or absence of additional REB.

The present experiments demonstrate that the use of a limiting transcriptional activator for enhancement of the expression of recombinant protein needs to be expressed specifically in the tissue used for production of the recombinant protein. In our experiments, the transgenic rice plants containing the *Reb* gene driven by the *Act* gene promoter show mutant phenotypes and are sterile. Presumably, this aberrant phenotype is because of the constitutive expression of the transcriptional activator REB in all plant cells, causing disturbances of the normal gene expression program during rice development. Comparable abnormalities of transgenic plants expressing a transcription factor have been observed in *Arabidopsis* (28). In both cases, it is concluded that a constitutive promoter is not suitable to drive a transcription factor gene with the aim to increase recombinant protein expression in the plants. The constitutive expression leads to untimely expression of the genes, as the same transcription factor is used in different cells and at different developmental stages. Its action is pleiotropic (28). To avoid this complication, we have used endosperm-specific promoters in the transgenic plants for both the recombinant protein-producing gene and for the REB transcription factor. Both the globulin gene and the native *Reb* gene are expressed in endosperm cells (21, 29); thus, their promoters are compatible for cell-specific interaction.

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