# The Rice *YABBY1* Gene Is Involved in the Feedback Regulation of Gibberellin Metabolism[1][C][W]

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Gibberellin (GA) biosynthesis is regulated by feedback control providing a mechanism for GA homeostasis in plants. However, regulatory elements involved in the feedback control are not known. In this report, we show that a rice (*Oryza sativa*) *YABBY1* (*YAB1*) gene had a similar expression pattern as key rice GA biosynthetic genes *GA3ox2* and *GA20ox2*. Overexpression of *YAB1* in transgenic rice resulted in a semidwarf phenotype that could be fully rescued by applied GA. Quantification of the endogenous GA content revealed increases of $GA_{20}$ and decreases of $GA_1$ levels in the overexpression plants, in which the transcripts of the biosynthetic gene *GA3ox2* were decreased. Cosuppression of *YAB1* in transgenic plants induced expression of *GA3ox2*. The repression of *GA3ox2* could be obtained upon treatment by dexamethasone of transgenic plants expressing a YAB1-glucocorticoid receptor fusion. Importantly, we show that YAB1 bound to a GA-responsive element within the *GA3ox2* promoter. In addition, the expression of *YAB1* was deregulated in GA biosynthesis and signaling mutants and could be either transiently induced by GA or repressed by a GA inhibitor. Finally, either overexpression or cosuppression of *YAB1* impaired GA-mediated repression of *GA3ox2*. These data together suggest that *YAB1* is involved in the feedback regulation of GA biosynthesis in rice.

The plant hormone GA regulates development throughout the life cycle of the plant. The biosynthesis of GA is regulated by both developmental and environmental stimuli (for review, see Hedden and Phillips, 2000; Olszewski et al., 2002). The genes encoding GA-biosynthetic enzymes have been identified in numerous species and are most highly expressed in rapidly growing tissues or organs (Olszewski et al., 2002). The final steps in the biosynthesis of the bioactive GAs, from $GA_{53}$ or $GA_{12}$ to $GA_1$ or $GA_4$, respectively, are catalyzed in parallel pathways by GA 20-oxidase (GA20ox) and GA3ox, whereas the bioactive $GA_1$ and $GA_4$ and their immediate precursors ($GA_{20}$ and $GA_9$, respectively) are converted to inactive forms by GA2ox (Hedden and Phillips, 2000; Olszewski et al., 2002). These enzymes, which are particularly important for the control of bioactive GA levels, are encoded by small gene families (Hedden and Phillips, 2000; Sakamoto et al., 2004). Overexpression or suppression of *GA20ox*, *GA3ox*, or *GA2ox* genes in transgenic plants often results in altered concentrations of active GA, emphasizing the importance of these genes in regulating GA content (for review, see Hedden and Phillips, 2000). These genes are also important in GA homeostasis. Transcript levels of some *GA3ox* and *GA20ox* genes are reduced, while *GA2ox* transcripts are increased by elevated GA signaling or GA treatment (Hedden and Phillips, 2000; Olszewski et al., 2002), indicating that GA biosynthesis is controlled by feedback regulation through the activity of the GA response pathway. When this pathway is derepressed as a result of loss-of-function mutations in the DELLA proteins that act as negative regulators, bioactive GAs as well as *GA20ox* and/or *GA3ox* mRNA are present at lower levels than in the wild-type plants (Croker et al., 1990; Martin et al., 1996; Dill and Sun, 2001; Silverstone et al., 2001). Conversely, gain-of-function mutations in the repressors or loss-of-function mutations in the positive components of the GA response pathway often result in higher levels of bioactive GA and up-regulation of *GA20ox* and *GA3ox* gene expression (Fujioka et al., 1988; Talon et al., 1990; Xu et al., 1995; Cowling et al., 1998; Ueguchi-Tanaka et al., 2000; Amador et al., 2001).

No signaling components downstream of the DELLA proteins have been clearly identified as mediators of GA homoeostasis, although several transcriptional regulators have been shown to modify GA metabolic gene expression. For example, the KNOX I class of homeodomain proteins repress *GA20ox* gene

[1] This work was supported by the National Special Key Program of Rice Functional Genomics, by the National Natural Science Foundation of China, and by the Biotechnology and Biological Sciences Research Council of the United Kingdom (grants to Rothamsted Research).

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www.plantphysiol.org/cgi/doi/10.1104/pp.107.096586

expression in the shoot apical meristem (SAM) in a number of species (for review, see Hay et al., 2004), while the FUS3 transcription factor down-regulates *GA3ox* in Arabidopsis (*Arabidopsis thaliana*) embryos (Curaba et al., 2004; Gazzarrini et al., 2004; for review, see Fleet and Sun, 2005).

In rice (*Oryza sativa*), GA3ox and GA20ox are encoded by two and four genes, respectively (Sakamoto et al., 2004). Loss-of-function mutations such as *d18* in *GA3ox2* and *sd1* in *GA20ox2* result in dwarf or semidwarf plants (for review, see Sakamoto et al., 2004). These genes are expressed in rapidly growing regions, such as leaf primordia, young leaves, elongating internodes, developing anthers, and embryo (Kaneko et al., 2003), but not in the vegetative SAM. The expression of both genes is also detected in reproductive meristems and stamen primordia during floral organogenesis (Kaneko et al., 2003). Their expression pattern suggests that the enzymes encoded by rice *GA3ox2* and *GA20ox2* play a major role in GA biosynthesis during both vegetative and reproductive development. In most cases, their expression in rice tissues overlaps with that of GA signaling genes, indicating that GA is synthesized at the sites of perception (Kaneko et al., 2003). The deactivating enzyme GA2ox is also encoded by a small gene family, two members of which (*GA2ox3* and *GA2ox4*) are broadly expressed in many organs or tissues (Sakamoto et al., 2004).

The plant genome encodes a large number of transcription factors, many of which are specific to plants. Among them, the YABBY family, which is characterized by the juxtaposition of two conserved domains (a $C_2C_2$ zinc finger domain toward the amino terminus and a putative helix-loop-helix ''YABBY'' domain conserved in HMG and other transcription factors toward the carboxyl terminus), is not found in yeast or animal cells (Bowman and Smyth, 1999; Golz and Hudson, 1999). The Arabidopsis genome contains at least six members of the *YABBY* gene family (*FILAMENTOUS FLOWER* or *YABBY1* [*YAB1*]; *YAB2*; *YAB3*; *INNER NO OUTER* or *YAB4*; *YAB5*; and *CRABS CLAW* [*CRC*]), all of which show a polar expression pattern, being confined to the abaxial side of one or more aboveground lateral organs (Bowman, 2000). Loss of expression of Arabidopsis *YABBY* genes results in loss of polar differentiation of abaxial cell types in the lateral organs, whereas ectopic expression of family members in the adaxial regions of developing cotyledons and leaves is sufficient to induce abaxialization of the adaxial cells (Eshed et al., 1999; Sawa et al., 1999; Siegfried et al., 1999).

The rice genome also seems to contain six members of the YABBY family. Recently, it has been shown that a rice *CRC*-related *YABBY* gene, *DL* (the mutation of which induces a *drooping leaf* phenotype), is required for carpel specification and leaf midrib development (Yamaguchi et al., 2004). Interestingly, none of the *YABBY* genes studied in rice shows an adaxial/abaxial polar expression pattern in lateral organs (Jang et al., 2004; Yamaguchi et al., 2004; Dai et al., 2007). Alteration of their expression by mutation or by ectopic expression produces no change in the adaxial/abaxial polarity of the lateral organs. These results suggest that rice *YABBY* genes may have functions different from their Arabidopsis homologs.

In this work, we studied the expression pattern and regulatory function of rice *YAB1* by transgenic approaches. Our data suggest that this gene is involved in the regulation of *GA* homeostasis in rice.

## RESULTS

### *YAB1* Expression Pattern

Reverse transcription (RT)-PCR analysis showed that *YAB1* was expressed in both shoots and developing panicles (Fig. 1A). In addition, *YAB1* expression was detected in leaf sheaths at a lower level (Fig. 1A). In situ hybridization experiments detected *YAB1* mRNA in the shoot apex and in young leaves (Fig. 1B, a and b). In the shoot apex, *YAB1* mRNA was detected only in leaf primordia but not in the SAM (Fig. 1B, a). The expression of *YAB1* in young leaves did not show any adaxial/abaxial polar distribution but was enhanced in both the upper and lower epidermis and in cells surrounding the vascular bundles (Fig. 1B, b). *YAB1* mRNA was also detected by in situ hybridization in rachis meristems and in lemma, palea, stamen, carpel primordia, and the embryonic axis (Fig. 1B, c–e). No adaxial/abaxial polar distribution of *YAB1* transcripts in the stamen or carpel primordia was observed (Fig. 1B, d). In addition, transgenic rice plants containing the GFP gene, under the control of the *YAB1* promoter region spanning from the initiation ATG codon to −1,662, showed GFP expression in elongating internodes and in leaf sheaths (Fig. 1C, a and b), in which GFP was detected mostly in sclerenchyma, vascular bundles and epidermal cells (Fig. 1C, c and d), confirming the in situ hybridization data (Fig. 1B, b). Therefore, *YAB1* was expressed widely in many different tissue or cell types, mostly in rapidly dividing or elongation tissues or organs but not in the SAM.

### Overexpression of *YAB1* Resulted in Semidwarf Phenotype

To study the function of *YAB1*, full-length cDNAs of the gene were isolated from a cDNA library of *indica* rice 'Minghui 63.' The *indica* cDNA presented a number of nucleotide substitutions resulting in seven amino acid changes of the protein compared to the published *japonica* sequence (Supplemental Fig. S1). This cDNA was cloned between the double cauliflower mosaic virus (CaMV) 35S promoter and the *NOS* (nopaline synthase) terminator in a rice transformation vector (see ''Materials and Methods''). The construct was introduced into 'Nipponbare' by *Agrobacterium*-mediated transformation, and more than 30 independent transgenic T0 plants were produced. A semidwarf phenotype occurred in many of the transgenic lines,

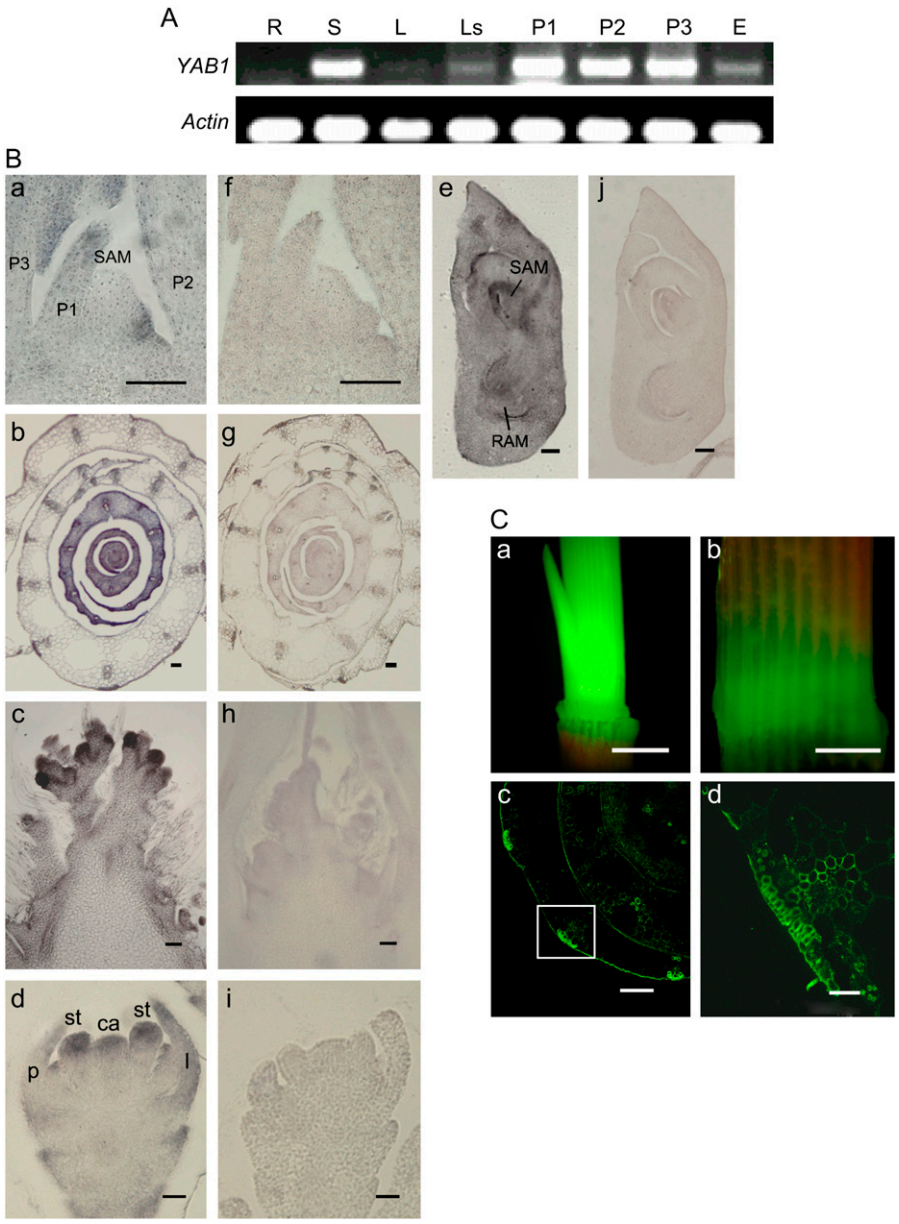


**Figure 1.** Expression pattern of *YAB1*. A, Detection of *YAB1* transcript by semiquantitative RT-PCR in roots (R), shoots (S), mature leaves (L), leaf sheaths (Ls), and panicles at three developmental stages (P1–P3; P1, primary panicle branch primordia; P2, secondary branch primordia; P3, stamen and carpel primordia) and developing embryo (E). B, In situ hybridization detection of *YAB1* transcripts in leaf primordia (a and f), young leaves (b and g), rachis meristem (c and h), floral meristem (d and i), and developing embryo (e and j) with antisense (a–e) or sense (f and g) cDNA of *YAB1* as probes. Bars in a, f, c, and h = 5 $\mu$m; bars in e and j = 10 $\mu$m; bars in b, g, and d, I = 20 $\mu$m. C, Detection of GFP expression under the control of the *YAB1* promoter (−1,662 to 1) in the elongating stem (a) and the leaf sheath (b). c, Cross section of sheaths showing GFP expression in sclerenchyma, vascular bundles, and epidermal cells. d, Enlargement of the boxed area in c. Bars in a and b = 1 cm, in c = 30 $\mu$m, in d = 100 $\mu$m. ca, Carpel primordium; l, lemma; p, palea; P1 to P3, leaf primordium; RAM, root apical meristem; st, stamen primordium.

with plant height about one-third that of the wild type together with about 3 times more tillers (Fig. 2A; Tables I and II). Further investigation of the semidwarf transgenic plants indicated that elongation of all the internodes, except internode V, was affected (Table I). Stem epidermal, elongating, and intercalary meristem cells in the transgenic plants were shorter than in the wild type (Fig. 2B), indicating that cell elongation was reduced. No alteration of leaf shape and flowering time was observed. The overexpression plants were partially fertile. Transgenic plants showing phenotypic changes were subsequently analyzed in the T1 generation for cosegregation between the phenotype and the transgene. Eight semidwarf transgenic plants were analyzed by RNA-gel blots. As shown in Figure 2C, all of the semidwarf plants overexpressed the transgene. Southern-blot analysis of these lines indicated that they contained a single transgene insertion in the genome (Supplemental Fig. S2).

### The Dwarf Phenotype of *YAB1*-Overexpressing Plants Could Be Suppressed by Exogenous GA Treatment

To determine whether or not the semidwarf phenotype of the *YAB1* overexpression plants was caused by GA deficiency, the response of the plants to exogenous GA was measured. Four-week-old (at four-leaf stage) transgenic and wild-type plants were transferred to a hydroponic culture medium supplemented with 10 $\mu$M paclobutrazol (an inhibitor of GA biosynthesis) for 1 d to reduce the endogenous GA to background levels, before adding $GA_3$ at different final concentrations

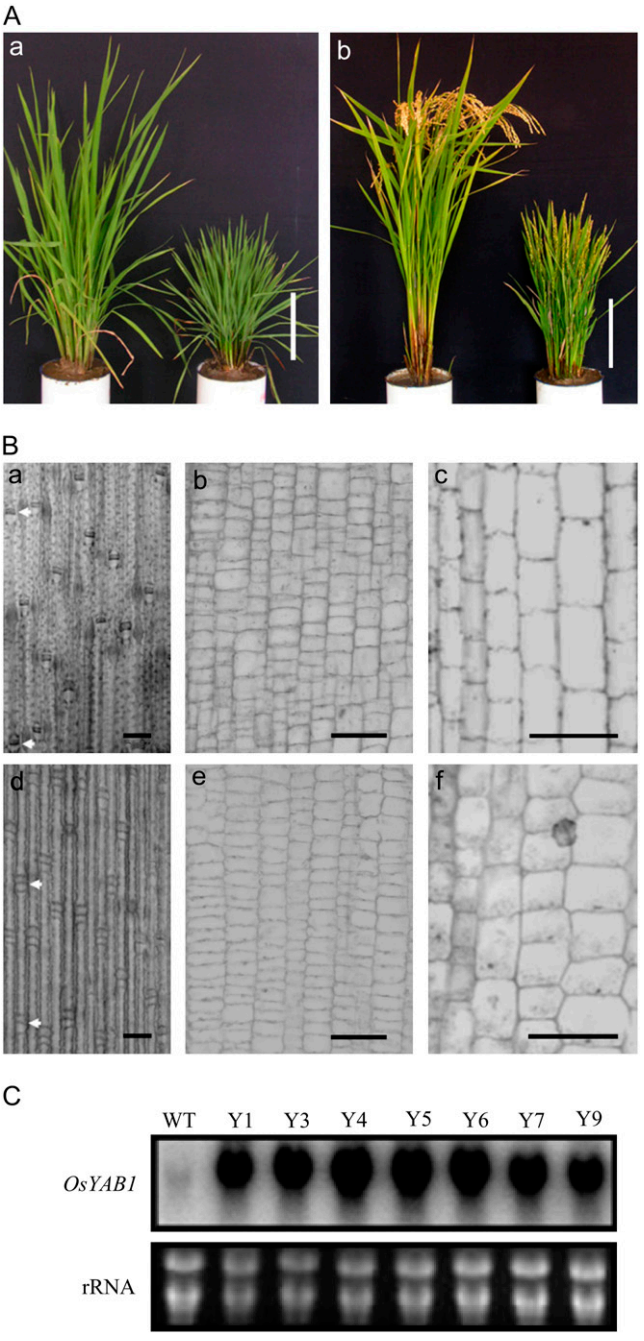


**Figure 2.** Overexpression of *YAB1* under the double CaMV 35S promoter induced semidwarf phenotypes in transgenic rice. A, Comparison between wild type and a *YAB1*-overexpressing line (Y4) at vegetative and reproductive stages. Bars = 15 cm. B, Overexpression of *YAB1* affected cell elongation in stems. a and d, Epidermal cells. Arrows indicate the upper or lower limits of epidermal cells. Bars = 2 $\mu$m. b and e, Cells in the intercalary meristem. Bars = 10 $\mu$m. c and f, Cells in elongating zone. Bars = 10 $\mu$m. a to c, Wild type; d to f, *YAB1*-overexpressing plants. C, RNA gel-blot hybridization showing that all examined semidwarf lines (Y1, Y3–Y7, and Y9) showed overexpression of *YAB1*.

ranging from 0.01 to 20 $\mu$M. Plant heights were measured 10 d after GA application. The data presented in Figure 3 showed that at 10 $\mu$M, GA could fully rescue the dwarf phenotype of the *YAB1* overexpression plants.

### Endogenous GA Contents in the Overexpression Plants

It has been shown that $GA_1$ is the major bioactive GA in the vegetative organs in rice (Kobayashi et al., 1988). Using gas chromatography-mass spectrometry, we measured endogenous levels of different 13-hydroxylated GAs within the $GA_1$ metabolic pathway (Fig. 4A) in both transgenic and wild-type seedlings. The data in Figure 4B shows that in the overexpression lines, the $GA_1$ content decreased to about one-half that of the wild type, whereas the levels of $GA_{20}$ (precursor of $GA_1$), $GA_8$ (deactivated form of $GA_1$), and $GA_{29}$ (deactivated form of $GA_{20}$) increased about 2-fold. Changes in the levels of more upstream precursors ($GA_{53}$ and $GA_{19}$) also occurred, including a slight decrease in $GA_{19}$ content in the transgenic lines.

### Overexpression of *YAB1* Altered GA Metabolic Gene Expression

Changes in the GA content in the *YAB1* overexpression lines may be due to altered expression of one or more of the genes encoding GA metabolic enzymes. We compared expression of six representative genes encoding four enzymes, *ent*-copalyl diphosphate synthase (CPS), GA3ox, GA20ox, and GA2ox, in young leaves of transgenic and wild-type plants by quantitative RT-PCR. As shown in Figure 4C, mRNA levels of *GA3ox2* in the *YAB1*-overexpressing lines (Y1, Y3, and Y4) were reduced to 10% to 20% of the wild-type levels. In contrast, *GA2ox3* was induced in the overexpression lines. It is suggested that GA3ox2 makes major contributions to the biosynthesis of $GA_1$ during vegetative development (Kaneko et al., 2003). The expression results are consistent with the observed increases of $GA_{20}$ (substrate for GA3ox), $GA_{29}$, and $GA_8$ (products of GA2ox) and decrease of $GA_1$ (product of GA3ox and substrate for GA2ox) in the overexpression plants.

### Analysis of *YAB1* Cosuppression Plants

Examination of transgene copy numbers by Southern blots revealed that two lines had at least two insertions of the *YAB1* transgene (Supplemental Fig. S2). These lines were found to contain lower *YAB1* mRNA levels compared to the wild type (Supplemental Fig. S3A), suggesting that expression of both endogenous and transgenic *YAB1* was being suppressed, as might be anticipated, because multiple copy transgenes have a high probability of inducing cosuppression and gene silencing (Schubert et al., 2004). The expression of two other *YABBY* genes (*YAB2* and *DL*) was not changed (Supplemental Fig. S3A). The cosuppression was

**Table I.** *Lengths of the internodes and relative contribution of each internode (in parentheses) to the total culm in YAB1-overexpressing lines in comparison with wild-type plants*

The average values were calculated from measures of at least 20 plants ±SD.

| Lines | Length of the Internodes | | | | | | Total Culm |
|---|---|---|---|---|---|---|---|
| | P | I | II | III | IV | V | |
| | | | *cm* | | | | |
| WT | 19.8 ± 1.0 (25.3) | 32.6 ± 2.9 (41.6) | 13.4 ± 1.0 (17.1) | 7.9 ± 1.3 (10.1) | 3.8 ± 1.7 (4.8) | 0.9 ± 0.3 (1.1) | 78.4 ± 3.4 |
| Y1 | 16.3 ± 2.7 (27.6) | 27.8 ± 2.3 (42.8) | 8.3 ± 1.2 (14.1) | 4.3 ± 0.8 (7.3) | 1.7 ± 0.6 (3.0) | 0.7 ± 0.2 (1.2) | 59.1 ± 2.8 |
| Y3 | 11.2 ± 0.6 (32.0) | 11.4 ± 2.1 (32.6) | 4.8 ± 1.2 (13.7) | 4.0 ± 1.3 (11.4) | 2.6 ± 0.7 (7.4) | 1.0 ± 0.4 (2.9) | 35.0 ± 4.0 |
| Y4 | 14.1 ± 2.0 (29.2) | 20.2 ± 2.0 (41.8) | 7.7 ± 2.0 (15.9) | 3.7 ± 1.4 (7.7) | 2.0 ± 1.0 (4.1) | 0.6 ± 0.1 (1.3) | 48.3 ± 4.0 |

therefore specific to *YAB1*. Examination of GA metabolic gene expression in cosuppression line Y8 by quantitative RT-PCR showed a clear increase in *GA3ox2* transcript levels compared to the wild type (Fig. 5A). However, the expression of *GA2ox3* was not altered (Fig. 5A). The results confirmed by RNA gel-blot hybridization (Supplemental Fig. S3B) suggested that *YAB1* was required for the expression of *GA3ox2* but not for *GA2ox3*. The induction of *GA2ox3* in YAB1 overexpression plants (Fig. 4C) might be achieved by an indirect mechanism (discussed later). In addition, *GA20ox2* mRNA levels were also increased (Fig. 5A; Supplemental Fig. S3B). Analysis of 13-hydoxylated GAs in plants showed slight increases in $GA_{19}$, $GA_{20}$, and $GA_1$ contents and reductions in $GA_{53}$ and $GA_{29}$ contents in the cosuppression plants (Fig. 5B). The changes in GA contents are consistent with the transcript levels of *GA20ox2*, *GA3ox2*, and *GA2ox3* present in line Y8.

The cosuppression plants did not show the slender phenotype, probably because of only slight increase of $GA_1$ or effects on other developmental aspects (see below) induced by the cosuppression. Instead, leaf blades of the cosuppression plants drooped outwards, forming an acute angle with the leaf sheaths (Fig. 6, A and B). In addition, leaves were rolled toward the abaxial side, forming a cylinder-like structure (Fig. 6D). Transverse sections showed increases in the number of the bulliform cells situated between the vasculatures on the adaxial side of the leaf blade (Fig. 6F). Bulliform cells deposited in the adaxial side of the leaf blade are responsible for the leaf rolling that may occur during drought stress in rice. These cells in Y8 plants had a rectangular or triangular shape rather than the spherical form observed in the wild-type plants. Other cells in the leaves appeared normal, with no alteration in adaxial-abaxial identity observed in the arrangement of the leaf inner structure. These observations suggested that the rolled leaf phenotype may be due to the increases in bulliform cell number and that *YAB1* may have a function in bulliform cell division and differentiation. The cosuppression plants were sterile due to defects in anther and carpel formation (Fig. 6H).

### YAB1 Is Directly Involved in the Regulation of *GA3ox2*

To determine whether YAB1 directly regulated the expression of the GA metabolic genes, we produced transgenic rice producing YAB1 fused with the glucocorticoid receptor (GR) that is used to sequestrate the fusion protein in the cytoplasm (Aoyama and Chua, 1997; Fig. 7A). Four analyzed lines that showed transgene expression (Fig. 7B) were assayed with dexamethasone (DEX), which induces conformational changes in GR allowing the fusion protein to be targeted into the nucleus. DEX treatment induced repression of *GA3ox2* expression in the transgenic plants (Fig. 7C). Furthermore, we assayed the DEX induction in the absence or presence of cycloheximide (CHX), which blocks de novo protein synthesis. The presence of DEX and DEX plus CHX had similar effects on the repression of *GA3ox2* transcript levels (Fig. 7C). In the wild-type plants, the expression of *GA3ox2* did not change significantly after treatment.

To know whether YAB1 could bind to the *GA3ox2* promoter, yeast one-hybrid assays revealed that that YAB1 interacted with a 0.8-kb promoter region of *GA3ox2* (not shown). This promoter fragment was subsequently cut into two segments (Fig. 8A) that were used for gel shift assays with *Escherichia coli*-produced YAB1 protein. The region from −662 to −329 was bound by YAB1 protein (Fig. 8B). Double-stranded oligonucleotides corresponding to seven overlapped regions of the DNA fragment were tested for binding to YAB1. One of the oligonucleotides (P3) retained the specific YAB1-binding activity (Fig. 8, C and D). Sequence analysis (with software PLANTCARE) revealed that this 50-bp region contained a GA-responsive element (GARE; 5′-TAACAGA; Fig. 8E). To test whether this element was responsible for the binding of YAB1, binding assays with oligonucleotides containing a pair of the wild-type or substituted GARE sequences showed that YAB1 bound specifically to the wild-type GARE (Fig. 8F).

These data together suggested a direct involvement of YAB1 in regulating the expression of *GA3ox2*.

**Table II.** *Comparison of average tiller number in transgenic and wild-type plants*

The average values were calculated from measures of at least 20 plants ±SD.

| Wild Type | Y1 | Y3 | Y4 |
|---|---|---|---|
| 18.7 ± 4.0 | 61.1 ± 24 | 68.2 ± 19 | 67.0 ± 25 |

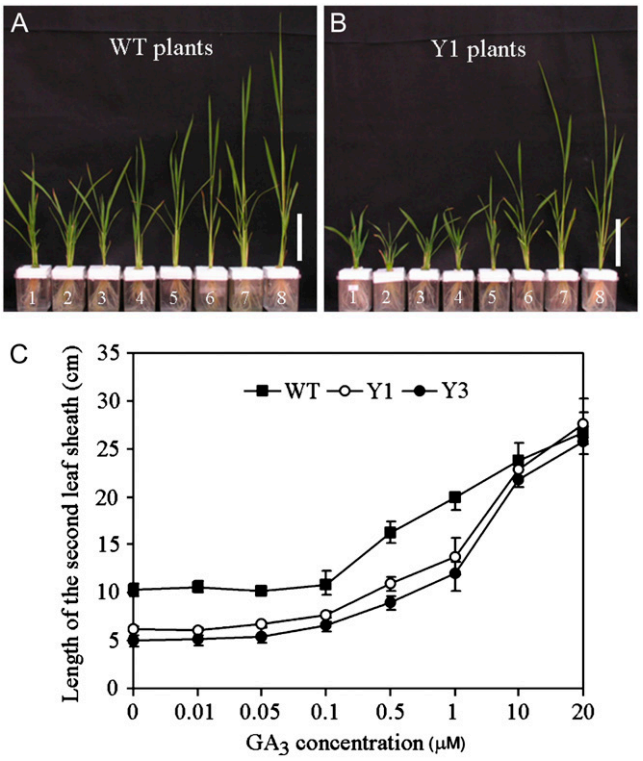


**Figure 3.** Rescue of semidwarf phenotype by $GA_3$ at different concentrations. Wild-type (A) and *YAB1* overexpression plants (B) at four-leaf stage were transferred to hydroponic culture media containing 10 $\mu$M paclobutrazol (an inhibitor of GA biosynthesis) for 1 d, then supplemented with $GA_3$ at the concentration of 0 (1), 0.01 (2), 0.05 (3), 0.1 (4), 0.5 (5), 1 (6), 10 (7), or 20 $\mu$M (8). The lengths of the second leaf sheath were measured 10 d after the treatment (C). Squares, white circles, and black circles represent wild type, Y1, and Y3, respectively. Error bars represent SD from at least 20 plants. [See online article for color version of this figure.]

### GA Treatment Transiently Induced *YAB1* Expression

The expression pattern of *YAB1* in shoot and floral meristems or in elongating internodes was similar to that of GA biosynthetic genes (Fig. 1; Kaneko et al., 2003). Therefore, we investigated whether GA regulated the expression of *YAB1*. Rice seedlings at the four-leaf stage were sprayed with 50 $\mu$M $GA_3$, and the aerial parts were harvested at different time points for quantitative RT-PCR analysis. As shown in Figure 9A, the *YAB1* mRNA level was increased 2-fold only 30 min after GA treatment and subsequently decreased to the control level at 2 h after the treatment. These data suggested that GA transiently induced the expression of *YAB1*. Interestingly, the expression of *YAB1* increased again from 8 to 12 h after the treatment, suggesting that additional regulators were involved in *YAB1* expression (M. Dai and D.-X. Zhou, unpublished data). These results were confirmed by RNA gel-blot hybridization (Supplemental Fig. S4).

We also investigated the effect of a low endogenous GA content on expression of *YAB1* by treating wild-type plants with the GA biosynthesis inhibitor paclobutrazol. Plants were harvested for RNA extraction at the same time points as for the GA treatment, and *YAB1* transcript was analyzed by real-time quantitative RT-PCR. As shown in Figure 9A, paclobutrazol treatment produced the opposite *YAB1* expression profile as obtained after $GA_3$ treatment.

### *YAB1* Was Deregulated in *eui*, *gid1*, and *slr1* Mutants

To confirm the observations in Figure 9A, we tested the *YAB1* mRNA levels in rice GA biosynthesis (*eui*) and signaling (*gid1* and *slr1*) mutants. The *eui* mutants

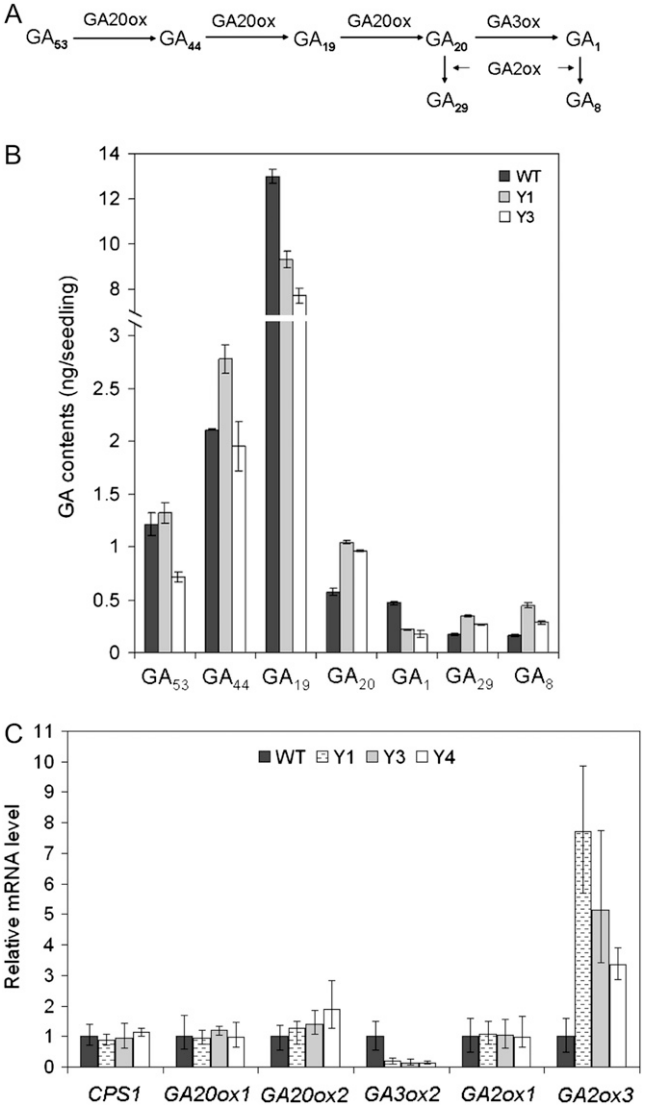


**Figure 4.** Overexpression of *YAB1* altered GA accumulation and GA metabolic gene expression. A, Schematic representation of 13-hydroxylated GA metabolic pathway, involving GA20ox, GA3ox, and GA2ox. B, Quantification of 13-hydroxylated GAs in four-leaf-stage seedlings. Data are means ± SD ($n = 3$). C, Quantification of transcripts of six GA metabolic genes by real-time RT-PCR. The PCR signals were normalized with that from actin transcripts. Transcript levels from wild type were set at 1. Data are means ± SD ($n = 3$).

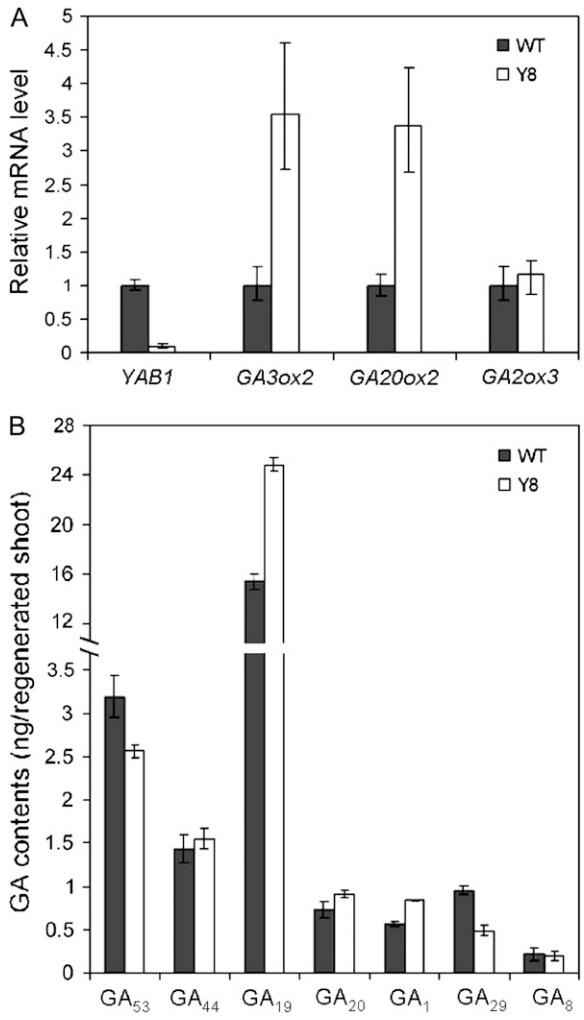


**Figure 5.** Cosuppression of *YAB1* altered GA biosynthetic gene expression and GA levels. A, Relative transcript levels by real-time RT-PCR of *GA3ox2*, *GA20ox2*, and *GA2ox3* transcript levels between wild-type and Y8 plants after normalization with *ACTIN* transcripts. B, Quantification of 13-hydraxylated GA forms. Data are means ± SD ($n = 3$).

accumulate biologically active gibberellins and show repression of *GA3ox2* (Zhu et al., 2006). In *eui-1*, the expression of *YAB1* was induced in agreement with the repression of *GA3ox2* in the same mutant (Fig. 9B; Zhu et al., 2006). The *gid1-1* mutants affecting GA reception have a GA-insensitive dwarf phenotype and show accumulation of bioactive GA but increased expression of GA biosynthetic genes *sd1* (*GA20ox2*; Ueguchi-Tanaka et al., 2005) and *GA3ox2* (Fig. 9B), indicating disruption of the feedback regulation. In this mutant, the expression of *YAB1* is repressed (Fig. 9B). *slr1-1* is a loss-of-function mutation of the rice *DELLA* gene that encodes a GA-signaling repressor (Ikeda et al., 2001). In *slr1-1*, the *YAB*1 expression was induced (Fig. 9B). The expression of *GA3ox2* in *eui*, *gid1*, and *slr1* showed an opposite expression pattern compared to *YAB1* (Fig. 9B).These data indicate GA signaling is required for GA-mediated repression of *YAB1* and YAB1 is a downstream negative feedback regulator of *GA3ox2*.

### Deregulation of *YAB1* Impaired GA-Mediated Repression of *GA3ox2*

Our results suggested that YAB1 may function as a negative regulator of *GA3ox2*. To know whether *YAB1* played a role in mediating GA-dependent down-regulation of *GA3ox2* expression, both *YAB1* overexpression and cosuppression plants along with wild type at the four-leaf stage were sprayed with 50 $\mu$M $GA_3$ and harvested at different time points for RNA extraction and quantitative RT-PCR analyses. In wild-type plants, the applied GA rapidly (1–2 h) suppressed the expression of *GA3ox2* to a much lower level (about a 5-fold decrease). In *YAB1* cosuppression plants (Y8), the applied GA had little effect on the expression of *GA3ox2*, suggesting that *YAB1* is required for GA-mediated *GA3ox2* repression. However, overexpression of *YAB1* also attenuated the GA-induced repression of *GA3ox2* (Fig. 10). The later observation could be explained by an already repressed expression level of *GA3ox2* in *YAB1* overexpression plants. Applied GA had less effect on its repression than in wild-type

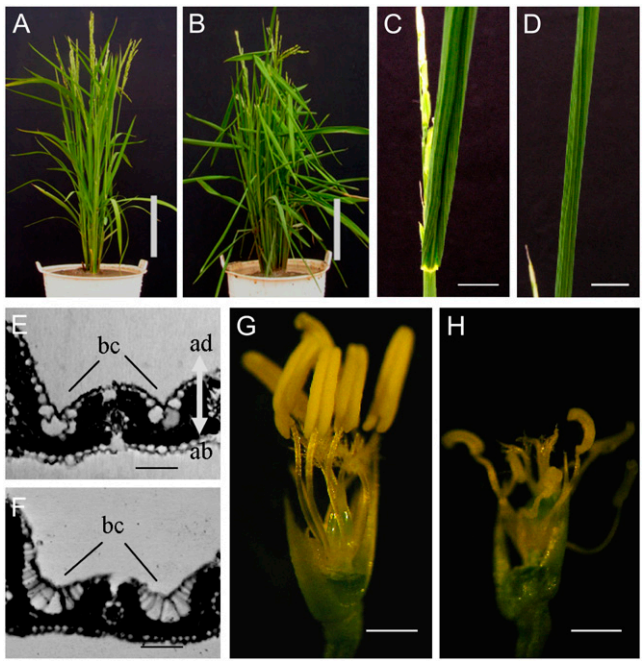


**Figure 6.** Cosuppression of YAB1 induces rolled leaves and abnormalities in flower development. A, Wild-type rice at heading stage. B, Y8 plant at the heading stage with rolled leaves dropped, forming an acute angle with the sheaths. C, Lower part of a rolled leaf. D, Upper part of a rolled leaf. E and F, Comparison of a leaf section in Y8 (F) with wild type (E) showing altered bulliform cells in Y8. G and H, Comparison of Y8 (H) stamens and carpels to wild type (G). Bar = 20 cm in A and B, 4 cm in C and D, 100 $\mu$m in E and F, and 5 mm in G and H. bc, Bulliform cells; ad, adaxial side; ab, abaxial side.

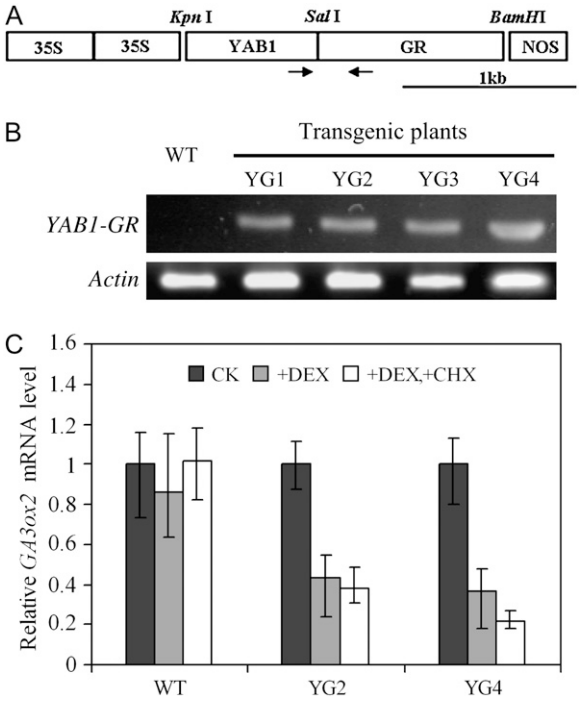


**Figure 7.** YAB1 is directly involved in the transcriptional regulation of *GA3ox2*. A, Schematic representation of construct in which YAB1 is fused to the GR. Arrows indicate the positions of primers used in B. B, RT-PCR detection of *YAB1-GR* transcripts in four independent rice transgenic lines. C, Effects of DEX on the transcription of *GA3ox2* in the presence of CHX (10 $\mu$M). CHX was added 1 h prior the addition of DEX. Samples were harvested after 2-h treatment. Data normalized with *ACTIN* transcripts are means from three biological repeats ±SD. The values from nontreated samples were assessed as 1.

plants. These data suggested that *YAB1* is required for the GA-mediated *GA3ox2* repression.

## DISCUSSION

In this work, we present several lines of evidence that *YAB1* is involved in the feedback regulation of GA biosynthesis. In contrast to the SAM-expressed *KNOX* genes that exclude the expression of *GA20ox* from the SAM (for review, see Hay et al., 2004), the expression domains of *YAB1* overlap with those of *GA3ox2*, *GA20ox2*, and GA-signaling genes, which are expressed in diverse organs or tissues of rice, including embryo, shoots, and floral meristems (Fig. 1; Kaneko et al., 2003). This suggests that *YAB1* have a function in modulating GA metabolic gene expression or GA signaling throughout the plant. The semidwarf phenotype, which can be suppressed by applied GA, and the changes in GA metabolic gene expression and GA levels induced by both overexpression and cosuppression of *YAB1* support a regulatory function for *YAB1* in GA biosynthesis (Figs. 2–4). Importantly, the expression of *YAB1* is GA responsive, being rapidly and transiently induced by GA or repressed by inhibition of GA biosynthesis (Fig. 9), suggesting that *YAB1*and GA form part of a regulatory loop in GA-responsive tissues. Finally, *YAB1* binds to the promoter of *GA3ox2* and is required for GA-mediated repression of the gene (Figs. 7, 8, and 10). These data together would support a feedback regulatory scenario; the rapid activation of *YAB1* by GA subsequently induces the repression of *GA3ox2* and consequently decreases bioactive GA levels, leading to the repression of *YAB1* itself. In addition, the effects of overexpression and cosuppression of *YAB1* on GA contents is relatively moderated (about 2-fold changes; Figs. 4 and 5) in favor of a feedback regulatory function of the gene in GA biosynthesis.

### Function of *YAB1* in the Regulation of *GA3ox2*, *GA2ox3*, and *GA20ox2*

We have shown that overexpression of *YAB1* induces repression of *GA3ox2* and, consequently, accumulation of its substrate $GA_{20}$ and a reduction in the amount of its bioactive product $GA_1$ (Fig. 4). Accordingly, cosuppression of *YAB1* results in increased *GA3ox2* expression (Fig. 5). The experiments with the inducible YAB1 construct (Fig. 7) showed that the repression of *GA3ox2* was obtained upon induction of the YAB1 activity. In addition, YAB1 interacts directly with a GARE sequence within the *GA3ox2* promoter (Fig. 8). These data, together with the requirement of a normal expression level of *YAB1* for GA-mediated *GA3ox2* repression (Fig. 10), indicate that *YAB1* is directly involved in the regulation of *GA3ox2*.

In contrast, *YAB1* overexpression induces *GA2ox3*, which is consistent with the accumulation of the 2-oxidized products $GA_8$ and $GA_{29}$ (Fig. 4). However, the cosuppression of *YAB1* did not decrease the expression of *GA2ox3* (Fig. 5; Supplemental Fig. S2), which helps to explain why there was only a slight increase of $GA_1$ accumulation and there was no typical slender phenotype. This discrepancy suggests that *YAB1* is not involved in *GA2ox3* regulation at natural expression levels in wild-type plants or that there is a baseline level of *GA2ox3* expression regulated independently of *YAB1*. It is also possible that a reduction of *GA2ox3* expression in the absence of YAB1 might be compensated for by feedback induction due to the increase of the active $GA_1$ in the cosuppression plants. However, failure to detect any YAB1-binding activity of the *GA2ox3* promoter where no GARE was found (not shown) suggests that YAB1 is not directly involved in the regulation of the gene. Conversely, overexpression of *YAB1* does not significantly alter *GA20ox2* expression (Fig. 4), but cosuppression of *YAB1* induces the expression of *GA20ox2* (Fig. 5). We favor the hypothesis that the expression of *GA20ox2* is indirectly induced in the cosuppression plants through an independent feedback mechanism required for coactivation of both of the key biosynthetic genes, *GA3ox2* and *GA20ox2*, because there was no direct binding of YAB1 to *GA20ox2* promoter that contains no GARE sequence (not shown). Therefore,

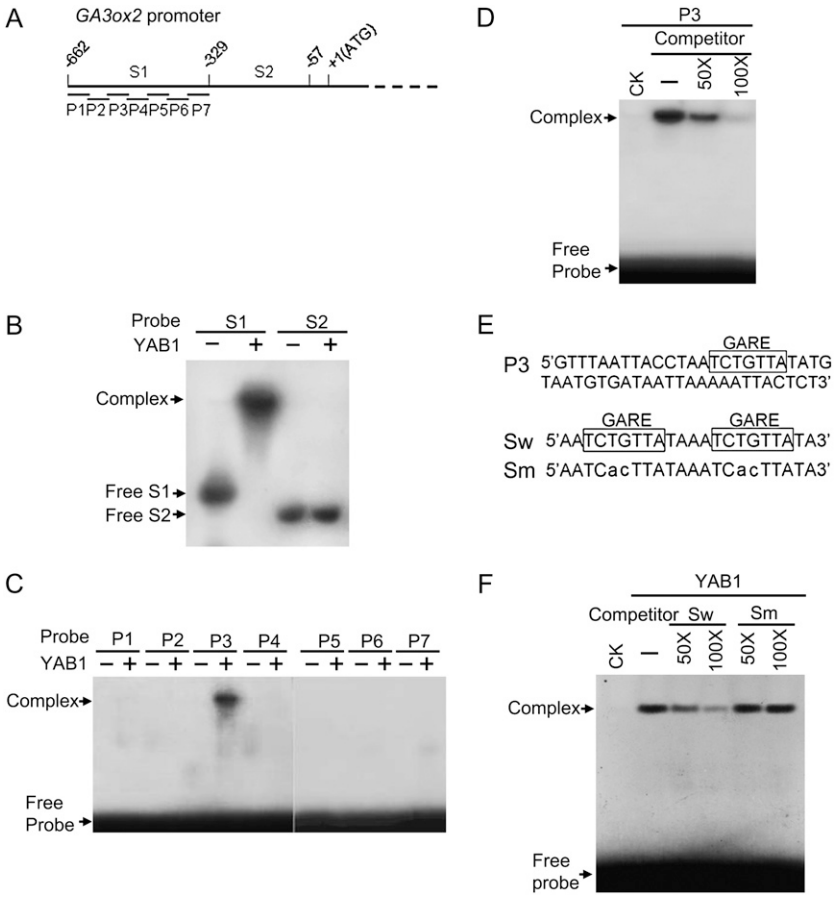


**Figure 8.** YAB1 binds to a GARE in *GA3ox2* promoter. Gel shift binding assays with *E. coli* produced rice *indica* YAB1 protein to *GA3ox2* promoter regions. A, Schematic representation of the *GA3ox2* promoter regions with indications relative to the ATG codon. B, Binding assays with *GA3ox2* promoter fragments S1 and S2 in the presence (+) or absence (−) of YAB1 protein. C, Binding assays with seven overlapping double-stranded oligonucleotides corresponding to S1 in the presence (+) or absence (−) of YAB1 protein. D, YAB1-binding assays with $^{32}$P-labeled P3 in the presence or absence (−) of cold P3 at 50 to 100 M excesses as indicated. E, Top, P3 contains a GARE. Bottom, Sequences of oligonucleotides corresponding to a doublet of the wild-type (Sw) or mutated (Sm) GARE used in F. F, YAB1-binding assays to $^{32}$P-labeled Sw in the presence or absence (−) of Sw or Sm at 50 to 100 M excesses as indicated. Positions of binding complexes and free probes are indicated by arrows.

our data show that YAB1 is a direct regulator of *GA3ox2*, but its deregulation has indirect impact on the other key GA metabolic gene expression.

### YAB1 Is Involved in the Negative Feedback Regulation of GA Homeostasis

GA biosynthesis is controlled by feedback regulation through the activity of the GA response pathway. DELLA proteins that act as negative regulators of the pathways are required for GA feedback regulation, as mutations in DELLA genes affect GA metabolic gene expression and bioactive GA homeostasis. GA induces DELLA protein degradation. It is suggested that DELLA proteins may directly or indirectly repress transcriptional activators of GA signaling pathways. However, no transcriptional regulator downstream of the DELLA proteins has been so far clearly identified as a mediator of GA homoeostasis, although it is shown that the tobacco (*Nicotiana tabacum*) protein REPRESSION OF SHOOT GROWTH (RSG), a bZIP transcription factor, binds to and activates the transcription of the early GA biosynthetic gene *ent-kaurene oxidase*. Overexpression of a dominant negative form of *RSG* results in a GA-deficient dwarf phenotype and prevents activation of an *NtGA20ox* gene in transgenic tobacco, suggesting that RSG plays a role in feedback regulation of *NtGA20ox* (Fukazawa et al., 2000). However, it is not known if RSG is directly involved in the regulation of *NtGA20ox*. Our present data show that YAB1 is likely a mediator of GA homeostasis downstream of the DELLA protein, as its expression is dependant on GA signaling and is derepressed in loss-of-function mutants of the rice DELLA gene (*slr*; Fig. 9) and deregulation of the expression impairs *GA3ox2* expression (Fig. 10).

### Binding of YABBY Proteins to GARE

The GARE (TAACAGA) motif has been identified as a cis-regulatory element involved in the GA induction of rice α-amylase genes (Gubler and Jacobsen, 1992). A MYB-type transcription factor named GAMYB initially identified from barley (*Hordeum vulgare*) and later from rice binds to GARE and transactivates the transcription from the promoters (Gubler et al., 1995, 1999; Sutoh and Yamauchi, 2003). GA-induced activation of α-amylase genes as well as a number of other hydrolase genes requires an additional cis-element (5′-CAACTC, called CARE) in addition to GARE (Gubler and Jacobsen, 1992; Cercós et al., 1999; Sutoh and Yamaochi, 2003). No CARE sequence was found in the *GA3ox2* promoter that is repressed by GA, suggesting that the GARE motif has either an activating or a repressing function, depending on promoter

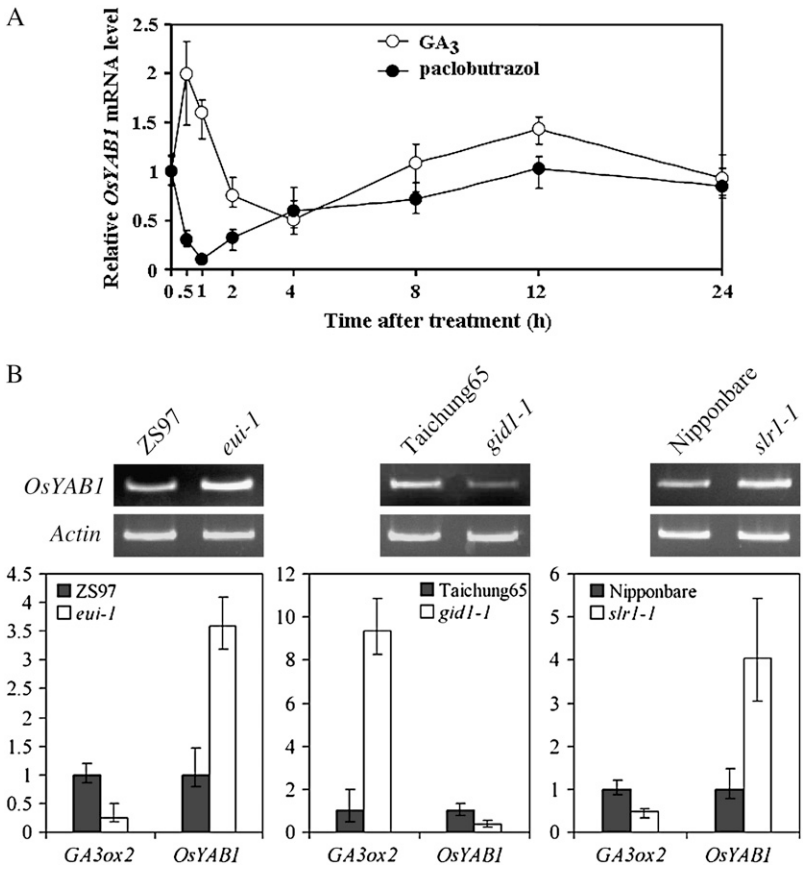


**Figure 9.** GA transiently induced expression of *YAB1* in seedlings. A, Real-time RT-PCR quantification of *YAB1* transcripts isolated from seedlings treated with 10 $\mu$M paclobutrazol or with 50 $\mu$M $GA_3$. Data normalized with actin transcripts are means from three biological repeats ±SD. B, YAB1 mRNA levels in eui-1, gid1-1, and slr1-1 compared to the respective wild-type plants ('ZS97', 'Taichung65', and 'Nipponbare') measured by both semiquantitative and real-time RT-PCR in which *YAB1* and *GA3ox2* mRNA levels are normalized with *ACTIN* mRNA levels (assessed as 1). Data are means from three biological repeats ±SD.

context or on the cognate binding factors. To our knowledge, no DNA-binding property has been described for YABBY proteins that belong to the zinc finger superfamily of transcription factors. In this study, we show that rice YAB1 binds to the GARE motif within the *GA3ox2* promoter and represses transcription of the gene. It has been previously shown that in barley, a zinc finger protein, HRT (*Hordeum* repressor of transcription), represses GA-inducible gene expression and also binds to the GARE motif (Raventós et al., 1998).

At this stage, it is not known whether the other members of YABBY proteins bind to GARE. Arabidopsis *YABBY* genes are expressed in the abaxial domain of the leaf and are required for specifying adaxial/abaxial polarity of the leaf, in addition to contributing to repression of *KNOX1* expression in the leaf primordium. Previous results and data from this study have shown that the rice *YABBY* genes are not expressed in a polar manner and that deregulation of expression by mutation or by transgenics did not affect the leaf adaxial/abaxial polarity (Jang et al., 2004; Yamaguchi et al., 2004; Dai et al., 2007), indicating that rice *YABBY* genes have distinct developmental function. The *CRC*-related rice *DL* gene is required for midrib formation in the leaf in addition to its requirement for flower development (Yamaguchi et al., 2004). The overexpression of *YAB1* did not induce deformation of leaves, whereas cosuppression of *YAB1* induced rolled leaf phenotype, but the midrib seemed to be normal (Fig. 6). The leaf phenotype observed in Y8 plants is unlikely to be related to *DL* and other *YABBY* genes, whose expression was not affected in Y8 plants. Therefore, we suggest that rice *YAB1* has distinct functions from the other *YABBY* members.

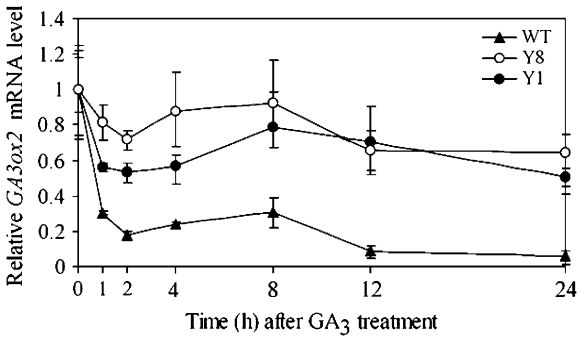


**Figure 10.** Deregulation of *YAB1* impaired GA-mediated repression of *GA3ox2*. Relative mRNA levels of *GA3ox2* in wild-type (WT, triangles), YAB1 overexpression (Y1, black circles), and YAB1 cosuppression (Y8, white circles) plants treated by $GA_3$ and harvested at different time points. The mRNA levels were measured by quantitative RT-PCR. The values at time point zero were assessed arbitrarily as 1. Data normalized with *ACTIN* transcripts are means from three biological repeats ±SD.

It has to be pointed out that the *YAB1* cDNA previous studied by Jang et al. (2004) was isolated from a *japonica* variety and the one of our study was isolated from an *indica* variety. The transgenic studies of the *japonica YAB1* do not reveal similar phenotypes shown in this study. There are seven changes of amino acid sequences between the two sequences, of which four are located in the zinc finger domain (Supplemental Fig. S1). It is not known at this stage whether the phenotypic differences were due to the sequence changes.

## MATERIALS AND METHODS

### Materials

Rice (*Oryza sativa* spp. *japonica*) 'Nipponbare' was used in this study. The full-length cDNAs of *YAB1* (accession no. AF098752) were isolated from a normalized cDNA library of the elite *indica* rice 'Minghui 63' (Zhang et al., 2005).

### Transgene Constructs and Rice Transformation

The binary vector for transformation was constructed based on pCAMBIA1301 (CAMBIA) and pRTL2 (Mason et al., 1992). A *Hin*dIII fragment with a double CaMV 35S enhancer/promoter and the NOS A terminator from pRTL2 was inserted into pCAMBIA1301. The new vector was named as pDS1301. For overexpression of *YAB1*, the full-length cDNA of the gene was inserted into pDS1301digested with *Bam*HI and *Kpn*I. To create the YAB1-GR fusion construct, the full-length coding sequence of *YAB1* was amplified by PCR. The stop codon of *YAB1* was removed and replaced with a *Sal*I site. A *Kpn*I site was added to the end of the forward primer. The amplified fragment was inserted into the plasmid pSport1 (CLONTECH), which served as a medium vector by *Sal*I and *Kpn*I. The primers used in PCR were YAB1 (GR)-F (5′-GGTACCCAATCGATCTCTTCGGTGAG) and YAB1 (GR)-R (5′-GTCGACAAGTTGCTGCCGCCGCCGCC). In the same way, the DNA fragment encoding the steroid-binding domain of the mouse GR was amplified using the plasmid pBI-ΔGR as template. The PCR primers were GR-F (5′-GTCGACAGATCCTGAAGCTCGAAAAAC) with a *Sal*I adaptor and GR-R (5′-GGATCCACCGGCAACAGGATTCAATG) with a *Bam*HI adaptor. The amplified GR fragment was inserted downstream to and in frame with *YAB1* in pSport1. The fused *YAB1-GR* sequence was then cut from pSport1 and inserted into p1301DS digested with *Kpn*I and *Bam*HI. The chimeric protein sequence was (YAB1, amino acids 1–169)-Leu-Ser-Thr-Asp-Pro-(GR, amino acids 507–794). *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation of rice plants was conducted according to Hiei et al. (1994).

### RNA Isolation, RNA Gel Blot, and RT-PCR

For expression analysis of *YAB1*, total RNA was extracted from different vegetative organs and panicles at different developmental stages using an RNA extraction kit (TRIzol Reagent, Invitrogen). For assaying expression of transgenes, total RNA was extracted from the flag leaves of the wild-type and transgenic plants. To quantify transcripts of *GAox* genes, total RNA was extracted from 1-month-old rice seedlings (four-leaf stage). For quantitative real-time PCR analysis, 4 $\mu$g total RNA was treated first with 2 U DNase I (Invitrogen) and then reverse transcribed in a total volume of 20 $\mu$L with 0.5 $\mu$g oligo(dT)$_{15}$, 0.75 mM dNTPs, 10 mM dithiothreitol (DTT), and 200 U SuperScript II RNase H– reverse transcriptase (Invitrogen). RT-PCR was performed using gene-specific primers (see below) in a total volume of 25 $\mu$L with 1.5 $\mu$L of the RT reactions, 0.25 $\mu$M gene-specific primers, and 12.5 $\mu$L SYBR Green Master mix (Applied Biosystems) on a 7500 real-time PCR machine (Applied Biosystems) according to the manufacturer's instructions. The rice *actin1* gene was used as the internal control. All primers were annealed at 58°C and run 45 cycles. The relative expression level of each gene in transgenic plants was compared to that in wild-type ones, after normalization using the *actin1* cDNA level and averaging over three replicates. The primers used in RT-PCR for *YAB1* were: YAB1-F (5′-CTTGCTCCTTTTCACCAAGC-3′) and YAB1-R (5′-ATGAGCCCAGTTCTTTGCAG-3′). The primers used in GA synthetic gene expression analysis were: CPS1-F (5′-GCGTGCATTTTCGAACCAA-3′), CPS1-R (5′-TTGGCCAGCACTGACACTCT-3′); GA20ox1-F (5′-GCCACTACAGGGCCGACAT-3′), GA20ox1-R (5′-TGGTTGCAGGTGACGATGAT-3′); GA20ox2-F (5-′CCAATTTTGGACCCTACCGC-3′), GA20ox2-R (5′-GAGAGAAGCCCAACCCAACC-3′); GA3ox2-F (5′-TCCTCCTTCTTCTCCAAGCTCAT-3′), GA3ox2-R (5′-GAAACTCCTCCATCACGTCACA-3′); GA2ox1-F (5′-TGACGATGATGACAGCGACAA-3′), GA2ox1-R (5′-CCATAGGCATCGTCTGCAATT-3′); and GA2ox3-F (5′-TGGTGGCCAACAGCCTAAAG-3′), GA2ox3-R (5′-TGGTGCAATCCTCTGTGCTAAC-3′). The *actin1* primers were: Actin1-F (5′-TGCTATGTACGTCGCCATCCAG-3′) and Actin1-R (5′-AATGAGTAACCACGCTCCGTCA-3′).

For semiquantitative RT-PCR analysis, 2 $\mu$g of total RNA was reverse-transcribed in a total volume of 20 $\mu$L with 0.5 $\mu$g oligo(dT)$_{15}$, 0.75 mM dNTPs, 10 mM DTT, and 100 U SuperScript II RNase H− reverse transcriptase (Invitrogen). PCR was performed in a total volume of 20 $\mu$L with 1 $\mu$L of the RT reactions, 0.2 $\mu$M gene-specific primers, and 1 U rTaq (TaKaRa). A total of 28 to 30 cycles was performed. The primers used in RT-PCR for *YAB1* were: YAB1-F (5′-CTTGCTCCTTTTCACCAAGC-3′) and YAB1-R (5′-ATGAGCCCAGTTCTTTGCAG-3′).

For northern-blotting analysis, 20 $\mu$g of total RNA was separated on 1.2% (w/v) denaturing agarose gel before being transferred to nylon membranes. Gene-specific probes were labeled with $^{32}$P-dCTP using a Random Primer kit (Invitrogen) and hybridized to the RNA blots. The probes were amplified by PCR using the following primers: GA3ox2-F (5′-CGACCTCTTCCACATCCTCACC-3′) and GA3ox2-R (5′-CGTCGGTGGAGACCATCTTGAG-3′); GA2ox3-F (5′-TTCTTCGTCAACGTCGGCGACTCGTTGC-3′) and GA2ox3-R (5′-TCTCAAACTGGGCCAGCCTGTTGTCTCC-3′); and GA20ox2-F (5′-GAGACCCTCTCCTTCGGCTTC-3′) and GA20ox2-R (5′-CATGGCGGGTAGTAGTTGCAC-3′).

### GA Quantification

The growing shoots with shoot apex of 4-week-old seedlings of *YAB1* overexpression transgenic and wild-type rice lines were harvested for quantitative GA analysis. After harvesting, the samples were immediately plunged into liquid $N_2$, freeze-dried, and ground into fine powder using a mill ball. The extraction and purification were carried out as previously described (Coles et al., 1999) with some modifications. Briefly, samples were extracted by stirring overnight at 4°C in 80% (v/v) methanol-water (100 mL) containing [17-$^2$H$_2$]GAs (5–45 ng, depending on anticipated endogenous GAs contents) as internal standards, and 833 Bq each of the following tritiated GA standards: [1,2-3H2]GA$_1$, [1,2-3H2]GA$_4$, 16,17-dihydro[15,16,17-3H4]GA$_{19}$, and [1,2,3-3H3]GA$_{20}$. After filtration, the residue was reextracted with methanol (100 mL) for 2 h and refiltrated. The combined methanol extracts were evaporated almost to dryness under reduced pressure at 4°C. The residue was resuspended in water, adjusted to pH 8.0 (1 M KOH), and purified by QAE Sephadex A-25 (Pharmacia) anion-exchange column and C$_{18}$ Solid Phase Extraction cartridge (500 mg; Thermo). The dried GAs were methylated twice with excess diazomethane, then dissolved in ethyl acetate. After partitioning against water, the organic phase was put through NH$_2$ Solid Phase Extraction cartridge (Altech Associates) and evaporated to dryness in vacuo. GAs were resolved by reverse-phase HPLC and fractions combined based on the location of tritiated GA$_1$, GA$_4$, GA$_{19}$, and GA$_{20}$. Samples were analyzed as methyl ester trimethylsilyl ethers using a Finnigan GCQ gas chromatography-mass spectrometry system in selected ion monitoring mode with instrument parameters described previously (Coles et al., 1999). Characteristic ions (mass-to-charge ratio value) monitored by mass spectrometer under selected ion monitoring mode were as follows: GA$_8$/[17-2H2]GA$_8$, 594/596, 448/450; GA1/[17-2H2]GA$_1$, 506/508, 448/450; GA$_{29}$/[17-2H2]GA$_{29}$, 506/508, 375/377; GA$_{20}$/[17-2H2]GA$_{20}$, 418/420, 375/377; GA$_{44}$/[17-2H2]GA$_{44}$, 432/434, 373/375; GA$_{19}$/[17-2H2]GA$_{19}$, 434/436, 374/376; and GA$_{53}$/[17-2H2]GA$_{53}$, 448/450, 389/391. The concentrations of GAs in the original extracts were determined from previously established calibration curves of the peak area ratios for unlabeled and deuterated GAs plotted against varying molar ratios of the two compounds. The same stock solutions of labeled GAs were used for production of the calibration curves.

### In Situ Hybridization

Plant materials were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) overnight at 4°C, dehydrated through a concentration grade of

ethanol, cleared through a xylene series, then infiltrated through a series of paraffin, and finally embedded in 100% paraffin melted at 52°C to 54°C. Then 10- to 15-$\mu$m-thick microtome sections were mounted on RNase free glass slides.

The *YAB1* probe was amplified using the gene-specific primers as mentioned above. The PCR fragments were inserted into pBlueScript SK–. The digoxigenin-labeled sense and anti-sense RNA probes were produced by T7 and T3 transcriptase, respectively. The reagents used in the experiments were purchased from Roche.

## GFP Imaging

For the construction of the fusion between the *YAB1* promoter and GFP, the GUS sequence in pCAMBIA1381Xb (CAMBIA) was replaced by the GFP gene. The *YAB1* promoter was amplified from 'Nipponbare' genomic DNA and inserted into the constructed pCAMBIA1381Xb-GFP at the *Eco*RI and *Bam*HI sites. The primers used in PCR amplification were YAB1prom-F (5′-GGGAATTCTGATCCTGTAGCCCCATCTC-3′) and YAB1prom-R (5′-GGGAGCTCCATGCTCCGATGTAAACTGG-3′) with an *Eco*RI adaptor at one end and a *Bam*HI adaptor at the other. Different tissues were harvested at different developmental stages of the transgenic plants, then fixed and sectioned for observation.

## Southern-Blotting Analysis

Genomic DNA was extracted from young seedlings. A total of 4 $\mu$g of DNA was digested with *Bam*HI overnight at 37°C, separated on 1% (w/v) agarose gel, then transferred to a nylon membrane and hybridized according to standard protocols.

## Chemical Treatments

DEX (Sigma) was dissolved in ethanol to 30 mM and stored at −20°C before use. Before experiment, DEX was diluted with growth medium to a final concentration of 10 $\mu$M. Transgenic siblings from the same lines were divided into three parts: one was treated with DEX, one was treated first with CHX for 1 h then with DEX, and the third received no treatment but an equal volume of ethanol was added to the medium. After 12 h of treatment, the aerial parts of the plants were harvested for RNA extraction, RT-PCR, and quantitative PCR analysis.

## Yeast One-Hybrid Analysis

Yeast one-hybrid analysis was performed by using a CLONTECH system. The promoter fragment of *GA3ox2* was amplified by PCR, which spanned from the initiation ATG codon to −862 and inserted into pHIS2. The primers used were: GA3ox2P-F (5′-GAATTCAGTGGCTGCATGTGAGAATG-3′), GA3ox2P-R (5′-GAGCTCCTGGCAGAAAGCCAGTAACA-3′). The full-length coding sequence of *YAB1* was amplified from the full-length cDNA clone using the primer pair YAB1CDS-F (5′-GAATTCGAAATGTCGGTCCAGTTTACATC-3′) and YAB1CDS-R (5′- GGATCCTATATCGTCGTCGTCCCTCC-3′). The PCR product was inserted into pGADT7-Rec2 to fuse with the GAL4 activation domain. The constructs were sequenced and used to transform the yeast strain Y187 for one-hybrid analysis.

## Gel Shift Assays

To produce the YAB1 protein, the full length of the *indica YAB1* cDNA was inserted into and translationally fused with the pET-32a expression vector (Novagen) and expressed in Rosetta-gami (DE3) *Escherichia coli* cells (Novagen). The target protein was purified with B-PER 6×His Spin Purification kit (Pierce). The 0.8-kb *GA3ox2* promoter fragment was cut into two segments by restriction digestion and used as probes in gel shift assays. Oligonucleotides corresponding to the five overlapping regions of the upstream segment of the promoter or to the GARE elements were synthesized and annealed into double strands. The sequence of wild-type GARE oligonucleotide was 5′-TA**TAACAGA**TTTA**TAACAGA**TT and that of the mutant GARE sites 5′-TATATGAGATTTATATGAGATT. The probes were end-labeled by filling with $^{32}$P-dCTP using the Klenow fragment. DNA-binding reactions were performed at room temperature for 20 min in 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol, and 50 mg $L^{-1}$ poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech) and separated on 6% polyacrylamide gels in Tris-glycin (0.3% Tris, 1.88% glycin) buffer.

## Growth Regulator Treatments

Transgenic and wild-type seedlings were grown on hydroponic culture media to the four-leaf stage before treatment. Paclobutrazol (at the final concentration of 10 $\mu$M) was added to the hydroponic culture 1 d before adding $GA_3$ at the concentrations of 0.01, 0.05, 0.1, 0.5, 1, 10, and 20 $\mu$M. The length of the second leaf sheath was measured 10 d after the treatment. The hydroponic culture media and treatments were repeated every 2 d.

For *YAB1* expression analysis, wild-type seedlings at the four-leaf stage were sprayed with 50 $\mu$M $GA_3$ or 10 $\mu$M paclobutrazol and harvested at different time points for RNA extraction and quantitative RT-PCR analysis. For expression analysis of *GAox* genes, transgenic and wild-type plants were sprayed with 50 $\mu$M $GA_3$ and harvested at different time points for RNA extraction and quantitative RT-PCR analysis. The expression levels of these genes in these samples were determined relative to time zero, normalized against *actin1*, and averaged over three biological replicates.

## Light Microscopy

Stems and leaves were harvested from the mature plants of wild type and transgenic ones. Stems were treated with 15% of hydrofluoric acid before dehydration. The procedures of dehydration, clearing, infiltration, and embedding were carried out as mentioned above. The microtome sections (15 $\mu$m) were mounted on glass slides for imaging.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF098752 and BAF12697.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Rice *YAB1* sequences from *indica* and *japonica* subspecies.

**Supplemental Figure S2.** Southern-blot analysis of *YAB1* transgene copy numbers.

**Supplemental Figure S3.** Cosuppression of *YAB1* expression induced *GA3ox2*.

**Supplemental Figure S4.** GA transiently induced expression of *YAB1*.

## ACKNOWLEDGMENTS

We thank Li Xianhua for technical assistance, Xu Chaiguo for rice field management, Dr. M. Matsuoka for providing *slr* and *gid1* mutants, and Dr. Z. He for sending *eui* mutants.

Received January 25, 2007; accepted March 8, 2007; published March 16, 2007.

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