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Phytochrome Regulation and Differential Expression of Gibberellin 3β-Hydroxylase Genes in Germinating Arabidopsis Seeds

Shinjiro Yamaguchi,[a,b,1] Maria W. Smith,[a] Robert G. S. Brown,[a,2] Yuji Kamiya,[a] and Tai-ping Sun[b]

[a]Frontier Research Program, Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan

[b]Developmental, Cell, and Molecular Biology Group, Department of Botany, Box 91000, Duke University, Durham, North Carolina 27708-1000

Despite extensive studies on the roles of phytochrome in photostimulated seed germination, the mechanisms downstream of the photoreceptor that promote germination are largely unknown. Previous studies have indicated that light-induced germination of Arabidopsis seeds is mediated by the hormone gibberellin (GA). Using RNA gel blot analyses, we studied the regulation of two Arabidopsis genes, *GA4* and *GA4H* (for *GA4* homolog), both of which encode GA 3β-hydroxylases that catalyze the final biosynthetic step to produce bioactive GAs. The newly isolated *GA4H* gene was expressed predominantly during seed germination. We show that expression of both *GA4* and *GA4H* genes in imbibed seeds was induced within 1 hr after a brief red (R) light treatment. In the phytochrome B–deficient *phyB-1* mutant, *GA4H* expression was not induced by R light, but *GA4* expression still was, indicating that R light–induced *GA4* and *GA4H* expression is mediated by different phytochromes. In contrast to the *GA4* gene, the *GA4H* gene was not regulated by the feedback inhibition mechanism in germinating seeds. Our data demonstrate that expression of GA 3β-hydroxylase genes is elevated by R light, which may result in an increase in biosynthesis of active GAs to promote seed germination. Furthermore, our results suggest that each GA 3β-hydroxylase gene plays a unique physiological role during light-induced seed germination.

INTRODUCTION

Gibberellins (GAs) are a group of diterpenoids, some of which are growth regulators in higher plants. Studies using GA-deficient mutants have shown that active GAs control many aspects of plant development, including seed germination, stem elongation, flowering, and seed development (for a recent review, see Ross et al., 1997). In the major GA biosynthetic pathway in higher plants, GA 3β-hydroxylase catalyzes the conversion of both GA_9 to GA_4 and GA_{20} to GA_1 (Hedden and Kamiya, 1997). Several GA-deficient dwarf mutants with reduced 3β-hydroxylase activities have been isolated. These include *dwarf1* in maize (Fujioka et al., 1988), *le* in pea (Ingram et al., 1984), *dy* in rice (Kobayashi et al., 1989), and *ga4* in Arabidopsis (Talon et al., 1990a). Biochemical studies using these mutants have shown that only GA_4 and GA_1, but not their precursors GA_9 and GA_{20}, are biologically active in stimulating stem elongation. Therefore, GA 3β-hydroxylase catalyzes the final step of the biosynthetic pathway to produce active GAs in these plant species.

Based on their function in GA biosynthesis, expression of GA 3β-hydroxylase genes is likely to play a key regulatory role in controlling the appropriate levels of active GAs during plant growth. Recently, the Arabidopsis *GA4* and pea *LE* genes have been cloned and shown to encode GA 3β-hydroxylase (Chiang et al., 1995; Lester et al., 1997; Martin et al., 1997; Williams et al., 1998). The transcript levels of both *GA4* and *LE* are controlled by a feedback inhibition mechanism, that is, they are upregulated in the GA-deficient mutant background and downregulated by applied GAs (Chiang et al., 1995; Martin et al., 1997). Moreover, the mRNA levels of these genes vary in different tissues (Chiang et al., 1995; Martin et al., 1997).

In addition to the endogenous developmental program, environmental stimuli also can affect GA biosynthesis and GA-mediated growth. In Arabidopsis, both de novo biosynthesis of GAs and appropriate light conditions are essential

[1]To whom correspondence should be addressed at Developmental, Cell, and Molecular Biology Group, Department of Botany, Box 91000, Duke University, Durham, NC 27708-1000. E-mail shinjiro @acpub.duke.edu; fax 919-613-8177.

[2]Current address: Integrated Approach to Crop Research–Long Ashton Research Station, Department of Agricultural Science, University of Bristol, BS41 9AF, UK.

for seed germination. The requirement for de novo biosynthesis of GAs is evident by the inhibitory effect of GA biosynthesis inhibitors on the germination of wild-type seeds, even under photoinductive conditions (Hilhorst and Karssen, 1988; Nambara et al., 1991). Because exogenous GAs can mimic the effects of light to promote seed germination (Hilhorst and Karssen, 1988), it was speculated that a seed's response to light is mediated by GA. The effect of light on germinating Arabidopsis seeds is mediated via the photoreceptor phytochrome, which undergoes photoreversible conformational changes between the red (R) and far-red (FR) light–absorbing forms (Pr and Pfr, respectively) (reviewed in Furuya, 1993; von Arnim and Deng, 1996; Chory, 1997; Shinomura, 1997). Pfr, resulting from irradiation with R light, is considered to be the active form, inducing seed germination. In Arabidopsis, phytochrome is encoded by a multigene family consisting of at least five members, *PHYA* to *PHYE* (Sharrock and Quail, 1989; Clack et al., 1994). Studies using the *phyB* mutant, which is deficient in the PHYB apoprotein (Somers et al., 1991; Reed et al., 1993), indicated that R/FR-reversible induction of seed germination is primarily regulated by PHYB (Shinomura et al., 1994, 1996).

Derkx et al. (1994) showed that the level of GA_4 (but not its precursor GA_9) in wild-type Arabidopsis seeds irradiated by continuous white light was higher than in dark-imbibed seeds. In addition, R light irradiation was shown to lower the concentration of applied GAs required to induce germination of both the GA-deficient *ga1* mutant (Hilhorst and Karssen, 1988) and wild-type seeds treated with the GA biosynthesis inhibitor uniconazole (Yang et al., 1995). These results suggest that light can alter both GA biosynthesis and tissue sensitivity to GA during seed germination in Arabidopsis. Regulation of GA biosynthesis by light during seed germination has been studied in more detail in lettuce (cv Grand Rapids) seeds. Toyomasu et al. (1993) demonstrated that the level of GA_1 (but not its precursors GA_{19} and GA_{20}) in lettuce seeds is photoreversibly regulated by a brief irradiation with R and/or FR light and suggested that 3β-hydroxylation of GA_{20} is likely to be regulated by the action of phytochrome.

In this study, we isolated and characterized a second gene (*GA4H* [for *GA4* homolog]) from Arabidopsis that encodes a 3β-hydroxylase and studied the regulation of GA 3β-hydroxylation in detail. RNA gel blot analyses showed that the *GA4H* gene is predominantly expressed in germinating seeds and in very young seedlings, whereas the *GA4* mRNA was detected in all tissues examined. We then focused on the regulation of *GA4* and *GA4H* expression by light and the feedback inhibition of expression by GA during seed germination. We showed that transcript levels of these genes are photoreversibly controlled by R and FR light through the action of different phytochromes. Moreover, their differential expression patterns after photoinduction suggest that these genes have separate roles in controlling levels of active GAs during seed germination and early seedling growth. We further showed that the *GA4* gene, but not the *GA4H* gene, is under feedback regulation during germination.

RESULTS

Isolation of a Second Gene Encoding GA 3β-Hydroxylase from Arabidopsis

The *ga4-2* allele is likely to be a null allele because it contains a T-DNA insertion in its single intron (Chiang et al., 1995). However, in contrast to the severe mutant alleles of other GA biosynthetic genes (e.g., *ga1-3* and *ga2-1*) that are nongerminating, male-sterile, extreme dwarfs (Koornneef and van der Veen, 1980), the *ga4-2* mutant is a semidwarf and does not require exogenous GAs to germinate, bolt, and set seeds (Chiang et al., 1995). This leaky phenotype of the *ga4-2* mutant suggests that additional genes encoding 3β-hydroxylase are present in the Arabidopsis genome.

To understand better the regulation of GA 3β-hydroxylation, we set out to isolate homologs of *GA4.* An Arabidopsis genomic library was screened using the *GA4* cDNA as a hybridization probe under low-stringency conditions, and two new clones were isolated. DNA sequence analysis showed that both clones contained an identical sequence that is different from the *GA4* sequence. We named this gene *GA4H.* The *GA4H* cDNA was isolated by reverse transcriptase–polymerase chain reaction (RT-PCR), using poly(A)$^+$ RNA prepared from 14-day-old wild-type rosette plants. The *GA4H* cDNA contained an open reading frame consisting of 347 amino acids. As shown in Figure 1, the deduced amino acid sequence of GA4H shares a high degree of similarity to other GA 3β-hydroxylases, including Arabidopsis GA4 (Chiang et al., 1995, 1997), pea LE (Lester et al., 1997; Martin et al., 1997), and the GA 2β,3β-hydroxylase from pumpkin (Lange et al., 1997). GA4H is the most closely related to GA4 (75.5% identity and 79.5% similarity).

In Vitro Functional Analysis of the GA4H Protein

To determine the enzymatic activity of GA4H, the coding region of the *GA4H* cDNA was expressed as a fusion protein with a maltose binding protein (MBP) in *Escherichia coli.* In Arabidopsis, both the early 13-hydroxylation and the non-13-hydroxylation pathways are present in flowering shoots (Talon et al., 1990a, 1990b) and possibly in germinating seeds (Derkx et al., 1994). Therefore, we used both GA_9 and GA_{20} as substrates to determine whether MBP–GA4H had 3β-hydroxylase activity (Figure 2A).

An *E. coli* lysate containing the MBP–GA4H fusion protein exhibited 3β-hydroxylase activity, converting 17-^{14}C-GA_9 to 17-^{14}C-GA_4 (Figure 2B) and converting 17-^{14}C-GA_{20} to 17-^{14}C-GA_1 (data not shown). The identity of the products was confirmed by full-scan gas chromatography–mass spectrometry

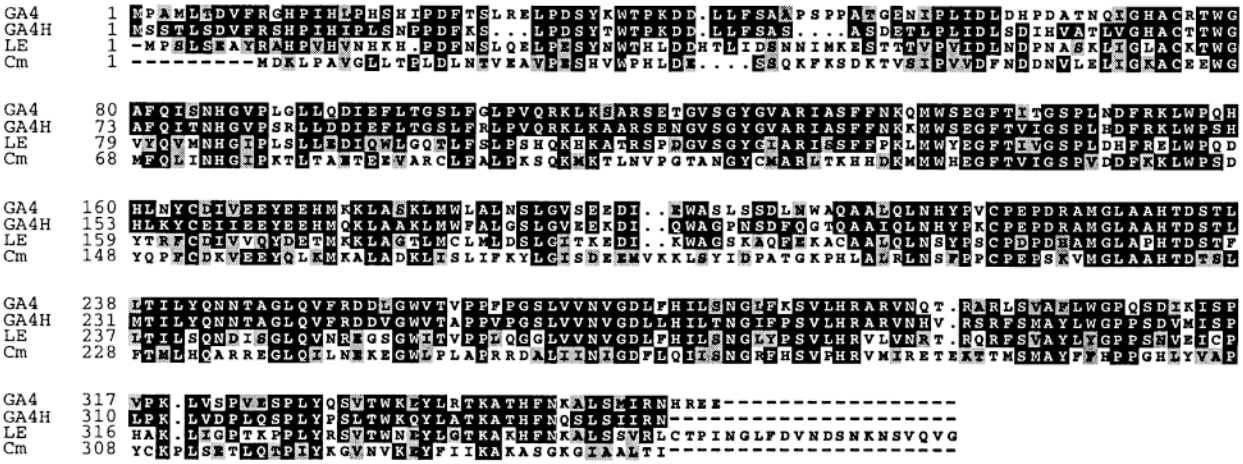

Figure 1. Sequence Alignment of GA 3β-Hydroxylases.

An alignment is shown for the deduced amino acid sequences of Arabidopsis GA4 (Chiang et al., 1995, 1997) and GA4H (GenBank accession number AF070937), pea LE (Lester et al., 1997; Martin et al., 1997), and GA 2β,3β-hydroxylase from pumpkin (Cm; Lange et al., 1997). Identical amino acid residues conserved between GA4H and at least one other protein are indicated in reverse type, and similar residues are in gray boxes. Gaps introduced to optimize the alignment are indicated as dots, and sequence truncations are indicated by dashes. The boxes were drawn using the BOXSHADE web site (http://ulrec3.unil.ch/software/BOX_form.html).

(data not shown). The control *E. coli* lysate containing the MBP did not show this enzymatic activity. No 2β-hydroxylase activity was detected in the lysate containing MBP–GA4H. These results indicate that the *GA4H* gene encodes a second GA 3β-hydroxylase in Arabidopsis.

The preferred substrate for the *E. coli*–expressed GA4 protein is GA_9, for which the K_m is 1.0 μM, whereas that for GA_{20} is 15 μM (Williams et al., 1998). To examine whether GA4H has a different substrate preference from GA4, we determined the K_m values of MBP–GA4H for GA_9 and GA_{20}. We found that the K_m values for GA_9 and GA_{20} were 1.0 and 13 μM, respectively (data not shown), which were very similar to those of GA4. These results indicate that GA4H also prefers GA_9 to GA_{20} as a substrate.

Developmental Regulation of the *GA4* and *GA4H* Genes

To investigate the roles of the *GA4* and *GA4H* genes during plant development, their expression patterns were compared by RNA gel blot analysis using gene-specific RNA probes (Figure 3). Because *GA4* and *GA4H* share a high degree of sequence similarity (Figure 1), we examined the degree of cross-hybridization of our antisense RNA probes by using *GA4* and *GA4H* sense RNAs synthesized in vitro as standards (see Methods). Under our hybridization conditions, the signals from cross-hybridization were below detection in RNA samples isolated from wild-type plants.

The *GA4* gene was expressed in every organ tested (Figure 3). The levels of *GA4* mRNAs were highest in 12-hr-old germinating seeds and siliques that contained mature green seeds and lowest in leaf tissues from both 14- and 35-day-old plants. In contrast, the *GA4H* mRNA level was high in germinating seeds (12 hr) and 48-hr-old young seedlings but was barely detectable in other tissues.

Effect of an R Light Pulse on *GA4* and *GA4H* Expression in Imbibed Wild-Type Seeds

Our results show that both *GA4* and *GA4H* genes are expressed at relatively high levels in germinating seeds under continuous white light (Figure 3). To examine whether expression of the *GA4* and/or *GA4H* genes is induced by light, we analyzed the levels of these mRNAs in imbibed seeds after different light treatments that either induce or inhibit seed germination. Wild-type Arabidopsis seeds germinate at a low frequency in the dark due to the active form of PHYB stored in dormant seeds (Reed et al., 1994; Shinomura et al., 1994). Therefore, in our experiments, the seeds were irradiated with an FR light pulse 1 hr after imbibition in the dark to inhibit PHYB-dependent dark germination (Figure 4A). Under this condition (dark control; D), the germination frequency of wild-type seeds was <3%. A subsequent R light pulse, which was given 24 hr after the FR light pulse, greatly induced seed germination (~90% at 48 hr; Figure 4C). Radicle emergence was the criterion used for scoring germination (Bewley, 1997).

As shown in Figure 4B, the levels of both *GA4* and *GA4H* mRNAs were dramatically higher in the seeds treated with

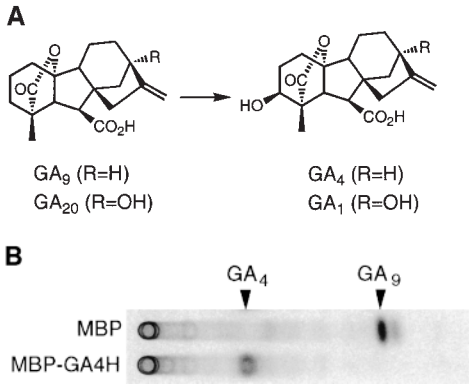

Figure 2. In Vitro Functional Analysis of the Recombinant GA4H Protein.

(A) Diagram showing conversion of GA_9 and GA_{20} to GA_4 and GA_1 by 3β-hydroxylation.

(B) Functional analysis of the GA4H protein. Lysates of *E. coli* containing the MBP (as a control) or MBP–GA4H were incubated with ^{14}C-GA_9. The reaction mixture was separated on a silica gel by thin-layer chromatography. The positions of authentic GA_4 and GA_9 are indicated by arrowheads.

an R light pulse than in the dark control seeds. Induction of *GA4* and *GA4H* mRNA accumulation was observed as early as 1 hr after the R light pulse, before germination occurred (Figure 4C). Interestingly, the patterns of *GA4* and *GA4H* expression were different from each other. The *GA4* mRNA level increased sharply after the R light pulse treatment, peaked at 4 hr, and then decreased rapidly as germination occurred. The amount of *GA4* mRNA was elevated again at 36 hr, when most of the seeds had germinated and etiolated seedling growth had begun (Figure 4C). In contrast, the *GA4H* mRNA increased more gradually after the R light pulse and reached its maximum level at 12 hr.

Expression of Both *GA4* and *GA4H* Genes Is Induced by an R Light Pulse in the Nongerminating *ga1-3* Seeds

Our data showed that an R light pulse induced both seed germination and accumulation of the *GA4* and *GA4H* mRNAs in wild-type seeds (Figure 4). Moreover, the elevated levels of these mRNAs were observed before germination (Figure 4C). These results support the hypothesis that R light increases the de novo biosynthesis of active GAs, which stimulate seed germination. To determine whether the increase in the *GA4* and *GA4H* mRNA levels is a direct effect of light, we tested whether R light treatment could still induce expression of these genes in the *ga1-3* mutant seeds (Figure 5).

Germination of the *ga1-3* mutant seeds is arrested due to deficiency in the copalyl diphosphate synthase that catalyzes an early step of the GA biosynthetic pathway (Sun and Kamiya, 1994). We found that cross-hybridization of the *GA4H* probe with the *GA4* mRNA in the *ga1-3* RNA samples was much higher than in the wild type (Figure 5B), because the *GA4* mRNA level is greater in the *ga1-3* mutant seeds than in wild-type seeds (see below). To determine the true amount of *GA4H* mRNA, we calculated the amount of cross-hybridization in each lane (see Methods), and this value was subtracted from the total hybridization signals.

As shown in Figure 5A, an R light pulse rapidly induced both *GA4* and *GA4H* expression in the *ga1-3* seeds as it did in wild-type seeds, and their expression patterns were similar to those in wild-type seeds from 1 to 24 hr for *GA4* and from 1 to 12 hr for *GA4H* (Figures 4C and 5C). However, the level of *GA4* mRNA did not increase at 36 hr as it did in wild-type seeds, and *GA4H* transcript accumulation declined dramatically by 24 hr.

Induction of *GA4H* Expression by an R Light Pulse Is Mediated by PHYB

Previous studies have suggested that PHYB is the major phytochrome in dormant seeds for the control of germina-

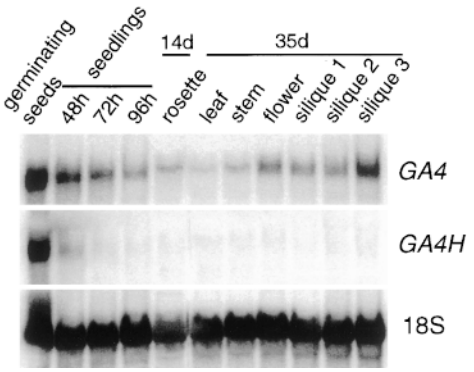

Figure 3. Developmental Regulation of *GA4* and *GA4H* Expression.

Autoradiography of RNA gel blots containing 25 μg of total RNAs isolated from different tissues, as labeled. Germinating seeds under continuous white light were harvested 12 hr (h) after imbibition. Young seedlings (harvested at 48, 72, and 96 hr after imbibition) and 14- and 35-day (d)-old plants were grown under 16-hr-light and 8-hr-dark cycles. Aerial tissues (rosette) were harvested from 14-day-old plants. Cauline leaves (leaf), main stems (stem), flower clusters (flower), and siliques (siliques 1, 2, and 3) were harvested from 35-day-old plants. Silique 1 has embryos at globular and heart-shaped stages. Embryos at torpedo to upturn U stages are contained in silique 2. Silique 3 has mature green seeds. The membrane was hybridized with the radiolabeled *GA4* or *GA4H* antisense RNA probe and then reprobed with the the radiolabeled 18S rDNA probe as a loading control.

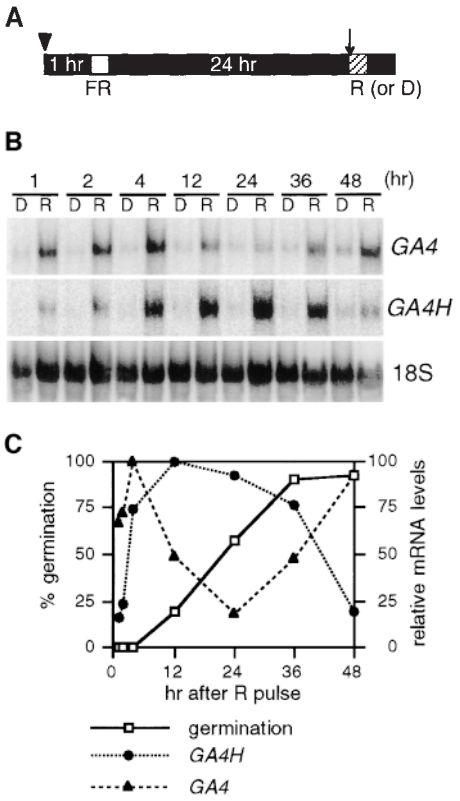

Figure 4. R Light–Induced Expression of the *GA4* and *GA4H* Genes and Germination in Wild-Type Arabidopsis Seeds.

(A) Diagram showing different light treatments. The seeds were imbibed in the dark and then irradiated with an FR light pulse 1 hr after imbibition. The seeds were then either irradiated with an R light pulse (stippled box) 24 hr after the FR light pulse (R in **[B]**) or incubated without the R light pulse in the dark (D in **[B]**). The triangle indicates the starting time of imbibition. The vertical arrow indicates the beginning of the R light pulse, which was set as 0 hr in the experiments shown in **(B)**.

(B) Autoradiography of RNA blots containing 12.5 μg of total RNAs prepared from germinating wild-type seeds under different light conditions as described in **(A)**. The time after the R light pulse is indicated above the blot. The membrane was hybridized with the *GA4* or *GA4H* antisense RNA probe and then reprobed with the radiolabeled 18S rDNA probe as a loading control.

(C) Germination frequency and the levels of *GA4* and *GA4H* mRNAs after the R light pulse. The highest mRNA levels for each gene were set as 100.

tion because *phyB* seeds germinated at a much lower frequency than did wild-type seeds in response to an R light pulse given shortly (i.e., 1 to 3 hr) after imbibition (Shinomura et al., 1994, 1996). However, germination of *phyB* seeds can be dramatically induced by an R light pulse after a longer imbibition period via the action of other phytochromes (Shinomura et al., 1996; Poppe and Schäfer, 1997). To examine the involvement of PHYB in the induction of *GA4* and *GA4H* expression, wild-type and *phyB-1* (a putative null allele; Reed et al., 1993) seeds were treated with or without an R light pulse after a shorter imbibition period (3 hr rather than 25 hr) (Figure 6A). *GA4* and *GA4H* transcript levels were analyzed after these treatments. Figure 6B shows that *GA4* expression was elevated by an R light pulse in both wild-type and *phyB-1* seeds. The *GA4H* mRNA level also increased after the R light pulse in wild-type seeds. However, no increase occurred after the R irradiation of *phyB-1* seeds. We determined the germination frequency of the wild type and the *phyB-1* mutant after the R light pulse to be 94 and 8%,

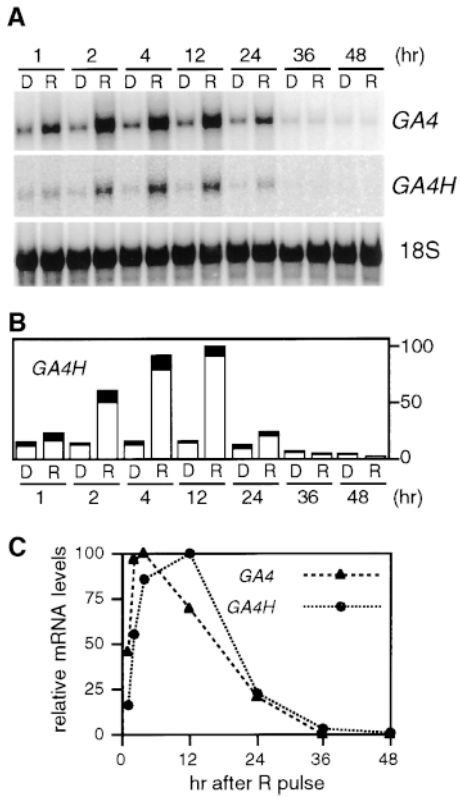

Figure 5. R Light–Induced Expression of the *GA4* and *GA4H* Genes in Imbibed *ga1-3* Seeds.

(A) Autoradiography of RNA blots containing 12.5 μg of total RNAs from imbibed *ga1-3* seeds after light treatments, as diagrammed in Figure 4A. The membranes were hybridized with the *GA4* or *GA4H* antisense RNA probe and then reprobed with the radiolabeled 18S rDNA probe as a loading control. Abbreviations are as given in Figure 4A.

(B) A diagram showing relative levels of cross-hybridization of the *GA4H* probe with the *GA4* mRNAs (black bars) and the net *GA4H* mRNA (open bars) in each lane in **(A)**. The highest level of the hybridization signal was set as 100.

(C) *GA4* and *GA4H* mRNA levels after the R light pulse. The highest mRNA levels for each gene were set as 100.

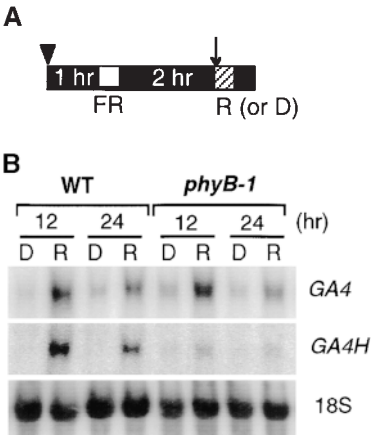

Figure 6. Effects of the *phyB-1* Mutation on *GA4* and *GA4H* Expression.

(A) Diagram showing different light treatments. The seeds were imbibed in the dark and then irradiated with an FR light pulse 1 hr after the start of imbibition. The seeds were then either irradiated with an R light pulse 2 hr after the FR light pulse (R in **[B]**) or incubated without an R light pulse in the dark (D in **[B]**). Symbols are as given in the legend to Figure 4A.

(B) Autoradiography of RNA gel blots containing 12.5 μg of total RNAs prepared from germinating wild-type (WT) or *phyB-1* seeds under the light conditions given in **(A)**. The membrane was hybridized with the *GA4* or *GA4H* antisense RNA probe and then reprobed with a radiolabeled 18S rDNA probe as a loading control.

respectively (measured 7 days after imbibition), which is consistent with the primary role of PHYB in controlling seed germination under the short imbibition condition (Shinomura et al., 1994, 1996). These results indicate that PHYB plays a major role in mediating the R light–induced transcript accumulation of *GA4H*, whereas PHYB is not essential for the induction of *GA4* expression by R light under this condition.

Expression of Both *GA4* and *GA4H* Shows R/FR Photoreversibility

Under the shorter imbibition condition (Figure 6A), the effect of R light on PHYB-mediated seed germination can be photoreversibly canceled by a subsequent irradiation with an FR light pulse (Shinomura et al., 1994). We determined the effect of an FR light pulse immediately after the R light pulse on *GA4* and *GA4H* expression (Figure 7A). The germination percentage after the second FR light pulse was nearly zero (data not shown), confirming a full reversibility of the effect of R by FR light on seed germination. Figure 7B shows that the accumulation of both *GA4* and *GA4H* transcripts after the R light pulse was canceled by a subsequent FR light pulse, which correlates with its inhibitory effect on seed germination. The levels of these mRNAs in seeds after the second FR light pulse were similar to those in dark control seeds at all time points examined (Figure 7B), which indicated a full reversibility by FR.

GA4 but Not *GA4H* Is under Feedback Regulation during Seed Germination

Previous studies have shown that the *GA4* mRNA level in rosette leaves of the *ga4-1* mutant and seedlings of the *ga1-3* mutant was higher than that of wild-type plants and that application of GA_3 downregulated the accumulation of *GA4* mRNA (Chiang et al., 1995; Silverstone et al., 1998). To determine whether expression of the *GA4* and *GA4H* genes during seed germination is also subject to the feedback inhibition mechanism, we examined their mRNA levels in a GA-deficient mutant background. As shown in Figure 8A, the *GA4* mRNA level in the dark-imbibed *ga1-3* seeds was much higher than in wild-type seeds. Indeed, the *GA4* mRNA level in the dark-imbibed *ga1-3* seeds was higher than its maximum level in the R light–treated wild-type seeds. This result suggests that expression of the *GA4* gene in germinating seeds is controlled by the feedback mechanism. In contrast, the level of *GA4H* mRNA in *ga1-3* was approximately the same as in the wild type (Figure 8A).

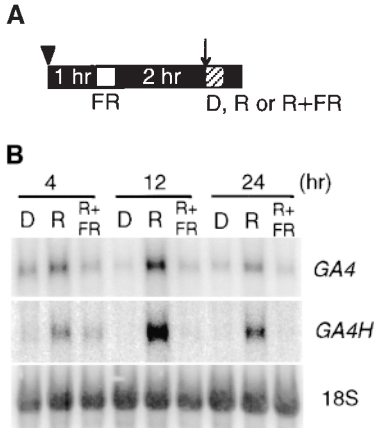

Figure 7. Photoreversibility of *GA4* and *GA4H* Expression.

(A) Diagram showing different light treatments. The seeds were imbibed in the dark and then irradiated with an FR light pulse 1 hr after imbibition. The seeds were then incubated in the dark (D), irradiated with an R light pulse (R), or treated with a second FR light pulse immediately after the R light pulse (R+FR). Symbols are as given in the legend to Figure 4A.

(B) Autoradiography of RNA blots containing 12.5 μg of total RNAs prepared from germinating wild-type seeds under the light conditions given in **(A)**. The membrane was hybridized with the *GA4* or *GA4H* antisense RNA probe and then reprobed with a radiolabeled 18S rDNA probe as a loading control.

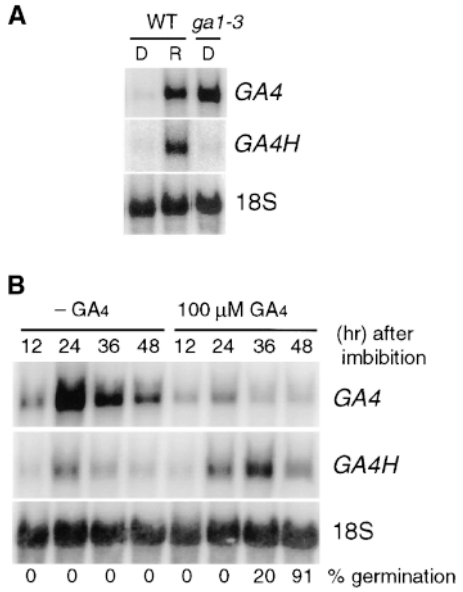

Figure 8. Effects of the *ga1-3* Mutant Background and GA_4 Application on *GA4* and *GA4H* Expression in Imbibed Seeds.

Shown is autoradiography of RNA gel blots containing 12.5 μg of total RNA from imbibed wild-type (WT) or *ga1-3* seeds.

(A) RNA was isolated from seeds at 4 hr after the light treatments, as shown in Figure 4A.

(B) The *ga1-3* seeds were imbibed in water ($-GA_4$) or in 100 μM GA_4 under continuous white light. Germination percentage is shown below the blots.

The membranes were hybridized with the *GA4* or *GA4H* antisense RNA probe and then reprobed with a radiolabeled 18S rDNA probe as a loading control.

Therefore, the *GA4H* gene is not likely to be regulated by the feedback mechanism during seed germination.

We also examined the effect of exogenous GA_4 on the expression of the *GA4* and *GA4H* genes in the imbibed *ga1-3* seeds. GA_4 is the major bioactive GA in germinating Arabidopsis seeds (Derkx et al., 1994) and has the highest germination-inducing activity among GAs examined thus far (Yang et al., 1995). In the presence of 100 μM GA_4, the *ga1-3* seeds started to germinate at ~36 hr (Figure 8B), and 91% of the seeds had germinated at 48 hr after imbibition under continuous white light. The levels of *GA4* mRNAs at all time points were considerably lower in the presence of GA_4 than in the untreated controls. In contrast, there was no striking difference between the levels of *GA4H* transcripts in GA_4-treated and nontreated seeds before germination (12 and 24 hr after imbibition). Interestingly, *GA4H* mRNA accumulated to higher levels in GA_4-treated seeds than in control seeds at 36 and 48 hr, when seeds had started to germinate (Figure 8B). These data further support the notion that the *GA4H* gene is not under the feedback control during seed germination.

DISCUSSION

GA4 and *GA4H* Genes Are Differentially Expressed, Although Their Products Have Similar Enzymatic Properties

We have isolated the *GA4H* gene, which encodes a second 3β-hydroxylase in Arabidopsis. Our in vitro enzyme assays have shown that the recombinant GA4H protein has enzymatic properties similar to GA4, including the substrate preference for GA_9 rather than GA_{20} (Williams et al., 1998). This observation may reflect the predominance of non-13-hydroxylated GAs in Arabidopsis (Talon et al., 1990a, 1990b; Derkx et al., 1994) and is consistent with more efficient conversion of the non-13-hydroxylated substrate than the 13-hydroxylated precursor by the Arabidopsis GA 20-oxidases, which catalyze the formation of GA_9 and GA_{20} (Phillips et al., 1995). In addition to the 3β-hydroxylation of GA_9 and GA_{20}, recombinant GA4 catalyzes 3β-hydroxylation of C_{20} GAs (GA_{15}, GA_{24}, and GA_{44}) and 2,3-epoxidation of 2,3-didehydrogibberellins with lower efficiencies (Williams et al., 1998). To characterize further its substrate specificity, these GAs also should be incubated with GA4H.

Although GA4 and GA4H have similar enzyme activity, their patterns of expression are different (Figure 3). High levels of *GA4H* mRNA in germinating seeds and in young seedlings suggest that it functions mainly in early developmental stages. However, we were able to detect a trace amount of *GA4H* mRNAs in both 14- and 35-day-old plants when using RNA gel blots containing poly(A)$^+$ RNA isolated from 200 μg of total RNA (data not shown). In fact, the *GA4H* cDNA was isolated by RT-PCR using RNA isolated from 14-day-old rosette plants. Therefore, the *GA4H* mRNA is present either at very low levels or in specific cell types in 14- and 35-day-old plants.

The isolation of the *GA4H* gene helps to explain the leaky phenotype of the *ga4-2* mutant carrying a putative null allele with respect to germination. However, the *ga4-2* mutant is also a semidwarf and is fertile in contrast to other severe GA-deficient mutants (e.g., *ga1-3*). These mutants are extreme dwarfs and male-sterile in addition to being nongerminating (Koornneef and van der Veen, 1980; Chiang et al., 1995). A BLAST search (Altschul et al., 1990) revealed another related Arabidopsis sequence that showed 70 and 65% identity to GA4 and GA4H, respectively, in a stretch of 40 amino acids (bacterial artificial chromosome end sequence of F20L14; GenBank accession number B08843). Characterization of this related sequence is necessary to determine whether it encodes a third 3β-hydroxylase in Arabidopsis.

Phytochrome Regulation of *GA4* and *GA4H* Expression

We have shown that *GA4* and *GA4H* expression in dark-imbibed wild-type seeds was induced by an R light pulse before seed germination (Figure 4). Our data support the hypothesis that R light stimulates the biosynthesis of active GAs, which promote seed germination (Figure 9). This rapid increase in *GA4* and *GA4H* mRNA levels after R light treatment also was observed in the nongerminating *ga1-3* seeds (Figure 5). This implies that it is not simply a secondary event resulting from physiological changes during germination but may be a direct effect of R light. However, in *ga1-3* seeds, the second peak of *GA4* transcript accumulation was absent, and the *GA4H* mRNA level declined much earlier than it did in wild-type seeds (Figures 4 and 5). These results suggest that expression of the *GA4* and *GA4H* genes at the later time points in the wild type is dependent on germination, which does not occur in the *ga1-3* mutant seeds, and may be important for the growth and development of young seedlings.

Using the *phyB-1* mutant, we showed that the R light–induced expression of the *GA4H* gene is mainly controlled via PHYB under the short imbibition condition (Figures 6 and 9). The fully reversible effect of R light on *GA4H* transcript levels by FR light (Figure 7) is consistent with the photoreversibility of PHYB-mediated seed germination (Shinomura et al., 1994).

Our results indicate that PHYB is not essential for R light–induced *GA4* expression under the shorter imbibition condition, in contrast to *GA4H* expression. However, the photoreversible effects of R and FR light pulses on *GA4* mRNA accumulation (Figure 7) imply the involvement of another phytochrome (Figure 9). This phytochrome is not likely to be PHYA, because PHYA was shown to be not involved in the R light–stimulated seed germination under the short imbibition condition (Shinomura et al., 1996). In fact, the elevated *GA4* expression by an R light pulse was not affected in the *phyA-201* mutant (Nagatani et al., 1993) (data not shown).

A non-PHYB–controlled photoreversible response was previously demonstrated by Yang et al. (1995), who showed that tissue sensitivity to exogenous GA_4 in *phyB* seeds is photoreversibly altered by R and FR light pulses that were given 2 hr after imbibition. Therefore, *GA4* mRNA levels could be controlled by the same unidentified light (phytochrome)-signaling pathway that may modulate the GA response pathway during seed germination (Figure 9). Because *phyB-1* seeds germinate at a low frequency (8%) after an R light pulse under the short imbibition condition, activation of the non-PHYB pathway(s), which controls *GA4* expression and tissue sensitivity to GA (Yang et al., 1995), is insufficient but may be promotive to induce complete seed germination. These results suggest that *GA4H* may play a more important role in seed germination than does *GA4* .

Recently, a gene encoding the GA 3β-hydroxylase in lettuce was isolated, and its mRNA level was shown to be photoreversibly regulated by R and FR light pulses in imbibed seeds (Toyomasu et al., 1998). Therefore, light-regulated expression of GA 3β-hydroxylase genes via phytochrome may be a common mechanism in plant species whose germination is dependent on a light stimulus. It remains to be investigated whether only GA 3β-hydroxylation is a rate-limiting step in response to light or whether other biosynthetic steps are modulated concertedly by light in germinating Arabidopsis seeds.

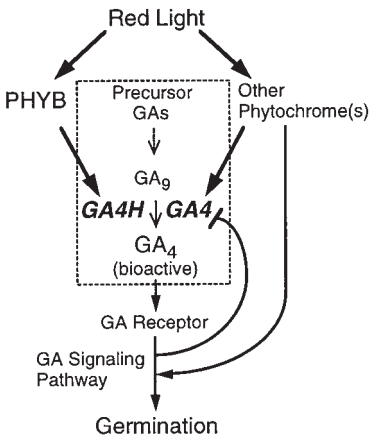

Figure 9. Proposed Model for the Regulation of *GA4* and *GA4H* Expression.

The last two steps of the GA biosynthetic pathway are shown in the dashed box. Arrows indicate positive regulation. The feedback inhibition is shown by the T-bar.

Feedback Regulation by GA Response in Germinating Seeds

Negative feedback regulation of GA 20-oxidase and GA 3β-hydroxylase mRNA levels has been illustrated in several plant species (reviewed in Hedden and Kamiya, 1997; Ross et al., 1997). In Arabidopsis, transcript levels of the *GA4* gene in rosette leaves and in seedlings (Chiang et al., 1995; Silverstone et al., 1998) and of three GA 20-oxidases in rosette leaves and in floral shoots (Phillips et al., 1995; Xu et al., 1995) were upregulated in the GA-deficient mutant background and were downregulated by the application of GAs. This feedback mechanism is probably modulated by the GA response pathway because the *gai* mutant, which is impaired in the GA response, contains elevated levels of *GA5* mRNA (encoding one of the GA 20-oxidases) and bioactive GAs (Talon et al., 1990b; Xu et al., 1995). In germinating seeds, feedback regulation of GA biosynthetic genes has not been demonstrated previously. However, the considerably higher levels of bioactive GAs (GA_1 and GA_4) in imbibed *gai* seeds relative to those in wild-type seeds (Derkx et al.,

1994) suggest that the feedback mechanism functions in germinating Arabidopsis seeds. In this study, we show that the *GA4* gene in imbibed seeds is under feedback regulation as occurs in rosette plants (Figures 8 and 9).

In contrast to *GA4*, the *GA4H* gene is not regulated by the feedback mechanism during seed germination (Figures 8 and 9). *GA4H* mRNA accumulated to an even higher level in the GA_4-treated *ga1-3* seeds than in the untreated seeds after the seeds began to germinate (Figure 8B). This increased *GA4H* expression is probably due to developmental regulation as a consequence of GA_4-induced seed germination rather than to a direct effect of GA_4, because the increase was not observed until germination occurred (Figure 8B).

Considering their distinct patterns of expression (Figure 4) and the different response to feedback regulation (Figure 8), *GA4* and *GA4H* are likely to have separate physiological roles during seed germination. The main function of GA in promoting seed germination in Arabidopsis is probably to facilitate breakage of the seed coat, because mechanically dissected *ga1-3* embryos are able to grow into rosette plants (Silverstone et al., 1997). Studies using the tomato GA-deficient mutant *gib-1* suggested that GAs synthesized in the embryo facilitate germination by inducing the production of enzymes that digest the tissue surrounding the radicle tip (Groot and Karssen, 1987). In addition, GA also may promote cell elongation in the growing radicle, because the tomato *gib-1* embryo had a reduced growth rate when compared with the wild type (Groot and Karssen, 1987). Feedback regulation of GA biosynthesis is likely to be a homeostatic mechanism to adjust the concentration of bioactive GAs in cells to an appropriate level. However, this mechanism could prevent accumulation of the increased level of bioactive GAs, which may be essential for seed germination. Therefore, the *GA4H* gene, which is not regulated by feedback inhibition, may be crucial to circumvent this potential problem and to produce sufficient bioactive GAs for seed germination. It is also possible that the *GA4H* gene might be expressed in specific cells in germinating seeds. Future studies on the cellular localization of *GA4* and *GA4H* mRNAs in germinating seeds by using in situ hybridization may help to understand the relative physiological function of these genes during seed germination.

METHODS

Plant Materials

Arabidopsis thaliana ecotype Landsberg *erecta* was used as the wild-type control in this study. Wild-type seeds used in Figure 4 were purchased from Lehle Seed (Round Rock, TX). The *ga1-3* seeds were originally obtained from Maarten Koornneef (Agricultural University, Wageningen, The Netherlands) and propagated in 1995. These seeds were stored and kept dry at 4°C in the dark. For the experiments depicted in Figures 6 and 7, we used wild-type and *phyB-1* mutant seeds, which were harvested in 1996 and stored at room temperature. The *phyB-1* seeds were originally obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus).

Growth Conditions

Red (R) light was obtained by passing light from a fluorescent tube (40 W) through a red plastic filter (Shinkolite A#102; Toray, Tokyo, Japan). Far-red (FR) light was obtained by filtering the output from a water-cooled incandescent bulb (100 W) through an FR filter (Delaglass A900; Asahikasei, Tokyo, Japan). The R light pulse was 9.3 μmol m^{-2} sec^{-1} (15 min), and the FR light pulse was 0.50 μmol m^{-2} sec^{-1} (20 min). Where necessary, all manipulations were conducted under a dim green safelight.

To harvest tissues for RNA extraction, 75 mg of dry seeds (wild type or *ga1-3*) were washed with 0.2% Triton X-100 and rinsed with sterile water three times. The seeds were resuspended in 1.2 mL of water, and each sample was spread in two Petri dishes (35-mm diameter) containing two layers of chromatography paper (Fisher Scientific, Pittsburgh, PA). During tissue harvesting, ~100 seeds were left in each dish to score for germination percentage, and the averages from the two different plates are presented in Figures 4C and 8B. The time when seeds were placed in 0.2% Triton X-100 was defined as the beginning of imbibition.

Isolation of *GA4H* Genomic and cDNA Clones

A 1.3-kb *GA4* cDNA fragment was amplified from cDNAs prepared from 14-day-old wild-type plants (Yamaguchi et al., 1998) by using *GA4*-specific primers 5′GA4 (5′-CACAAACATCTATCAAATTTAC-3′) and 3′GA4 (5′-ACAAATCATATTGCTGAAATC-3′). The 1.3-kb polymerase chain reaction (PCR) fragment was cloned into pBluescript KS− (Stratagene, La Jolla, CA), and the nucleotide sequence was determined to confirm its identity. This plasmid (pGA4-1) was digested with HindIII to excise a 0.4-kb *GA4* cDNA fragment, which was used as a probe to screen an Arabidopsis (Columbia) genomic library (courtesy of M. Matsui, RIKEN Institute). Hybridization was performed using Hybond N+ membranes (Amersham) in Rapid-Hyb buffer (Amersham) at 55°C, and washes were performed in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate; Sambrook et al., 1989) containing 0.1% SDS at 60°C.

Two positive clones (λGA4-32 and λGA4-34) were characterized further. DNA gel blot analysis showed that a 1.6-kb DNA fragment, present in both λGA4-32 and λGA4-34, hybridized with the *GA4* probe under the same conditions used for the library screening. The 1.6-kb DNA fragments were cloned into pUC118 (Toyobo, Osaka, Japan), and analysis of partial sequence showed that these two DNA fragments were identical and that they shared a high degree of sequence similarity to *GA4*. Exonuclease III and the S1 nuclease (Gibco-BRL) were used to generate a series of deletions from this 1.6-kb *GA4H* genomic DNA clone (pgGA4-3.7) for DNA sequence analysis. pgGA4-3.7 contains most of the coding sequence of the *GA4H* gene, except that its 5′ end is truncated.

To isolate the complete 5′ region of *GA4H* cDNA by rapid amplification of cDNA ends (RACE), *GA4H*-specific primers (3β2R, 5′-CGTGGAGGGGAGAGCCAATAAC-3′; 3β3R, 5′-CAGTATTTGAGGTGGTGGCT-3′) were synthesized. 5′ RACE was conducted with poly(A)+ RNA from 14-day-old wild-type plants by using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA), according to the

manufacturer's instructions. A 0.5-kb RACE product was cloned into the pCRII vector (Invitrogen, San Diego, CA) to create plasmid p5′/GA4H, and the nucleotide sequence was determined. To obtain the full-length coding region by PCR, oligonucleotide primers 3β5′BamHI (5′-TTGGATCCATGAGTTCAACGTTGAGCGATG-3′) and 3β3′SalI (5′-TGTCGACAGTTAATTTCTAATAATGGAAAG-3′) were synthesized based on the DNA sequence from the 5′ RACE product and the genomic clone pgGA4-3.7, respectively. PCR was performed with the Expand high-fidelity PCR system (Boehringer Mannheim) by using the cDNA pool (see above) as a template. The resulting PCR product (1.1 kb) was digested with BamHI and SalI and inserted into the BamHI-SalI site of pMAL-c2 vector (New England Biolabs, Beverly, MA) to generate pMAL/GA4H. The 1.1-kb cDNA from pMAL/GA4H was subcloned into pBluescript KS− (pKS/GA4H), and a series of unidirectional deletion clones was generated as described above for sequence analysis of the 1.1-kb *GA4H* cDNA.

DNA Sequence Analyses

DNA sequences were determined using a DNA sequencer (model ABI377; Applied Biosystems, Foster City, CA). The BLAST (Altschul et al., 1990) program was used to search for homologous sequences in the databases. The PILEUP program of the Genetics Computer Group (Madison, WI) was used to generate sequence alignments.

Heterologous Expression of *GA4H* in *Escherichia coli* and 3β-Hydroxylase Activity Assays

A maltose binding protein (MBP)–GA4H fusion protein was synthesized in *E. coli* JM109 or BL21 harboring pMAL/GA4H. Production of the fusion protein was induced by addition of isopropyl β-D-thiogalactopyranoside, as described previously (Yamaguchi et al., 1996).

E. coli lysates (50 μL) in 20 mM Tris-HCl, pH 7.5, were assayed in a final volume of 100 μL, containing 4 mM 2-oxoglutarate, 5 mM L-ascorbate, 0.5 mM Fe_2SO_4, and 0.24 nmol 17-^{14}C-GA_9 (30,000 dpm). Assays were incubated at 30°C for 90 min, and reactions were stopped by the addition of 0.9 mL of 0.1 N HCl. Samples were loaded onto Bond Elut C_{18} columns (Varian, Harbor City, CA) and then eluted with methanol. The eluates were loaded onto a 0.2-mm silica gel thin-layer chromatography plate (No. 5553; Merck, Darmstadt, Germany), which was developed with ethyl acetate–*n*-hexane–acetic acid (50:50:1 [v/v]). The dried plate was exposed to a BAS 2000 plate (Fuji Film, Tokyo, Japan) to visualize radioactivity. To identify the products by full-scan gas chromatography–mass spectrometry, 60 ng of cold GA_9 or GA_{20} was incubated for 2 hr, using the same conditions as described above. The eluate from the C_{18} column step was loaded onto a DEA column (Varian) and eluted with methanol containing 0.5% acetic acid. After derivatization with diazomethane and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, the samples were analyzed on a GCQ gas chromatography–mass spectrometer (Finnigan MAT, San Jose, CA).

Incubations for kinetic studies with MBP–GA4H were performed as described by Martin et al. (1997), using varying concentrations of 17-^{14}C-GA_9 (0.4 to 9.1 μM) or 17-^{14}C-GA_{20} (1.7 to 45.6 μM). Each product, GA_4 or GA_1, was determined by HPLC with on-line radiomonitoring using a C_{18} column (Hypersil; 5 μm, 250 × 4.6-mm internal diameter; Phenomenex, Cheshire, UK). Michaelis-Menten curves were obtained using Enzfitter (Biosoft, Cambridge, UK). The K_m values were determined twice with similar results.

RNA Gel Blot Analysis

RNA gel blot analyses were performed using antisense RNA probes. A 0.6-kb EcoRV *GA4* cDNA fragment isolated from pGA4-1 was subcloned into the SmaI site of pBluescript SK+ (pGA4-2). The *GA4* and *GA4H* cDNA fragments were amplified from pGA4-2 and p5′/GA4H, respectively, by PCR using the forward and reverse M13 primers. These PCR products were used as templates to synthesize antisense RNA probes with T7 RNA polymerase (Pharmacia).

RNA samples were subjected to electrophoresis in 1% agarose–2.2 M formaldehyde gels. After gel blotting, the membranes (Hybond N; Amersham) were hybridized at 65°C in a buffer containing 50% formamide, 1 × Denhardt's solution (0.02% Ficoll [Pharmacia], 0.02% PVP, and 0.02% BSA; Sambrook et al., 1989), 1% SDS, 5 × SSPE (1 × SSPE is 0.2 M NaH_2PO_4, pH 7.4, 0.3 M NaCl, 0.02 M EDTA; Sambrook et al., 1989), 0.1 M sodium phosphate, pH 7.0, and 0.2 mg/mL calf liver RNA. Two membranes were prepared for each set of samples and hybridized separately with the *GA4* or *GA4H* probe. The membranes were washed in 0.1 × SSPE and 0.1% SDS at 68°C, then exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA), and analyzed on a PhosphorImager (model 400E; Molecular Dynamics) by using ImageQuant (version 4.1) software (Molecular Dynamics). After autoradiography, the membranes were stripped and reprobed with a radiolabeled 1.0-kb fragment from soybean 18S rDNA as a loading control. For each set of two blots containing identical samples, we show only one blot reprobed with 18S rDNA in Figures 3 to 8.

To examine cross-hybridization, full-length *GA4* and *GA4H* sense RNAs were synthesized in vitro by using T3 and T7 RNA polymerases (Pharmacia) with pGA4-1 and pKS/GA4H as the templates, respectively. RNA gel blots containing dilutions of both *GA4* and *GA4H* sense RNAs (1, 10, and 100 pg per lane) were hybridized with the radiolabeled *GA4* and *GA4H* antisense probes separately, and standard curves were generated based on radioactivity quantified as described above. We confirmed that the standard curves were linear within this range. In each RNA blot, a known amount of in vitro–synthesized sense *GA4* and *GA4H* RNAs was included to determine the absolute amount of endogenous *GA4* and *GA4H* mRNA in each RNA sample. The signal derived from cross-hybridization with the other RNA species was calculated from the standard curve for cross-hybridization. To determine the amount of net *GA4H*–*GA4H* hybridization in Figure 5B, the signal from cross-hybridization with *GA4* RNA was subtracted from the total hybridization signal. Except for the data shown in Figure 5, cross-hybridization of the *GA4* or *GA4H* probe with the other RNA species was below detection for all of the data presented in this study.

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