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

Shinjiro Yamaguchi, Maria W. Smith, Robert G. S. Brown, Yuji Kamiya, and Tai-ping Sun

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 GA9 to GA4 and GA20 to GA1 (Hedden and Kamiya, 1997). Several GA-deficient dwarf mutants with reduced 3β-hydroxylase activities have been isolated. These include dwarf1 in maize (Fujikawa 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 GA4 and GA1, but not their precursors GA9 and GA20, 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
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 Arim 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).

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 (Derks et al., 1994). Therefore, we used both GA4 and GA20 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 17-14C-GA20 to 17-14C-GA8 (Figure 2B) and converting 17-14C-GA20 to 17-14C-GA1 (data not shown). The identity of the products was confirmed by full-scan gas chromatography–mass spectrometry.
Phytochrome Regulation of GA4 and GA4H (data not shown). The control E. coli lysate containing the MBP did not show this enzymatic activity. No 2β-hydroxylation 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 GA9, for which the Km is 1.0 μM, whereas that for GA20 is 15 μM (Williams et al., 1998). To examine whether GA4H has a different substrate preference from GA4, we determined the Km values of MBP–GA4H for GA9 and GA20. We found that the Km values for GA9 and GA20 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 GA9 to GA20 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). Radi- cal 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
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-
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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). 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 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.

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 mRNA (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.
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 \( \text{GA4H} \), whereas PHYB is not essential for the induction of \( \text{GA4} \) expression under this condition.

Expression of Both \( \text{GA4} \) and \( \text{GA4H} \) 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 \( \text{GA4} \) and \( \text{GA4H} \) 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 \( \text{GA4} \) and \( \text{GA4H} \) 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 \( \text{GA4H} \) Is under Feedback Regulation during Seed Germination

Previous studies have shown that the \( \text{GA4} \) mRNA level in rosette leaves of the \( \text{ga4-1} \) mutant and seedlings of the \( \text{ga1-3} \) mutant was higher than that of wild-type plants and that application of \( \text{GA3} \) downregulated the accumulation of \( \text{GA4} \) mRNA (Chiang et al., 1995; Silverstone et al., 1998). To determine whether expression of the \( \text{GA4} \) and \( \text{GA4H} \) genes during seed germination is also subject to the feedback inhibition mechanism, we examined their mRNA levels in a \( \text{GA} \)-deficient mutant background. As shown in Figure 8A, the \( \text{GA4} \) mRNA level in the dark-imbibed \( \text{ga1-3} \) seeds was much higher than in wild-type seeds. Indeed, the \( \text{GA4} \) mRNA level in the dark-imbibed \( \text{ga1-3} \) seeds was higher than its maximum level in the R light–treated wild-type seeds. This result suggests that expression of the \( \text{GA4} \) gene in germinating seeds is controlled by the feedback mechanism. In contrast, the level of \( \text{GA4H} \) mRNA in \( \text{ga1-3} \) was approximately the same as in the wild type (Figure 8A).
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Therefore, the GA4H gene is not likely to be regulated by the feedback mechanism during seed germination.

We also examined the effect of exogenous GA4 on the expression of the GA4 and GA4H genes in the imbibed ga1-3 seeds. GA4 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 GA4, 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 GA4 than in the untreated controls. In contrast, there was no striking difference between the levels of GA4H transcripts in GA4-treated and nontreated seeds before germination (12 and 24 hr after imbibition). Interestingly, GA4H mRNA accumulated to higher levels in GA4-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 GA9 rather than GA20 (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 GA9 and GA20 (Phillips et al., 1995). In addition to the 3β-hydroxylation of GA9 and GA20, recombinant GA4 catalyzes 3β-hydroxylation of C20 GAs (GA15, GA24, and GA44) 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 mRNA 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 occur in the later time points in the wild type is dependent on germination, which 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 photoresponsive response was previously demonstrated by Yang et al. (1995), who showed that tissue sensitivity to exogenous GA4 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 regulated by light in germinating Arabidopsis seeds.

**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 (GA1 and GA4) in imbibed gai seeds relative to those in wild-type seeds (Derkx et al.,

**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.
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 GA4-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 GA4-induced seed germination rather than to a direct effect of GA4, 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 (Delaplanss 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′-CACAAACATCTATCAAAATTTAC-3′) and 3′GA4 (5′-ACAAATCATATGGCTGAAAATC-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 35°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′-CGTGAGGGAGGGAGCAATAAC-3′; 3β3R, 5′-CTGATTTTGAGTGTTGCT-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 3p5′-BamHI (5′-TTGGATCCATGATTACCTGGACGTGAGTG-3′) and 3p3′-SalI (5′-TGGTCGACGATTATTTTCTAATAAGGAAAG-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 FeSO4, and 0.24 mM nmo1 17-α-C-GA3 (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 C18 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–hexane–acetic acid (50:50:1 [v/v]). The dried plate was exposed to a BAS 2000 plate (FujiFilm, Tokyo, Japan) to visualize radioactivity. To identify the products by full-scan gas chromatography–mass spectrometry, 60 ng of cold GA9 or GA20 was incubated for 2 hr, using the same conditions as described above. The eluate from the C18 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-trimethylsilyl trifluoroacetamide, the samples were analyzed on a GC 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-α-C-GA3 (0.4 to 9.1 μM) or 17-α-C-GA20 (1.7 to 45.6 μM). Each product, GA20 or GA20, was determined by HPLC with on-line radio-monitoring using a C18 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 blots were performed using antisense RNA probes. A 0.6-kb EcoRI GA4 cDNA fragment isolated from pGA4-1 was subcloned into the Smal 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 blotted, 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 NaH2PO4, 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). After autoradiography, the membranes were stripped and reprobed with a radiolabeled 1.0-kb fragment from soybean 18S rRNA as a loading control. For each set of two blots containing identical samples, we show only one blot reprobed with 18S rRNA 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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