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Mini Review

Gibberellin Biosynthesis: Its Regulation by Endogenous and Environmental Signals

Shinjiro Yamaguchi [1] and Yuji Kamiya [2]

[1] *Developmental, Cell and Molecular Biology Group, Department of Botany, Box 91000, Duke University, Durham, North Carolina, 27708-1000, U.S.A.*

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

The hormone gibberellin (GA) plays an essential role in many aspects of plant growth and development, such as seed germination, stem elongation and flower development. In recent years, exciting progress has been made in understanding how the biosynthesis of this hormone is regulated by endogenous and environmental factors. This has resulted from isolation of genes encoding enzymes involved in GA biosynthesis and metabolism, which also enabled us to manipulate the pathway by modifying the expression of these genes in transgenic plants. In addition, new GA response mutants provided information about how signaling components are involved in feedback regulation of the GA biosynthetic pathway.

Key words: Biosynthesis — Feedback regulation — Gibberellin — Phytochrome — Plant hormone.

Introduction—GAs are a group of tetracyclic diterpene carboxylic acids. Among numerous different GAs identified from plants, a few act as endogenous plant growth regulators (bioactive GAs), but most are precursors or inactivate forms. The biological functions of GAs have been well demonstrated by the phenotype of mutants with reduced GA production (GA-deficient mutants) and the effects of chemical inhibitors of GA biosynthesis enzymes on plant growth (Graebe 1987, Hedden and Kamiya 1997, Ross et al. 1997, Hedden and Proebsting 1999). The best characterized GA function in a number of plant species is the promotion of stem elongation. GA plays an essential role in controlling other developmental processes as well. For example, severe GA-deficient mutants of *Arabidopsis thaliana* are non-germinating, male sterile and non-flowering under short-day conditions, besides being dwarfed.

The GA biosynthetic pathway has three different classes of enzymes: terpene cyclases, Cyt P450 monooxygenases, and 2-oxoglutarate-dependent dioxygenases (Graebe 1987), as illustrated in Fig. 1. Geranylgeranyl diphosphate (GGDP) is a common precursor for diterpenes and carotenoids. To initiate GA biosynthesis, GGDP is converted to a tetracyclic hydrocarbon, *ent*-kaurene, by two terpene cyclases, copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). These cyclases are located in plastids (Sun and Kamiya 1997). In the second stage of the pathway, *ent*-kaurene is oxidized by Cyt P450 monooxygenases associated with the endoplasmic reticulum to form GA_{12}-aldehyde. In the final stage of this pathway, GA_{12}-aldehyde is converted to bioactive GAs by 2-oxoglutarate dependent dioxygenases, which may be cytosolic enzymes. These include GA 7-oxidase, GA 20-oxidase, GA 3β-hydroxylase, and GA 2-oxidase (Fig. 1).

Genes encoding GA biosynthesis enzymes were initially isolated from pumpkin (*Cucurbita maxima*) and *Arabidopsis* (Hedden and Kamiya 1997). Endosperm from immature pumpkin seeds is a rich source of GA biosynthetic enzymes. Therefore, this material was utilized for the conventional purification of enzymes, which resulted in the isolation of cDNAs for KS and GA 20-oxidase (Lange et al. 1994, Yamaguchi et al. 1996). On the other hand, a molecular genetic approach has been successfully used to isolate genetic loci involved in GA biosynthesis, such as the *GA1, GA3* and *GA4* genes of *Arabidopsis* (Sun et al. 1992, Chiang et al. 1995, Helliwell et al. 1998). The *GA1, GA3* and *GA4* genes were then shown to encode CPS, *ent*-kaurene oxidase and GA 3β-hydroxylase, respectively, by the enzyme activity of the recombinant proteins (Sun and Kamiya 1994, Williams et al. 1998, Helliwell et al. 1999). Recently, Lange (1997) developed a new approach to screen a cDNA expression library, which utilizes enzyme assays of pooled recombinant *E. coli* cells. This method was used to isolate cDNA clones for GA 7-oxidase (Lange 1997) and GA 2-oxidase (Lester et al. 1999, Martin et al. 1999, Thomas et al. 1999). GA biosynthesis enzymes for which cDNA clones have been isolated are shown in Fig. 1.

Isolation and functional characterization of these genes provided new information on GA biosynthesis. First, several GA biosynthesis enzymes have been shown to be

Abbreviations: CDP, copalyl diphosphate; CPS, copalyl diphosphate synthase; FR, far-red light; GA, gibberellin; GGDP, geranylgeranyl diphosphate; KS, *ent*-kaurene synthase; R, red light; PHY, phytochrome; WT, wild-type.

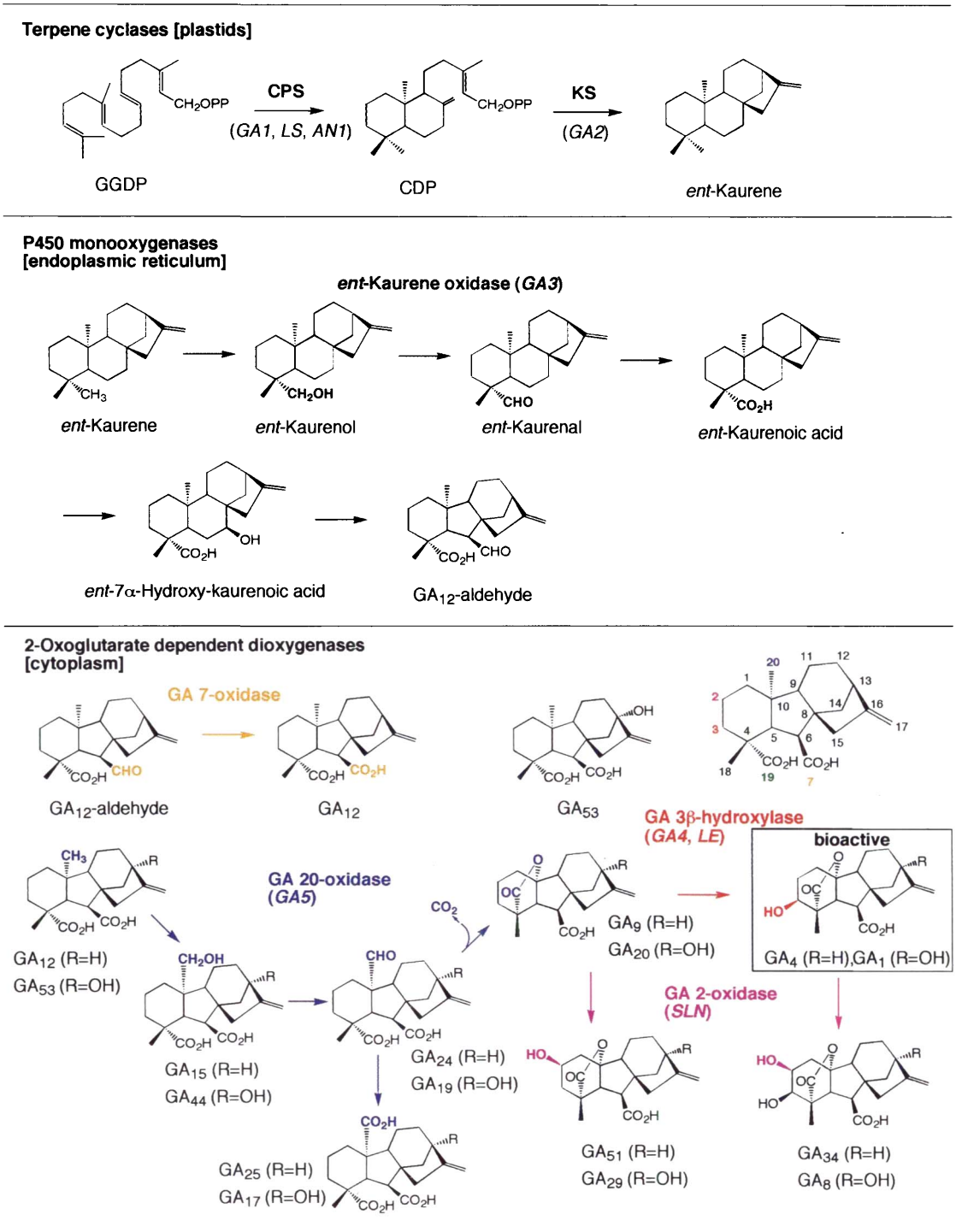

Fig. 1 The major GA biosynthetic pathway in plants. The pathway is divided into three stages based on the nature of the enzymes involved (Graebe 1987). Enzymes for which cDNA clones have been isolated are shown. Oxidases and their reactions are highlighted with different colors (green, *ent*-kaurene oxidase; yellow, GA 7-oxidase; blue, GA 20-oxidase; red, GA 3β-hydroxylase; purple, GA 2-oxidase). Genetic loci encoding GA biosynthesis enzymes are indicated in parentheses (*GA1-GA5*, Arabidopsis; *LS, LE* and *SLN*, pea; *AN1*, maize). GA 2-oxidase catalyzes the conversion of 2β-hydroxylated GAs to the corresponding catabolites (Thomas et al. 1999, Lester et al. 1999), which are not shown here for simplification. CPS, copalyl diphosphate synthase; KS, *ent*-kaurene synthase.

multifunctional. For example, both *ent*-kaurene oxidase and GA 20-oxidase can catalyze three consecutive oxidation reactions (Fig. 1). In addition, many enzymes can catalyze the same reaction for different, but structurally similar, substrates (e.g., *Arabidopsis* GA 3β-hydroxylase catalyzes both $GA_9 \rightarrow GA_4$ and $GA_{20} \rightarrow GA_1$; Williams et al. 1998). Because of these features, two enzymes, GA 20-oxidase and 2β, 3β-hydroxylase, can catalyze the conversion of GA_{12} to more than 10 different GAs in pumpkin endosperm in vitro (Lange et al. 1994, 1997). Second, several enzymes are encoded by small gene families (e.g., Phillips et al. 1995). Each gene may have unique biological roles in controlling GA biosynthesis.

The availability of genomic and cDNA clones for GA biosynthetic genes have allowed the analysis of their promoter activity, mRNA accumulation and protein levels in plants. This provides an opportunity to better understand how each step of the pathway is regulated to modulate the bioactive GAs' concentration and the sites of GA biosynthesis. These problems had been difficult to study based solely on measurements of GAs because they are present at very low levels in plants.

In this article, we summarize how GA biosynthesis is regulated by endogenous and environmental signals, with an emphasis on recent studies using molecular tools.

Developmental regulation—Recent data indicate that GA biosynthetic genes are tightly regulated during plant growth and development. Developmental regulation has been studied in detail for the *Arabidopsis GA1* gene, which encodes CPS (Silverstone et al. 1997). Because the *GA1* mRNA and protein levels in plant tissues are extremely low, cellular localization of this gene was analyzed by *GA1* promoter-*GUS* reporter gene expression and reverse transcription-PCR. The *GA1* promoter activity is limited to specific cell types, e.g., shoot apices, root tips, anthers and immature seeds. The sites of *GA1* expression correlate well with the tissues where the *ga1* mutant phenotype is observed, suggesting that the *GA1* gene is expressed at or near the sites responding to GA. Whether later GA biosynthetic genes are also expressed in the same cells as *GA1* needs to be investigated.

The regulation of expression of the *CPS* and *KS* genes has been examined during vegetative growth of pumpkin using RNA blot analysis (Smith et al. 1998). The two *CPS* mRNAs are abundant in very young tissues and declined with aging. In contrast to the change in *CPS* mRNA levels, the level of the *KS* mRNA is constant in the same tissue at different ages. These results suggest that *CPS* expression is more critically regulated than *KS* is. This is consistent with the finding in *Arabidopsis* that the *GA2* (encoding KS) mRNA levels do not fluctuate as much as *GA1* in different organs (Yamaguchi et al. 1998).

Rebers et al. (1999) studied transcript accumulation of several GA biosynthetic genes during flower and early fruit development in tomato (*Lycopersicon esculentum*). GA biosynthesis is required for tomato flower development because flower buds of the GA-deficient *gib-1* mutant are developmentally arrested. The data from RNA blot analysis showed that all the genes examined, including those for CPS, three GA 20-oxidases, and GA 3β-hydroxylase, are highly regulated but with different patterns of mRNA accumulation (Rebers et al. 1999). These results indicate a complex regulatory mechanism for controlling GA biosynthesis during flower development.

Regulation by GA activity—Accumulating evidence shows that GA biosynthesis is negatively regulated by GA activity. This feedback regulation was first noted in a class of semidominant dwarf mutants that are insensitive to GA, such as *gai* of *Arabidopsis, D8* of maize and *Rht* of wheat (Hedden and Kamiya 1997, Ross et al. 1997). These mutants exhibit a phenotype similar to GA-deficient dwarfs, but contain abnormally high levels of bioactive GAs, suggesting that reduced GA response results in elevated biosynthesis of GA. Thus, GA activity would lower the biosynthesis of GAs. Recent studies have shown that mRNA levels of genes encoding GA 20-oxidase and 3β-hydroxylase are down-regulated by GA (Hedden and Kamiya 1997). No evidence has been shown for the feedback regulation of mRNA levels of CPS, KS and *ent*-kaurene oxidase (Helliwell et al. 1999). Therefore, this feedback inhibition targets mRNA levels of later GA biosynthesis enzymes.

Besides the synthesis of bioactive GAs, recent cloning of GA 2-oxidase cDNAs revealed that deactivation steps of GAs are also controlled by GA activity. The major deac-

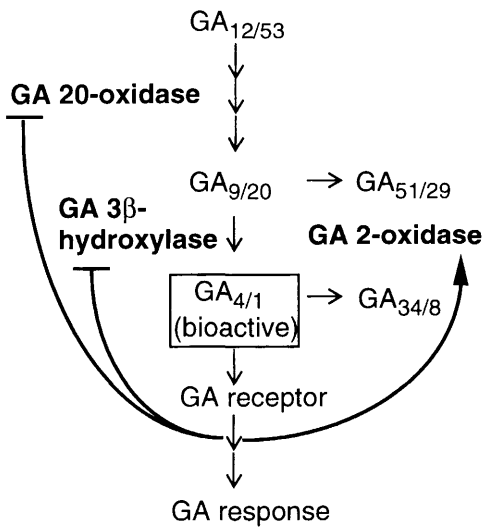

Fig. 2 Model showing the homeostatic regulation of GA biosynthesis. Feedback inhibition is shown by the T-bar. The arrow with a closed triangular arrowhead indicates feedforward up-regulation.

tivating reaction of bioactive GAs is 2β-hydroxylation, catalyzed by GA 2-oxidase. Thomas et al. (1999) noted that the amount of GA 2-oxidase mRNAs is up-regulated after the application of bioactive GA to the flower buds of the *Arabidopsis ga1-2* mutant. This contrasts with the down-regulation of GA 20-oxidase and 3β-hydroxylase mRNA levels by GA activity, and suggests that GA 2β-hydroxylation is controlled by a type of feedforward regulation (Thomas et al. 1999). Thus, the level of bioactive GAs is likely to be maintained by modulating both their synthesis and catabolism (Fig. 2).

Recent characterization of new GA response mutants indicates which signaling components are involved in the feedback inhibition of GA biosynthesis. The recessive *rga* mutation partially suppresses phenotypic defects of the *Arabidopsis* GA-deficient mutant *ga1-3*, and therefore RGA is proposed to be a negative regulator of the GA response pathway (Silverstone et al. 1998). Transcript accumulation of the *GA4* gene (encoding a GA 3β-hydroxylase) is elevated in the *ga1-3* mutant, but not as much in the *rga/ga1-3* mutant. This result indicates that the *rga* mutation can partially suppress up-regulation of *GA4* expression in the GA-deficient mutant background. The *Arabidopsis shi* (short internodes) mutant exhibits reduced GA responsiveness due to overexpression of the *SHI* gene (Fridborg et al. 1999). Similar to the *gai* mutant, the *shi* mutant contains reduced C_{20}-GAs (substrates for GA 20-oxidase), but has increased C_{19}-GAs, which indicates up-regulation of 20-oxidation. Peng et al. (1999) characterized two extragenic suppressors of the semidominant *gai* mutant, *spy-7* and *gar2-1*. These mutations suppress the defects of the *gai* mutant in a wide range of GA responses, including seed germination, stem elongation and flowering. In the *gai* mutant background, these mutations increase the levels of C_{20}-GAs, but reduces C_{19}-GAs such as bioactive GA_1 and GA_4. These data indicate that the *spy-7* and *gar2-1* mutations result in enhanced GA responses during plant growth and reduced GA 20-oxidation in the *gai* mutant background (Peng et al. 1999).

Cowling et al. (1998) took a different approach to suggest that feedback regulation and hypocotyl elongation are controlled by similar GA recognition and signal transduction processes. Different GA derivatives (GAs with modified 3β-hydroxyl group, 7-carboxylic acid or C/D rings) were tested for the ability to reduce the *GA4* mRNA level and to induce hypocotyl elongation of *Arabidopsis* seedlings. There was a correlation between the degree of activity of each GA structure in the two assays (Cowling et al. 1998).

Feedback and feedfoward regulation are likely to help to maintain the concentrations of bioactive GAs within a limited range. However, this homeostatic mechanism may be circumvented when the increased production of bioactive GAs is desired during plant development. During *Arabidopsis* seed germination, at least two GA 3β-hydroxylase genes (*GA4* and *GA4H*) are expressed. Interestingly, only the *GA4* gene, but not the *GA4H* gene is regulated by the feedback inhibition mechanism (Yamaguchi et al. 1998). In addition, both mRNAs are localized in the same cell types in germinating seeds (S. Yamaguchi and T. -p. Sun, unpublished data). Therefore, the *GA4H* gene, which is not down-regulated by GA activity, may be important to maintain active GA levels to promote seed germination.

Homeobox genes—Recently, *KNOTTED*-type homeobox genes have been implicated in controlling GA biosynthesis in tobacco (*Nicotiana tabacum*) (Kusaba et al. 1998, Tanaka-Ueguchi et al. 1998). KNOTTED-type proteins are a class of transcriptional regulators with a conserved homeodomain, and are involved in controlling plant morphology. Transgenic tobacco plants overexpressing either the *NTH15* or *OSH1* gene, encoding the KNOTTED-type homeodomain protein from tobacco and rice (*Oryza sativa*), respectively, are dwarfs with abnormal leaf and flower morphology. These morphological changes are partially restored by the application of bioactive GAs, which suggests the involvement of GA in this morphological alteration (Kusaba et al. 1998, Tanaka-Ueguchi et al. 1998).

Relative to WT, the transgenic plants overexpressing the *NTH15* gene contain reduced GA_{20} and GA_1, but increased GA_{19}, in leaves, suggesting that the conversion of GA_{19} to GA_{20} is decreased. This is supported by the observation that application of GA_{20}, but not GA_{19}, can restore the leaf morphology. Furthermore, in different transgenic lines, positive correlation is observed between the morphological severity and the reduction in the GA 20-oxidase transcript levels (Tanaka-Ueguchi et al. 1998). In situ hybridization showed that, in WT shoot apical regions, GA 20-oxidase mRNA is predominantly expressed in the rib meristem, but not in the tunica and corpus, where the *NTH15* mRNA is localized. Thus, NTH15 may suppress GA 20-oxidase expression in these cells in WT.

The involvement of a *KNOTTED*-type homeobox gene in controlling GA biosynthesis during stem growth was further studied in the dwarfed transgenic tobacco plants overexpressing the rice *OSH1* gene (Kusaba et al. 1998). In stem tissues, these transgenic plants contain a much lower amount of GA_{19}, GA_{20} and GA_1 than WT. Exogenous application of GA_{20} is effective to promote stem elongation of the dwarfed transgenic plants, but GA_{53} is not, indicating that GA 20-oxidation is reduced. In addition, transcript accumulation of GA 20-oxidase in stem is greatly reduced in the transgenic plants. Taken together, these data suggest that the *KNOTTED*-type homeobox genes regulate plant development in part by modifying GA levels.

Regulation by light—Many light-regulated developmental processes, such as seed germination, stem elonga-

tion and flowering, are also controlled by GA. In some cases, the effects of light on growth can be mimicked by altering GA levels. For example, the requirement of appropriate light conditions for seed germination can be replaced by exogenously applied GAs in lettuce (*Lactuca sativa*) and *Arabidopsis*. Thus, light has been studied as a potential regulator of GA biosynthesis (Hedden and Kamiya 1997). Regulation of GA biosynthesis by light is recently covered in more detail by Kamiya and García-Martínez (1999) In this review, we will discuss light-regulated expression of GA biosynthetic genes during seed germination and stem elongation.

Germination of lettuce seeds (cv. Grand rapids) is critically dependent on light. The red (R) and far-red light (FR) photoreceptor phytochrome (PHY) was first recognized in this system by the observation that R promotes, but FR photoreversibly inhibits seed germination. In dark-imbibed lettuce seeds, the level of the bioactive GA_1, but not its precursor GA_{20}, is elevated after a R treatment (Toyomasu et al. 1993). These results, together with the accumulation of GA_{20} (about 100 times higher than GA_1), suggest that the conversion of GA_{20} to GA_1 is the limiting step in germinating lettuce seeds. Supporting this hypothesis, mRNA for GA 3β-hydroxylase is not detected in dark-imbibed seeds, but is increased after a R treatment (Toyomasu et al. 1998). The elevated mRNA accumulation by R is photoreversibly canceled by a subsequent FR treatment, which indicates the involvement of PHY. Interestingly, mRNA levels of two GA 20-oxidase are induced by imbibition alone in the dark, consistent with the accumulation of GA_{20}. These results indicate that PHY controls 3β-hydroxylation of GA_{20} through GA 3β-hydroxylase expression in germinating lettuce seeds (Toyomasu et al. 1998).

Germination of *Arabidopsis* seeds is also regulated by light through PHY. Among five PHYs in this species, PHYB is the major PHY controlling seed germination in response to R and FR shortly after the start of imbibition (Shinomura et al. 1996). In germinating *Arabidopsis* seeds, at least two genes for GA 3β-hydroxylase, *GA4* and *GA4H*, are expressed with different temporal patterns of mRNA accumulation (Yamaguchi et al. 1998). Both *GA4* and *GA4H* mRNA accumulation is elevated by a brief R treatment, which can be reversed by FR. In the PHYB-deficient *phyB-1* mutant, *GA4H* expression is not induced by R, showing that the induction by R is primarily mediated by PHYB under this condition. *GA4* and *GA4H* transcript accumulation is still induced by R in the non-germinating *ga1-3* seeds, which supports that the induction is not a consequence of seed germination, but may be a direct effect of light (Yamaguchi et al. 1998). It is yet to be determined if other biosynthetic steps are also regulated by PHY and whether bioactive GA levels are in fact elevated in response to R in germinating *Arabidopsis* seeds.

Unlike in germinating seeds, light may reduce GA levels in dark-grown seedlings because light inhibits stem elongation. Recent works from two independent labs have shown that light alters GA biosynthesis during deetiolation of pea (*Pisum sativum*) seedlings (Gil and García-Martínez 1998, Ait-Ali et al. 1999). Within 2 h after light irradiation, the amount of GA_1 (the major bioactive GA in pea) sharply decreases in etiolated seedlings. However, GA 20-oxidase and GA 3β-hydroxylase mRNA levels remain constant until they increase temporally after the reduction in the GA_1 level. This suggests that the reduced GA_1 level is not achieved through altered mRNA accumulation of GA 20-oxidase and GA 3β-hydroxylase (Ait-Ali et al. 1999). Instead, because the amount of GA_8 (the deactivated GA_1 by 2β-hydroxylation) increases transiently after irradiation, 2β-hydroxylation may be modulated to decrease the GA_1 level during deetiolation (Kamiya and Garcia-Martinez 1999). The elevated mRNA accumulation of GA 20-oxidase and GA 3β-hydroxylase could be a consequence of feedback up-regulation due to the reduced GA_1 level. Experiments using PHYA- and PHYB-deficient pea mutants indicated that both PHYA and PHYB are involved in the increased mRNA accumulation of GA 20-oxidase (Ait-Ali et al. 1999).

Manipulation of the pathway—Manipulation of the GA biosynthetic pathway is not only a target of genetic engineering to modify plants' stature, but also informative to understand the regulation of this pathway. For example, overproduction of individual enzymes in transgenic plants can test the influence of a particular biosynthetic step on modulating the levels of bioactive GAs, and the resulting plant growth.

Conversion of GGDP to CDP is a possible step limiting the flow of GA biosynthesis, because GGDP is a common substrate for other pathways (carotenoids and other diterpenes) and is likely to be present in a large pool. Also, the *GA1* gene (encoding CPS) is expressed at a very low level in specific cell types in *Arabidopsis*. Sun and Kamiya (1994) have generated transgenic *Arabidopsis* plants that accumulate the GA1 protein at a much higher level than WT does. However, these transgenic plants did not show a GA-overproducing phenotype. This result may be due to the regulation of the downstream pathway, including feedback inhibition of later biosynthetic steps and feed-forward induction of deactivating enzymes.

There is some evidence to suggest that GA 20-oxidase activity is limiting the concentration of bioactive GAs in *Arabidopsis* shoots. GA 20-oxidase mRNA levels in *Arabidopsis* are regulated by feedback inhibition, and one of the GA 20-oxidase genes, *GA5*, is photoperiodically modulated in stem (Xu et al. 1997). In shoots, GA_{24} and GA_{19}, the substrates for GA 20-oxidase, accumulate at a higher level relative to other GA intermediates. Recently, the effects of overexpression of GA 20-oxidase genes on

plant growth have been tested in transgenic *Arabidopsis* plants carrying the GA 20-oxidase cDNA driven by a strong constitutive promoter (Huang et al. 1998, Coles et al. 1999). These transgenic plants exhibit the GA-overdose phenotype, such as longer hypocotyl, earlier flowering and increased final stem height compared to WT. Coles et al. (1999) have shown that, in fact, the level of GA_4 (the major active GA) in rosettes is elevated in different transgenic lines, which demonstrates that GA 20-oxidase activity can modulate the levels of active GA in *Arabidopsis*.

Xu et al. (1999) has attempted to modify GA biosynthesis in *Arabidopsis* by overproducing a heterologous GA 20-oxidase (P16) from pumpkin endosperm. Unlike other GA 20-oxidases, the major products of P16 from GA_{12} are tricarboxylic GAs (GA_{17} and GA_{25}) (Lange et al. 1994), which are biologically inactive (Fig. 1). Therefore, overexpression of the *P16* gene is expected to reduce the concentration of bioactive GAs. As predicted, the transgenic lines accumulated GA_{17} and GA_{25}, and contained reduced GA_4 (20–30% of WT). However, stem height of these plants are either only slightly reduced or comparable to WT. The weak effect of P16 on plant growth is, at least in part, attributed to the feedback up-regulation of GA biosynthesis, as shown by elevated mRNA accumulation of endogenous GA 20-oxidase and 3β-hydroxylase in the transgenic plants (Xu et al. 1999).

Concluding remarks—The isolation of genomic and cDNA clones for GA biosynthesis enzymes has increased our understanding of the complex regulation of this pathway. Evidence is accumulating in a variety of plants that endogenous and environmental factors regulate mRNA levels of GA biosynthetic genes. Lange et al. (1997) reported that mRNA levels for GA dioxygenases in germinating pumpkin seeds did not always correlate with the enzyme activities, which points the importance of analyzing protein levels. Localization of early and late GA biosynthetic enzymes could demonstrate the possible transport of GAs between different tissues. Cellular localization of enzymes might be also crucial when other aspects of regulation (e.g. light) are investigated if only limited cells or tissues respond to the signal.

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