B2. Gibberellin Biosynthesis and Inactivation

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INTRODUCTION

The gibberellins (GAs[1]) are defined by chemical structure. Naturally-occurring tetracyclic diterpenoid acids with structures based on the *ent*-gibberellane carbon skeleton (Fig. 1) are assigned gibberellin "A numbers" in chronological order of their identification (45) (http://www.plant-hormones.info/gibberellin_nomenclature.htm). At the present time there are

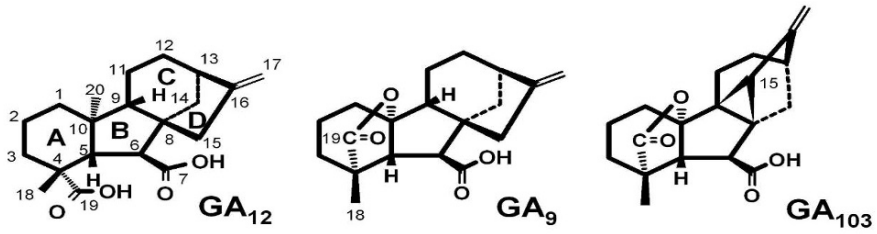

Figure 1. Structure of GA_{12}, a C_{20}-GA with the *ent*-gibberellane skeleton, showing the carbon atom numbering system and the assignment of the four rings. Also shown are GA_9, the simplest C_{19}-GA, which has an *ent*-20-norgibberellane skeleton, and GA_{103}, which has an extra cyclopropane ring.

[1] Abbreviations: BR, brassinosteroid; CDP-ME, 4-diphosphocytidyl-methylerythritol; CDP-MEP, CDP-ME 2-phosphate; CMK, CDP-ME kinase; CMS, CDP-ME synthase; CPS, *ent*-copalyl diphosphate synthase; DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; FPP, farnesyl diphosphate; GA, gibberellin; GA-Glc ester, GA-glucosyl ester; GA-*O*-Glc, GA-*O*-glucosyl ether; GA2ox, GA 2-oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 3-oxidase; GA_n, gibberellin A_n; GFP, green fluorescent protein; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; GUS, β-glucuronidase; HDS, HMBPP-synthase; HMBPP, hydroxymethyl-butenyl 4-diphosphate; IAA, indole 3-acetic acid; IDS, IPP/DMAPP synthase; IPP, *iso*pentenyl diphosphate; KAO, *ent*-kaurenoic acid oxidase; KO, *ent*-kaurene oxidase; KS, *ent*-kaurene synthase; MCS, ME-cPP synthase; MEP, methylerythritol 4-phosphate; ME-cPP, ME 2,4-cyclodiphosphate; MVA, mevalonic acid.

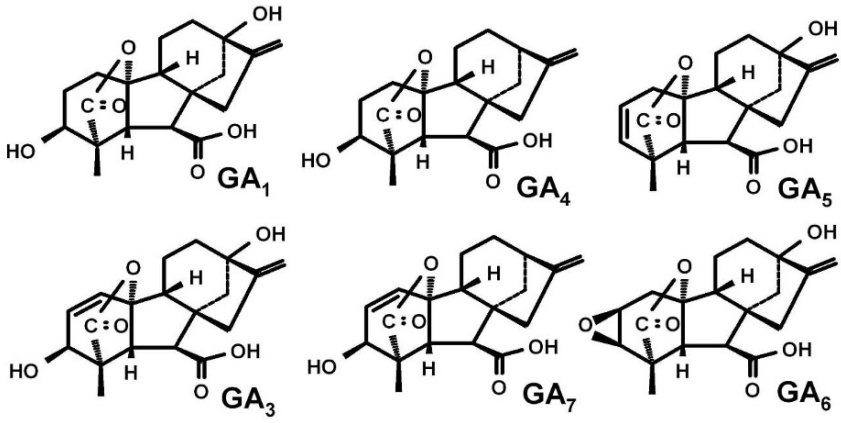

Figure 2. The structures of the main growth-active GAs.

136 fully characterized GAs, designated gibberellin A_1 (GA_1) through GA_{136}, that have been identified from 128 different species of vascular plants, and also from seven bacteria and seven fungi (44) (http://www.plant-hormones.info/ga1info.htm).

Gibberellins were first isolated from the pathogenic fungus *Gibberella fujikuroi* from which they derive their name (50). The presence of large quantities of GAs as secondary metabolites in this fungus leads to the extensive overgrowth of infected rice plants. Thus, from the time of their discovery GAs were known to be effective in promoting stem elongation and their characterization from the fungus was followed shortly by their identification as natural components of non-infected plants.

Gibberellins are now known to be regulators of many phases of higher plant development, including seed germination, stem growth, induction of flowering, pollen development and fruit growth. The concentration of bioactive GAs in plants is in the range 10^{-11} – 10^{-9} g/g fresh weight, depending on the tissue and species, and is closely regulated. This chapter describes the biosynthesis and catabolism of GAs, and examines the regulatory processes that optimize the levels of bioactive GAs within plant tissues.

Only a few of the 136 known GAs have intrinsic biological activity. Not surprisingly, many of the GAs that were identified in the earliest years of GA research are the ones which possess the highest biological activity, and are candidates for the role of an active hormone. These include GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7 (Fig. 2). The bioactive GA(s) present in a particular plant species is/are accompanied by a dozen or more GAs that are likely to be inactive precursors or deactivation products of the active forms. Experiments utilizing single gene dwarf mutants have provided convincing evidence that GA_1 is the major active GA for stem elongation in *Zea mays* and *Pisum sativum*. Moreover, GA_1 has been identified in 86 plants, more than any other GA, implying that it has an important and widespread role as a

GA "hormone." However, GA_4 co-occurs with GA_1 in many species and in some members of the Cucurbitaceae and in Arabidopsis, it is the major bioactive GA. Gibberellin A_3, which is also known as gibberellic acid, has been identified in 45 plants. It is the major GA accumulating in *G. fujikuroi*, from which it is produced commercially. Gibberellic acid is used to promote seed germination, stem elongation, and fruit growth in a variety of agronomically and horticulturally important plants.

By definition, GAs possess tetracyclic *ent*-gibberellane (C_{20}) or 20-nor-*ent*-gibberellane (C_{19}) skeletons, with the rings designated A through D as shown in Fig. 1. The C_{20}-GAs (e.g., GA_{12}, Fig. 1) have the full complement of 20 carbon atoms, whereas the C_{19}-GAs (e.g., GA_9, Fig. 1) possess only 19 carbon atoms, having lost carbon-20 by metabolism. In almost all the C_{19}-GAs the carboxyl at C-4 forms a lactone at C-10. In some cases, there is a bond between C-9 and C-15 to form an additional cyclopropane ring (e.g., GA_{103}, Fig. 1). Other structural modifications can be made to the *ent*-gibberellane skeleton of both C_{20}- and C_{19}-GAs, such as the insertion of additional functional groups. Both the position and stereochemistry of these substituents can have a profound effect on the biological activity of the GA. For example, a hydroxyl (OH) group in the 3β-position is required for growth-promoting activity, whereas the insertion of an OH in the 2β-position will substantially reduce the bioactivity of an active GA. In either instance the insertion of an OH in α-orientation has little effect.

The C_{20}-GAs do not normally have biological activity *per se*, but can be

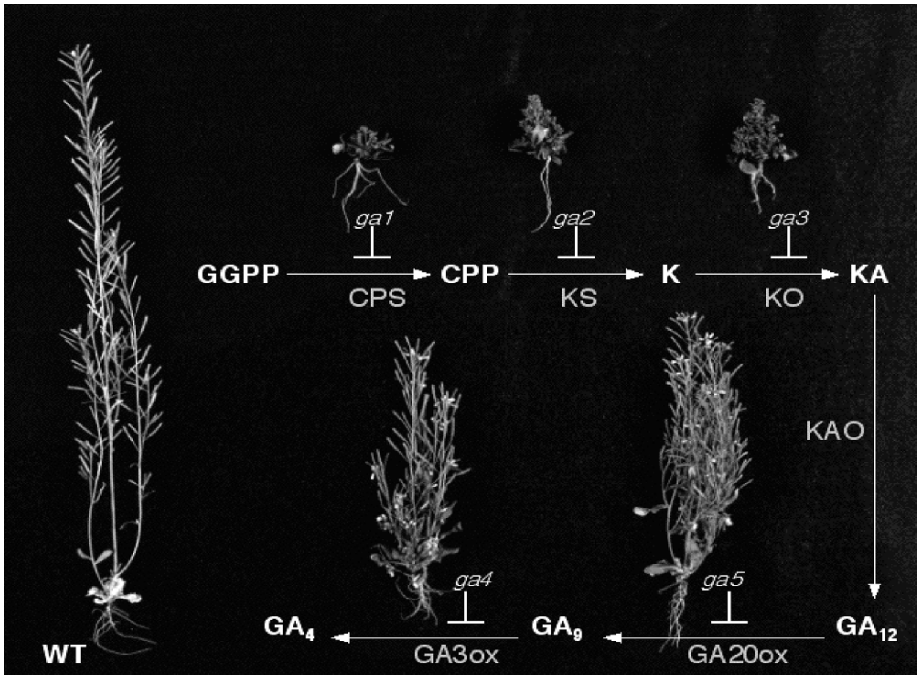

Figure 3**.** Gibberellin-responsive dwarf mutants of Arabidopsis (ecotype Landsberg *erecta*) showing the position of the lesion in the GA-biosynthetic pathway for each locus. (From 65) (©Academic Press).

metabolized to C_{19}-GAs that may be bioactive. Many assays determine bioactivity in terms of stem elongation in dwarf seedlings (rice, maize), or hypocotyl elongation in seedlings whose growth is inhibited by light (lettuce, cucumber). However, because an applied GA may be metabolized in the assay plant, the observation that it has bioactivity does not establish that it is active *per se*. More sophisticated studies, most often using mutants in which specific steps in GA metabolism are blocked, are required to assess intrinsic activity. For example, in mutant seedlings in which 3β-hydroxylation is blocked, applied GA_9 may be inactive, but applied GA_4 (3β-hydroxyGA_9) may elicit a growth response. From this result one can infer that GA_9 does not have activity *per se*, but must be metabolized to GA_4. A series of GA-biosynthetic mutants of Arabidopsis with lesions at different points of the pathway (Fig. 3) has been extremely valuable in elucidating the genetics of GA-biosynthesis in this species. As discussed later, the different severities of dwarfism for mutations at each locus is due to the different sizes of gene families for each enzyme.

The requirements for intrinsic biological activity for growth stimulation are that it is a C_{19}-GA, with a 4,10 lactone and a carboxylic acid group on C-6, that it possesses a 3β-hydroxyl group, or some other functionalization at C-3, such as a 2, 3-double bond (as in GA_5) and that it does not possess a 2β-hydroxyl group. All the GAs shown in Fig. 2 fulfill these requirements. Since 2β-hydroxylation is a deactivating mechanism, a GA that has some functionality at C-2 that prevents 2β-hydroxylation may have enhanced activity. Thus GA_3 and GA_7, which possess a 1, 2 double bond, are not substrates for the 2β-hydroxylating enzyme and have longer lasting activity than their 1, 2-dihydro counterparts (GA_1 and GA_4). There is evidence that the structural requirements for florigenic activity may be subtly different than those for stem elongation. For example, GA_5 and GA_6 are more active than GA_1 and GA_4 in enhancing flowering in *Lolium*, whereas GA_1 and GA_4 show enhanced activity over GA_5 and GA_6 in promoting stem growth in this genus (37).

THE BIOSYNTHETIC PATHWAY

As diterpenoids, GAs are synthesized from geranylgeranyl diphosphate (GGPP) *via* isopentenyl diphosphate (IPP), which is the 5-carbon building block for all terpenoid/isoprenoid compounds (Fig. 4). The GA-biosynthetic pathway can be divided into three parts. The first part, which occurs in plastids, leads to the synthesis of the tetracyclic hydrocarbon, *ent*-kaurene (Figs. 5 and 6). In the second part of the pathway, which occurs in the endoplasmic reticulum, *ent*-kaurene is sequentially oxidized to yield the first-formed GA, GA_{12} and its 13-hydroxylated analog GA_{53} (Fig. 7). In the third part of the pathway, which occurs in the cytosol, GA_{12} and GA_{53} are further oxidized to other C_{20}-GAs, and C_{19}-GAs (Fig. 9).

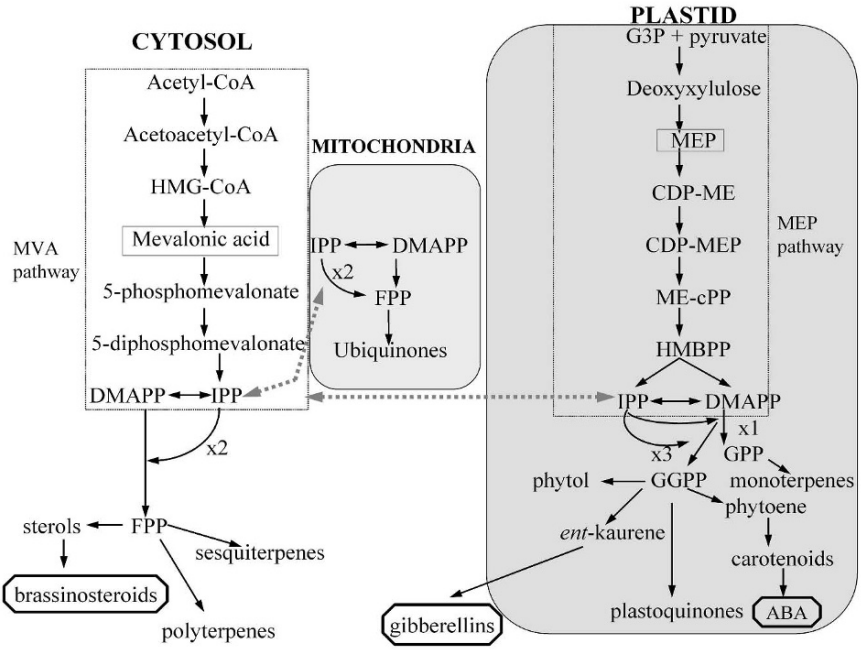

Figure 4. Pathways for isoprenoid biosynthesis and their compartmentalization in plant cells, showing the origin of plant hormone and other products.

The Pathway to *ent*-Kaurene.

For four decades IPP was thought to be formed exclusively *via* the acetate/mevalonate (MVA) pathway. Since the last edition of this book a novel biosynthetic route to IPP has been elucidated (55). The new pathway has been referred to in the literature by several names: the non-mevalonate, the Rohmer (after one of its discoverers), the deoxyxylulose phosphate (DXP) and the methylerythritol phosphate (MEP) pathway. The latter name has gained most recent acceptance, since MEP is considered to be the first committed intermediate in the pathway. The MEP pathway was first characterized in Eubacteria, and is now known to occur in plastids of eukaryotic organisms.

While most organisms possess either the MVA pathway (fungi and animals) or the MEP pathway (most eubacteria and many algae), plants and their immediate algal ancestors, the Charophyceae, possess both pathways. The two pathways exist in different cellular compartments and the resulting IPP has different fates (Fig. 4). In plants the MVA pathway is cytosolic, and IPP formed by this route is further metabolized to sesqui- (C_{15}), and tri-terpenoids (C_{30}), including sterols. In contrast, the MEP pathway is plastidic, and IPP formed in plastids is converted to monoterpenes (C_{10}), diterpenes

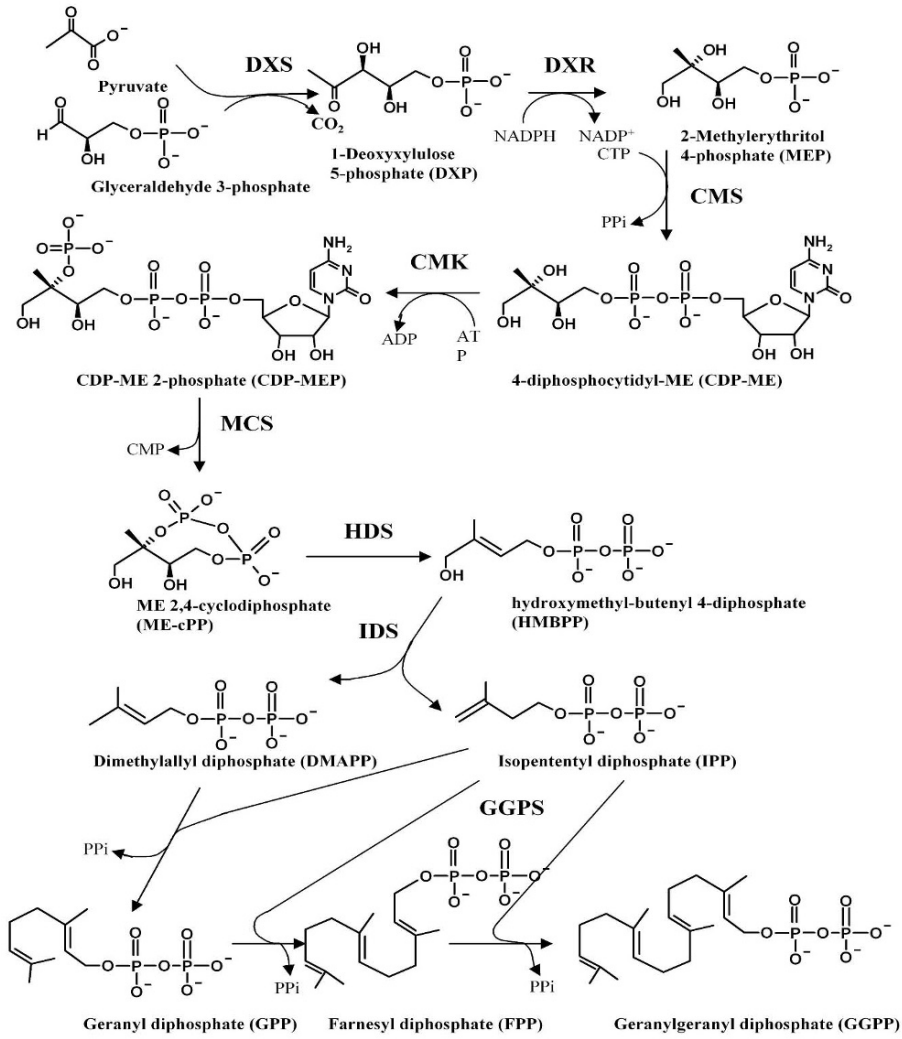

Figure 5**.** Biosynthesis of geranylgeranyl diphosphate (GGPP) in plastids *via* the MEP pathway. Enzymes in biosynthetic sequence are: DXS, deoxyxylulose 5-phosphate synthase; DXR, deoxyxylulose 5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl methylerythritol synthase; CMK, 4-diphosphocytidyl methylerythritol kinase; MCS, methylerythritol 2,4-cyclodiphosphate synthase; HDS, hydroxymethyl-butenyl 4-diphosphate synthase; IDS, isopentenyl diphosphate/dimethylallyl diphosphate synthase; GGPS, GGPP synthase.

(C_{20}), including GAs and the phytyl side-chain of chlorophyll, and tetraterpenes (C_{40}), including carotenoids. The co-occurrence and compartmentation of the two pathways is unique to plants and their immediate ancestors. Thus in fungi the MVA pathway provides IPP for synthesis of all terpenoids, including GAs. Although there was considerable

indirect evidence to suggest that the MEP pathway provides the IPP for GA biosynthesis in plants (47, 64), this has now been confirmed by feeding studies (34). This latter study also addressed the question of whether MEP is the only source of IPP for GA biosynthesis, or whether there is cross-over of one or more intermediates from the MVA pathway.

The MEP pathway for the production of *trans*-geranylgeranyl diphosphate (GGPP) in plastids is shown in Fig. 5, and its elucidation in Eubacteria is described in several reviews (12, 55). Briefly, a two-carbon unit (derived by decarboxylation of pyruvate) is added to glyceraldehyde 3-phosphate to give deoxyxylulose 5-phosphate (DXP). The condensation is catalyzed by DXP synthase (DXS), which requires thiamin diphosphate, indicating a transketolase type reaction. Although this is not the first committed reaction in the pathway, for DXP is also a precursor of thiamine, it is known to be a regulated step (see below). The next reaction, catalyzed by DXP reductoisomerase (DXR), involves the reduction and intramolecular rearrangement of the linear DXP to give the branched structure, methylerythritol 4-phosphate (MEP), which is subsequently converted to ME 2,4-cyclodiphosphate (ME-cPP) in three stages: the formation of 4-diphosphocytidyl-ME (CDP-ME), catalyzed by CDP-ME synthase (CMS), ATP- dependent phosphorylation of CDP-ME to CDP-ME 2-phosphate (CDP-MEP), catalyzed by CDP-ME kinase (CMK), and then conversion of CDP-MEP to ME-cPP with elimination of CMP in a reaction catalyzed by ME-cPP synthase (MCS). Reductive ring opening of ME-cPP to give hydroxymethyl-butenyl 4-diphosphate (HMBPP) is catalyzed by HMBPP-synthase (HDS). The final step in the pathway yields both IPP and its isomer dimethylallyl diphosphate (DMAPP), in a reaction catalyzed by IPP/DMAPP synthase (IDS). In *E.coli* the products, both of which are required for subsequent isoprenoid synthesis, are formed in a 5 to 1 ratio (56). This branch-point in the MEP pathway is in contrast to the MVA pathway in which DMAPP is formed from IPP, by the action of IPP isomerase. Analysis of this final step in the MEP pathway in plants is eagerly awaited.

Although the enzymes in the MEP pathway, and genes encoding them, were first identified in *E. coli*, orthologous genes and expressed sequence tags for all the enzymes in this pathway have now been found in Arabidopsis databases (e.g., DXS: (14); DXR: (62); MCS: (57); HDS: (51)). The enzymes have also been studied in peppermint leaves, which synthesize high levels of monoterpenes, and fruits of tomato and pepper, which are excellent systems for studying carotenoid biosynthesis. All the plant enzymes are encoded by nuclear genes, possess N-terminal sequences for putative plastid targeting, and conserved cleavage motifs. Direct plastid targeting has been demonstrated for DXS (3, 43), DXR (9) and HDS (formerly known as GCPE) (51). Cleavage of the signal peptides give enzymes similar to the bacterial enzymes except in the case of HDS, which contains a large plant-specific domain of unknown function (51).

DXP synthase is rate-limiting for IPP synthesis in all systems studied (13, 43). In Arabidopsis it is encoded by *CLA1* (chloroplastos alterados or

altered chloroplasts), mutations in which result in an albino phenotype with very low levels of chlorophyll and carotenoids. Feeding of ^{13}C DXP to *cla1* mutants led to the identification of labeled *ent*-kaurene and GA_{12}, with very little dilution, confirming, for the first time, that the MEP pathway is the major source of IPP for GA biosynthesis (34). Despite the severe phenotype of *cla1* mutants there are two other *CLA* homologs in Arabidopsis. Both have plastid targeting sequences, but whether they encode functional proteins is not yet known. Other enzymes in the MEP pathway are encoded by single-copy genes in the Arabidopsis genome. T-DNA insertional mutants of DXR and CMS have seedling-lethal albino phenotypes (8). DXR is competitively inhibited by fosmidomycin, an antibiotic from *Streptomyces lavedulae* that acts as an effective herbicide (38).

The conversion of MEP-derived IPP and DMAPP to isoprenoids proceeds in the plastids, and is catalyzed by prenyl transferases. Repetitive addition of IPP to DMAPP in a head-to-tail fashion gives geranyl diphosphate (GPP), the C_{10} precursor of all monoterpenes, and GGPP. Both GPP synthase and GGPP synthase have been characterized from several plant sources, and shown to be plastid localized (7). In Arabidopsis GGPP synthases are encoded by a small family of five nuclear genes, the products of which are targeted to specific subcellular locations (48). Two of them, GGPS-1 and GGPS-3, become plastid localized and catalyze the formation of GGPP for use in plastid pathways (see below).

At GGPP the pathway in plastids branches in several directions, with separate pathways leading to the *ent*-kaurenoids and GAs, the phytyl side-chain of chlorophyll, phytoene and carotenoids, and the nonaprenyl (C_{45}) side-chain of plastoquinone (Fig. 4). It should be noted any regulation in the MEP pathway before GGPP will affect all branches, which is why *cla1*, *dxr* and *cms* mutants have albino, seedling-lethal phenotypes, and why fosmidomycin, which blocks DXS, is a herbicide. Also it should be remembered that since there is a shared precursor pool of GGPP, manipulation of one of the branch pathways can have significant effects on the flux through other branches. Thus, up- and down-regulation of phytoene synthase (*PSY*) to increase and decrease carotenoid biosynthesis causes reciprocal changes in GA levels (16).

Cyclization of the linear GGPP to the tetracyclic *ent*-kaurene occurs in two stages (Fig. 6). GGPP is converted first to the bicyclic compound, *ent*-copalyl diphosphate, by *ent*-copalyl diphosphate synthase (CPS), which was

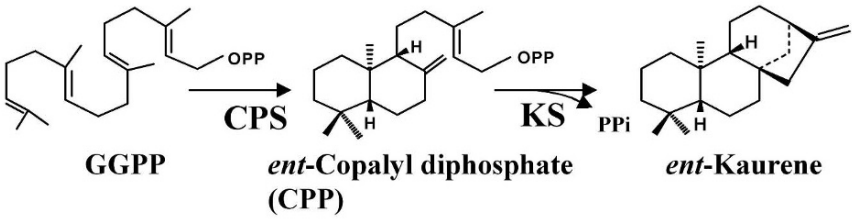

Figure 6. Two-step cyclization of GGPP to *ent*-kaurene *via ent*-copalyl diphosphate, catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS).

previously called *ent*-kaurene synthase A. In Arabidopsis CPS is encoded by the *GA1* gene, which is present as a single-copy. However, *ga1* null mutants, although very severely dwarfed (Fig. 3), contain traces of GA, suggesting either the presence of an additional unrecognized *CPS* gene in the Arabidopsis genome, or an additional minor pathway to GA. *ent*-Copalyl diphosphate is converted to *ent*-kaurene by *ent*-kaurene synthase (KS) previously called *ent*-kaurene synthase B. KS, like CPS, is encoded by a single-copy gene (*GA2*) in Arabidopsis, mutations in which cause severe dwarfism (Fig. 3). The enzyme has been shown to be localized in plastids in Arabidopsis (30). *Stevia rebaudiana*, which produces large quantities of stevioside (glycosides of 13-hydroxykaurenoic acid) in mature leaves, has two *KS* gene copies (54). It has been suggested that gene duplication and differential regulation allows for tightly-regulated GA biosynthesis in rapidly dividing tissues, and the much greater flux to stevioside in mature leaves.

In the fungi, *G. fujikuroi* and *Phaeosphaeria* spp., CPS and KS activities reside in the same protein, though at separate catalytic sites (35). In plants, the occurrence of two separate proteins gives more opportunity for independent regulation of the two activities than exists in the fungi. Thus *CPS* transcript levels are much lower than those of *KS* in Arabidopsis, indicating that *CPS* may have a "gate-keeper" function on the branch of the terpenoid pathway that is committed to *ent*-kaurenoids and GAs.

Conversion of *ent*-Kaurene to GA_{12} and GA_{53}

All metabolic steps after *ent*-kaurene are oxidative (26). Initially, *ent*-kaurene is converted by a membrane-associated cytochrome P450 monooxygenase, *ent*-kaurene oxidase (KO), to *ent*-kaurenoic acid, which is oxidized by a second P450, *ent*-kaurenoic acid oxidase (KAO), to GA_{12}. The formation of GA_{12} from *ent*-kaurene requires six steps, the two enzymes involved each catalyzing three reactions (Fig. 7): KO catalyzes the sequential oxidation of the C-19 methyl group of *ent*-kaurene *via* the alcohol and aldehyde to the carboxylic acid, while KAO oxidizes C-7 of *ent*-kaurenoic acid to produce *ent*-7α-hydroxykaurenoic acid[2], which is then oxidized by this enzyme on C-6 to form GA_{12}-aldehyde. Finally, KAO oxidizes GA_{12}-aldehyde on C-7 to produce GA_{12}. The conversion of *ent*-7α-hydroxykaurenoic acid to GA_{12}-aldehyde involves the contraction of ring B from six C atoms to five, thereby transforming the *ent*-kaurane carbon skeleton to the *ent*-gibberellane structure. Arabidopsis contains a single *KO* gene (*GA3*) and two *KAO* genes. Whereas loss-of-function *ga3* mutants are severely dwarfed (Fig. 3) and, in common with the *ga1* and *ga2* null mutants, are male sterile, and need GA treatment to produce seeds that themselves do not germinate unless treated with GA, no *kao* mutants have been identified in

[2] Using IUPAC nomenclature the precursor of GAs is the enantiomeric form of kaurene, designated *ent*-kaurene. By convention α-substituents are designated *ent*-β, and β-substituents are designated *ent*-α.

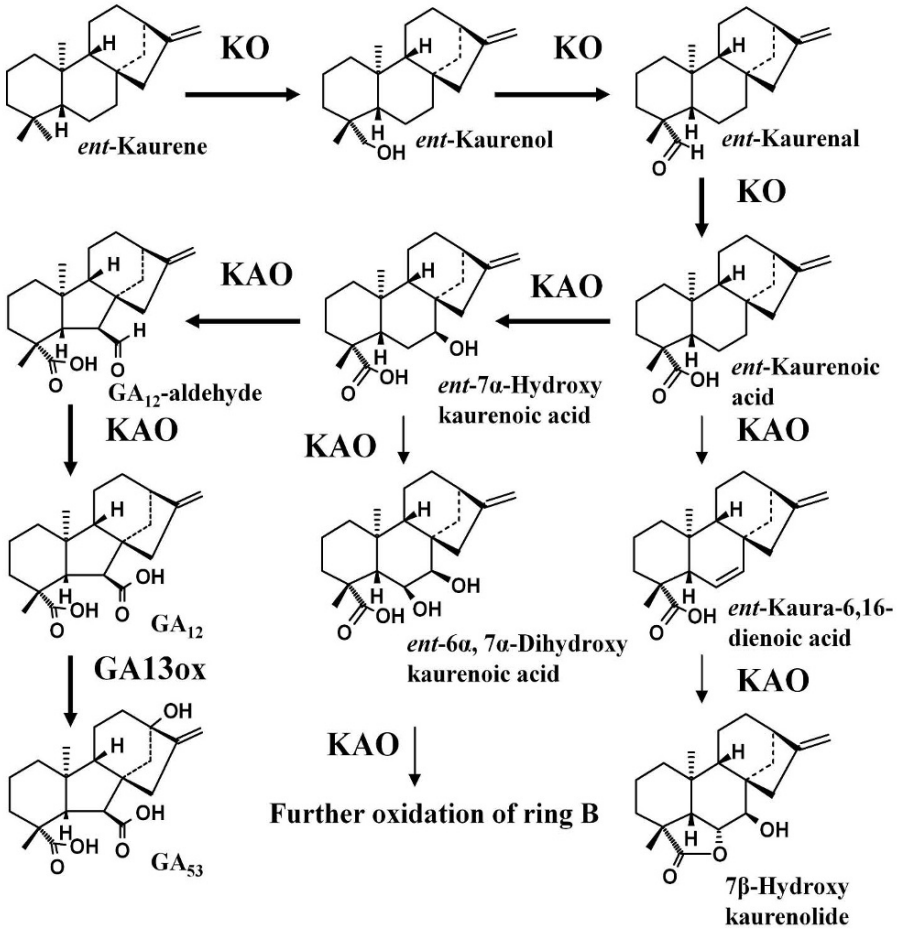

Figure 7. Reactions catalyzed by cytochrome P450-dependent mono-oxygenases. GA_{12} is formed from *ent*-kaurene by the sequential action of *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). GA_{12} is converted to GA_{53} by 13-hydroxylases (GA13ox). Also shown are side reactions of KAO noted in some systems that result in the formation of kaurenolides and seco ring B compounds (in which ring B has been oxidatively cleaved between C-6 and C-7).

Arabidopsis because the two genes are fully redundant with the same expression patterns (29). However, *kao* mutants of barley (29) and pea (11) are GA-deficient dwarfs since most of the KAO activity present in vegetative tissues of these plants is due to expression of single genes. Pea has a second *KAO* gene, which is expressed in developing seeds (11).

GA_{12} is the common precursor for all GAs in higher plants. It lies at a branch-point in the pathway, undergoing either oxidation at C-20, or hydroxylation on C-13 to produce GA_{53}. GA_{12} and GA_{53} are precursors for the so-called non-13-hydroxylation and 13-hydroxylation pathways, respectively, which will be discussed in the following section. Genes

encoding 13-hydroxylases have not yet been identified and there is still some uncertainty about the nature of this enzyme, with evidence suggesting the involvement of P450s in developing and germinating seed tissues (32, 39), but of a soluble dioxygenase in vegetative tissues of spinach (19).

Experiments using subcellular fractionation (22) and transient expression of enzyme fused to green fluorescent protein (GFP) (30)(see later) show that KAO is located in the endoplasmic reticulum (Fig. 8). Work with KO-GFP fusions transiently expressed in tobacco leaves indicate that KO is located on the outer membrane of the plastid (Fig. 8) and may thus participate in the translocation of *ent*-kaurene from its site of synthesis in plastids to the endoplasmic reticulum (30). However, as is discussed in more detail later, other experiments based on the use of reporter genes and *in situ* hybridisation suggest that *ent*-kaurene synthesis and oxidation may occur in different cell types in young germinating Arabidopsis embryos (73). Movement of the highly hydrophobic *ent*-kaurene between cells would require the assistance of a carrier protein. The discrepancy between these results may be due to the different experimental systems used, but clearly more work is required to resolve this disparity.

The fungus *G. fujikuroi* contains P450 monooxygenases that are functionally equivalent to the plant KO and KAO, although they have very low sequence similarity with the plant enzymes and are probably not closely related in evolutionary terms (28). The fungal *ent*-kaurene oxidase (P450-4) catalyzes the same reactions as KO, while the KAO equivalent possesses 3β-hydroxylase activity in addition to the other activities, such that it converts *ent*-kaurenoic acid to GA_{14} (3β-hydroxyGA_{12}). The substrate for 3β-hydroxylation is GA_{12}-aldehyde, which is converted to GA_{14} *via* GA_{14}-aldehyde. This remarkably multifunctional enzyme is also responsible for the formation of kaurenolides, *via ent*-kaura-6, 16-dienoic acid, and of seco-ring B compounds, such as fujenal, *via ent*-6α, 7α-dihydroxykaurenoic acid. Kaurenolides and *ent*-6α, 7α-dihydroxykaurenoic acid are also by-products of KAOs from pumpkin (23) and pea (11), but these enzymes do not have 3β-hydroxylase activity.

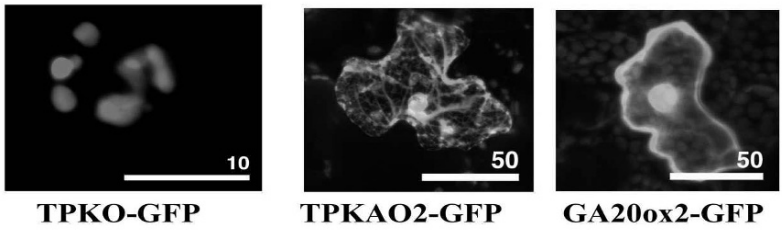

Fig. 8 (Color plate page CP3). Confocal images of tobacco leaves after microprojectile bombardment with the following constructs: TPKO-GFP, N-terminal region of Arabidopsis *ent*-kaurene oxidase (AtKO) fused to green fluorescent protein (GFP); TPKAO2-GFP, N-terminal region of *ent*-kaurenoic acid oxidase AtKAO2 fused to GFP; GA20ox2-GFP, full coding region of an Arabidopsis GA 20-oxidase (AtGA20ox2) fused to GFP. TPKO-GFP is associated with plastids, while TPKAO2-GFP is associated with the endoplasmic reticulum and GA20ox2-GFP is in the cytosol. Images are modified from (30).

Conversion of GA_{12} and GA_{53} to C_{19}-GAs

Reactions in the third part of the biosynthetic pathway, illustrated in Fig. 9, are catalyzed by soluble 2-oxoglutarate-dependent dioxygenases. The first enzyme, GA 20-oxidase (GA20ox), is responsible for the removal of C-20 in the formation of the C_{19}-GA skeleton. GA_{12} and GA_{53} are converted by this enzyme in parallel pathways to GA_9 and GA_{20}, respectively, by sequential oxidation of C-20 to the alcohol and aldehyde, and then removal of this C atom with formation of the 4, 10-lactone. Each of the C_{20}-GA intermediates in the reaction sequence is converted by GA20ox, although the alcohol intermediate must be present as the free alcohol. These alcohol intermediates form δ-lactones with the 19-carboxyl group when extracted from plant tissues and are then no longer oxidized by GA20ox. It is unclear whether the δ-lactones form naturally *in planta*, but it is of interest that vegetative tissues, but apparently not seeds, contain an enzyme capable of converted the δ-lactones to the aldehydes (71). This enzyme may serve to ensure formation of C_{19}-GAs when GA20ox activity is low, as in vegetative tissues, when lactone formation may compete with further oxidation of the alcohol intermediate. In most systems C_{20}-GAs containing a 20-carboxylic acid group are formed by GA20ox as minor biologically inactive by-products, which are not converted to C_{19}-GAs. However, these tricarboxylic acid GAs are major products of the GA20ox present in endosperm and immature embryos of *C. maxima* that was the first *GA20ox* to be cloned (40). The function of this pumpkin enzyme is considered abnormal as this type of activity has not been encountered in other species or, indeed, in vegetative tissues of pumpkin. The chemical mechanism for the loss of C-20 has not been elucidated. There is evidence that it is lost as CO_2 and that both O atoms in the lactone function originate from the 19-carboxyl group. Direct removal of C-20 as CO_2 requires the formation of an intermediate between the aldehyde and final C_{19}-GA product, but none has been identified and it may remain bound to the enzyme.

The growth-active GAs GA_4 and GA_1 are formed by 3β-hydroxylation of GA_9 and GA_{20}, respectively, catalyzed by GA 3-oxidases (GA3ox). The major GA3ox of Arabidopsis (AtGA3ox1) is highly regiospecific, producing a single product, while enzymes from certain other species also oxidize neighboring C atoms to a small extent (26). For example, oxidation of both C-2 and C-3 produces a 2, 3-double bond, as in the conversion of GA_{20} to GA_5. Further oxidation of GA_5, initially on C-1 and then on C-3, by the same enzyme results in the formation of GA_3 (2, 66). While most GA 3-oxidases are specific for C_{19}-GAs, some plants, and particularly seeds, produce 3β-hydroxylated C_{20}-GAs. For example, an enzyme from pumpkin endosperm 3β-hydroxylates C_{20}-GAs more readily than C_{19}-GAs (41).

A third class of dioxygenase, GA 2-oxidase (GA2ox), is responsible for the irreversible deactivation of GAs by 2β-hydroxylation, so ensuring GA turnover, which is necessary for effective regulation of GA concentration. In some tissues, such as the cotyledons and, particularly, the testae of

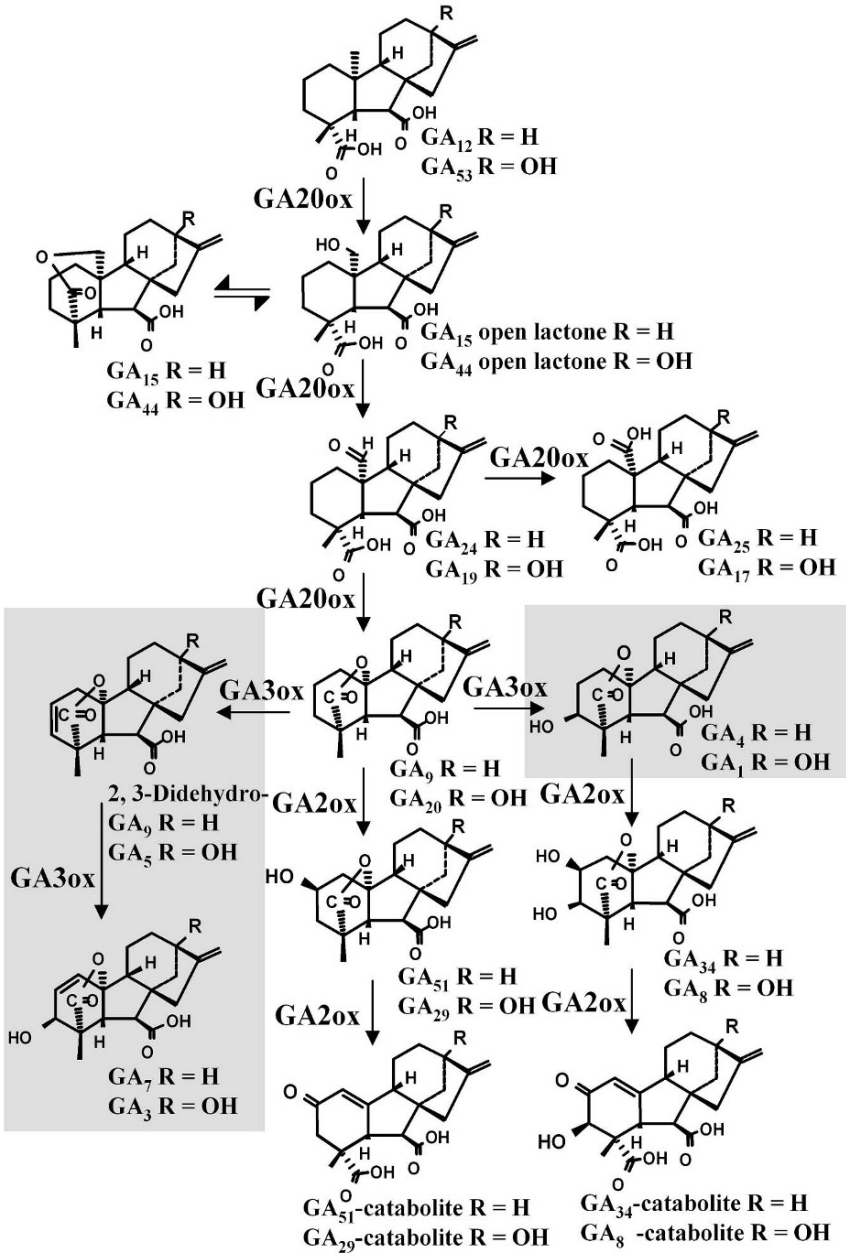

Figure 9**.** Reactions catalyzed by soluble 2-oxoglutarate-dependent dioxygenases. GA_{12} and GA_{53} are converted in parallel pathways to the active GAs, GA_4 and GA_1, respectively, by the sequential action of GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox). The tricarboxylic acid GAs GA_{25} and GA_{17} are bi-products of GA 20-oxidases, while 2, 3-didehydroGA_9/GA_5 and GA_7/GA_3 are by-products of some GA 3-oxidases. Gibberellin 2-oxidases (GA2ox) act mainly on C_{19}-GAs to form inactive products. The shaded regions indicate GAs with biological activity.

developing pea seeds, C-2 is oxidized further to the ketone, giving rise to the so-called GA-catabolites. As detected by GC-MS, the catabolites appear as dicarboxylic acids in which the lactone has opened (Fig. 9), although this rearrangement is possibly an artefact occurring during the analytical procedure. Studies of GA2ox function using recombinant protein prepared in *E. coli* reveal that these enzymes are capable of both reactions, 2β-hydroxylation and ketone formation (67). However, ketone production is relatively inefficient and occurs only when enzyme levels are very high, as in pea seeds. Most 2-oxidases are specific for C_{19}-GAs, and will accept the 3β-hydroxy bioactive GAs and their non-3β-hydroxylated precursors as substrates (Fig. 9). C_{20}-GAs may also be 2β-hydroxylated and Arabidopsis has been shown to contain GA 2-oxidases that are specific for these compounds (61). Such enzymes may be important for maintenance of GA homeostasis (see later) when levels of C_{20}-GA precursors become very high.

In contrast to the enzymes responsible for the earlier steps of the pathway that are encoded by single or small numbers of genes, the dioxygenases are encoded by multigene families, members of which differ in their positional and temporal patterns of expression. The phylogenetic relationship between the Arabidopsis *GA20ox*, *GA3ox* and *GA2ox* genes that have been identified on the basis of their derived amino acid sequences are shown in Fig. 10. Five *GA20ox* and four *GA3ox* genes have been identified. So far mutant phenotypes have been recognized only for *AtGA20ox1* (*ga5*) and *AtGA3ox1* (*ga4)*, in both cases the mutants growing as semi-dwarfs (Fig. 3) with normal seed germination and flower fertility. There is, therefore, partial redundancy between the gene family members, which may have overlapping expression or, alternatively, GA products from other family members may move between tissues leading to partial rescue.

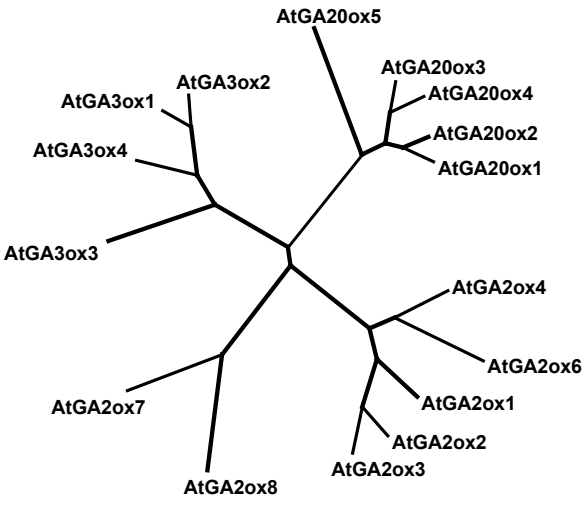

Figure 10. Unrooted phylogenetic tree of the Arabidopsis GA dioxygenases, produced using the Phylip programs PROTDIST and NEIGHBOR and displayed using TREEVIEW.

In Arabidopsis there are two classes of *GA2ox* genes (Fig. 10). One class contains six genes (*AtGA2ox1–6*), although one of these (*AtGA2ox5*) contains a large DNA insert and is apparently not expressed. Functional analysis of the expressed genes indicate that they are all C_{19}-GA 2-oxidases, whereas two further genes (*AtGA2ox7 and*

-*8*) encode enzymes that are specific for C_{20}-GAs (61). These last two genes, which were identified by activation tagging, are not closely related to the other 2-oxidases and their function could not have been predicted on the basis of sequence. It is, therefore, possible that further dioxygenses involved in GA biosynthesis in Arabidopsis have yet to be discovered.

Dioxygenases are not utilized for GA biosynthesis by *G. fujikuroi*, in which the GA 20-oxidase that converts GA_{14} to GA_4 is a P450 monooxygenase (28). Although the potential C_{20}-GA intermediates in this conversion are present in low levels in fungal cultures they are not metabolised to C_{19}-GAs so, in contrast to plants, the reaction sequence is unclear. In the next step in the fungal pathway, GA_4 is converted to GA_7 by a desaturase that has little homology to known enzymes (68). Finally, another P450 catalyzes the 13-hydroxylation of GA_7 to GA_3 (68). In contrast to plants, *G. fujikuroi* does not deactivate GAs by 2β-hydroxylation or conjugate formation (see below). Deactivation would be unnecessary since GAs have no physiological function in the fungus, but may act on the host by inducing the production of hydrolytic enzymes to aid infection or facilitate the acquisition of nutrients (28). A physiological role for the fungal GAs on the host rather than the fungus itself is supported by the large amounts of active GAs produced and their efficient secretion by the fungus.

Gibberellin Conjugates

In order to reduce the pool of bioactive GA, GAs can either be deactivated by 2β-hydroxylation and further catabolism, as described above, or they can be converted into conjugates (60). Conjugation to glucose is found most commonly for GAs and this can occur either *via* a hydroxyl group to give a GA-*O*-glucosyl ether (GA-*O*-Glc), or *via* the 7-carboxyl group to give a GA-glucosyl ester (GA-Glc ester). The most common sites within the GA molecule for –*O*-glc conjugation are C-2, C-3 and C-13. When applied to bioassay plants GA-*O*-Glcs show little or no activity, whereas GA-glc esters can exhibit bioactivity in certain assays, although this is unlikely to be activity of the conjugate *per se*. Instead it appears that if a bioassay plant, or microbial contaminant of the plant, possesses the requisite hydrolytic enzyme to cleave the glucose moiety and if the resulting aglycone is a potentially active GA, then the GA-conjugate will appear to have bioactivity. Feeding studies suggest that GA-Glc-esters sequester bioactive GAs, often quite rapidly, and release the free GA as required. On the other hand, the fate of GA-*O*-Glcs appears to be determined by the nature of the parent GA. GA-2-*O*-glcs, upon hydrolysis, will yield inactive GAs, whereas GA-3-*O*-glcs are hydrolyzed to bioactive GAs. Thus the enzymes for the synthesis and hydrolysis of GA-3-*O*-glcs have higher specificity than those catalyzing the hydrolysis of GA 2-*O*-glcs, reflecting their more direct role in maintaining the pool size of active GA.

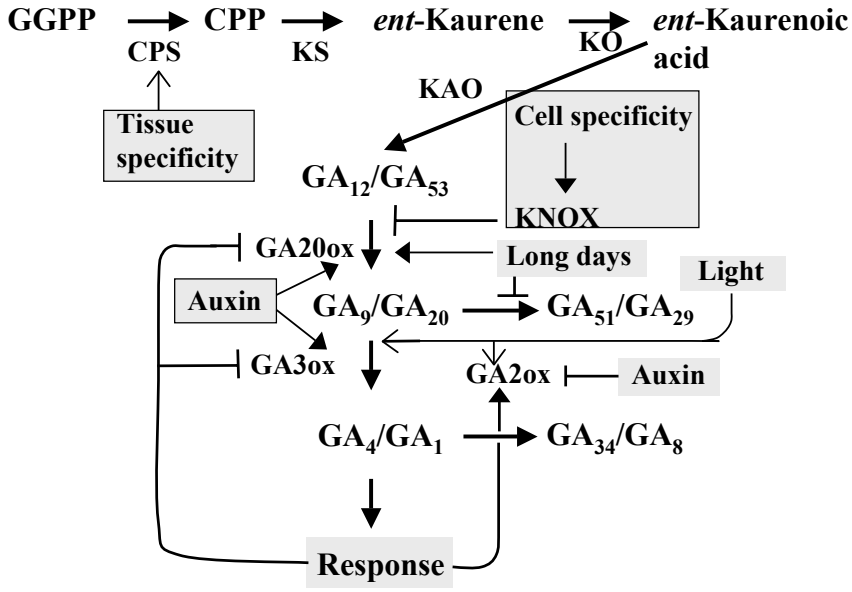

Figure 11. Major points of regulation of GA biosynthesis. Environmental or endogenous stimuli that modify expression of GA-biosynthetic genes are shaded. Solid arrows (→) indicate positive regulation (stimulation of gene expression), while bars (⟝) show negative regulation (suppression of gene expression). Open arrows (→) indicate that regulation can be positive or negative depending on the tissue or the nature of the signal.

REGULATION OF GA BIOSYNTHESIS AND CATABOLISM

With the identification of most of the genes involved in GA biosynthesis and catabolism, considerable advances are being made in our understanding of their tissue/cell specificity and their regulation by environmental factors, such as light and temperature. Furthermore, it is becoming evident that not only is GA metabolism moderated by its own action in order to achieve homeostasis, but that GAs participate in complex interactions with other hormones. The main sites of regulation in the GA-biosynthetic pathway are indicated in Fig. 11.

Developmental Regulation – Sites of GA Biosynthesis

Many different methods of assessing sites of GA biosynthesis have been used. The demonstration that all reactions in GA biosynthesis can be demonstrated in cell-free systems from seeds or seed parts (for instance liquid endosperm of Cucurbits) provided definitive evidence that developing seeds are sites of GA biosynthesis. However, GA biosynthesis in seeds is stage-specific. A surge in GA biosynthesis accompanies and is necessary for seed and early fruit growth, whereas studies with inhibitors of GA

biosynthesis has shown that GAs that accumulate to high levels later in seed maturation may not have a physiological role (17).

For vegetative tissues, studies using cell-free systems have provided evidence that rapidly growing regions of the plant are sites of GA biosynthesis. For instance, *ent*-kaurene synthesizing activity was demonstrated only in immature plastids in plant parts undergoing active growth, rather than in mature chloroplasts (1). These and other studies have raised the possibility that GA biosynthesis and action occur in the same tissues, and perhaps even in the same cells. This contention is contrary to the classical definition of a hormone, which states that its synthesis and action are remote from one another. Certainly, long distance transport of GAs has been reported in some systems, but a wealth of new information is providing strong evidence for GA biosynthesis and action occurring in close proximity.

The advent of reporter genes and their use to localize the expression of genes encoding enzymes in GA biosynthesis has provided the technology to study GA metabolism in individual tissues and cells. In these experiments a reporter gene encoding, for example, β-glucuronidase (GUS) or GFP is fused to the promoter and regulatory sequences of the gene of interest. Use of promoter-reporter gene fusions has been instrumental in not only identifying sequences necessary for tissue- or developmentally-specific expression, but in defining the sites and stages that the expression occurs. In several instances it appears that the sites of GA synthesis and action are close-by each other, or may even be coincident. Two comprehensive series of studies, one using Arabidopsis and the other rice, have been selected from the considerable literature on this subject, and are described below.

In Arabidopsis, expression of *GA1*, encoding CPS, was observed in shoot apices, root tips, anthers and immature seeds (63). Importantly, these sites of *GA1* expression in wild-type plants coincide with sites where the *ga1* mutant phenotype is evident. Thus, Silverstone *et al*. (63) concluded that CPS is present in tissues and cells that are sites of GA action. This work has now been extended to look at the sites of expression of several other genes coding for enzymes of GA biosynthesis (73). In addition to CPS this study includes AtKO, encoded by the *GA3* gene in Arabidopsis, and two different 3β-hydroxylases, AtGA3ox1 (encoded by *GA4*) and AtGA3ox2. Interestingly within germinating seeds the gene encoding CPS is expressed in the provasculature, whereas those encoding KO, AtGA3ox1 and AtGA3ox2 are expressed in the cortex and endodermis of the embryonic axis (Fig. 12). The results imply that an intermediate, most likely *ent*-kaurene is transported intercellularly from the provasculature to the endodermis and cortex during GA biosynthesis, which, given the hydrophobic nature of *ent*-kaurene, would presumably require the assistance of a carrier (as previously discussed). Since cortical cells expand on GA treatment it was assumed that the *ent*-kaurene oxidation and 3β-hydroxylation occur at or close to the site of GA action. Following on from this work Ogawa *et al.* (46) used *in situ* hybridization to study the site(s) of GA response, by examining the cellular

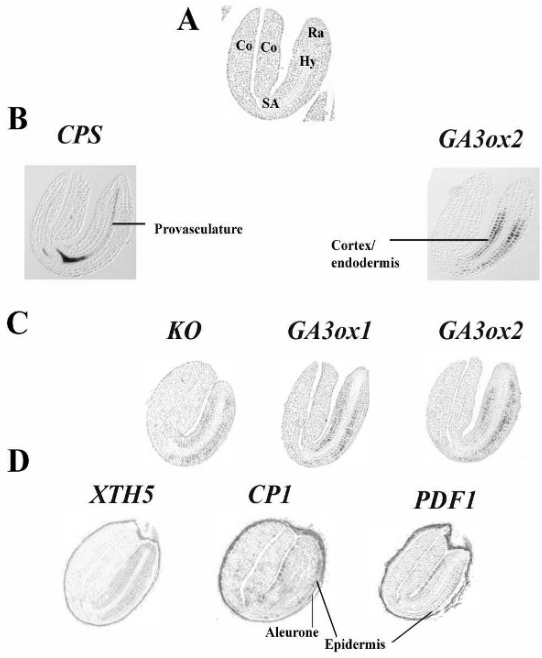

Figure 12. (Color plate page CP3). Cellular localization of GA-biosynthesis and GA-regulated genes in germinating Arabidopsis (ecotype Landsberg *erecta*) seeds. A, longitudinal section showing component organs: Co, cotyledon; Hy, hypocotyl; Ra, radicle; SA, shoot apex. B, X-gluc staining of GUS activity in transgenic seeds expressing the reporter genes *AtCPS-GUS* and *AtGA3ox2-GUS*, showing expression in the provasculature and cortex/endodermis of the embryonic axis, respectively. C and D, *in situ* hybridization of (C) the biosynthetic genes *AtKO*, *AtGA3ox1* and *AtGA3ox2* and (D) the GA-regulated genes *AtXTH5* (encoding xyloglucan endotransglycosylase/ hydrolase), *AtCP1* (encoding a putative cysteine proteinase) and *PDF1* (PROTODERMAL FACTOR1). The GA-biosynthetic genes are expressed in the cortex/endodermis of the embryonic axis, while the GA-regulated genes show different expression patterns: *XTH5* is expressed in the cortex/endodermis of the embryonic axis, including the radicle, *CP1* is expressed in the epidermis and provasculature of the embryonic axis, and in the aleurone, while *PDF1* is expressed mainly in the epidermis. The images are from (73)(A-C) and (46)(D).

distribution of transcripts of three genes whose expression is dramatically up-regulated by endogenous GA_4 during germination. The genes *AtXTH5*, encoding a xyloglucan endotransglycosylase / hydrolase, *AtCP1*, which encodes a putative cysteine proteinase, and *PDF1*, PROTODERMAL FACTOR 1, showed unique exprression profiles (Fig. 12). *AtXTH5*, whose gene product is likely to be involved in the wall loosening that is a prerequisite for cell expansion, was co-expressed with *AtGA3ox1* in the cortex of the embryonic axis, but was also expressed in the radicle. *AtCP1* transcripts were abundant in the epidermis and provasculature of the embryonic axis, and in the aleurone layer where *AtGA3ox1* transcripts were undetectable. The expression of *PDF1* was also in the epidermal cells of the axis. Thus GA_4 can up-regulate the expression of genes in cellular locations both at and separate from its site of synthesis, suggesting that it or a downstream signal must move between different cell types in the germinating seed.

The potential co-localization of GA biosynthesis and response has been studied further in rice, using reporter genes, and *in situ* hybridization. The expression patterns of two GA 20-oxidase genes (*OsGA20ox1* and

OsGA20ox2), and two 3-oxidase genes (*OsGA3ox1* and *OsGA3ox2*), and the expression of two genes involved in GA signaling have been monitored throughout development (33). The GA-signaling genes are *Gα*, which encodes a subunit of a heterotrimeric G protein and is a positive regulator of GA signaling in rice, and *SLR1*, which encodes a negative regulator. The results provide a comprehensive picture of the timing and precise locations of GA metabolism, in relation to the sites of action. The two 20-oxidase and 3β-hydroxylase genes show tissue- and cell-specific patterns of expression in rice. *OsGA20ox1* and *OsGA3ox1* are expressed in just two cell layers- the epithelium of the embryo and the tapetum of the pollen sacs, suggesting that they have very specific functions in GA metabolism. In germinating grain, *OsGA20ox2* and *OsGA3ox2* are expressed in both the epithelium (like *OsGA20ox1*) and in the developing shoot of the embryo, whereas the response genes, *Gα* and *SLR* are expressed at both these locations, and also in the aleurone. Therefore the embryo seems to be a site of GA biosynthesis and response, whereas the aleurone is a site of response only. From early work with cereal half-seeds and isolated aleurone layers the movement of active GA from embryo to aleurone has long been implicated in the *de novo* synthesis of amylase and other hydrolytic enzymes in this cell layer, for use in mobilizing endosperm reserves.

OsGA20ox2 and *OsGA3ox2* are also expressed in young leaf primordia of developing seedlings and elongating stems, and in seedling root tips, whereas expression of the other genes encoding each enzyme is low to absent in these sites. *Gα* and *SLR* are expressed in both locations inferring that young leaf primordia and root tips are sites of GA synthesis and action. During the development of floral organs *OsGA20ox2* and *OsGA3ox2*, and the response genes, are expressed in stamen primordia. Each of the genes, including *OsGA20ox1* and *OsGA3ox1* and the response genes are expressed in the tapetal layer. However the observation that *Gα* and *SLR1* are also expressed in other floral organs, suggests that the tapetum is a source of bioactive GAs whose site of action includes floral tissues separate from the site of their biosynthesis.

In summary, it appears that together OsGA20ox2 and OsGA3ox2 catalyze the production of bioactive GA(s) that have a site of action within the cells of the vegetative tissues in which they are formed. The fact that the loss of function *OsGA3ox2* mutant *d18* is a severe dwarf with normal levels of GA_4 in its reproductive tissue supports a role for this gene only in vegetative growth. In contrast *OsGA20ox1* and *OsGA3ox1* show high expression only in cell layers (embryo epithelium and anther tapetum) that provide bioactive GA for nearby, but separate locations (aleurone layer and other floral tissues, respectively). This and other work suggest that for genes encoded by small families, the time and site of expression of individual members is tightly regulated. Furthermore, the expression of these genes appears to be located in cells and tissues which respond to GA, or in close proximity, suggesting that GAs are produced at or very near to their site of action.

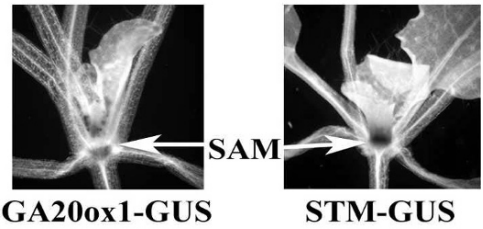

Fig. 13 (Color pate page CP3). The GA 20-oxidase (*AtGA20ox1*) and KNOX (*STM*) genes have non-overlapping expression patterns at the Arabidopsis shoot apex. Transgenic plants were produced containing the reporter genes *AtGA20ox1-GUS*, in which the *AtGA20ox1* promoter including part of the coding region was fused to the β-glucuronidase (*GUS*) coding region, and *STM-GUS*, in which the *STM* promoter was fused to *GUS*. GUS expression (blue staining) in the *AtGA20ox1-GUS* line is in the leaf primordia and sub-apical region, but is excluded from the shoot apical meristem (SAM), where *STM-GUS* is expressed. The images are modified from (25).

The molecular mechanisms underlying cell-specific expression of the GA-biosynthetic genes are beginning to emerge. For example, Chang and Sun (10) identified one *cis*-regulatory region in the Arabidopsis *CPS* promoter (between –997 and –796 with respect to the translation initiation site) that is required for its expression in developing seeds. Other regions provided either positive regulation in all tissues, or regulation in all tissues except seeds, as well as negative regulation.

A number of transcription factors have been described that alter expression of GA-biosynthesis genes and may be involved in their regulation and/or cell-specific expression. The most fully characterized of these in terms of their involvement with GA biosynthesis are the KNOTTED-1-like homeobox (KNOX) transcription factors. Ectopic expression of KNOX genes in tobacco causes dwarfism due, at least in part, to suppression of *GA20ox* gene expression (58). This effect was specific to the *GA20ox* gene with *GA3ox* expression apparently not affected. The tobacco KNOX protein NTH-15 was shown to bind to a *cis* element within the first intron of the *GA20ox* gene thereby inhibiting its expression. Consequently, *KNOX* and *GA20ox* genes have non-overlapping expression patterns in the shoot apex, with *KNOX* expressed in the meristem and *GA20ox* in the leaf primordia and sub-meristem region (25, 58) (Fig. 13). It is proposed that KNOX functions to exclude GA biosynthesis from the meristem so that the cells in this region remain indeterminate. It has also been suggested that leaf morphology may be determined by the presence or absence of KNOX expression in the developing leaf resulting in uneven GA distribution (25). However, GA movement to meristematic regions would compromise this control mechanism so that GAs from external sources would need to be removed from the meristems by, for example, deactivation. In rice, expression of a GA 2-oxidase gene (*OsGA2ox1*) is localized to a ring of cells at the base of the shoot apical meristem (59). Expression of *OsGA2ox1* was reduced on flower initiation leading Sakamoto *et al*. (59) to suggest that this enzyme controls the amount of active GAs reaching the shoot apical meristem; its reduction would allow more GA to reach the meristem and thereby contribute to its transition to a meristem inflorescence.

Gibberellin Homeostasis

Observations that certain GA-insensitive dwarf mutants contain abnormally high amounts of C_{19}-GAs provided the first clues for a link between GA biosynthesis and response (27). It is now clear that part of a plant's response to GAs is to depress GA biosynthesis and stimulate catabolism in order to establish GA homeostasis. This is achieved through down-regulation of GA 20-oxidase and 3-oxidase gene expression (negative feedback regulation) and up-regulation of GA 2-oxidase expression (positive feedforward regulation)(Fig. 11), the relative importance of each gene to this process varying with species and tissue. Mutations that disrupt GA response (as in GA-insensitive dwarfs) or severely reduce levels of endogenous GAs such that the GA response pathway does not occur (as in GA-deficient dwarfs) have similar effects on expression of genes encoding enzymes of GA metabolism. Numerous studies have demonstrated that regulation occurs, at least partly, at the level of mRNA accumulation so that GA-deficient plants accumulate transcript for *GA20ox* and *GA3ox* genes, but have lower levels of *GA2ox* transcript, as for example in Arabidopsis (67)(Fig. 14). However, effects on enzyme levels have been little investigated. The homeostatic mechanism allows changes in GA content in response to developmental or environmental signals, but probably serves to restore GA concentrations to normal levels, particularly after gross changes (70). The existence of this close association between GA metabolism and response provides further evidence that at least the final stages of GA biosynthesis must occur at a site of GA action.

Regulation by other hormones

Recently it has been shown that other hormones also regulate GA biosynthesis, with the most compelling evidence being provided for auxin. Most of this evidence has come from work with pea and is discussed in detail by Reid et al. (Chapter B7). In summary, auxin acts as a long-distance signal, between pea seeds and the pod or between the shoot apex and the internodes, to promote cell enlargement in the target tissues *via* enhanced GA production. In the first example, 4-chloro-IAA up-regulates GA 20-oxidase and 3-

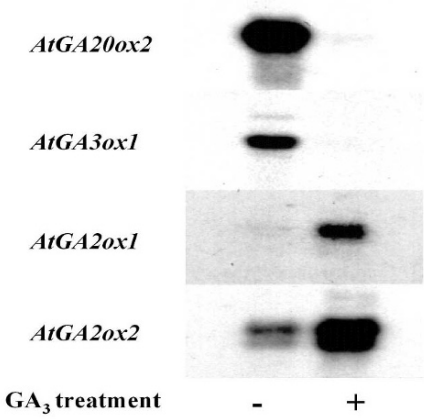

Fig. 14. Northern blot analysis of the GA-biosynthetic genes *AtGA20ox2* and *AtGA3ox1*, and the GA-catabolic genes *AtGA2ox1* and *AtGA2ox2* in the shoot apex of the Arabidopsis GA-deficient mutant *ga1-2* with and without treatment with GA_3. Expression of the biosynthetic genes is strongly depressed by GA treatment while expression of the GA 2-oxidase genes is stimulated by GA. From (67). (© 1999, National Academy of Sciences, U.S.A).

oxidase gene expression in the pod (49, 69), while, in pea internodes, IAA stimulates GA 3-oxidase expression and suppresses GA 2-oxidase expression. Removal of the source of auxin prevents growth of the target tissues unless auxin or GA is supplied. The interaction between auxin and GA in stem elongation was also demonstrated in tobacco, in which removal of the apical bud resulted in reduced GA_1 concentration in internodes (72). Application of IAA to the cut end of the stem restored GA_1 levels. In this case the major effect of IAA was to promote GA 20-oxidase activity and to reduce 2-oxidase activity; although 3-oxidase activity was also reduced by removal of the apical bud it could not be restored by IAA. Thus, although regulation of GA biosynthesis by auxin is probably a general phenomenon, the target enzymes may vary from species to species. In Arabidopsis, expression of the GA 20-oxidase gene *AtGA20ox1* (*GA5*) was shown to be up-regulated by application of epibrassinolide to a BR-deficient mutant, indicating that BR may also promote GA biosynthesis (6).

Environmental Regulation

Plants are extremely sensitive to their environment, reacting to external stimuli by changing their patterns of growth and development. Gibberellins are intermediaries for a number of environmental signals, which may induce changes in GA concentration and/or sensitivity. In particular, light quality, quantity and duration (photoperiod) can all influence the rate of GA biosynthesis and catabolism through effects on expression of specific genes. Examples of developmental processes that are regulated particularly by light-induced changes in GA biosynthesis will be discussed below (31). Further examples from pea are provided in chapter B7, while the role of light and GAs in potato tuberization is discussed in chapter E5.

Seed germination

In dicotyledonous species such as lettuce and Arabidopsis, seed germination is stimulated by exposure to light, which acts to promote production of GAs. Germination in such species has an absolute requirement for *de novo* biosynthesis of GAs, which induce the production of enzymes to digest the endosperm that would otherwise form a mechanical barrier to radicle emergence. Red light acts to promote specifically the 3β-hydroxylation step in the pathway; in Arabidopsis expression of two genes, *AtGA3ox1* (*GA4*) and *AtGA3ox2* (*GA4H*), is induced in imbibed seeds from about 4 hours following exposure to red light (74) (Fig. 15). Regulation of *AtGA3ox2* by light is mediated by phytochrome B, whereas *AtGA3ox1* expression is regulated by a different photoreceptor, which has not been identified. The two genes differ also in their developmental patterns of expression, with *AtGA3ox2* being expressed only during seed germination up to about 2 days following imbibition, in contrast to *AtGA3ox1*, which is induced more rapidly, but less strongly and is involved in most of the other GA-regulated developmental processes. Furthermore, *AtGA3ox2*, unlike *AtGA3ox1*, is not

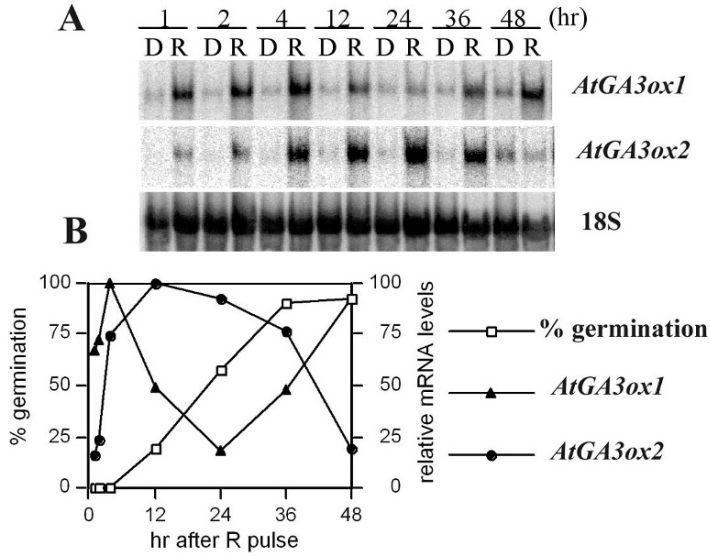

Fig. 15. Red light-induced expression of *AtGA3ox1* (*GA4*) and *AtGA3ox2* (*GA4H*) genes and germination in Arabidopsis (ecotype Landsberg *erecta*) seeds. Seeds were imbibed in the dark and irradiated with a pulse of far-red light one hour after imbibition. They were then either irradiated with a pulse of red light (R) 24 hours later, or kept in the dark (D). A, northern blot analysis of RNA from germinating seeds at different times following the red-light pulse or the equivalent time in the dark. B, germination frequency of red light-irradiated seeds (non-irradiated seeds do not germinate) at different times following irradiation, and the mRNA levels for *AtGA3ox1* and *AtGA3ox2* relative to the 18S rDNA loading control. After (74).

subject to feedback regulation. Expression of *AtGA3ox1*is also enhanced by cold treatment of the seeds prior to imbibition, a process known as stratification that stimulates germination in many species, including Arabidopsis (75).

De-etiolation

Plants growing in low light conditions are characterized by an etiolated growth habit with long, thin stems and small, chlorotic cotyledons and leaves. On exposure to normal light conditions, the growth rate of the stem is reduced, the stems thicken and leaves expand and green, in a process known as de-etiolation. After many years of controversy about whether or not de-etiolation involved changes in GA content it has now been clearly established by several groups working with etiolated pea seedlings that exposure to light results in a rapid, but temporary reduction in GA_1 content. The light, which is detected by phytochrome A and possibly a blue light receptor, causes down-regulation of a *GA3ox* gene (*PsGA3ox1*) and up-regulation of a *GA2ox* gene (*PsGA2ox2*) within 0.5 hours after exposure (53). After about four hours there is a strong increase in expression of the *GA3ox* gene and the *PsGA20ox1* gene, presumably due to feedback regulation, leading eventually to recovery of the GA_1 concentration. The lower growth rate in light in the longer term is thought to be due to reduced responsiveness

to GA in the light compared with the dark. Related mechanisms may explain the control of plant growth by phytochrome in the light, such as in the shade avoidance response in which plants respond to a change in the light spectrum. Plants also increase their GA content in low light conditions without changes in light quality, for example in the shade of a building. There is evidence that pea seedlings respond to reduced light by enhancing production of C_{19}-GAs (18), presumably due to higher GA 20-oxidase activity, but the detailed mechanism has not been determined.

The induction of flowering

Plants use changes in day-length (photoperiod) as cues for the initiation of numerous development changes. Gibberellins have been shown to act as secondary messengers in processes induced by long photoperiods, such as flowering in long-day rosette plants, the breaking of bud dormancy in woody plants (24), and the induction of stolons rather than tubers in potato (Chapter E5).

The interaction of photoperiod and GAs in reproductive development is complex, although significant progress in dissecting the various signaling pathways in Arabidopsis, a facultative long day plant, has been made utilizing mutants affecting flowering time, floral meristem identity and floral organ identity. In Arabidopsis a facultative long-day pathway exists which is separate from a GA-dependent pathway that promotes flowering in short days. Thus the *ga1-3* mutant will not flower in SD unless treated with GA, whereas this GA-deficient mutant will flower in LD without GA treatment, albeit after a slight delay compared to wildtype plants. Mutations in *CONSTANS* (*CO*), a component of the long-day pathway, together with *ga1-3* prevent flowering in LDs too. However, there is an interaction between the long-day and GA pathways, so that LDs do in fact enhance GA 20-oxidase expression in Arabidopsis, leading to rapid stem extension known as bolting and accelerated flowering. In spinach (*Spinacia oleracea*) too, which has an absolute requirement for long days, bolting requires the presence of bioactive GA(s), synthesis of which is induced when plants are transferred from short days to long days. Enhanced GA biosynthesis in long days is associated with higher levels of *ent*-kaurene production and of GA 20-oxidase activity, the latter resulting from greatly increased transcription of the *SoGA20ox1* gene in shoot tips (42). Exposure to long days also depressed expression of *SoGA2ox1* but had little effect on GA 3-oxidase gene expression.

In *Lolium temulentum*, ryegrass, which requires only 1 long photoperiod of at least 16 h duration for induction, flowering occurs without much stem extension, allowing the efficacy of GAs for both processes to be measured separately. GA_5 and GA_6 have high florigenic activity, a property that may be related to their failure to be 2β-hydroxylated and hence deactivated. GA_5 levels have been shown to increase five-fold in illuminated leaves at the end of a single 16 h photoperiod, and to double in the shoot apex, along with GA_6, on the following day (36). King et al. (37) have suggested that these values reflect the transport of GA_5 from leaf to apex at a rate consistent with

the proposed movement of the hypothetical flowering stimulus, florigen, from the site of illumination to the site of floral initiation. Changes in expression of a MADS-box-containing transcription factor, which is functionally related to the Arabidopsis *AP1* gene, within hours after the purported arrival of GA_5 at the shoot apex is considered as strong evidence for a relationship between GA_5 and floral induction (36). Interestingly GA_1 and GA_4 appear to be excluded from the apical meristem at this time perhaps, by analogy with rice, because of a transient zone of high 2β-hydroxylating activity surrounding the apical meristem. Thus these GAs are not thought to be associated with the earliest events of floral evocation in *Lolium*, although they can promote stem elongation, and may also be involved in inflorescence initiation 2 or 3 d later when they gain access and accumulate in the shoot meristem.

In Arabidopsis, for which the most complete data are available, both the photoperiod and GA pathways to flowering converge with the activation of *LFY*, a floral meristem identity gene. *LFY* expression is only observed in SD-grown *ga1-3* mutants that have been treated with GA, though constitutive *LFY* expression from a 35S promoter will allow SD-grown *ga1-3* to flower without GA treatment (4). However, the up-regulation of *LFY* expression in *ga1-3* plants transferred from SD to LD is quite weak, and occurs more slowly than in WT plants, providing additional evidence for the biosynthesis of bioactive GA being important even in the long-day pathway to flowering. Using deletion analysis, Blasquez and Weigel (5) identified a 8 bp motif in the *LFY* promoter which is necessary for GA responsiveness, but not for response to daylength. This sequence is a potential target for GAMYB transcription factors, and candidate MYBs, whose expression increases in the shoot apex in response to exogenous GA_4, or to elevated levels of native GA_4, have been identified in both Arabidopsis and *L. temulentum* (20, 21).

CHEMICAL CONTROL OF GIBBERELLIN BIOSYNTHESIS

Growth retardants, synthetic chemicals that inhibit GA biosynthesis, have been used for several decades for the manipulation of agronomic and horticultural crops (52). The importance of these retardants may depend on their selectivity to inhibit only GA biosynthesis. Most often their commercial use is to reduce vegetative growth, without toxicity, and without decreasing crop yield. Since much of the GA-biosynthetic pathway is common to other diterpenoids, and tetraterpenoids, a chemical which blocks the pathway before GGPP (for example fosmidomycin which blocks DXR in the MEP pathway) will most likely be lethal, and thus be an effective herbicide. For inhibitors that block later in the pathway, there is the greater potential to inhibit GA biosynthesis selectively. However, many of the inhibitors target a particular category of enzyme (terpene cyclases/synthases, monooxygenases, or dioxygenases), and do in fact also affect other pathways

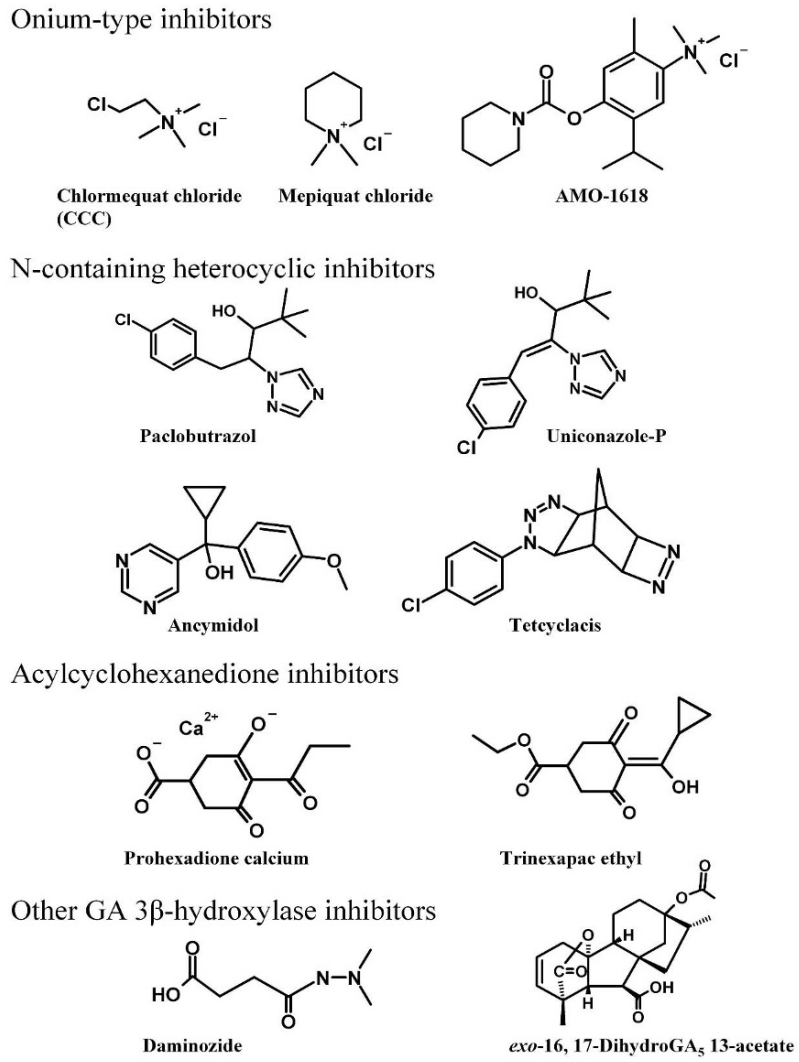

Figure. 16. Structures of growth retardants that act as inhibitors of GA biosynthesis.

that utilize these types of enzymes. The most selective action comes from inhibitors whose chemical structure is modeled on that of GAs, and are therefore likely to block specifically the active site of GA-metabolizing enzymes. There are four main types of GA biosynthesis inhibitors, which are discussed briefly below. The most recently developed retardants are considered in more detail. Selected structures for each type of retardant are shown in Fig. 16.

The type exemplified by chlormequat chloride, mepiquat chloride and AMO-1618 may exert their growth retarding activity by primarily blocking CPS activity, though there is a minor inhibition of KS too. These quaternary

ammonium compounds, which are positively charged at cellular pH, may mimic cationic intermediates in the cyclization of GGPP to CPP. They have been used extensively for reducing stem length in wheat, thereby preventing lodging and subsequent decrease in yield. They are also used to reduce vegetative growth in cotton, and to control the growth habit of ornamental plants.

The second class of GA biosynthesis inhibitors include the triazoles, exemplified by paclobutrazol and uniconazol, and other heterocyclic compounds such as ancymidol and tetcyclasis (Fig. 16). These retardants are inhibitors of KO, which catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid, and other monooxygenases. There is some overlap of activities with triazole-type fungicides such as triadimenol and metconazole whose main function is to inhibit sterol biosynthesis. Thus the growth retardants also have some fungicidal activity, and the fungicides have some growth-retarding properties. Of the two diastereoisomers in commercial samples of paclobutrazol, the 2S, 3S form is the more effective growth retardant whereas the 2R, 3R-enantiomer is more active in blocking ergosterol biosynthesis in fungi, by inhibiting lanosterol C-24 demethylation. The commercial use of these growth retardants is, amongst other things, for the reduction of stem growth in rice seedlings, and the restriction in vegetative growth of fruit trees and ornamentals.

The acylcyclohexanedione growth retardants, for example prohexadione-calcium and trinexapac, block the dioxygenase enzymes. These inhibitors have some structural features in common with 2-oxoglutarate, the co-substrate for this class of enzymes, and have been shown to inhibit the dioxygenases competitively. However, since these compounds inhibit both the biosynthetic enzyme GA 3-oxidase and the deactivating enzyme GA 2-oxidase, their activity might be somewhat unpredictable. Although on most plants these compounds act as growth retardants, there are a few examples where they may enhance growth though inhibition of GA-catabolism. The mode of action of daminozide, which was developed many years before the acylcyclohexanediones and has been used to control the stature of potted ornamentals such as chrysanthemum, has only recently been demonstrated. It has been shown to inhibit 2-oxogutarate-dependent dioxygenases in the same manner as prohexadione-Ca.

The most recently developed growth retardants are 16, 17-dihydro-GAs. These compounds were evaluated initially for activity as inducers of flowering in *Lolium temulentum*, but were found to act as effective growth retardants on Graminaceous species (15), with *exo*-16, 17-dihydro-GA_5 (applied as the 13-acetate) being one of the most effective examples. Like the acylcyclohexanediones these compounds also inhibit 2-oxoglutarate-dependent dioxygenases, but are more specific for the GA-metabolizing enzymes since the retardant mimics the GA substrate, rather than 2-oxoglutarate, which is common for all reactions. The activity of these compounds is somewhat puzzling, for their impressive efficacy on grasses is accompanied often by inactivity in dicot species.

CONCLUSIONS

Since the previous edition of this book, published almost ten years ago, progress in our understanding of GA biosynthesis and its regulation has been spectacular. This has occurred largely because advances in molecular genetics have enabled the isolation of most of the genes encoding the enzymes of GA biosynthesis and catabolism. In particular, the completion of the Arabidopsis genome sequence has allowed almost all the genes of GA metabolism to be identified in this species. It should be pointed out, however, that this rapid progress was possible to a large extent because of the earlier detailed metabolism studies that delineated the pathways and determined the nature of the enzymes involved. An important recent advance has been the recognition that GAs are biosynthesized predominantly *via* the MEP, rather than the MVA, pathway in plants, coupled with the identification of the Arabidopsis genes for the enzymes in this pathway. Notable omissions from the identified GA-biosynthesis genes are those encoding the 13-hydroxylase. It has, therefore, not been possible to assess the significance of this step in the production of the active hormones. In addition, little is known about the other enzymes that modify GAs on rings C and D, for example, those that hydroxylate at C-11, C-12 and C-15. Although GAs oxidized at these positions are found mainly in developing seeds, it is possible that they have roles in specific developmental processes, such as flowering, in some species.

The availability of the GA-metabolic genes is allowing the molecular mechanisms underlying the regulation of GA metabolism to be deciphered. Such studies have reinforced the importance of GAs as mediators of environmental signals, and are also revealing previously unsuspected cross-talk between the GA signaling pathways and those of other hormones. Furthermore, GA signal transduction has been shown to impinge directly on GA metabolism in the establishment of homeostasis for this hormone. Progress in understanding this process is likely to accelerate with the continuing impressive advances in our knowledge of GA signal transduction (Chapter D2).

Another benefit accruing from the availability of the GA-metabolic genes is the ability to manipulate GA biosynthesis and catabolism by altering expression of these genes (Chapter E7). Genetic manipulation of GA metabolism could provide an alternative to chemical treatments for control of plant growth and development and, furthermore, can be targeted to specific tissues or stages of development. It will also provide an important experimental tool to further understanding of GA biosynthesis and the function of GAs in plant development.

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