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Gibberellin Biosynthesis in Plants and Fungi: A Case of Convergent Evolution?

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Abstract

As well as being phytohormones, gibberellins (GAs) are present in some fungi and bacteria. Indeed, GAs were first discovered in the fungus *Gibberella fujikuroi,* from which gibberellic acid (GA_3) and other GAs are produced commercially. Although higher plants and the fungus produce structurally identical GAs, there are important differences in the pathways and enzymes involved. This has become particularly apparent with the identification of almost all of the genes for GA-biosynthesis in *Arabidopsis thaliana* and *G. fujikuroi,* following the sequencing of the *Arabidopsis* genome and the detection of a GA-biosynthesis gene cluster in the fungus. For example, 3β-hydroxylation occurs early in the pathway in *G. fujikuroi* and is catalyzed by a cytochrome P450 monooxygenase, whereas it is usually the final step in plants and is catalyzed by 2-oxoglutarate-dependent dioxygenases. Similarly, 20-oxidation is catalyzed by dioxygenases in plants and a cytochrome P450 in the fungus. Even where cytochrome P450s have equivalent functions in plants and *Gibberella,* they are unrelated in terms of amino acid sequence. These profound differences indicate that higher plants and fungi have evolved their complex biosynthetic pathways to GAs independently and not by horizontal gene transfer.

Key words: *Arabidopsis thaliana*; *Gibberella fujikuroi*; Gibberellin biosynthesis

Introduction

The fungus *Gibberella fujikuroi* has played a crucial role in the history of gibberellin (GA) research (Phinney 1983). The over-growth symptoms exhibited by rice infected with GA- producing strains of *G. fujikuroi* led Japanese scientists to the discovery of this group of natural products, culminating in the structural determination of gibberellic acid (GA_3) at the ICI Laboratories in the United Kingdom (Cross and others 1959). Later, the ability of GA_3 to restore normal growth to dwarf mutants of several plant species and to induce bolting in rosette plants prompted the suggestion that GAs may be natural regulators of growth and development in higher plants (Lang 1956; Radley 1956).

The biosynthetic origin of GAs was investigated first in *G. fujikuroi,* as soon as the structure of GA_3, the major GA produced by the fungus, was known. By determining incorporation patterns from ^{14}C-labeled acetate and mevalonate into GA_3 in fungal

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cultures, Birch and co-workers (Birch and others 1958, 1959) showed that GAs were diterpenoids. Subsequently, in the 1960s and early 1970s, the basic biosynthetic pathway to GA_3 was determined by isolating potential intermediates from fungal cultures and demonstrating their incorporation into GA_3 (reviewed by Bearder 1983). This work profited from the availability of a GA-deficient mutant of *G. fujikuroi,* B1-41a, in which GA biosynthesis is blocked at an early point in the pathway (Bearder and others 1974), allowing the incorporation of potential intermediates into GA_3 and other GAs to be demonstrated without the requirement for isotopically labeled substrates. The development of combined gas chromatography-mass spectrometry (GC-MS) for GA analysis at about this time (Binks and others 1969; MacMillan and others 1967), was a breakthrough in the identification of products essential to all further progress in GA research.

Initially, research on GA biosynthesis in higher plants profited from the knowledge gained with the fungus, but advanced more slowly. Most progress was made using cell-free systems from developing seeds, particularly those from *Marah macrocarpus* (formerly *Echinocystis macrocarpa)* (Graebe and others 1965), *Cucurbita maxima* (pumpkin) (Graebe and others 1974), and *Pisum sativum* (garden pea) (Kamiya and Graebe 1983). Work with the cell-free systems established the biosynthetic pathways in these tissues and enabled the nature of the enzymes involved to be determined, thereby providing essential information needed for the isolation of the corresponding genes that began in the mid 1990s (Lange and others 1994; Phillips and others 1995; Sun and others 1992; Sun and Kamiya 1994; Xu and others 1995). In some cases the availability of dwarf mutants, particularly of *Arabidopsis thaliana,* with lesions in the GA-biosynthetic pathway (see reviews by Hedden and Proebsting 1999; Ross and others 1997) also aided gene isolation. Almost all the genes that encode GA-biosynthetic enzymes in *Arabidopsis* have now been identified, and there is considerable information on how the expression of these genes is regulated by endogenous and environmental factors (Hedden and Phillips 2000; Kamiya and García-Martínez 1999; Yamaguchi and Kamiya 2000).

Although research on GA biosynthesis in plants made spectacular progress following the gene isolation, there had been little advance on the fungal system in almost 20 years. One reason for this was the inability to determine the nature of the enzymes for the later steps in the pathway (see below). Very recently, however, the breakthrough came with the discovery that the genes for the pathway were clustered on the *G. fujikuroi* genome (Tudzynski and Holter 1998). This discovery is leading rapidly to the identification of these genes, from which information on the character of the participating enzymes and their regulation is following. What is already clear is that GA biosynthesis in *G. fujikuroi* differs fundamentally from that in plants at the chemical (pathway), biochemical (enzyme), and genetic levels. In this article, we will compare the information from plants and *G. fujikuroi* to support the conclusion that GA production in this fungus arose independently from that in higher plants.

Pathways of Gibberellin Biosynthesis

The biosynthetic pathways to the GAs in plants and fungi have been the subject of many reviews, most recently by MacMillan (1997). The pathways to the biologically active GAs, GA_1 and GA_3, in higher plants and *G. fujikuroi* are compared in Figure 1. Both GAs are produced by the fungus, although GA_1 is a minor product compared with GA_3. In contrast, GA_1 is the major biologically active form of GA in many higher plant species (MacMillan 2002, this issue) and is probably ubiquitous, whereas GA_3 is not produced in all plant species, and, when present, is usually at lower concentrations than GA_1.

The pathways are identical as far as GA_{12}-aldehyde, which is formed from the tetracyclic diterpene, *ent*-kaurene, by sequential oxidation at C-19 to give *ent*-kaurenoic acid, followed by 7β-hydroxylation to give *ent*-7α-hydroxykaurenoic acid, and finally oxidation at C-6β, resulting in contraction of ring B. *ent*-Kaurene is produced in two steps from the common diterpene precursor geranylgeranyl diphosphate (GGPP); proton-initiated cyclization gives rise to rings A and B in *ent*-copalyl diphosphate (CPP), which is converted to *ent*-kaurene by further cyclization and rearrangements following ionization of the diphosphate bond (MacMillan and Beale 1999). After GA_{12}-aldehyde, the pathways in higher plants and *G. fujikuroi* differ. In vegetative tissues of most plant species that have been studied, GA_{12}-aldehyde is converted to GA_{12}, which may be oxidized at C-20 to form the C_{19} product, GA_9, or, as is illustrated in Figure 1, GA_{12} is first 13-hydroxylated to give GA_{53}, which is then converted to GA_{20} by oxidation of C-20. GA_9 and GA_{20} thus are formed in parallel pathways involving oxidation of C-20 to the alcohol and aldehyde, from which this C atom is lost in the formation of the γ-lactone function in C_{19}-GAs. Although not shown in Figure 1, the C-20 alcohols and aldehydes are

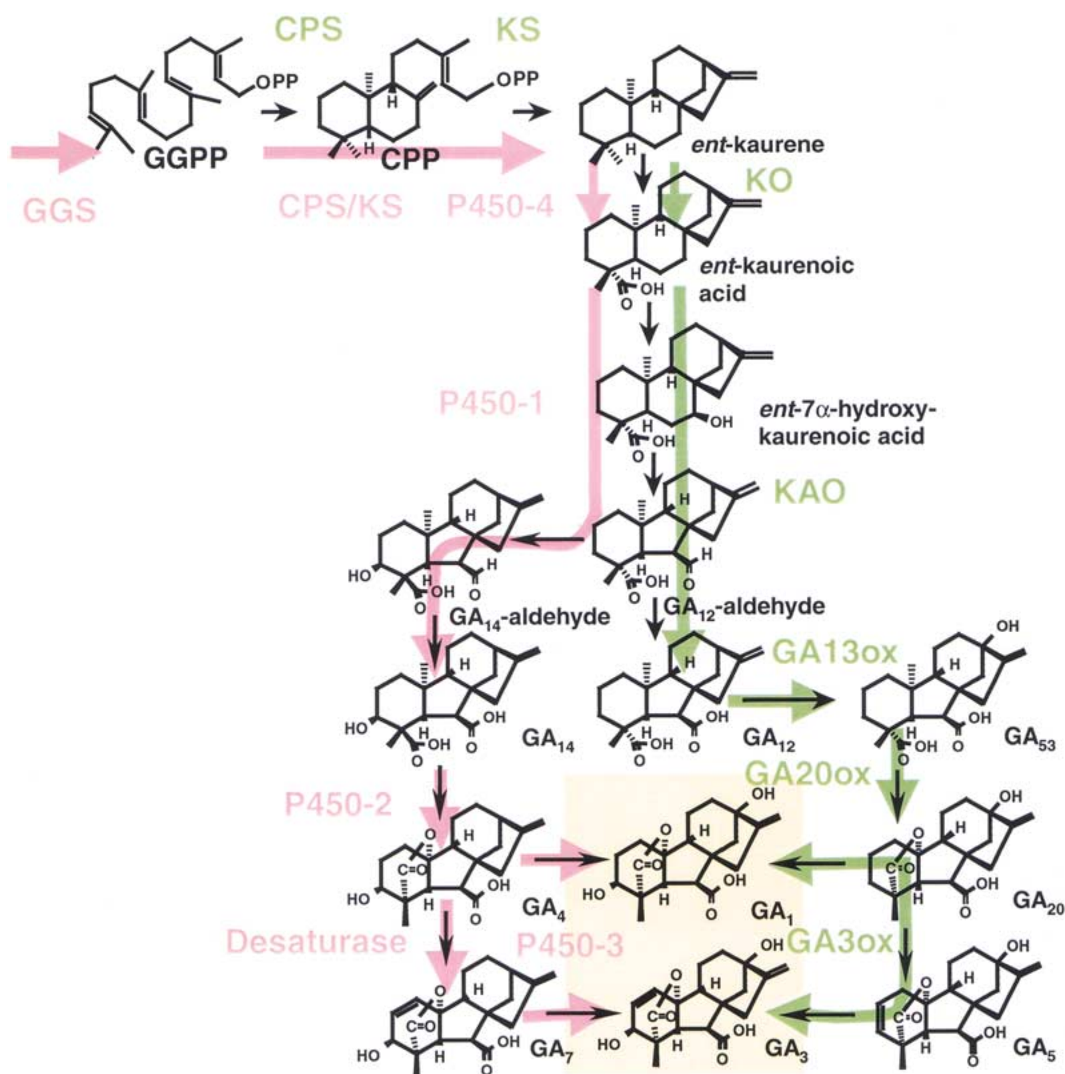

Figure 1. Biosynthetic pathways to GA_1 and GA_3, highlighted in yellow, in higher plants and the fungus *Gibberella fujikuroi*. The higher plant enzymes and the reactions they catalyze are shown in green; for *G. fujikuroi*, they are shown in red.

formed as true intermediates that are both products and substrates for the multifunctional GA 20-oxidases that catalyze the removal of C-20 (Lange and others 1994; Phillips and others 1995)(see below). The final step in the formation of growth-active GAs in plants is the introduction of a 3β-hydroxyl group, whereby GA_9 and GA_{20} are converted to GA_4 and GA_1, respectively. In some plants, GA_3 is formed from GA_{20} *via* GA_5 as a product of side reactions of the 3β-hydroxylase (Albone and others 1990; Fujioka and others 1990; Itoh and others 2001).

A major difference between the pathways in *G. fujikuroi* and plants is the stage at which the hydroxyl groups are introduced. In the fungus, GA_{12}-aldehyde is 3β-hydroxylated to GA_{14}-aldehyde, which is then oxidized at C-7 to form GA_{14} (Hedden and others 1974; Urrutia and others 2001). The subsequent conversion of GA_{14} to GA_4 by 20-oxidation is analogous to the formation of GA_9 and GA_{20}. However, although the putative C-20 alcohol and aldehyde intermediates in this reaction (GA_{37} and GA_{36}, respectively) are found in fungal cultures, there is as yet no evidence that they are converted to GA_4 and are thus true precursors (Bearder and others 1975). Desaturation of GA_4 at C-1,2 results in the formation of GA_7, which is converted to GA_3 by 13-hydroxylation. GA_1 is formed in a minor side reaction by 13-hydroxylation of GA_4 and is not converted efficiently to GA_3 (Bearder and others 1975). Apart from G. *fujikuroi,* a number of other fungal species produce GAs at high concentrations (mg quantities per liter of culture

Table 1. *Arabidopsis* Genes Involved in GA Biosynthesis, Including the Function of their Encoded Proteins and Mutants

Gene	Enzyme function	Mutant	Reference
CPS	CPP synthase (GGPP to CPP)	ga1	Sun and Kamiya 1994
KS	*ent*-Kaurene synthase (CPP to *ent*-kaurene)	ga2	Yamaguchi and others 1998b
KO	*ent*-Kaurene oxidase (*ent*-kaurene to *ent*-kaurenoic acid)	ga3	Helliwell and others 1998
KAO1	*ent*-Kaurenoic acid oxidase (*ent*-kaurenoic acid to GA_{12})		Helliwell and others 2001
KAO2	*ent*-Kaurenoic acid oxidase		Helliwell and others 2001
GA20ox1	GA 20-oxidase (GA_{12}/GA_{53} to GA_9/GA_{20})	ga5	Phillips and others 1995; Xu and others 1995
GA20ox2	GA 20-oxidase		Phillips and others 1995
GA20ox3	GA 20-oxidase		Phillips and others 1995
GA20ox4	Undetermined[a]		
GA20ox5	Undetermined[a]		
GA3ox1	GA 3β-hydroxylase (GA_9/GA_{20} to GA_4/GA_1)	ga4	Chiang and others 1995; Williams and others 1998
GA3ox2	GA 3β-hydroxylase		Yamaguchi and others 1998a
GA3ox3	GA 3β-hydroxylase		Phillips and Hedden,
GA3ox4	GA 3β-hydroxylase		unpublished information
GA2ox1	GA 2-oxidase (GA_1/GA_4/GA_9/GA_{20} to GA_8/GA_{34}/GA_{51}/GA_{29} and corresponding catabolites)		Thomas and others 1999
GA2ox2	GA 2-oxidase		Thomas and others 1999
GA2ox3	GA 2-oxidase		Thomas and others 1999
GA2ox4	GA 2-oxidase		Thomas, Phillips and Hedden, unpublished
GA2ox5	Probably pseudogene[b]		
GA2ox6	GA 2-oxidase		Woolley, Phillips and Hedden, unpublished

[a]*Classified as GA 20-oxidases on the basis of predicted amino acid sequences.*
[b]*The gene contains a large insertion and is apparently not expressed.*

medium) (Rademacher 1994). Several species of *Sphaceloma* produce GA_4, but not GA_3 (Rademacher 1994) and therefore lack 1,2-desaturase and 13-hydroxylase activities. A species of *Phaeosphaeria* produces GA_1 by a pathway in which 3β-hydroxylation occurs after C_{19}-GA formation, as in plants (Kawaide and others 1995). However, unlike in plants and similar to *G. fujikuroi,* 13-hydroxylation occurs late in the pathway. Thus the major pathway to GA_1 in *Phaeosphaeria* involves the intermediacy of GA_9, which is 3β-hydroxylated to GA_4 and then 13-hydroxylated (Kawaide and others 1995).

In plants, but not in fungi, GAs are 2β-hydroxylated and in some cases, oxidized further to the 2-ketones known as GA-catabolites (Sponsel and Macmillan 1978) (illustrated for GA_1 in Figure 2). As 2β-hydroxy GAs have no biological activity, this serves as a deactivating, and therefore catabolic, reaction that allows turnover of the bioactive GAs, and is necessary for the regulation of GA concentrations. The GA-catabolites, as identified after GC-MS analysis, are dicarboxylic acids in which the γ-lactone has opened with formation of a double bond, but it seems likely that these structures are formed by rearrangement of the true enzymatic products, the simple 2-ketones, as a result of derivatization for GC-MS analysis. Although *G. fujikuroi* does not produce 2β-hydroxy or 2-keto GAs, 2α-hydroxyGAs are found at low levels in cultures of the fungus. Because introduction of the 1,2-double bond involves removal of the 1α and 2α hydrogen atoms (Albone and others 1990; Evans and others 1970), it is possible that 2α-hydroxyGAs are formed as by-products of the desaturation. In contrast, introduction of the 1,2-double bond in plants involves elimination of the 1β, 2β hydrogen atoms (Albone and others 1990).

Genes and Enzymes

In higher plants, GA_1 is biosynthesized from GGPP in 12 steps, which require the action of seven enzymes, comprising terpene cyclases, cytochrome

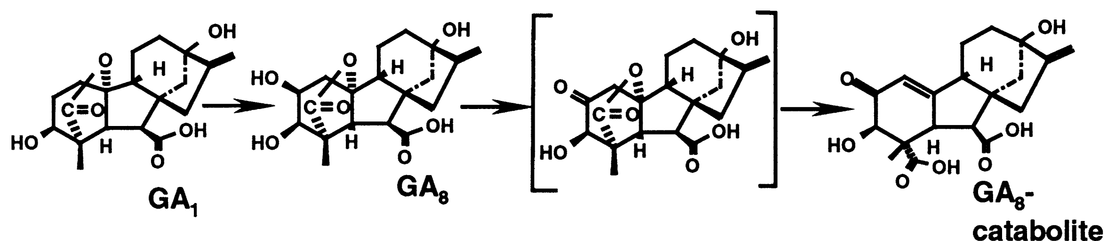

Figure 2. Catabolism of GA$_1$ in higher plants, catalyzed by GA 2-oxidases. The structure in square brackets is thought to be formed *in planta*, but probably decomposes to the open-lactone structure during derivatization prior to GC-MS analysis.

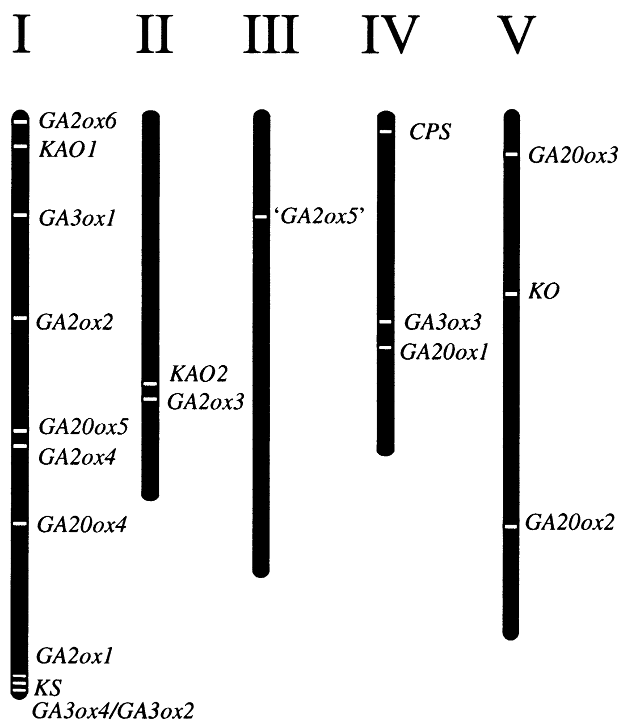

Figure 3. Position of GA-biosynthesis genes on the *Arabidopsis* genome.

P450 monooxygenases, and 2-oxoglutarate-dependent dioxygenases (Hedden and Kamiya 1997; Hedden and Phillips 2000). *G. fujikuroi,* in which only five enzymes are required for GA$_1$ synthesis from GGPP (six for GA$_3$ synthesis), also utilizes a terpene cyclase and cytochrome P450 monooxygenases, but dioxygenases are apparently not involved. Much of the information on the nature of the enzymes has been obtained from work with cell-free extracts, although in plants, this has been mainly restricted to tissues, usually from developing seed, that produce relatively large amounts of GAs. Further understanding of the enzymes and their distribution has followed the identification of the genes.

The completion of the *Arabidopsis* genome sequencing project (The Arabidopsis Genome Initiative 2000) has enabled almost all the genes for GA biosynthesis to be identified in this species; the function of the products of many of these genes has been demonstrated by expression of cDNAs in *E. coli* or *yeast,* whereas others are provisionally assigned on the basis of derived amino acid sequence similarity to genes of known function. The genes and the proposed function of their products are listed in Table 1, which also includes reported mutations. As shown in Figure 3, the genes are distributed throughout the *Arabidopsis* genome with little clustering, with the exception of two adjacent *GA3ox* genes on chromosome 1. In *Arabidopsis,* the enzymes catalyzing early reactions in the pathway are encoded by single genes, while those for later steps, particularly the dioxygenases, are encoded by gene families (see Table 1) (Hedden and Phillips 2000).

In *G. fujikuroi,* the genes for GA-biosynthesis are clustered on chromosome 4 (Linnemannstons and others 1999; Tudzynski and others 2001; Tudzynski and Holter 1998). The arrangement of six of the genes is shown in Figure 4. The cluster contains a geranylgeranyl diphosphate synthase gene *(ggs2),* which is co-ordinately regulated with most of the other genes of the cluster and may serve to provide GGPP exclusively for GA biosynthesis (Tudzynski and Holter 1998). A second GGPP synthase gene *(ggsl)* that lies elsewhere on the genome is constitutively expressed and is thought to be responsible for the biosynthesis of essential diterpenoids (Mende and others 1997). The cluster also contains *ent*-kaurene synthase and four cytochrome P450 genes. A seventh gene belonging to the cluster lies to the left of *P450-4,* but has not yet been fully characterized (B. Tudzynski, unpublished information). The function of the genes, which will be discussed in detail below, is being determined by gene disruption and by expression of individual genes in mutants lacking the gene cluster so that enzyme function can be studied in isolation (Rojas and others 2001; Tudzynski and others 2001).

The enzymes in plants and *G. fujikuroi* are compared in the following sections, in which the path-

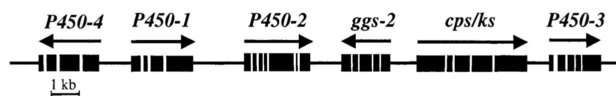

Figure 4. Six genes of the GA-biosynthesis gene cluster in *G. fujikuroi,* showing position of introns. Arrows denote direction of transcription.

way is divided into three phases on the basis of the types of enzyme employed in plants.

ent-Kaurene Synthesis

Two enzymes are required for the conversion of GGPP to *ent*-kaurene in plants: CPP synthase (CPS) converts GGPP to CPP, which is then converted to *ent*-kaurene by *ent*-kaurene synthase (KS). In fungi, both of these activities are contained in a single bifunctional polypeptide, CPS/KS. Genes encoding this enzyme have been isolated from *Phaeosphaeria* (Kawaide and others 1997) and *G. fujikuroi* (Tudzynski and others 1998); the function of the enzymes, which share 48% identity, was demonstrated by expressing the cDNAs in *E. coli* (Kawaide and others 1997; Toyomasu and others 2000). Kinetic and site-directed mutagenesis experiments indicate that the *Phaeosphaeria* enzyme contains two overlapping sites for the CPS and KS activities at the N- and C-terminal regions, respectively, of the protein (Kawaide and others 2000).

ent-Kaurene synthesis in plants occurs in plastids (Aach and others 1997), which are major sites of GGPP production (Gray 1987). The products of the *CPS* and *KS* genes contain N-terminal transit peptides, which, in the case of CPS, have been shown to be cleaved after import of the protein into chloroplasts (Sun and Kamiya 1994). It appears that CPS utilizes the same plastidic GGPP pool as phytoene synthase, the first enzyme of carotenoid biosynthesis, as overexpression of a phytoene synthase gene in tomato resulted in reduced GA content (Fray and others 1995). On the other hand, it has been suggested on the basis of the differential incorporation of precursors into the major terpenoids that, in *G. fujikuroi,* carotenoids and GAs, as well as sterols, are each produced in separate cell compartments (Domenech and others 1996). This is consistent with the existence of a GGPP synthase gene that is exclusive for GA biosynthesis. Although there is substantial evidence that GAs are produced in *G. fujikuroi via* the mevalonate pathway (Birch and others 1959; Domenech and others 1996; Evans and others 1975), the situation in plants is not clear. GGPP is derived from isopentenyl diphosphate, which is synthesized from mevalonate in the cytosol of plant cells, but from glyceraldehyde 3-phosphate and pyruvate in plastids (Lichtenthaler 1999). The latter pathway and its possible involvement in GA biosynthesis are discussed by Sponsel (2002) in this issue.

Formation of 10-Methyl GAs

All reactions of GA biosynthesis after *ent*-kaurene are oxidative (Hedden 1997). The early steps, comprising the conversion of *ent*-kaurene to GA_{12} and GA_{53} in higher plants, are catalyzed by membrane-associated monooxygenases, most of which have now been confirmed as cytochrome P450s. Mutant studies and functional expression in yeast of the genes from *Arabidopsis* and other species have revealed that only two enzymes are required for the conversion of *ent*-kaurene to GA_{12}: *ent*-kaurene oxidase (KO) catalyzes the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Helliwell and others 1998; Swain and others 1997), which is converted by a second enzyme, named *ent*-kaurenoic oxidase (KAO), to GA_{12} *via ent*-7α-hydroxykaurenoic acid and GA_{12}-aldehyde (Helliwell and others 2001). The 13-hydroxylation of GA_{12} to GA_{53} is also catalyzed by a monooxygenase (Grosselindemann and others 1992; Hedden and others 1984), but the gene(s) that encodes this enzyme has not yet been identified. In *Arabidopsis* there is a single *KO* gene *(CYP701A1),* but two *KAO* genes *(CYP88A3* and *CYP88A4),* which would appear to be fully redundant since no mutant in KAO has been identified. However, *kao* mutants of barley (Helliwell and others 2001) and probably also maize (Winkler and Helentjaris 1995) are GA-deficient dwarfs, indicating that these species contain a single *KAO* gene *(CYP88A5* and *CYP88A1,* respectively) or multiple genes with different expression patterns. Pumpkin contains a 2-oxoglutarate-dependent dioxygenase that converts GA_{12}-aldehyde to GA_{12} (Lange 1997). The function of this soluble GA 7-oxidase (GA7ox) is unclear since pumpkin also possesses monooxygenase GA 7-oxidase activity (Hedden and others 1984) that is presumably due to a multifunctional KAO. An apparent *KAO* orthologue *(CYP88A2)* has been cloned from pumpkin seed, although its function has not been demonstrated (Helliwell and others 2000). The *Arabidopsis* genome does not contain sequences similar to the pumpkin *GA7ox,* which may not, therefore, have general significance in GA biosynthesis in higher plants.

A comparison of the derived amino acid sequences of the three *Arabidopsis* cytochrome P450s, KO, KAO1, and KAO2, with those of the four P450s encoded by genes in the G. *fujikuroi* gene cluster (Figure 5 and Table 2) indicates that the plant and

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AtKO      : --MAFFSMISILLG-FVISSFIFIFFKKLLSFS-RKNMSEV--ST-LPSVPVVPGLPVIGNLL----Q-LKEKKPHKTF : 68
AtKAO1    : --ITEGLILMLFPLIIIGLLVLKWVLKKVNVWIYVSKLGEK--KHYLP--PGDLGWPVIGNMWSFL-RAFKTSDP-ESF : 72
AtKAO2    : -MAETLSWIPVLFPLMVLGCLGLNWLVRKVNVWLYESSLGEN--RHYLP--PGDLGWPFIGNMSFL-RAFKTSDP-DSF : 73
GfP450-1  : MANHSSSYYHEPYK--DHSHTVLTLVSEKPVILP-SLILGTC--AVLLC-----IQLLKPQPLLMVNGRKRGELSN-VRA : 69
GfP450-2  : --MSIFNMLTSLAGSQLLPFVLAIFVFTLVPWAIRFSWLELRKG---------SVVLLANPPD----SLLGLGKTRRSF : 64
GfP450-3  : ------------------------------------------------------------------------------- :  -
GfP450-4  : --MSKSNSMNSTSHETLFQQLVLGLDRMPLMDVHWLIYVAFG-----AWLCSYVIHVLSSSSTVKVPVVGYRSVFEPTWL : 73

AtKO      : TR-WSEIYGP--IYSIKMGSSS--LIVLNSTETAKEAMVTRFSSISTRKLS-NALTVLTCDKSMVATSDYDDFHKLVKRC : 142
AtKAO1    : IQSYITRYGRTGIYKAHMFGYP--CVLVTTPETCRRVLTDD-DAFHIGWPK-STMKLIG-RKSFVGIS-FEE-HKRLRRL : 145
AtKAO2    : TRTLIKRYGPKGIYKAHMFGNP--SIIVTTSDTCRRVLTDD-DAFKPGWPT-STMELIG-RKSFVGIS-FEE-HKRLRRL : 146
GfP450-1  : KR--DFTLGARQLLEKGLKMSPDKPFRIMGDVGELHILPPK-YAYEVRNNEKLSFTMAAFKWFYAHLPGFEGFREGTNES : 146
GfP450-2  : VKLSREILAK----ARSLFPNE--PFRLITDWGEVLILPPE-FADEIRNDPRLSFSKAAMQDNHAGIPGFETVALVGRED : 137
GfP450-3  : ------------------------------------------------------------------------------- :  -
GfP450-4  : LGLRFVWEGGS-LIGQGYNKFKDSIFQVRKLGTDIVIIPPN-YIDEVRKLS--QDKTRSVEPFINDFAGQYTRGMVFLQS : 149

AtKO      : LLNGLLGANAQKRKR-HYRDALIENVSSKLHAHARDHP---QEPVNFRAIFEHELFGVALKQAFGKDVESIYVKELGVTL : 218
AtKAO1    : TSAPVNGPEALS----VYIQFIEETVNTDLEKWSK------MGEIEFLSHLRKLTFKVIMYIFLSSESE--HVMD---SL : 210
AtKAO2    : TAAPVNGHEALS----TYIPYIEENVITVLDKWTK------MGEFEFLTHLRKLTFRIIMYIFLSSESE--NVMD---AL : 211
GfP450-1  : HLMKLVARHQLTHQLTLVTGAVSEECALVLKDVYTDSPE--WHDITAKDANMKLMARITSRVFLGKEMC--RNPQ-WLRI : 221
GfP450-2  : QLIQKVARKQLTKHLSAVIEPLSRESTLAVSLNFGETTE--WRAIRLKPAILDIIARISSRIYLGDQLCR---NPAWLKI : 212
GfP450-3  : -----MKYTTCQMN--ILPSLWSMKTSFRWPRTSK------KSSVSLYDMMLRTVALLSGRAFVGLPLCR---DEGWLQA :  64
GfP450-4  : DLQNRVIQQRLTPKLVSLTKVMKEEIDYALTKEMPDMKNDEWVEVDISSIMVRLISRISARVFLGPEHCR--NQE---WL : 224

AtKO      : SKDEIFKVLVHDMMEGAIDVDWRDF-FPYLKWIPNKSFE-ARIQQKHKRRLAVMNALIQDR----LKQNGSESDDDC--- : 289
AtKAO1    : EREYTNLNYGVR--AMGINLPG--FAYHRALKAR----KKLVAAFQSIVTNRRNQRKQN----ISSNRKDMLDN---- : 272
AtKAO2    : EREYTALNYGVR--AVAVNIPG--FAYHRALKAR----KTLVAAFQSIVTERRNQRKQN----ILSNKKDMLDN---- : 273
GfP450-1  : TST--YAVIAFRAVE-ELRL-WPSWLRPVVQWFMPHCTQSRALVQEARDLINPLLERRREEK-AEAERTGEKMTYND--- : 293
GfP450-2  : TKT--YTTNFYTAS--TNLRMFPRSIRPLAHWFLPECRKLRQERKDAIGIITPLLERRRELRRAAMAAGQPLPVFHD--- : 285
GfP450-3  : SIG--YTVQCVSIR--DQLFTWSPVLRPIIGPFLPSVRSVRRHLRFAAEIMAPLISQALQDE--KQHRADTLLADQTEGR : 138
GfP450-4  : TTTAEYSESLFITG--FILRVVPHILRPFIAPLLPSYRTLLRNVSSGRRVIGDIIRSQQGD----------GNED----- : 287

AtKO      : --YLNFLMSEAK----TLTKEQIAILVWETIIETADTTLVTTEWAIYELAKHPSVQDRLCKEIQNVCG-----GEK-FKE : 357
AtKAO1    : --LLDVKDENGR----VLDDEKIIDLLLMYLNAGHESSGHLTMWATILMQEHPMILQKAKEEQERIVKKRAP-GQK-LTL : 344
AtKAO2    : --LLNVKDEDGK----TLDDEEIIDVLLMYLNAGHESSGHTIMWATVFLQEHPEVLQRAKAEQEMILKSRPE-GQKGLSL : 346
GfP450-1  : --AMEWLDDLAREK--GVGYDPACAQLSLSVAALHSTTDFFTQ-VMFDIAQNPELIEPLREEIIAVLG-----KQG-WSK : 362
GfP450-2  : --AIDWSEQEAEAAGTGASFDPVMFQLTLSLLAIHTTYDLLQQ-TMIDLGRHPEYIEPLRQEVVQLIR-----EEG-WKK : 356
GfP450-3  : GTFISWLLRHLP-EELRTPEQVGLDQMILVSFAAIHTTTMALTK-VVWELVKRPEYIEPLRTEMQDVFGPDAVSPDICIN : 216
GfP450-4  : --ILSWMRDAATGE--EKQIDNIAQRMLILSLASIHTTAMTMTHAMYDLCACPEYIEPLRDEVKSVVG------ASGWDK : 357

AtKO      : EQLSQVPYLNGVFHETLRKYSPAPLVPIR-YAHEDTQI-GGYHVPAGSEIAINIYGCNMDKKR-----------WERPED- : 424
AtKAO1    : KETREMEYLSQVIDETLRVIT-FSLTAFR-EAKSDVQM-DGYIIPKGWKVLTWFRNVHLDPEI-----------YPDPKK- : 410
AtKAO2    : KETRKMEFLSQVVDETLRVIT-FSLTAFR-EAKTDVEM-NGYLIPKGWKVLTWFRDVHIDPEV-----------FPDPRK- : 412
GfP450-1  : NSLYNLKLMDSVLKESQRLKP-IAIASMARFTTHNVKLSDGVILPKN-KLTLVSAHQHWDPEY-----------YKDPLK- : 429
GfP450-2  : TTLFKMKLLDSAIKESQRLKP-GSIVTMRRYVTEDITLSSGLTLKKGTRLNVDNRRLDD-EKI-----------YDNPEV- : 423
GfP450-3  : EALSRLHKLDSFIREVQRWCPSTFVTPSR-RVMKSVTLSNGIKLQRGTSIAFPAHAIHMSEETPTFSPDFSSDPENPSPR : 295
GfP450-4  : TALNRFHKLDSFLKESQRFNP-VFLLTFNRIYHQSMTLSDGTNIPSGTRIAVPSHAMLQDSAHVPG---------PTPPT- : 427

AtKO      : -WWPERFLEDGK--------YETSDLHKTMAFGAGKRVCAGALQASLMAGIAIGRLVQEFEWKLRDGEEENVDTYGLTSQ : 495
AtKAO1    : -FDPSRWEG------------YTPKAGTFLPFGLGSHLCPGNDLAKLEISIFLHHFL--LKYRVERSNPGCPVMFLPHNR : 475
AtKAO2    : -FDPARWDNG-----------FVPKAGAFLPFGAGSHLCPGNDLAKLEISIFLHHFL--LKYQVKRSNPECLVMYLPHTR : 478
GfP450-1  : -FDGYRFFNMRREPGKESKAQLVSATPDHMGFGYGLHACPGRFFASEEIKIALSHILLKYDFKPVEGSSMEPRKYGLNMN : 508
GfP450-2  : -YNPYRFYDMRSEAGKDHGAQLVSTGSNHMGFGHGQHSCPGRFFAANEIKVALCHILVKYDWKLCPDTETKPDTRGMIAK : 502
GfP450-3  : IFDGFRYLNLRSIKGQGSQHQAAMTGPDYLIFNHGKHACPGRFFAISEIKMILIELLAKYDFRLEDGKPG-PELMRVGIE : 374
GfP450-4  : EFDGFRYSKIRSDSNYAQKYLFSMTDSSNMAFGYGKYACPGRFYASNEMKLTLAILLLQFEFKLPDGKGR-PRNITIDSD : 506

AtKO      : KLYPLMAIINPRRS-------- : 509
AtKAO1    : PKDNCLARITRTMP-------- : 489
AtKAO2    : PTDNCLARISYQ---------- : 490
GfP450-1  : ANPTAKLSVRRRKEEIAI---- : 526
GfP450-2  : SSPVTDILIKRRESVELDLEAI : 524
GfP450-3  : TRLLTKAGLEMRRR-------- : 388
GfP450-4  : MIPDPRARLCVRKRSLRDE--- : 525
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Figure 5. Amino acid alignment *of Arabidopsis* and *G. fujikuroi* cytochrome P450s involved in GA biosynthesis, produced using Clustal W. Heavy, intermediate and light shading denotes 91, 67, and 50% conservation, respectively, of amino acid residues within the seven sequences.

fungal enzymes are highly diverged. Two enzymes from *G. fujikuroi* are required for the conversion of *ent*-kaurene to GA_{14}. *P450-4* encodes an *ent*-kaurene oxidase with the same function as the plant enzymes (Tudzynski and others 2001). However, it has only 10% amino acid sequence identity with the *Arabidopsis* enzyme (Table 2) and it is placed in a different P450 family, CYP503, as opposed to CYP701 for the plant KOs. The B1-41a mutant of *G. fujikuroi,* which was reported to be defective in *ent*-kaurenoic acid synthesis (Bearder and others 1974), could be rescued by transformation with the wild-type *P450-4* gene (Tudzynski and others 2001). The gene from B1-41a was found to contain a single base substitution at an intron-exon boundary resulting in incorrect splicing of the second intron and, therefore, highly reduced levels of normal transcript.

A second enzyme, encoded by *P450-1,* oxidizes *ent*-kaurenoic acid to GA_{14} (Rojas and others 2001).

Table 2. Percentage Identities between the Cytochrome P450s of GA Biosynthesis in *Arabidopsis* (Unshaded) and *G. fujikuroi* (Shaded)

	AtKO	AtKAO1	AtKAO2	GfP450-1	GfP450-2	GfP450-3
AtKAO1	11					
AtKAO2	13	75				
GfP450-1	6	12	14			
GfP450-2	12	9	10	35		
GfP450-3	7	13	10	25	25	
GfP450-4	10	10	12	25	22	29

AtKO and GfP450-4 (ent-kaurene oxidase), and AtKAO1/2 and GfP450-1 (ent-kaurenoic acid oxidase) gave similar functions.

This remarkable enzyme fulfills a similar role to the KAOs identified in *Arabidopsis* and barley, but has, in addition, 3β-hydroxylase activity. The 3β-hydroxyl is in fact introduced into GA_{12}-aldehyde to form GA_{14}-aldehyde before oxidation at C-7 (Hedden and others 1974). Work with microsomal preparations indicates that the 3β-hydroxylase and 7-oxidase activities have different requirements for pyridine and flavin nucleotides, suggesting that different electron transport proteins are involved in these reactions despite the fact that they are catalyzed by the same enzyme (Urrutia and others 2001).

In addition to its role in GA biosynthesis, the product of *P450-1* is apparently also responsible for the production of a number of *ent*-kaurenoid metabolites, including kaurenolides, derived from *ent*-kaurenoic acid and fujenoic acid, derived from *ent*-7α-hydroxykaurenoic acid (Rojas and others 2001). Despite their related function, the plant KAOs and *G. fujikuroi* P450-1 have low sequence homology (Table 2) and belong to different cytochrome P450 families; the plant enzymes are assigned to subfamily CYP88A, and P450-1 is in CYP68.

Formation and Metabolism of C_{19}-GAs

The final stages of GA biosynthesis in plants, comprising the removal of C-20 by GA 20-oxidases (GA20ox) and the introduction of the 3β-hydroxyl group by GA 3-oxidases (GA3ox), are carried out by soluble dioxygenases that are assumed to be localized in the cytosol. A third group of dioxygenases, GA 2-oxidases (GA2ox), are responsible for GA deactivation. The dioxygenases are encoded by multigene families, members of which show distinct tissue-specific patterns of expression (Carrera and others 1999; García-Martínez and others 1997;

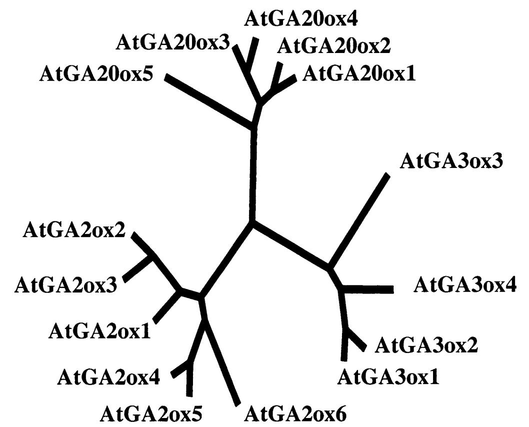

Figure 6. Unrooted phylogenetic tree of the *Arabidopsis* GA dioxygenases, produced using the Philip programs PROTDIST and NEIGHBOR (Felsenstein 1989), and displayed using TREEVIEW (http:\\taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Phillips and others 1995; Rebers and others 1999) and, in some cases are differentially regulated by endogenous or environmental signals (Yamaguchi and others 1998). As shown in Table 1 and Figure 6, the *Arabidopsis* genome contains potentially five *GA20ox,* four *GA3ox*, and six *GA2ox* genes. The functions of three of the *GA20ox* genes (Phillips and others 1995), all four *GA3ox* genes (Williams and others 1998; Yamaguchi and others 1998; Phillips and Hedden, unpublished information) and five of the *GA2ox* genes (Thomas and others 1999; Thomas, Woolley, Phillips and Hedden, unpublished) have been confirmed by expressing their cDNAs in *E. coli. GA2ox5* is not expressed and is assumed to be a pseudo gene. The GA 20-oxidases catalyze the sequential oxidation of C-20 including the cleavage of the C-10 – C-20 bond and the formation of the γ-lactone that is characteristic of C_{19}-GAs. The enzymes will accept as substrates only C_{20}-GAs containing a free C-19 carboxyl group, which becomes the lactone function of the final product.

The GA 3-oxidases and 2-oxidases that have been studied to date as recombinant enzymes act on GAs containing the lactone function so that the preferred substrates are the C_{19}-GAs or C_{20}-GAs, such as GA_{15}, that contain a δ-lactone (Itoh and others 2001; Thomas and others 1999; Williams and others 1998). GA 3-oxidases from *Arabidopsis* (AtGA3ox1; Williams and others (1998)) and pea (PsGA3ox1; Lester (1997); Martin and others (1997)) show tight regiospecificity and function solely as 3β-hydroxylases, whereas other GA 3-oxidases, such as those from *Phaseolus vulgaris* (French bean) (Smith and others 1990) and rice (Itoh and others 2001) possess de-

saturase and even 2β-hydroxylase activities in addition to their main function as 3β-hydroxylases. The rice enzyme also converts GA_{20} to GA_3, for which GA_5 is thought to be the intermediate. In the equivalent reactions in *Marah macrocarpus,* 2,3-didehydro GA_9 was shown to be an intermediate in the conversion of GA_9 to GA_7 (MacMillan and others 1997), to which it is metabolized by abstraction of the 1β-H and migration of the 2,3-double bond to the C-1,2 position (Albone and others 1990). An alternative route to GA_3 could involve 3β-hydroxylation of GA_{121} (1,2-didehydro GA_{20}), which is present in *Prunus persica* (Nakayama and others 2001). The type of enzyme responsible for the introduction of the 1,2-double bond in GA_{121} is unclear.

The properties of the enzymes that convert GA_{14} to GA_3 in *G. fujikuroi* were, until recently, unknown. Although GA_{14} formation could be shown in cell-free extracts of fungal mycelia (Urrutia and others 2001; West 1973), the later activities could not be demonstrated in such extracts. It has now been possible by gene disruption experiments to identify the cytochrome P450 genes, P450-2 and P450-3, as being responsible for the removal of C-20 and the introduction of the 13-hydroxyl group, respectively (B. Tudzynski, M. C. Rojas, P. Gaskin, and P. Hedden, unpublished). The desaturase that introduces the 1,2-double bond in the conversion of GA_4 to GA_7 is thought to be encoded by the final (seventh) gene of the cluster. Thus, GA 20-oxidase activity in *G. fujikuroi* is due to the action of a cytochrome P450 (P450-2), whereas the same activity in plants is the responsibility of dioxygenases. Although the properties of the fungal GA 20-oxidase are currently being determined, there are indications from earlier work for mechanistic differences between the fungal and plant enzymes. As discussed above, in work with cultures of the GA-deficient mutant B1-41a, no intermediates could be demonstrated, either as products or substrates, in the multi-step reaction sequence required for the removal of C-20 (Bearder and others 1975), although the assumed intermediates are present in fungal cultures. It would seem unlikely, in the light of the large range of applied substrates that are metabolized by *G. fujikuroi,* that the failure of the fungus to metabolize C_{20}-GAs, such as GA_{37} and GA_{36}, is due to inefficient uptake. One explanation is that the oxidation sequence occurs without the intermediates, or their enzyme-bound equivalents, leaving the enzyme active site, the presence of the proposed intermediates in cultures being due to their release from the enzyme at low rates, perhaps as a result of hydrolysis. Despite the nature of the fungal GA 20-oxidase being known, there is still no explanation for the failure to observe P450-2 activity *in vitro* while the related enzyme, P450-1 (GA_{14} synthase), is active in membrane preparations.

Regulation of Gene Expression

In plants, GAs mediate responses to developmental and environmental cues, which modify the rate of GA biosynthesis and/or the extent to which tissues respond to the hormone. There is now considerable information on how these cues regulate expression of the genes encoding GA-biosynthetic enzymes. As is discussed in detail in other contributions to this issue (García-Martinez and Gil 2002, this issue; Martínez-García and others 2002, this issue), light is the principal environmental signal that has been shown to modify GA-biosynthetic gene expression, acting mainly on the dioxygenase genes. Tight developmental control is important at several points in the pathway. In *Arabidopsis,* the *CPS* gene is expressed in growing tissues and vascular elements (Silverstone and others 1997), consistent with the report that *ent*-kaurene synthesis occurs in proplastids rather than mature chloroplasts (Aach and others 1997). As noted above, members of the GA dioxygenases gene families show distinct tissue-specific patterns of expression and are thus involved in different developmental processes. For example, differential expression of *GA3ox* genes has been described for *Arabidopsis* seedlings (Yamaguchi and Kamiya 2002, this issue; Yamaguchi and others 1998). Although expression of GA biosynthesis genes is high at the shoot apex, GA biosynthesis is apparently excluded from the central zone of the meristem, where GAs may be detrimental to the maintenance of the meristem. It has been shown in tobacco that a homeodomain transcription factor encoded by a *KNOX* gene, which is expressed within the meristem and is important in meristem maintenance, represses the expression of a *GA20ox* gene (Sakamoto and others 2001). The *GA20ox* gene is expressed in peripheral regions of the meristem where GAs may function in leaf development.

The plant GA dioxygenase genes participate in GA homeostasis. Expression of *GA20ox* and *GA3ox* genes is suppressed by bioactive GAs, whereas *GA2ox* expression is up-regulated (Hedden and Phillips 2000; Thomas and others 1999). The mechanism for this feedback and feedforward regulation is not understood in detail, but it appears to share at least part of the same signal transduction pathway as the growth responses (Cowling and others 1998). Expression of

GA dioxygenase genes is also regulated by auxin in a clear example of cross-talk between plant hormone pathways. This subject is reviewed by Ross and others (2002), in this issue.

The conditions for optimal production of GAs by *G. fujikuroi* are of commercial importance and, as such, have been studied extensively over the last 50 years (Borrow and others 1955; Borrow and others 1961; Brückner and Blechschmidt 1991; Candau and others 1992; Darken and others 1959; Fuska and others 1961; Geissman and others 1966). The most striking regulatory principle is that GA production is repressed by a high nitrogen content of the culture medium. Only a low level of *ent*-kaurene biosynthetic activity was measured during the exponential growth phase of the fungus, whereas *ent*-kaurene and GA_3 biosynthesis reached a much higher level after the exogenous nitrogen, as ammonium or glutamine was exhausted (Munoz and Agosin 1993). When the conversion of ammonium into glutamine was inhibited, only glutamine inhibited GA production, indicating that glutamine was the effector of GA-biosynthesis inhibition. After the GA-biosynthetic genes were cloned, an intensive study of the molecular mechanism of the nitrogen regulation has been started. Expression studies on the genes in the GA-biosynthesis cluster demonstrated that the transcript level of six of the seven genes increases dramatically under conditions of ammonium limitation (Tudzynski and Holter 1998; Tudzynski and others, unpublished). In order to show if the increased expression of the genes is mediated by the major nitrogen regulatory protein AREA (NIT2), which in *Neurospora crassa* and *Aspergillus nidulans* positively affects the expression of many genes involved in the utilization of nitrogen sources other than ammonium (Marzluf 1996), the corresponding regulatory gene, *areA-Gf*, was cloned from *G. fujikuroi*. Inactivation of this gene in *G. fujikuroi* resulted in a very large reduction of GA production to about 10%–20% of that in the wild-type. Complementation of the *areA-Gf*-deficient mutants with the intact gene fully restored the ability to produce GAs in amounts comparable to the wild-type strain (Tudzynski and others 1999). There is a corresponding reduction in transcript levels for the GA-biosynthesis genes in the mutants, and restoration of gene expression after complementation with the wild type *areA-Gf* gene (Tudzynski, unpublished data).

Many authors have described a significant repression of GA production in *G. fujikuroi* by glucose and other carbon sources that can be metabolized rapidly (Fuska and others 1961; Jefferys 1970). However, expression studies on the GA-biosynthesis genes revealed the same high transcript levels in mycelia grown in nitrogen-limited media with glucose or plant oil as carbon sources (Tudzynki and others 2000). In contrast to the GA dioxygenase genes of higher plants, no feed-back or light regulation was observed for any GA-biosynthetic gene from *G. fujikuroi.* The addition of 1 g/L of the final product, GA_3, to the culture did not repress the biosynthesis of GAs nor the transcript level of the GA-biosynthetic genes (Tudzynski unpublished information). These results are consistent with GAs having no hormonal role in the fungus.

Summary and Conclusion

The recent identification of almost all the genes for GA biosynthesis in *Arabidopsis* and the fungus *G. fujikuroi* has revealed profound differences in this process between plants and the fungus. For example, a single terpene cyclase converts GGPP to *ent*-kaurene in fungi whereas two enzymes are required for this transformation in plants. Removal of C-20 and the introduction of the 3β-hydroxyl group are catalyzed by cytochrome P-450-dependent monooxygenases in *G. fujikuroi* but by 2-oxoglutarate-dependent dioxygenases in plants. Even when P450s have similar functions in both plants and fungi the enzymes are only distantly related. It seems likely, therefore, that GA biosynthesis in plants and *G. fujikuroi* have evolved independently. While the genes of GA-biosynthesis are dispersed throughout the genome *of Arabidopsis,* they are clustered in *G. fujikuroi,* as is common for secondary metabolite biosynthesis in fungi (Keller and Hohn 1997). The ability to produce GAs has been acquired by a number of fungal and bacterial species (MacMillan 2002, this issue; Rademacher 1994) and, at least for the fungi, it is likely to have a common origin in these organisms.

GAs do not have a clearly identifiable function in fungi, and strains of *G. fujikuroi* that lack the GA-biosynthesis gene cluster grow normally in culture, although their pathogenicity has not been examined. It is conceivable that, by induction of hydrolytic enzymes in the host, GAs may aid infection by the fungus and improve its nutrient supply. The very large amounts of GAs produced by some fungal species, including *G. fujikuroi,* and their efficient secretion would indicate that they act on the host rather than the fungus itself. Whatever the function of the fungal GAs, *G. fujikuroi* has played a leading part in the history of research into these phytohormones by being a valuable source of material and above all, the trigger for their discovery.

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