

# Gibberellin metabolism: new insights revealed by the genes

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**The identification of most of the genes involved in the metabolic pathways for gibberellin hormones has helped us to understand these pathways and their regulation. Many of these enzymes are multifunctional and therefore fewer enzymes than might be expected are required to synthesize the various gibberellin structures. However, several of the enzymes are encoded by multiple genes that are regulated differently, adding unexpected genetic complexity. Several endogenous and environmental factors modify the expression of gibberellin biosynthesis genes, including developmental stage, hormonal status and light. A future challenge will be to dissect the complex, interacting pathways that mediate the regulation of gibberellin metabolism.**

The key role that gibberellins (GAs) play in plant growth and development is clearly evident from observing GA-deficient mutants, which are usually much shorter than the wild type. In the 1950s, the height of dwarf mutants of pea and maize were restored to normal by applying gibberellic acid (GA<sub>3</sub>), which was newly available from cultures of the fungus *Gibberella fujikuroi*. This was the first indication that GAs were endogenous growth regulators in plants<sup>1</sup>. This proposal was supported by the finding that plant extracts could also stimulate the growth of the mutants and was later confirmed by the chemical identification of GAs from higher plants.

The use of sensitive physicochemical methods of analysis, which principally combined gas chromatography and mass spectrometry (GC-MS), has revealed that the GAs are a bewilderingly large group of natural products, with 126 different compounds currently known in higher plants, fungi and bacteria. However, it is clear from work with GA-deficient mutants that relatively few GAs have intrinsic biological activity and therefore possess a hormonal function. Such work has also shown that GAs are not only required for stem elongation but indeed participate in most, if not all, stages of plant development. Furthermore, they mediate between certain environmental signals (e.g. light quality and photoperiod) and the induced physiological responses (e.g. stem extension and flowering).

A broad range of endogenous and environmental factors thus clearly impinge on the GA signalling pathways, including the biosynthesis and catabolism of the hormones as well as their signal transduction. Isolation of most of the participating genes is the reason for the substantial recent progress in our understanding of GA metabolism, giving us insight into the mechanisms by which GA content is regulated and also providing a rationale in biochemical terms for the apparent chemical complexity of the GA family.

## Pathways

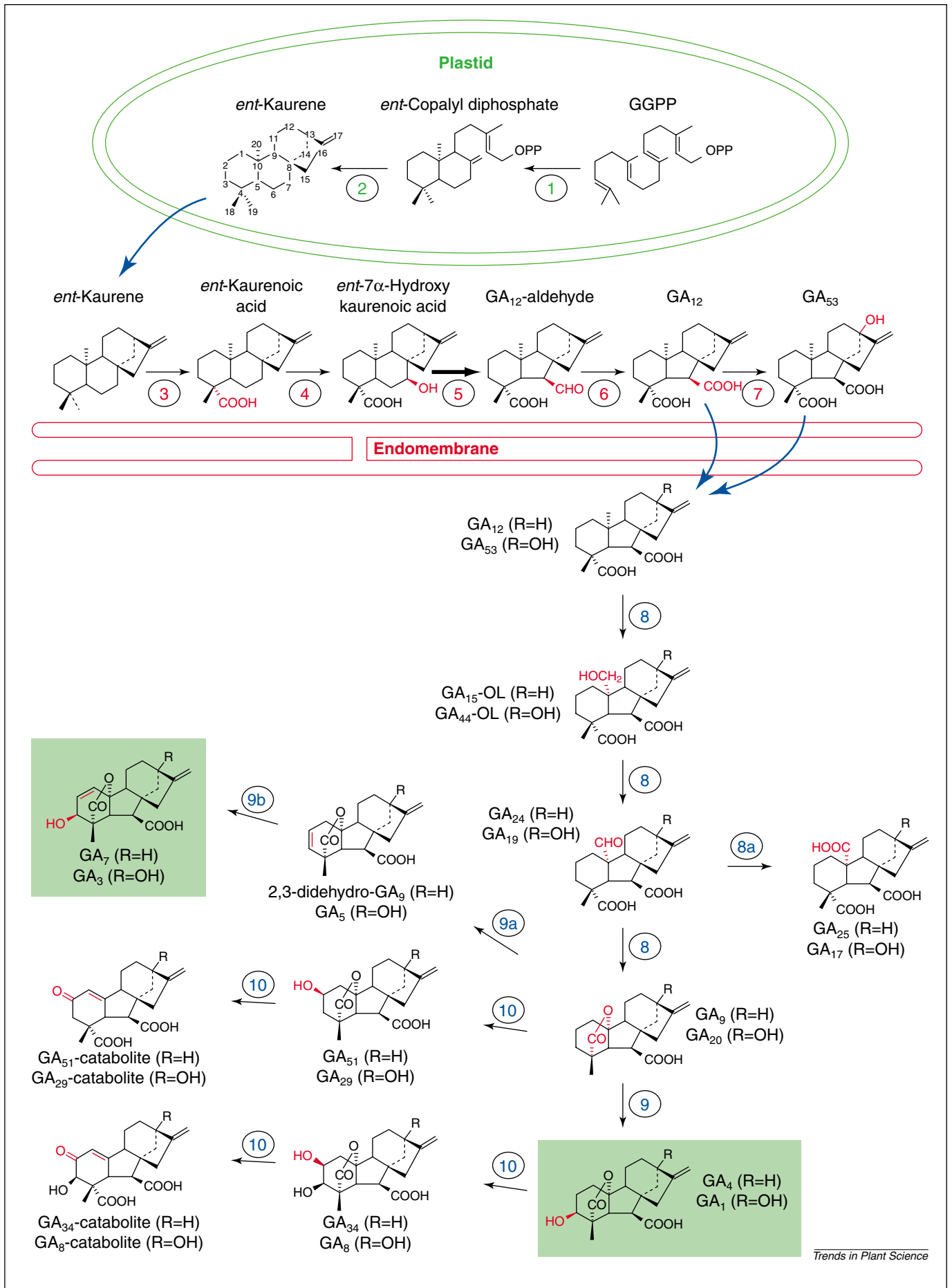
The complex pathways of GA metabolism were elucidated first in *G. fujikuroi* and then in higher plant species. This involved painstaking work in which putative intermediates, usually isotopically labelled, were applied to intact organisms or cell-free extracts and the products identified by GC-MS. The results of these studies, which have been reviewed comprehensively<sup>2</sup>, laid the foundation for the recent advances in the field.

The major pathways for the formation and catabolism of the bioactive compounds GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub> and GA<sub>7</sub> in higher plants are shown in Fig. 1. The early pathway, from *trans*-geranylgeranyl diphosphate (GGPP) to GA<sub>12</sub>-aldehyde, is common to all systems that have been studied. GGPP is converted via *ent*-copalyl diphosphate

(CPP) to the tetracyclic hydrocarbon *ent*-kaurene, which is then modified by sequential oxidations on C-19, C-7 and C-6 to produce GA<sub>12</sub>-aldehyde. In plants, *ent*-kaurene formation from GGPP occurs in plastids and requires two diterpene cyclases, CPP synthase (CPS) and *ent*-kaurene synthase<sup>3</sup> (KS), whereas a single enzyme catalyses both steps in fungi<sup>4,5</sup>. The cyclization catalysed by CPS is initiated by protonation of the C-14–C-15 double bond of GGPP. This is classified as a Type-B cyclization<sup>6</sup>, whereas conversion of CPP to *ent*-kaurene is initiated by ionization of the diphosphate (Type-A cyclization).

The origin of the GGPP that is destined for *ent*-kaurene biosynthesis in plastids is unclear. Although it has been shown that other GGPP-derived metabolites, such as carotenoids and the phytol residue of chlorophyll, originate from 1-deoxyxylulose-5-phosphate rather than mevalonate<sup>7</sup>, there is as yet no published information on the biochemical origin of the GAs. Mevalonate is incorporated into *ent*-kaurene in intact immature pumpkin (*Cucurbita maxima*) cotyledons – non-photosynthetic tissues in which *ent*-kaurenoids and GAs are major terpenoid products (P. Hedden, unpublished). It is possible that either or both pathways contribute to GA biosynthesis, their relative contribution depending on the tissue and developmental stage.

GA<sub>12</sub>-aldehyde is formed from *ent*-kaurene via *ent*-kaurenoic acid and *ent*-7 $\alpha$ -hydroxykaurenoic acid by the action of cytochrome-P450-dependent mono-oxygenases. The reactions occur on membranes outside the plastid, requiring the transport of *ent*-kaurene from the organelle by an unknown mechanism. Mono-oxygenases also catalyse the further oxidation of GA<sub>12</sub>-aldehyde at C-7 to give GA<sub>12</sub> and the 13-hydroxylation of GA<sub>12</sub> to GA<sub>53</sub>. These last two intermediates are substrates for the final stage of GA biosynthesis, involving soluble 2-oxoglutarate-dependent dioxygenases. They are converted by GA 20-oxidase (GA20ox) to GA<sub>9</sub> and GA<sub>20</sub>, respectively, by oxidation of C-20 to an aldehyde followed by the removal of this C atom and the formation of a lactone. The bioactive GAs, GA<sub>4</sub> and GA<sub>1</sub>, are then formed from GA<sub>9</sub> and GA<sub>20</sub>, respectively, by the action of GA 3 $\beta$ -hydroxylase (GA3ox). In some species, GA<sub>9</sub> and GA<sub>20</sub> are also converted to GA<sub>7</sub> and GA<sub>3</sub>, respectively, via 2,3-didehydroGA<sub>9</sub> and GA<sub>5</sub>, probably as side reactions of GA3ox activity<sup>8,9</sup>. A third dioxygenase, GA 2-oxidase (GA2ox), deactivates GAs by 2 $\beta$ -hydroxylation and, in some cases, further oxidation to a ketone function at C-2. Pumpkin contains a dioxygenase [GA 7-oxidase, (GA7ox)] that oxidizes GA<sub>12</sub>-aldehyde to GA<sub>12</sub> in addition to the mono-oxygenase 7-oxidase activity<sup>10</sup>, but this dioxygenase has not yet been found in other species.



**Fig. 1.** The principal pathways of gibberellin (GA) metabolism in higher plants, showing subcellular compartmentalization between plastids, the endomembrane system and cytoplasm. The functional groups that are introduced or modified at each step are indicated in red and the biologically active GAs are highlighted in green. The enzymes catalysing these reactions are: (1) *ent*-copalyl diphosphate synthase (CPS); (2) *ent*-kaurene synthase (KS); (3) *ent*-kaurene 19-oxidase (EKO); (4) *ent*-kaurenoic acid 7 $\beta$ -hydroxylase; (5) GA<sub>12</sub>-aldehyde synthase; (6) GA 7-oxidase (GA7ox); (7) GA 13-hydroxylase (GA13ox); (8) GA 20-oxidase (GA20ox); (9) GA 3 $\beta$ -hydroxylase (GA3ox); (10) GA 2-oxidase (GA2ox). A GA20ox from developing pumpkin seeds (8a) catalyses the three-step oxidation of GA<sub>12</sub> to GA<sub>25</sub> and of GA<sub>33</sub> to GA<sub>17</sub>. The biosynthesis of GA<sub>7</sub> from GA<sub>9</sub> and of GA<sub>3</sub> from GA<sub>20</sub> (9a,b) is probably catalysed as a side reaction by a subset of GA 3-oxidases. GA<sub>15</sub>-OL and GA<sub>44</sub>-OL are the open lactone forms of the C-20-hydroxylated GAs, GA<sub>15</sub> and GA<sub>44</sub>.

The dioxygenases belong to a large class of Fe-containing enzymes found in plants and fungi that share a common mechanism<sup>11</sup>. Most of these enzymes, including all of those involved in GA biosynthesis, use 2-oxoglutarate as a cosubstrate. The X-ray crystal structure of a fungal 2-oxoglutarate dioxygenase, desacetoxycephalosporin C synthase, has confirmed that Fe is bound to two H residues and a D residue, and that the 5-carboxyl group of 2-oxoglutarate binds to R and S (Ref. 12). These residues are conserved in all the GA dioxygenases (Fig. 2), with S occasionally replaced by T.

The two parallel pathways of GA-biosynthesis described above (Fig. 1) operate in the vegetative tissues of most plant species that have been studied, the predominance of one over the other presumably depending on the strength of 13-hydroxylase activity. However, this description is an oversimplification of GA biosynthesis generally, which is better represented after GA<sub>12</sub>-aldehyde as a complex metabolic grid. The routes through the grid depend on the order in which the oxidations at C-7, C-13, C-20, C-3 and C-2 occur, and can generate many possible intermediates, as can be seen in developing seeds of certain species.

Further complexity is present in some species because of oxidation at various positions in rings C and D, but nothing is known about the enzymes involved in these reactions. The pathway after GA<sub>12</sub>-aldehyde in *G. fujikuroi* diverges significantly from those in higher plants, even though the major end product is GA<sub>3</sub> (Refs 2,13). In contrast with plants, 3 $\beta$ -hydroxylation is catalysed by a monooxygenase, for which GA<sub>12</sub>-aldehyde is the major substrate, and 13-hydroxylation occurs at the end of the pathway. There is no evidence for the involvement of 2-oxoglutarate-dependent dioxygenases in GA biosynthesis in fungi.

### Genes and gene families

GAs are present in most vegetative and floral tissues at low concentrations (0.1–100.0 ng g<sup>-1</sup> fresh weight) and their biosynthetic enzymes are similarly low in abundance, precluding the purification of the enzymes from these sources. However, the developing seeds of several species have exceptionally high rates of GA biosynthesis and these have been the materials of choice both for characterization of the enzymes

and as a traditional biochemical route to isolate the genes<sup>14–16</sup> (Table 1). An alternative approach, which is based on the functional screening of cDNA expression products derived from these GA-rich seed tissues, has been used to identify genes for several dioxygenases from the later stages of the pathway<sup>10,17–19</sup>. Genetic strategies for the identification of GA-biosynthetic genes involving the isolation and characterization of GA deficient mutants in the model species *Arabidopsis* and maize have been equally successful. *Arabidopsis* mutants have been generated at several dwarf (*ga1*, *ga2*, *ga3*) and semi-dwarf (*ga4*, *ga5*) loci<sup>20</sup>, and several different approaches (e.g. genomic subtraction<sup>21</sup>, insertional mutagenesis<sup>22</sup> and map-based cloning<sup>23</sup>) have been used to isolate the genes involved (Table 1). More details of the methods used to isolate the GA-biosynthesis genes are given in Box 1.

These different strategies have led to the identification of genes representing all the terpene cyclases and dioxygenases from the early and final stages, respectively, of the GA-biosynthetic pathway, and their functions have been confirmed by heterologous expression in *E. coli*. Progress with the cytochrome-P450 enzymes that catalyse up to seven steps in the central part of the pathway (Fig. 1) has been slower, partly because of the difficulty of biochemical purification or heterologous expression in bacteria. One recent success is the cloning of the *GA3* gene from *Arabidopsis*, which has been expressed in yeast and been shown to encode *ent*-kaurene oxidase (EKO); this catalyses three oxidation reactions at C-19 (Ref. 24). The *D3* gene of maize (mutations in which yield a GA-deficient dwarf phenotype) encodes an unrelated enzyme of the P450 class<sup>25</sup> but no progress in demonstrating catalytic activity has been reported.

In *Arabidopsis*, enzymes early in the pathway [CPS (Ref. 21), KS (Ref. 26) and EKO (Ref. 23)] appear to be encoded by single-copy genes, and loss-of-function mutations at these loci consequently produce a severe dwarf phenotype. However, the deletion mutant *gal-3* accumulates a small amount of GA (Ref. 27), even though it is unlikely to encode a functional enzyme. This suggests that a small amount of CPP, or perhaps *ent*-kaurene directly, might be produced by a related diterpene cyclase in *Arabidopsis*. By contrast, the dioxygenases that catalyse the later reactions in the pathway are each encoded by a small multigene family: GA20ox and GA3ox are each encoded by at least four genes, and GA2ox by at least six genes, although one of these

|                 |   |
|-----------------|---|
| AtGA20ox1 (243) | GTGPHCDPTSLTILHQD---HVNGLQVFNENQ-WRSI           |
| AtGA3ox1 (228)  | GLAAHTDSTLLTILYQN---NTAGLQVFRDDLGVVTV           |
| AtGA2ox1 (193)  | GFGEHTDPQIISVLRSN---NTSGLQINLNDGSIWISV          |
| CmGA7ox1 (178)  | GLIHHEEDANCTILVIQD---DAGGLQVQKQDSE-WIPV         |
| ScDAOCS (179)   | RMAPHYDLSMVTLIQQTTPCANGFVSLQAEVGGGA-FTDL        |
|                 |   |
| RPNPKAFVFN      | GDTFMALSNDRYKSLHRAVVNSK-----SERKSLAFF (319)     |
| PPFPGLVNVG      | DLFHILSNGLFKSVLHRRARVNQT-----RARLSVAFL (305)    |
| PPDHTSFFFV      | GDLSQVMTNGREFKSVRHRVLANCK-----KSRVSMIYF (270)   |
| TPVEGAIIVNV     | GDIIQVLSNKKFKSATHRVVRQKG-----KERYSFVAF (254)    |
| EYRPDAVLVFC     | GAIALTLVTVGQVKAAPRHVVAAAPRRDQIAGSSRTSSVFF (256) |

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**Fig. 2.** Amino acid sequences of the active site core of 2-oxoglutarate-dependent dioxygenases involved in gibberellin (GA) biosynthesis from *Arabidopsis* [AtGA20ox1 (Ref. 36), AtGA3ox1 (Ref. 22), AtGA2ox1 (Ref. 17)] and pumpkin [CmGA7ox1 (Ref. 10)], aligned with desacetoxycephalosporin C synthase (ScDAOCS) from *Streptomyces clavuligerus*<sup>12</sup>. Residues in DAOCS that bind the active-site Fe are highlighted in red and those that interact with the 5-carboxylate of 2-oxoglutarate in green.

**Table 1. GA-biosynthetic enzymes whose genes have been cloned from plants**

| Enzyme                                   | Gene name     | <i>Arabidopsis</i> locus | First cloned from                          | Method   |
|--|---------------|--------------------------|--|--|
| <i>ent</i> -Copalyl diphosphate synthase | <i>CPS</i>    | <i>GA1</i>               | <i>Arabidopsis</i>                         | Genomic subtraction <sup>21</sup> and heterologous expression <sup>32</sup>      |
| <i>ent</i> -Kaurene synthase             | <i>KS</i>     | <i>GA2</i>               | Pumpkin ( <i>Cucurbita maxima</i> )        | Enzyme purification, degenerate RT-PCR and heterologous expression <sup>16</sup> |
| <i>ent</i> -Kaurene 19-oxidase           | <i>EKO</i>    | <i>GA3</i>               | <i>Arabidopsis</i>                         | Map-based cloning <sup>23</sup> and heterologous expression <sup>24</sup>        |
| GA <sub>12</sub> -aldehyde 7-oxidase     | <i>GA7ox</i>  | <sup>a</sup>             | Pumpkin <sup>b</sup>                       | Functional screening of expression library <sup>10</sup>                         |
| GA 20-oxidase                            | <i>GA20ox</i> | <i>GA5</i>               | Pumpkin                                    | Protein purification and immuno-screening of expression library <sup>15</sup>    |
| GA 3β-hydroxylase                        | <i>GA3ox</i>  | <i>GA4</i>               | <i>Arabidopsis</i>                         | Insertional mutagenesis <sup>22</sup> and heterologous expression <sup>63</sup>  |
| GA 2-oxidase                             | <i>GA2ox</i>  | <sup>a</sup>             | Runner bean ( <i>Phaseolus coccineus</i> ) | Functional screening of expression library <sup>17</sup>                         |

<sup>a</sup>No mutation at this locus has been identified in *Arabidopsis*.

<sup>b</sup>Note that the *GA7ox* gene cloned from pumpkin is a 2-oxoglutarate-dependent dioxygenase, whereas in most cases studied this reaction is catalysed by a cytochrome P450 mono-oxygenase.

appears not to encode a functional protein (A.L. Phillips and P. Hedden, unpublished).

This functional redundancy in the GA dioxygenases explains why null mutations in *AtGA20ox1* (*GA5*) and *AtGA3ox1* (*GA4*) exhibit a semi-dwarf phenotype: there is overlap between expression patterns of the isozymes and the GA products are, to different extents, mobile within the plant. Although the three dioxygenase classes act on similar GA substrates, cluster analysis shows that they are no more closely related to each other than to any other plant dioxygenase (Fig. 3). The GA-biosynthesis genes are distributed throughout the *Arabidopsis* genome and are not clustered in any way, with the exception of *AtGA3ox2* (*GA4H*) and *AtGA3ox4*, which are adjacent on chromosome 1. This is markedly different from *G. fujikuroi*, in which several, if not all, of the genes encoding the GA-biosynthesis enzymes are closely linked in a gene cluster<sup>28</sup>.

#### Regulation of gibberellin metabolism

The multiple roles of GAs in plant development suggest that the regulation of their levels is likely to be complex, particularly given the large numbers of enzymes and genes involved in the biosynthetic pathway. Several factors that influence GA metabolism have been identified (Fig. 4), of which the most studied are tissue type, developmental stage, light and response to GAs. This last effect, which provides a mechanism for GA homeostasis, might form part of a broader process that includes the action of other hormones.

The signalling pathways involved in the regulation of GA biosynthesis are unknown, but clues to their nature might be emerging. Overexpression of *KNOTTED*-type homeobox genes from tobacco<sup>29</sup> or rice<sup>30</sup> suppressed *GA20ox* expression, reduced GA<sub>1</sub> content and produced dwarfism in tobacco. However, it is not known whether these transcriptional regulators interact directly with the *GA20ox* gene. Recently a basic leucine zipper transcription factor known as RGS (repression of shoot growth) was shown to bind and activate the *Arabidopsis* *GA3* gene, which encodes *EKO* (Ref. 31). Expression of a dominant negative form of RGS, which binds but does not activate the *EKO* promoter, resulted in severe reductions in growth and GA content in tobacco. However,

it remains to be seen whether the transcription factors interact with these targets when expressed at normal levels in wild-type plants.

#### Developmental regulation

The conversion of GGPP to CPP by *CPS* appears to be under tight developmental control. Although, in *Arabidopsis*, most of the *CPS* activity is encoded by a single gene<sup>32</sup>, at least two differently expressed genes are present in pumpkin<sup>33</sup>. The *Arabidopsis* *CPS* gene is expressed in rapidly growing tissues and in the vascular elements of expanded leaves, which might act as a source of GAs or precursors<sup>34</sup>. This distribution is consistent with the finding that *ent*-kaurene formation from GGPP in pea and wheat occurs in proplastids within the shoot meristem<sup>35</sup>. Control of *ent*-kaurene synthesis appears to reside predominantly with *CPS* because *KS* expression shows no tissue specificity<sup>33</sup> and, in *Arabidopsis*, is at much higher levels than that of *CPS* (Ref. 26).

Members of the gene families encoding the dioxygenases of GA biosynthesis are expressed differently, as determined by transcript accumulation, and might be involved in different developmental processes. Expression of the *GA20ox* gene family members has been studied in several species<sup>36–39</sup> but few general patterns have been discerned so far: it has only been possible to identify *GA20ox* orthologues with similar expression patterns in closely related species. The *GA20ox1* and *GA3ox1* genes of *Arabidopsis* are expressed in growing vegetative tissues, with some expression also in flowers and, for *GA3ox1*, in developing siliques<sup>36,40</sup>. A second gene for *GA3ox* (*GA3ox2* or *GA4H*) is expressed only in young seedlings and is thought to be involved in germination<sup>40</sup>. The *GA20ox3* gene might have a similar function because antisense expression of this gene caused reduced growth only in young seedlings<sup>41</sup>.

#### Hormonal regulation

There are numerous examples of plants with reduced GA content (either as a result of mutation in the GA-biosynthesis pathway or after treatment with chemical inhibitors of GA biosynthesis) that contain elevated levels of transcript for *GA20ox* genes<sup>36,39,42–45</sup> and *GA3ox* genes<sup>22,40,45–47</sup>. Conversely, treatment of plants with bioactive GAs depresses the levels of these transcripts<sup>17,36,39,40,42–44,47</sup>.

**Box 1. Methods used for cloning GA biosynthetic genes****Degenerate reverse-transcription PCR**

Mixtures of oligonucleotide primers, designed on the basis of amino acid sequences from purified enzymes<sup>16</sup> or from conserved regions of related enzymes<sup>36</sup>, are used to amplify cDNA by PCR. Full-length cDNA sequences are obtained by screening cDNA libraries with the PCR products<sup>16,36</sup> or by extending the sequences using rapid amplification of cDNA ends<sup>62</sup> (RACE).

**Functional screening**

Aliquots of a cDNA library are expressed in *E. coli* and clones expressing the target enzymes selected using screens based on the enzyme catalytic activity<sup>10,17-19</sup>.

**Genomic subtraction**

This method exploits the existence of a deletion mutation in the target gene<sup>21</sup>. An excess of genomic DNA from the mutant is biotinylated and hybridized with wild-type DNA. Common sequences between wild-type and mutant are then removed by adding avidin-coated beads. After several rounds of hybridization, the remaining, enriched wild-type sequences are amplified by PCR.

**Heterologous expression**

The function of proteins encoded by genes of interest can be confirmed by expressing cDNA clones in *E. coli*<sup>36</sup> or yeast<sup>24</sup> and assaying the expressed protein in extracts or intact cells.

**Immunoscreening**

In a method similar to functional screening, a cDNA library is screened for expression of the target protein using antibodies raised against a peptide sequence present in the purified enzyme<sup>15</sup>.

**Insertional mutagenesis**

Target genes are tagged by inserting T-DNA<sup>22</sup> or a transposon<sup>25</sup>. The sequences flanking the insertion are then cloned and used as probes to isolate the genes from wild-type plants.

**Map-based cloning**

The target gene (*GA3* in this case<sup>23</sup>) was mapped in the *Arabidopsis* genome using restriction fragment length polymorphism (RFLP) analysis with a mapping population of recombinant lines segregating for mutant and wild-type alleles of the target and marker genes. A bacterial artificial chromosome containing the target locus was identified and searched for cytochrome-P450-like sequences by random sequencing of subclones. A putative P450 sequence was shown to cosegregate with *GA3* and to contain mutations in DNA from the mutant lines.

Altered mRNA expression from these genes is also found in plants in which GA signal transduction is impaired<sup>42,43,47,48</sup>. These observations provide strong evidence for negative feedback control of the expression of *GA20ox* and *GA3ox* genes by GA. However, the effects of feedback regulation on enzyme content have not yet been reported, although the anticipated changes in GA metabolism and profiles of endogenous GAs have been observed<sup>43,45</sup>.

Recently, it was reported that treatment of the GA-deficient *gal-2* mutant of *Arabidopsis* with GA resulted in an increase in mRNA content for two *GA20ox* genes. This suggests the existence of positive feedforward regulation of these genes, which would result in increased rates of deactivation of C<sub>19</sub>-GAs (Ref. 17). Feedback and feedforward regulation, which provide a mechanism for GA homeostasis, are restricted to the dioxygenase genes in GA biosynthesis and do not occur earlier in the pathway. Indeed, not all the dioxygenase genes are regulated by GA; in germinating *Arabidopsis* seeds, the *GA3ox2* (*GA4H*) gene, in contrast with *GA3ox1*, is not subject to feedback regulation<sup>40</sup>. It was suggested that *GA3ox2* might contribute to the provision of GA for germination, which requires high levels of GA for a short period, and thus limiting its expression through feedback regulation would not be necessary or appropriate.

There are indications that other endogenous signals regulate the expression of the dioxygenase genes of GA biosynthesis. The application of 4-chloro-indole-3-acetic acid (4-Cl-IAA) to deseeded pea ovaries increases *GA20ox* expression<sup>49</sup>. The effect is specific for 4-Cl-IAA; the synthetic auxin 2,4-D has little effect on *GA20ox* expression in unpollinated pea ovaries<sup>37</sup>. Recently, it has been reported that expression of the pea gene for *GA3ox* (*PsGA3ox1*) in stem internodes requires indole-3-acetic acid (IAA) from the shoot apex, and that *GA20ox* expression is reduced by IAA (Ref. 50). Interactions between hormones might also explain the huge accumulation of *GA20ox* transcript in unpollinated pea ovaries after removal of the shoot apex<sup>37</sup>. These interactions might form part of a broad homeostatic mechanism that coordinates and moderates plant growth.

**Regulation by light**

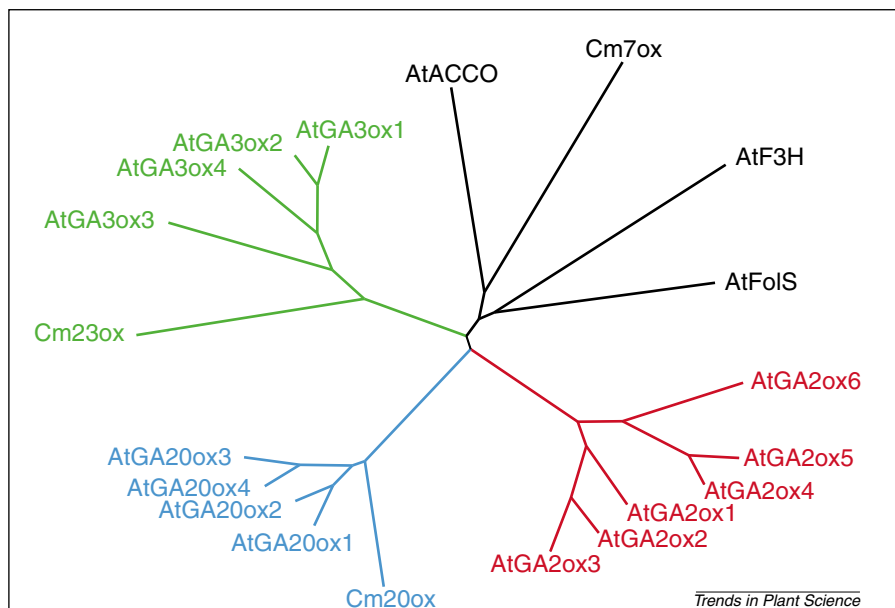
The dioxygenase genes are major targets for light regulation of GA metabolism<sup>51</sup>. Increased *GA20ox* expression in the rosette plants *Arabidopsis*<sup>52</sup> and spinach (*Spinacia oleracea*)<sup>53</sup> after exposure to long days might be an important factor in the induction of stem elongation in these species by photoperiod. Induction of tuber formation in potato by short days is also partially mediated by the photoperiod regulation of GA biosynthesis. Tuberization is inhibited by GA, the formation of which in leaves is less when grown under a photoperiod of short days than under long days<sup>54</sup>. The expression of *GA20ox* in leaves of potato grown under a photoperiod of short days was found to increase during the light period and to decrease during the dark period<sup>39</sup>. Interruption of the dark period with a light break prolonged the duration of expression of *GA20ox*.

Indirect evidence from grafting experiments indicates that phytochrome B mediates the photoperiod regulation of GA production in potato<sup>55</sup>. Phytochromes are also involved in light-induced seed germination and were shown to mediate the red-light induction of *GA3ox* production in imbibed seeds of lettuce<sup>56</sup> and *Arabidopsis*<sup>40</sup>. In *Arabidopsis* seeds, two genes for *GA3ox* are induced by red light, one of which [*AtGA3ox2* (*GA4H*)] specifically requires the intervention of phytochrome B, although other phytochromes are responsible for induction of *AtGA3ox1* (*GA4*) expression.

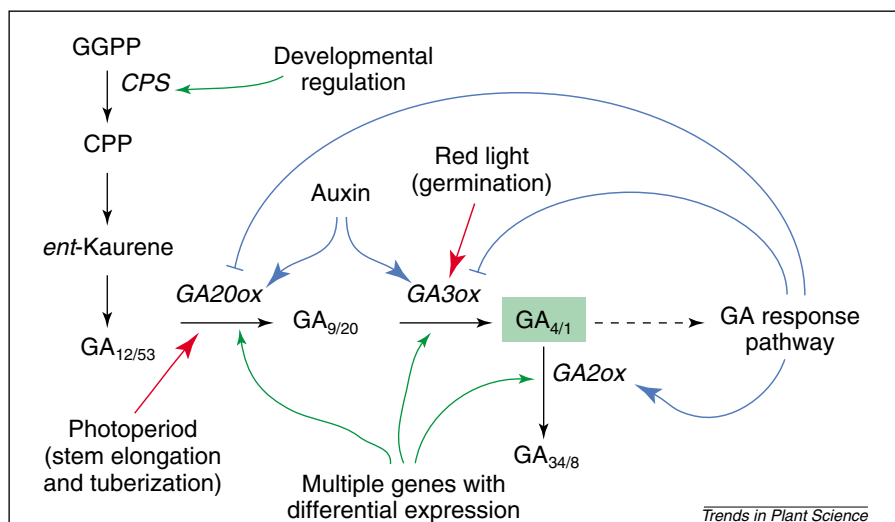
De-etiolation in pea seedlings is accompanied by a rapid reduction in GA<sub>1</sub> content in the shoot apex<sup>57,58</sup>. However, in this case, light regulation of GA metabolism does not appear to act through *GA20ox* or *GA3ox*. The transcript levels for these increase when plants are transferred from dark to light, presumably because of the effects of feedback regulation. The mechanism for the decrease in GA<sub>1</sub> concentration is unknown but, because it is accompanied by an increase in GA<sub>8</sub> content, regulation of 2β-hydroxylation might play a role<sup>58</sup>.

**Genetic modification of GA metabolism**

Chemical manipulation of the GA status of plants is widely practised in agriculture and horticulture. For example, seedless grapes



**Fig. 3.** Cluster analysis of *Arabidopsis* 2-oxoglutarate-dependent dioxygenases, including all known members of the gibberellin 20-oxidase (GA20ox), gibberellin 3 $\beta$ -hydroxylase (GA3ox) and gibberellin 2-oxidase (GA2ox) enzyme classes. The following enzymes from developing pumpkin seeds are also included: gibberellin 7-oxidase (Cm7ox), gibberellin 20-oxidase (Cm20ox) and gibberellin 2 $\beta$ ,3 $\beta$ -hydroxylase (Cm23ox). Also included for comparison are *Arabidopsis* sequences for ACC oxidase (AtACCO), flavanone 3 $\beta$ -hydroxylase (AtF3H) and flavonol synthase (AtFolS). The amino acid sequences were aligned using ClustalW (<http://www2.ebi.ac.uk/clustalw/>). The unrooted tree was generated using the PROTDIST and NEIGHBOR programs of the PHYLIP Package (<http://evolution.genetics.washington.edu/phylip.html>) and displayed using the Treeview program (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).



**Fig. 4.** An overview of the regulatory mechanisms known to affect expression of the genes encoding enzymes for gibberellin (GA) metabolism. Hormone and light regulation are indicated in red and blue, respectively, with large arrow heads denoting enhanced gene expression and bars denoting suppressed expression. The green arrows indicate genes that have been shown to exhibit tissue-specific patterns of expression. The biologically active GAs are highlighted in green. Abbreviations: CPP, *ent*-copalyl diphosphate; CPS, CPP synthase; GA2ox, gibberellin 2-oxidase; GA3ox, gibberellin 3 $\beta$ -hydroxylase; GA20ox, gibberellin 20-oxidase; GGPP, *trans*-geranylgeranyl diphosphate.

enzymes in the GA-biosynthesis pathway. An alternative approach that involves the genetic manipulation of GA biosynthesis or turnover would have several advantages. In addition to reducing the use of chemicals, the modification of GA metabolism can be targeted to specific tissues, permitting control over individual GA-regulated developmental processes such as stem elongation or fruit development.

It is widely accepted that the control of flux through biosynthetic pathways is usually distributed between several catalytic steps and that changes in the level of any one enzyme might have little effect on the overall rate of biosynthesis. Thus, the overproduction of CPS, an enzyme early in the pathway, had no dramatic effects on growth and development of *Arabidopsis*<sup>32</sup>. By contrast, there is abundant evidence, discussed above, that GA20ox, GA3ox and GA2ox catalyse regulatory steps in GA biosynthesis. Genetic manipulation of their expression might result in changes in flux or in altered levels of intermediates in the later stages of the pathway, including the bioactive GAs.

Overexpression of *GA20ox* under the CaMV-35S promoter yielded *Arabidopsis* plants with elevated levels of bioactive GAs, with effects on several aspects of development, including reduced seed dormancy, longer hypocotyls, pale-green leaves and early flowering<sup>41,59</sup>. A similar effect was achieved in potato, in which the transgenic plants overexpressing a potato *GA20ox* cDNA also required a longer period of short days to induce tuberization<sup>60</sup>. Overexpression of an *Arabidopsis* *GA20ox* in hybrid aspen (*Populus tremula*  $\times$  *Populus tremuloides*) increased the levels of GA<sub>1</sub> and GA<sub>4</sub>, which resulted in an increased growth rate and also in an increased number and length of xylem fibres, which are important traits for paper production<sup>61</sup>.

Several different approaches have been used to reduce the levels of bioactive GAs in transgenic plants. Suppression of *Arabidopsis* *GA20ox* expression by antisense RNA has been partially successful in identifying different roles for the individual members of the gene family. Suppression of *AtGA20ox1* was effective in two transgenic lines, which had a semi-dwarf habit and reduced levels of bioactive GAs (Ref. 41). Suppression of *AtGA20ox2* could not be shown but two lines were isolated that had shorter floral internodes under short days. A single line expressing antisense *AtGA20ox3* had a transient phenotype of short hypocotyls but this character

are treated with GA<sub>3</sub>, obtained from cultures of *G. fujikuroi*, to increase berry size and to stimulate elongation of the panicle. Conversely, many crops and ornamental plants are treated with a range of chemical growth retardants that act by inhibiting different

was lost in succeeding generations. A strong suppression of a *GA20ox* gene using antisense RNA was reported in potato, causing reduced stem elongation and increased tuberization, with an increased yield of tubers<sup>60</sup>.



**Fig. 5.** Overexpression of a gibberellin-catabolizing enzyme results in a gibberellin-deficient phenotype with a range of degrees of severity. Wheat cv. Canon was transformed with the bean gibberellin 2-oxidase cDNA<sup>17</sup> under the control of the maize ubiquitin promoter; the plants are 11 weeks old with a control (untransformed) plant on the left.

A *GA20ox* in developing pumpkin seeds (*CmGA20ox*) produces mainly C-20-carboxylic acid GAs instead of the C<sub>19</sub> precursors of bioactive GAs<sup>14</sup> (Fig. 1). Production of this enzyme in GA-biosynthesizing tissues should divert C<sub>20</sub> intermediates into inactive byproducts, reducing the flux into bioactive products. Overexpression of *CmGA20ox* in *Arabidopsis* resulted in the expected large increase in C-20 carboxylic acid GAs and a reduction in the level of GA<sub>4</sub> (Ref. 44). Surprisingly, the height of the plants was only slightly reduced, although increased expression of the endogenous genes for GA biosynthesis (*AtGA20ox1* and *AtGA3ox1*) indicated that the plants had responded to the reduced GA<sub>4</sub> concentration. By contrast, overexpression of *CmGA20ox* in *Solanum dulcamara* resulted in plants with reduced rates of stem elongation and dark-green leaves<sup>62</sup>. The plants accumulated GA<sub>17</sub>, formed by 13-hydroxylation of GA<sub>25</sub>, there was a substantial reduction in the level of GA<sub>1</sub> in leaves but little change in the level of GA<sub>4</sub>. This might reflect the preference of the pumpkin *GA20ox* for non-13-hydroxylated substrates, diverting C<sub>20</sub> intermediates away from the normal early-13-hydroxylation pathway.

GA levels can also be reduced by increasing the rate of inactivation. Overexpression of a bean (*Phaseolus coccineus*) *GA2ox* in *Arabidopsis* resulted in a range of phenotypes: plants with high-level expression of the transgene had an extreme dwarf phenotype with essentially no stem elongation (S.G. Thomas *et al.*, unpublished), whereas plants with lower levels of transgene expression had a semi-dwarf or wild-type phenotype, owing to increased expression of endogenous *GA20ox* and *GA3ox* genes through the operation of the feedback pathway. Similar results were achieved in a range of ornamental and crop species, including wheat, in which overexpression of the bean *GA2ox* yielded a similar range of extreme dwarf and semi-dwarf phenotypes (M. Wilkinson *et al.*, unpublished) (Fig. 5).

### The way forward

Although genes encoding most of the enzymes in the GA metabolic pathways have been identified, there are some important omissions. The 'gap' in the biosynthetic pathway to the common bioactive GAs for which no genes have been assigned comprises the four steps between *ent*-kaurenoic acid and GA<sub>53</sub>. It is unclear how many enzymes and genes are responsible for these steps. Their identification, which is essential for a full understanding of how GA content is regulated, awaits the development of an efficient system for functional expression of these mono-oxygenases.

There are several areas in which ongoing work on GA metabolism should result in progress within the next few years. Structural studies on recombinant enzymes, particularly the cyclases and dioxygenases, should provide basic information on function and might enable the design of enzymes with altered substrate specificities, product profiles and sensitivities to inhibitors. Knock-outs of individual isozymes should determine their influence on GA biosynthesis and plant development, and tissue-specific modification of GA content in transgenic plants might reveal new roles for GAs. The localization of transcripts and proteins should determine the extent of compartmentalization of the GA metabolic pathway between tissues and the importance of transport of GAs between sites of synthesis and action. Perhaps most significantly, a clearer picture of the molecular mechanisms involved in the regulation of GA metabolism should emerge, adding to our basic understanding of plant development.

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