Manipulation of hormone biosynthetic genes in transgenic plants

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Modification of plant hormone biosynthesis through the introduction of bacterial genes is a natural form of genetic engineering, which has been exploited in numerous studies on hormone function. Recently, biosynthetic pathways have been largely elucidated for most of the plant hormone classes, and genes encoding many of the enzymes have been cloned. These advances offer new opportunities to manipulate hormone content in order to study their mode of action and the regulation of their biosynthesis. Furthermore, this technology is providing the means to introduce agriculturally useful traits into crops.

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Abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ACCO	ACC oxidase
ACCS	ACC synthase
AOS	allene oxide synthase
CK	cytokinin
GA	gibberellin
IAA	indole-3-acetic acid
IAN	indole-3-acetonitrile
JA	jasmonic acid
NIT	nitrilase
SAM	*S*-adenosyl-methionine

Introduction

Crown gall formation by *Agrobacterium tumefaciens* is an example of natural genetic engineering of hormone biosynthesis in plants [1]. The insertion of genes that induce abnormal synthesis of indole-3-acetic acid (IAA) and cytokinins (CKs) in the host results in the proliferation of opine-producing cells in which the bacterium can prosper. Investigation of the transfer of these and other genes from the T-DNA of *A. tumefaciens* and *Agrobacterium rhizogenes* has enabled the development of a valuable vehicle for inserting foreign genes into plants, as well as spawning numerous studies on the physiological consequences of increased production of auxin and/or CK.

The biosynthetic pathways for the major plant hormone classes have been largely elucidated and genes are available for many of the enzymes involved. Changing expression of these genes, ideally in a tissue-specific manner, is providing new opportunities to study hormone function and regulation of their synthesis. Furthermore, it should be possible to introduce agriculturally useful traits into crops, in some cases reproducing the effects obtained by application of chemical growth regulators. In this review, we describe recent developments in engineering plant hormone biosynthesis and include a discussion of indirect changes in hormone biosynthesis resulting from genetic manipulation of other processes.

Auxin

Production of IAA is commonly increased in transgenic plants by expression of bacterial genes: the *tms* genes of *A. tumefaciens*; the *aux* genes of *A. rhizogenes*; or the *iaaM* and *iaaH* genes of *Pseudomonas syringae* pv. *savastanoi* [2]. These genes encode tryptophan 2-monooxygenase and indole-3-acetamide hydrolase, which catalyse the conversion of tryptophan to IAA by a pathway (Figure 1) not known to be present in higher plants. It is usually sufficient to express only *iaaM*, which encodes the monooxygenase, as its product, indole-3-acetamide, is converted to IAA in plant tissues [2]. For example, ovule-specific expression of *iaaM* driven by the *Antirrhinum majus DefH9* promoter allowed parthenocarpic fruit development in tobacco, eggplant and tomato [3,4•], presumably as a result of elevated IAA content in the unfertilised ovules.

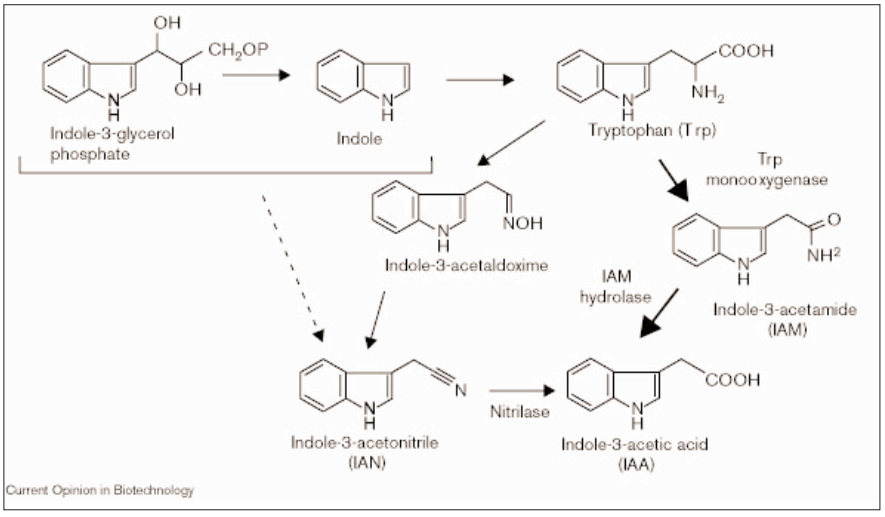
Several biosynthetic pathways to IAA are possible in plants, some involving tryptophan as an intermediate and others branching from indole or earlier precursors [5•]. In *Arabidopsis*, IAA is thought to be formed from, among other things, indole-acetonitrile (IAN; Figure 1) by the action of nitrilase (NIT), which is encoded by at least four genes [6]. Overexpression of the *Arabidopsis NIT2* gene in tobacco [7] and *Arabidopsis* [8•] had little effect on plant development or IAA content unless exogenous IAN was supplied, implying that IAN formation was limiting. Expression of antisense *NIT1* or *NIT2* in *Arabidopsis* resulted in a large decrease in total IAA (free IAA plus conjugates), whereas free IAA content in seedlings was little affected [8•]. Most IAA in plant tissue is in the form of inactive conjugates, some of which may be hydrolysed to free IAA. A number of hydrolases with specificity for particular IAA–amino-acid conjugates have been cloned from *Arabidopsis*. Overexpression of genes encoding an IAA–Ala hydrolase (*IAR3*) and a hydrolase with specificity for IAA–Leu and –Phe (*ILR3*) in *Arabidopsis* caused no morphological changes, but increased sensitivity to the corresponding IAA–amino-acid conjugates in terms of inhibition of root elongation [9•]. The large number of possible pathways for the biosynthesis and conjugation/deconjugation of IAA suggests a complex homeostatic mechanism for this hormone.

Cytokinins

Elevated CK concentrations in transgenic plants are obtained by expression of the *ipt* gene from the T-DNA of

Figure 1

Biosynthetic intermediates and pathways for IAA biosynthesis. The pathway from tryptophan (Trp) to IAA via indole-3-acetamide (thick arrows) has been demonstrated so far only in bacteria. Indole-3-acetonitrile, which is converted to IAA by nitrilase, is thought to be a biosynthetic intermediate for IAA in *Arabidopsis* and may be formed from Trp and by Trp-independent pathways.



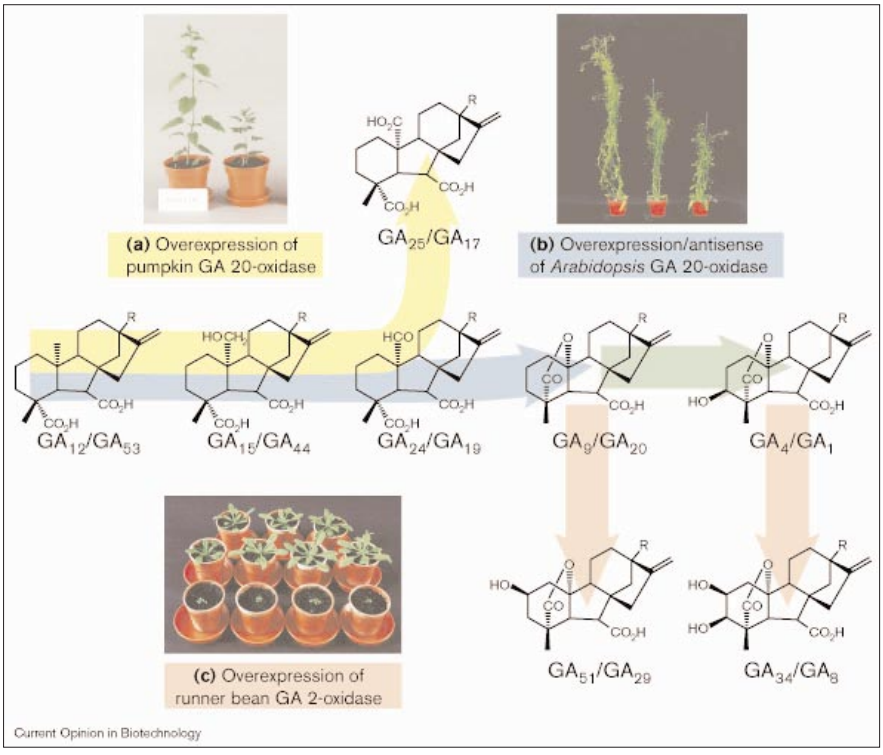
A. tumefaciens. The gene encodes an *iso*pentenyltransferase catalysing the formation of *iso*pentenyladenosine-5-phosphate from adenosine-5-phosphate and *iso*pentenyl diphosphate [10]. Plants are able to convert this intermediate into other CKs, including *trans*-zeatin and its riboside, which are considered to be the most important CKs in most plants. In tobacco plants expressing *ipt* under control of a tetracycline-inducible promoter, zeatin riboside accumulated first, followed by zeatin, dihydrozeatin and then glucosides, indicating that zeatin riboside, produced via the 5-phosphate [11], may form a CK pool [12]. The effects of CK overproduction in a transgenic tobacco rootstock, such as growth of lateral buds and retarded leaf senescence, were not transmitted to a grafted scion, placing in question the role of CKs as long-distance signalling molecules [12]. CK biosynthesis in plants is not well understood and as it has not yet been possible to identify an *ipt* homologue, the role of *iso*pentenyladenosine-5-phosphate as a CK intermediate in plants cannot be assumed.

There are numerous examples of *ipt* expression in plants using a range of constitutive, tissue-specific or inducible promoters. The physiological effects of the resulting abnormally high CK levels include stunted growth, adventitious shoot formation (loss of apical dominance), delayed senescence and reduced root formation. Heat-shock promoters have been employed extensively for inducible expression and, in a recent example, a *hsp70–ipt* construct was used to produce zeatin-type CKs in *Arabidopsis* [13•]. The induced plants showed higher levels of expression of the homeobox genes *KNAT1* and *STM*, which may act downstream of CKs in maintenance of the shoot apical meristem. Interestingly, overexpression of *KNAT1* in lettuce resulted in enhanced CK production [14•], indicating that this homeobox gene may participate in feedback regulation of CK production. A copper-inducible promoter gave a lower basal level of *ipt* expression in tobacco than normally obtained with heat shock promoters [15], although endogenous responses to this toxic metal must limit the specificity and utility of this system. The requirement for CK to induce shoot formation from callus was utilised in the development of an antibiotic-free selectable marker [16••]. The *ipt* gene was placed under the control of a promoter that is inducible by dexamethasone, application of which would thereby stimulate CK production and, hence, shoot regeneration in transformed callus. The *A. tumefaciens* gene *tzs*, which is present on the nopaline Ti plasmid but is not transferred to plants, is homologous to *ipt* and has a similar function. Heat-induced expression of a *hsp70–tzs* construct in oilseed rape resulted in increased amounts of zeatin-type CKs with associated symptoms, including increased seed numbers per silique [17].

Gibberellins

Chemical manipulation of GA levels is widely practised in agriculture, through application of growth retardants, such as chlormequat, that inhibit GA biosynthesis and of GAs to promote growth in crops such as seedless grapes. As several genes encoding GA biosynthetic enzymes are now available, genetic engineering of GA content has become practicable. Biochemical evidence indicates that GA 20-oxidase, which catalyses several consecutive steps resulting in the loss of C-20 (Figure 2), is modulated by factors such as feedback by bioactive GAs and daylength [18,19]. Overexpression of *Arabidopsis* GA 20-oxidase in transgenic *Arabidopsis* results in longer hypocotyls, early flowering, increased stem elongation and reduced seed dormancy [20•,21••] associated with an increase in GA_4, the main bioactive GA. These results indicate that growth throughout the life-cycle of *Arabidopsis* is limited by GA content, for which GA 20-oxidase activity is a limiting factor. As GA 20-oxidase is encoded in *Arabidopsis* by at least three genes with differential patterns of expression [22], specific reduction in mRNA levels for individual genes

Figure 2



The final stages of GA biosynthesis, catalysed by 2-oxoglutarate-dependent dioxygenases. The gibberellin numbers (e.g. GA_{12}/GA_{53}) refer to the non-13-hydroxy (R = H) and 13-hydroxy (R = OH) forms, respectively. *Arabidopsis* GA 20-oxidase (blue arrow) catalyses multiple, sequential reactions between GA_{12}/GA_{53} and GA_9/GA_{20}, including the loss of C-20 as CO_2, whereas the pumpkin seed GA 20-oxidase (yellow arrow) produces inactive C-20 tricarboxylic acid GAs as its final products. GA 3β-hydroxylase (green arrow) is responsible for the formation of the bioactive GAs GA_4 and GA_1. GA 2-oxidase (pink arrows) deactivates the bioactive GAs but also takes GA_9 and GA_{20} as substrates. Some examples of manipulation of these enzymes are shown. **(a)** Overexpression of pumpkin cotyledon 20-oxidase in *Solanum dulcamara* yields a dwarf phenotype (right) [25]. **(b)** Overexpression of *Arabidopsis* GA 20-oxidase (*AtGA20ox1*) in *Arabidopsis* (left) gives a slender, GA-overproduction phenotype, whereas antisense suppression of this gene yields a semi-dwarf (right), compared to a control plant (centre) [21••]. **(c)** Overexpression of the runner bean GA 2-oxidase in *Arabidopsis* gives an extreme dwarf phenotype (front) [27].

may identify the developmental role of each enzyme. Plants antisense for *At20ox1* had short hypocotyls and reduced stem elongation, resulting in a semi-dwarf phenotype (Figure 2b); the amount of GA_4 in the rosette was reduced to approximately one third of that in control plants [21••]. The single *At20ox2* antisense plant identified grew normally in long days, whereas it was slightly shorter than controls in short days due to a reduction in floral internode length and appeared to produce fewer and smaller siliques. An antisense *At20ox3* plant had reduced hypocotyl length early in seedling development, but grew normally thereafter. Thus, it is possible to target particular tissues or developmental processes without the requirement for specific promoters.

Alternative approaches to reducing GA content involve enhanced catabolism or sequestration of bioactive GAs or their precursors. A GA 20-oxidase from pumpkin seed that produces mainly inactive tricarboxylic acid GAs [23] was overexpressed in *Arabidopsis* [24•]. As anticipated, the plants contained substantially reduced levels of C-20 intermediates and very large increases in the tricarboxylic acids GA_{25} and GA_{17} (Figure 2). The amount of GA_4 was also reduced by up to 80% but the plants were only slightly dwarfed; implying that the concentration of GA_4 in the responsive tissues of the stem may be less affected than total GA levels measured in the whole plant. Overexpression of the pumpkin 20-oxidase in *Solanum dulcamara* was similarly successful in reducing GA content, yielding a reduction in stem height [25]. Hydroxylation of GAs at C-2β results in irreversible inactivation; a cDNA encoding the enzyme that catalyses this reaction, GA 2-oxidase, was isolated from runner bean [26•] and overexpressed in *Arabidopsis*, resulting in an extreme dwarf phenotype (Figure 2c) [27]. A non-enzymatic approach to reducing GA levels has been demonstrated by the expression in tobacco of a single-chain antibody against GA_{24}/GA_{19} [28•]. The antibody accumulated to about 3.6% of the total soluble protein when targeted to the endoplasmic reticulum, resulting in reduced levels of GA_1 and a dwarf stature.

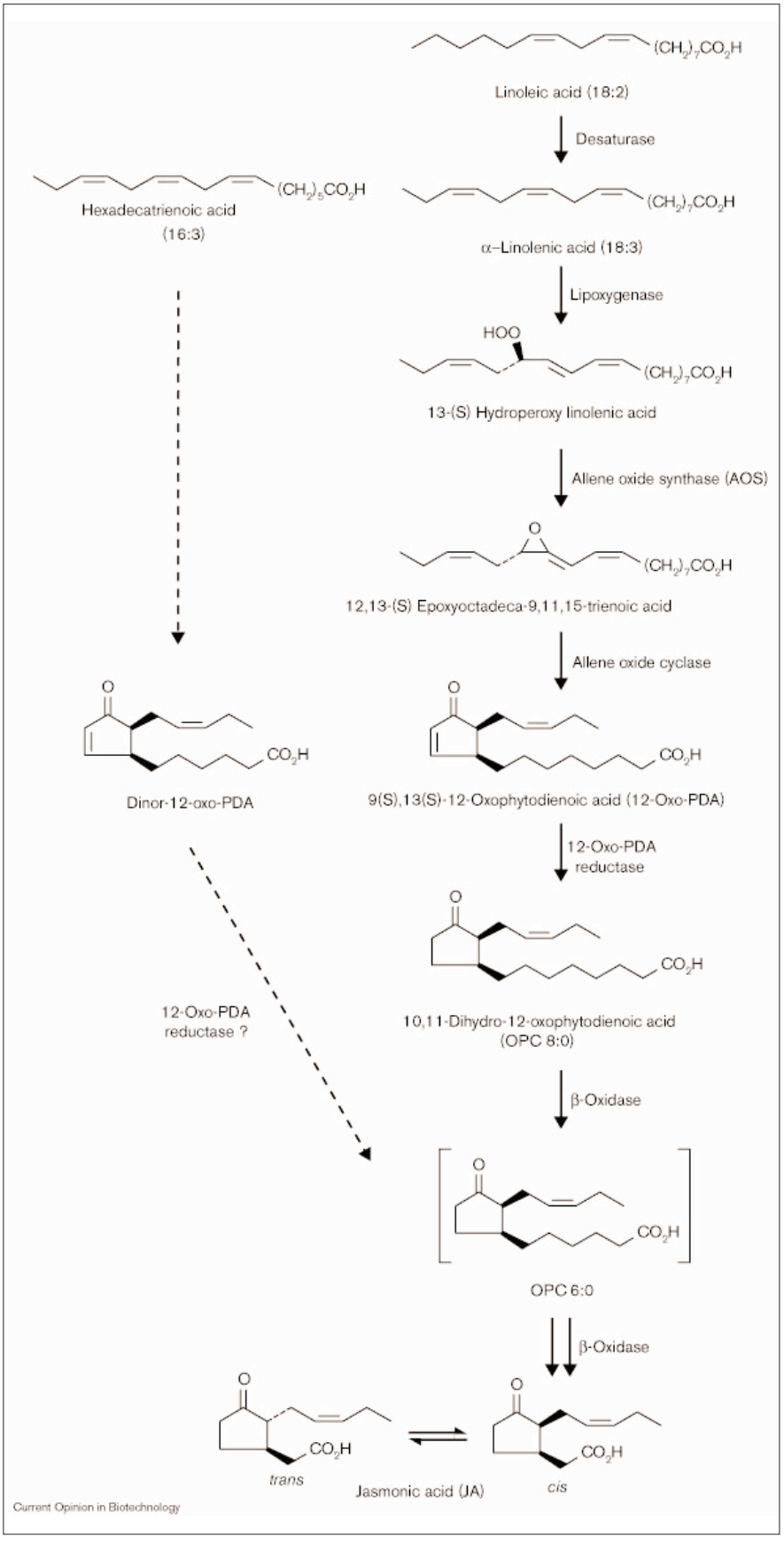
The later stages of GA biosynthesis are subject to a complex homeostatic mechanism whereby bioactive GAs repress transcription of GA 20-oxidase and 3β-hydroxylase genes and promote expression of the inactivating enzyme GA 2-oxidase [26•]. Attempts to manipulate GA status may be hampered by the ability of this homeostatic system to compensate for changes in bioactive GA levels. For example, diversion of GA intermediates into inactive products by overexpression of the pumpkin seed GA 20-oxidase in *Arabidopsis*, as described above, is partially compensated by up-regulation of the endogenous 20-oxidase and 3β-hydroxylase genes [24•].

Ethylene

Ethylene is produced in plants from *S*-adenosyl-methionine (SAM) via 1-aminocyclopropane-1-carboxylate (ACC) by the actions of ACC synthase (ACCS) and ACC oxidase (ACCO). The role of ethylene in promoting fruit ripening and flower

Figure 3

Biosynthetic pathways to jasmonic acid (JA). The active *cis* form slowly isomerises to the *trans* form to give an equilibrium mixture containing approximately 5% *cis* JA.



senescence provided a commercial incentive for early, successful approaches to down-regulation of its production by antisense suppression of ACCS [29] or ACCO [30] gene expression in transgenic tomato. Antisense suppression of ACCO expression has recently been used to reduce ethylene biosynthesis in melon [31], broccoli [32], and the ornamental flower *Torenia fournieri* [33]. Overexpression of ACCS in tobacco led to increased production of ethylene, whereas no increase was observed in plants overexpressing ACCO [34], in line with ACC synthesis being rate-limiting in most tissues. Expression of sense ACCO leads to co-suppression of the host ACCO gene in some plants [34], the efficacy of which was enhanced in tomato by inclusion of additional reversed copies of its 5′ untranslated region that appear to promote the generation of co-suppressing RNAs from adjacent sequences wihtin the transgene [35]. This approach has yet to be assessed, however, for general applicability. Different members of the ethylene biosynthetic gene families are associated with separate ethylene responses. For example, suppression of a pistil-specific ACCO in tobacco has revealed a role for ethylene in ovule development and fertilisation [36••]. A novel approach to increasing ethylene levels was described by Lu *et al.* [37], who fused the coding regions of the soybean ACCS and tomato ACCO genes; yeast cells expressing this gene produced a bifunctional enzyme that produced ethylene from ACC. The chimaeric gene might be useful as a replacement for treatment of plants with the ethylene-releasing compound ethephon in applications such as lodging prevention in barley, increasing latex flow in rubber trees and promoting leaf abscission of crops prior to harvesting.

Alternative strategies for manipulating ethylene levels in transgenic plants involve the removal of precursors. Polyamines and ethylene share a common precursor, SAM, and a reduction in polyamine biosynthesis engineered by reducing SAM decarboxylase activity in potato resulted in increased ethylene production [38]. Because SAM is involved in other metabolic pathways, this approach may be unfeasible, as overexpression of SAM decarboxylase was shown to be lethal. Degradation of ACC by overexpression of a ACC deaminase gene from *Pseudomonas* sp. resulted in a substantial reduction of ethylene generation by mature tomato fruit and may be more generally applicable [39].

Jasmonate

The jasmonates form a group of signalling compounds, derived from linolenic acid or hexadecatrienoic acid (Figure 3), that are involved in plant developmental processes, such as pollen growth and tuberisation, and mediate responses to wounding [40]. When expression of a plastid-localised ω3 desaturase, which converts dienoic to trienoic fatty acids, was reduced in potato by antisense technology, wound-induced jasmonic acid (JA) was substantially decreased, whereas the basal level was unchanged [41•]. Similarly, co-suppression-mediated depletion of a plastidic isoform of lipoxygenase, which catalyses the first step in the conversion of trienoic acids to JA, abolished wound-induced JA production in *Arabidopsis*, but did not affect basal levels [42]. It is possible that the levels of desaturase and lipoxygenase activities were not reduced sufficiently in these experiments to affect basal JA production or that other enzymes are involved in this case. Antisense-depletion of a lipoxygenase in potato, although resulting in reduced expression of the wound- and JA-inducible *PIN2* gene, had no effect on JA production on wounding, suggesting a broader role for lipoxygenases in plant defence [43•].

In contrast to the implied importance of plastidic enzymes in wound-inducible JA biosynthesis, wound-induced JA levels were increased in tobacco by overexpressing a cytosolic allene oxide synthase (AOS) gene from flax [44•]. This unusual cytochrome P450 catalyses the formation of epoxides, which are converted to JA by cyclization, reduction and a series of β-oxidations (Figure 3). The cytosolic AOS failed to increase JA levels in healthy tissues, unlike a chloroplast-targeted AOS in transgenic potato [45]. One interpretation of these results is that plastidic AOS activity limits JA production in intact tissues, whereas after induction of the JA pathway by wounding an earlier reaction, perhaps the synthesis or release of trienoic acids, becomes limiting. Furthermore, wounding would breakdown the compartmentation of the biosynthetic enzymes.

Other hormones

Abscisic acid (ABA) regulates seed development and germination, as well as being involved in responses to environmental stress. It is synthesised by oxidative cleavage of the xanthophylls (oxygenated carotenoids), violaxanthin or neoxanthin [46]. Violaxanthin is formed in two steps from zeaxanthin by the action of zeaxanthin epoxidase, which is encoded by the *ABA2* gene of *Nicotiana plumbaginifolia* [47]. CaMV-35S-driven expression of *ABA2* in *N. plumbaginifolia* resulted in increased ABA content in mature seeds and delayed germination, whereas expression of antisense *ABA2* reduced the seed ABA content, resulting in abnormally rapid seed germination [48•]. Overexpression of *ABA2* should cause an accumulation of the substrate for the cleavage enzyme, which is thought to catalyse a rate-limiting reaction in ABA biosynthesis. The gene encoding the cleavage enzyme has been cloned from several species (e.g. [46]) and is a prime target for manipulation of ABA biosynthesis. Expression of a single-chain antibody that binds ABA has been shown to modify concentrations of this hormone in transgenic tobacco, producing a wilty phenotype when expression was driven by the CaMV 35S promoter [49], and inducing precocious germination when expression was targeted to seeds [50].

Recent genetic studies have enabled impressive progress in understanding the function of the brassinosteroids and in the isolation of genes for their biosynthesis [51•]. Despite verbal reports that genetic manipulation of brassinosteroid biosynthesis produces severe, predictable changes in plant morphology, few examples have been published (see Update).

Indirect modification of plant hormone biosynthesis

Plant hormone biosynthesis forms part of a signalling network in which there are feedback loops [52] as well as crosstalk between the different pathways. Changes in the content of one class of hormone may result in altered levels of others, such that the physiological consequences are often complex and difficult to interpret. For example, IAA and CK suppress each other's synthesis [53], whereas IAA stimulates ethylene production [54]. In transgenic tobacco, auxin induced post-translational processing of an introduced *O*-xylosyltransferase, thereby producing an active enzyme that sequestered CKs [55]. Many other factors impinge on these pathways; for example, phytochromes act, in part, through altered GA biosynthesis [56•]. Products of the *rol* genes of *A. rhizogenes* modify hormone metabolism, with recent evidence that *rolA* expression reduces GA content by blocking GA 20-oxidase activity [57]. Possible clues to the signalling pathways by which hormones and other factors regulate hormone biosynthesis have emerged from recent experiments involving homeobox genes. A potential role for the *Arabidopsis KNAT1* gene in CK biosynthesis [14•] was discussed previously. Overexpression in tobacco of *KNOTTED*-type homeobox genes from tobacco [58•] or rice [59•] caused dwarfism, reduced GA_1 content and lower levels of GA 20-oxidase gene expression. Whether this reflects a function for these transcription factors in the regulation of hormone biosynthesis in wild-type plants remains unclear.

Conclusions

The genetic manipulation of enzymes involved in plant hormone biosynthesis and catabolism demonstrates the feasibility of producing transgenic plants with higher or lower levels of the active hormones. The resulting plants, with altered development or environmental responses, may have commercial applications, and these will be enhanced by the use of tissue-specific and inducible promoters that exert more control over expression of the transgene. Plants with modified hormone biosynthesis are also important research tools, yielding information on the mechanisms that regulate the biosynthetic pathways. Significantly, homeostasis, particularly in auxin, GA and brassinosteroid metabolism, appears to be a feature of hormone biosynthetic pathways. This may reduce the effectiveness of attempts to alter hormone levels through genetic engineering, and research on these regulatory mechanisms must be a high priority. Similarly, interactions between different hormones may result in unexpected pleiotropic effects in the transgenic plants. Elucidating the complexities of these interactions will be an important component of future research on hormone biosynthesis.

Update

As described in a paper published after this review was submitted [60], the *Arabidopsis BAS1* gene was isolated by activation tagging in a screen for suppressers of the long hypocotyl phenotype of a phytochrome B-defective mutant. *BAS1* encodes a cytochrome P-450 that deactivates brassinolide by hydroxylation on C-26. Overexpression of this gene caused dwarfism in *Arabidopsis* and tobacco as a result of reduced levels of bioactive brassinosteroids (shown for *Arabidopsis*), whereas suppression by antisense resulted in hypersensitivity to applied brassinolide in light-grown *Arabidopsis* seedlings.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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Indole-3-acetic acid (IAA)–amino-acid conjugates are hydrolysed to free IAA in plant tissues by hydrolases that are specific for particular amino acid conjugates. The *IAR3* (IAA–Ala-resistance) gene was isolated and shown to encode a hydrolase with specificity for IAA–Ala and IAA–Gly. CaMV35S-mediated expression in *Arabidopsis* of *IAR3* and *ILR1*, which is specific for IAA–Leu and IAA–Phe, resulted in plants with increased sensitivity to IAA–Ala and IAA–Leu/Phe, respectively, in terms of inhibition of root growth.

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• **steady state mRNA levels of the *STM* and *KNAT1* homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem.** *Plant J* 1999, **18**:557-563.

This paper provides a good example of the use of genetic manipulation to study hormone function. The content of zeatin-type cytokinins was increased in *Arabidopsis* by heat-induced expression of the *ipt* gene. The transgenic plants contained higher levels of transcripts for the *KNAT1*and *KN1* homeobox genes. On the basis of this and other evidence, the authors suggest that cytokinins act upstream of these genes in maintenance of the shoot meristem.

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The *Arabidopsis* homeobox gene *KNAT1* was expressed in lettuce under control of the pea plastocyanin promoter. The resulting morphological changes were consistent with elevated cytokinin concentration, which was confirmed by immunocytological analysis. The result suggests a possible role for homeobox genes in regulating cytokinin biosynthesis.

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The authors describe the development of an antibiotic-free selectable marker for plant transformation. Tobacco and lettuce were transformed with a gene containing the *ipt* coding region combined with a dexamethasone-inducible promoter. Regeneration of shoots from transformed callus or explants was induced in cytokinin-free medium by application of dexamethasone.

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Arabidopsis overexpressing a gibberellin 20-oxidase gene had increased levels of C-19 gibberellins and had reduced seed dormancy, elongated hypocotyls and flower earlier than wild type. Possible applications in promoting growth rate and accelerating the life cycle of plants are discussed.

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Showed that constitutive overexpression of gibberellin (GA) 20-oxidase in *Arabidopsis* resembled the effects of repeated application of GA_3 to wild-type plants. Transgenic plants had elevated levels of C-19 GAs, including a 2–3-fold increase in GA_4 in rosettes. Plants expressing antisense RNA for each isozyme had distinct phenotypes that reflected the expression patterns of the genes. The result demonstrates the feasibility of modulating specific GA-dependent processes in plants by antisense suppression of individual GA 20-oxidase genes.

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Gibberellin (GA) 20-oxidase from developing pumpkin seeds produces inactive C-20 carboxylate GAs. Overexpression of this gene should result in diversion of GA intermediates and reduction in bioactive GAs. Transgenic *Arabidopsis* expressing this gene had much lower levels of C-20 intermediates in the GA biosynthetic pathway, whereas tricarboxylic acid GAs accumulated to high levels. Only a mild phenotype was observed, however, because of homeostatic mechanisms that increased expression of the endogenous GA 20-oxidase and 3β-hydroxylase genes.

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