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Gibberellins: perception, transduction and responses

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Introduction

Gibberellins (GAs) are a class of plant hormones that exert profound and diverse effects on plant growth and development. The chemistry and metabolism of GAs have been studied for several decades and this has led to a detailed understanding of the pathways involved in their biosynthesis and catabolism. Attempts to understand the perception and mechanism of action of GAs have been based heavily on studies with the cereal aleurone as a model system and have drawn extensively on parallels with the molecular mechanism of action of mammalian steroid hormones. During the past few years, the established view of GA-perception has been challenged and new techniques for identifying GA receptors have been developed. As our understanding of GA-regulated events in aleurone cells has advanced through molecular and cell biology approaches, other GA-responsive plant tissues have also proved to be tractible for studying GA-action. This has led to a greater awareness of the diversity of cellular events that can be modulated by GAs. These currently range from ion channel activity to gene expression and present multiple targets for GA-signalling. Complementary to these approaches, molecular genetic analyses of GA-response mutants seems poised to provide insight into the identity of genes involved in GA signal transduction. While understanding of the GA perception-transduction-response pathway is in its infancy, there are promising prospects for understanding the molecular mechanisms whereby GAs invoke a variety of cellular and developmental events in plants.

GAs and plant development

It is clear from studies of GA-deficient mutants, and from the effects of the application of exogenous GAs to plants or plant parts, that this class of plant hormones are essential and potent regulators of growth and development. They affect a broad range of events during the normal growth and development of higher plants and, for the purpose of this article, I have divided the types of response of plant cells and tissues to GAs into three categories: (1) cell growth in vegetative tissues, (2) flower and fruit development and (3) seed reserve mobilisation by aleurone cells (Fig. 1). While there may very well be common elements between these different classes of response, the division provides a useful framework within which to consider the GA perception-transduction-response pathways. It is important to emphasise, however, that GAs are not unique regulators of these events. Other plant hormones and environmental stimuli affect plant cell growth

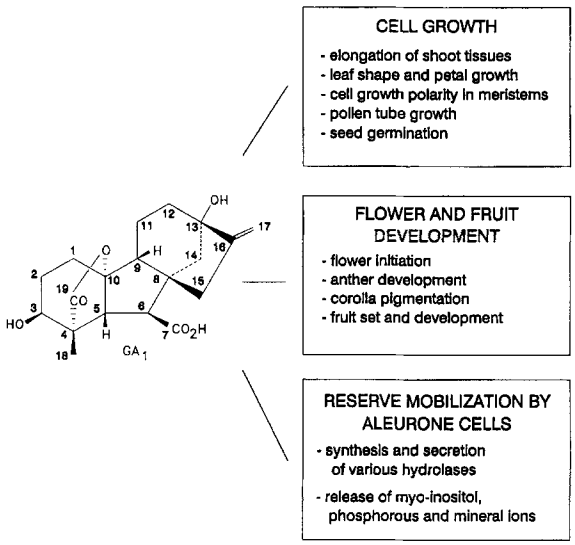

Fig. 1. Gibberellins and plant development. Structure of GA_1 with carbon atoms numbered and a summary of the three GA-response categories.

and the development of flowers and fruit. Seed reserve mobilisation, on the other hand, is under a more specific positive regulation by GAs.

Cell growth in vegetative tissues

Cell growth responses to GAs occur in a range of vegetative tissues. This can be confirmed by comparing the phenotype of a GA-deficient mutant with the wild-type or GA-treated mutant. Perhaps the most obvious of the GA-regulated growth responses in vegetative tissues is the elongation growth of shoots, particularly stem internodes, as well as hypocotyls, coleoptiles, mesocotyls and epicotyls. Other cell growth responses to GA are more subtle and occur only during specific stages of growth of the plant. For example, the transition from juvenile to adult leaf shape is affected by GA in a range of plant species, such as ivy [120], sweet pea [123] and tomato [71]. Cell growth polarity in root tips and shoot apical meristems is also markedly influenced by GAs [5]. During fruit set, GA may stimulate pollen germination and tube growth [106]. In petunia flowers, GA stimulates the growth of petals [154].

GAs promote elongation growth through effects on both cell division and expansion, although the latter process makes a greater contribution to increases in plant stature. The biophysics of GA promotion of elongation growth is complex and not well understood. It probably involves a combination of changes in the hydraulic properties of the cell and alterations in cell wall extensibility. However, the precise nature of these changes, their coordination and the primary action of GA on these processes are not understood [76].

The regulation of orientation of cell wall cellulose microfibril deposition is of primary importance in determining cell growth polarity and, therefore, a potential target for GA-action. The alignment of newly synthesised cellulose microfibrils appears to be mediated by the arrangement of underlying cortical microtubules. GA has been shown to induce transverse orientation of microtubules in a range of different cell types, including some in which the hormone does not affect the rate of elongation growth [5, 130]. GA regulation of the orientation of microtubules is an auxin-dependent process, although the nature of this cooperative response is not clear. Possible mechanisms underlying microtubule reorientation are being examined at the biochemical level and indicate that early changes in microtubule dynamics in response to GA are associated with modification of a specific tubulin isotype [25].

Changes in the expression of a number of genes are now known to accompany GA-induced elongation growth and present molecular targets for studies of GA-regulation of cell growth [95, 115, 129].

Exogenous GA is required for germination of GA-deficient mutants of tomato and Arabidopsis, suggesting that GA may be required for germination of wild-type seed of these species [86, 87]. In dicots, the radicle emerges through the endosperm and there is good evidence that GA promotes radicle emergence in tomato seed by enhancing the growth capacity of the embryo and by weakening the endosperm. The latter effect is caused by GA-induction and stimulation of the cell wall hydrolases endo-β-mannanase and mannohydrolase [104].

Flower and fruit development

Recent studies with developmental mutants of tomato and *Arabidopsis*, combined with the use of GAs and inhibitors of GA biosynthesis in horticulture and citriculture, implicated GAs as important regulators of flower and fruit development.

Flower induction

The transition from vegetative growth to flowering in long-day or cold-requiring plants and in conifers can be promoted by application of GAs. In fact, it is thought that vernalisation and long-day photoperiod promote stem elongation and flowering by stimulating GA biosynthesis [11]. Whether or not GAs influence stem elongation and floral initiation independently is still the subject of some debate, although the balance of evidence currently favours independent regulation of these events [11]. A definitive role for GA during flower induction has been established in *Arabidopsis* through studies with the extreme GA-deficient mutant *gal-3* [161]. In short days, this mutant does not flower unless GA is applied. Because other less extreme GA-deficient mutants show only a delay in, rather than prevention of, flowering under short days, yet still have a dwarf phenotype, it seems possible that flower induction may have a lower GA response threshold than stem elongation. Whether or not GAs are required for flowering of *Arabidopsis* in long days is not clear. A delay in flowering in the extreme GA-deficient *gal-3* grown under long days may be interpreted in favour of this, although the phenotype of other GA-deficient mutants and the GA-insensitive *gai* mutant do not. Leakiness of mutants can, however, cloud a definitive implication of cause and effect.

Photoperiod also influences floral development in *Arabidopsis* mutants that have lesions in a set of genes that collectively determine floral meristem development [108]. Recent evidence suggests that, in these mutants, GA can overcome the effect of short days on floral meristem identity and development [108]. Thus, in short days, GAs are important regulators of *Arabidopsis* flower initiation and may influence the expression of floral homeotic genes, such as *LFY* and *AG*.

Flower development

GAs affect the development of anthers which are a source of GAs that appear to influence corolla development and pigmentation [94, 114]. Inhibition of GA biosynthesis can delay anthesis, while exogenous GAs have the opposite effect. The GA-deficient *gib-1* and *gib-2* mutants of tomato show normal floral initiation but are arrested in anther development at a premeiotic G1 phase [67]. The extreme GA-deficient mutant of *Arabidopsis*, *gal-3*, is male-sterile and has poorly developed stamens and petals [161]. GA-deficient mutants of maize exhibit abnormal flower development. In the terminal flowers, stamen development is not suppressed (as it is in the wild type), resulting in bisexual flowers with pistils and anthers. Flowers of lateral shoots develop anthers but not pistils. Male sterility in GA-deficient mutants of *Arabidopsis* and maize does not appear to be caused by the same arrest in anther development exhibited by the *gib-1* and *gib-2* mutants of tomato, since pollen is formed in these mutants but is not released [67].

In a number of species, the GA content of stamens increases prior to anthesis [114]. In petunia, removal of stamens or anthers at an early stage in corolla development causes a marked inhibition in the pigmentation of corolla tissues. GA applied to the site of stamen removal can, to a large extent, overcome this effect [154].

Fruit development

Together with auxin and cytokinins, GAs appear to be involved at a number of stages in fruit development [35]. Exogenous GAs can promote fruit set and influence development in a range of horticultural species [41, 114]. Developing seeds contain the highest cellular concentrations of GAs in plants. Whether or not these endogenous GAs are essential for fruit growth and development is not entirely clear. There is evidence for promotive effects on cell division and expansion, and on the development of ovaries [6, 35, 114]. The clearest evidence that endogenous GAs are important for

seed development has come from the lh^i mutant of pea which is GA-deficient in the shoot and developing seeds. Seeds of the lh^i mutant abort unless their endogenous GA level is restored to near that of the wild type [118]. At the present time, little insight into GA-perception and signal transduction has come from studies with developing seeds and fruits.

Seed reserve mobilisation by aleurone cells

Aleurone cells are the most widely used experimental system for studying the cellular and molecular biology of GA-action. Aleurone cells of the Gramineae are a highly specialised tissue that differentiates from peripheral endosperm cells during seed development and forms a layers one or three cells thick enclosing the endosperm. Shortly after the seed has germinated, aleurone cells begin to synthesise and secrete a variety of hydrolytic enzymes that participate in the systematic breakdown of starch and protein reserves of the endosperm to provide nutrients for the growing seedling. In addition, aleurone cells contain the majority of the seed's stored reserves of myo-inositol, phosphorous and mineral cations, such as K^+ and Mg^{2+}. These are released into the endosperm after germination and provide the growing seedling with carbohydrate for cell wall synthesis, phosphorous and essential cations. Reserve mobilisation by aleurone cells appears to be coordinated to a large extent by GA produced by the embryo [31, 73].

Cellular responses to GAs

It is clear that GAs, like other plant hormones, have pleiotropic effects during plant growth and development. This variety is also reflected at the cellular level. In attempting to understand GA signal transduction, it is relevant to consider the full spectrum of cellular responses that GAs are able to invoke. The diversity of known cellular responses to GAs illustrates the heterogeneity of signalling possibilities that can be propagated by GAs (Fig. 2).

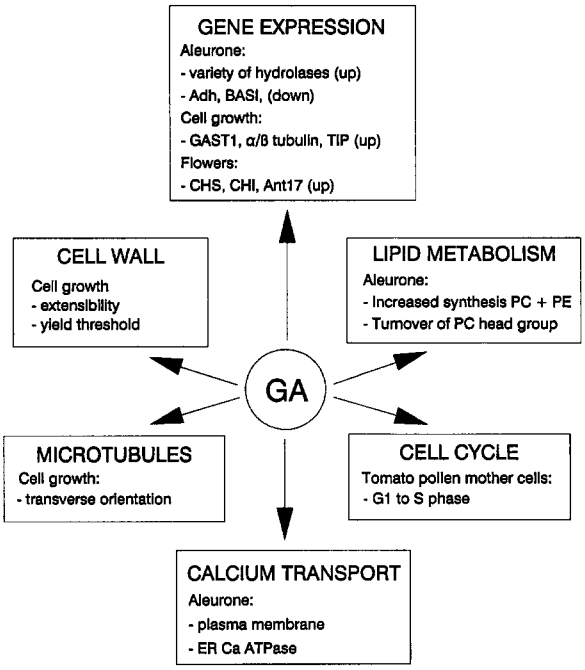

Fig. 2. Cellular responses to gibberellins. Different types of cellular responses to GAs together with brief examples that are elaborated further in the text.

GA structure-activity relations

Although 94 different GAs have been identified in higher plants and fungi, the majority of these are intermediates in a number of closely related biosynthetic pathways and only a relatively small number are biologically active *per se*. Structure-activity analysis of GAs has relied on bioassays in which exogenous GAs are applied to plants, or to parts thereof, and to characterisation of GA biosynthesis mutants. Bioassays suffer from the disadvantage that the ligand is usually applied at a position remote from its site of action. Thus, uptake, transport and metabolism can complicate assessment of the intrinsic activity. However, aleurone protoplasts are a particularly sensitive bioassay system since the GA is applied at, or very near to, its site of action [55, 136].

The biological activities of GAs measured in a number of assay systems are remarkably consistent, with only a handful displaying high biological activity [53, 127, 128, 136]. GAs that are highly active in promoting stem or hypocotyl

elongation are almost always highly active in other cell growth responses and in inducing α-amylase gene expression in aleurone. Similarly, GAs that are inactive in cell growth responses are also inactive in aleurone.

The carboxyl group on C-6 of the B-ring, the 19,10-lactone and the hydroxylation status at C-2 and C-3 are particularly important in determining high biological activity of GAs. 3β hydroxylation is generally associated with high biological activity, while 2β-hydroxylated GAs are inactive in all assays. The hydroxylation status at other positions in the molecule are less critical in determining biological activity. It appears, therefore, that structural features in the A/B ring area of GAs are important for determining high biological activity.

An exception to this principle appears to be the induction of flowering in long-day plants. Bioassay data with *Lolium temulentum* suggest that GAs with high florigenic activity are less effective at stimulating elongation growth, and *vice versa*. A double bond in the A ring, either at C-1,2 or C-2,3, is essential for florigenic activity. While 3β hydroxylation is of major importance in promoting activity of GAs for stem elongation and α-amylase induction, 3β-hydroxylated GAs are less active than non-3β-hydroxylated GAs in flower induction in *Lolium*. Hydroxylation at C-12, -13 and -15 enhances flowering relative to stem growth [26, 27]. These observations raise the possibility that GA perception associated with flower induction may involve receptors with specificities for GAs different from those involved in cell growth and seed reserve mobilisation responses. Alternatively, the possibility that uptake, transport and metabolism may obscure the intrinsic activity of GAs is a problem that is difficult to resolve [26].

Except for this example, the balance of evidence suggests fairly conserved structure-activity relationships for all classes of responses to GAs. This can be interpreted in favour of a degree of conservation in the GA perception mechanism and, consequently, diversity in at least some of the downstream signalling components. This situation contrasts quite markedly with other classes of hormones. For example, mammalian steroid hormones show substantially greater diversity in their range of biologically active structures than do GAs. Nevertheless, in spite of this diversity, steroids mediate the majority of their responses through specific interactions with members of a receptor superfamily [111]. Based on our current knowledge of the structure-activity relationships of GAs, it seems likely that, with the possible exception of flower induction, structural features of the receptor(s) involved in the molecular recognition of ligand will be highly conserved between the different classes of response to the hormone. This does not, however, exclude the possibility that other features of the receptor(s) which may, for example, be involved in coupling to signal transduction pathways, might show structural diversity related to the response chain, or chains, in which they are involved.

Cellular site of GA perception

The structural similarity between GAs and mammalian steroid hormones has led to the suggestion that GAs might act on plant cells by a mechanism similar to steroids in mammals [137]. There is good evidence that at least some components of the steroid-signalling pathway are either present in plant cells, or that proteins which can substitute for these functions are [125]. Nevertheless, the question of whether GAs signal through such a pathway is not clear.

GAs are hydrophobic weak acids with pK_as in the range 4.0 to 4.2 [147]. They can enter plant cells by passive partitioning of undissociated molecules that become trapped as relatively impermeant anions in alkaline compartments within the cell. GA_1 uptake by spinach cell suspension cultures appears to have, in addition, a carrier-mediated component [107]. Whether or not GA transport proteins occur in other plant tissues is a question that has not been examined in detail. GA uptake by plant cells will be reduced substantially as the pH of the medium bathing them is increased above the pK_a [107]. Notwithstanding the complication of GA metabolism [98, 102],

it can be argued that, if GAs are perceived by receptors inside plant cells, conditions that reduce GA uptake should decrease the response to the hormone. Some evidence supports this theory [44, 134], while other information opposes it [40, 136]. The interpretation of data produced under physiological extremes of pH however, may be complicated by nonspecific effects on the physiology of cells. For example, extracellular pH has a substantial effect on cytoplasmic Ca^{2+} in barley aleurone protoplasts [15].

GA perception at the plasma membrane

Membrane impermeant GAs are active in aleurone
We have attempted to identify the site of GA perception in aleurone protoplasts of *Avena fatua* with an impermeant derivative of GA_4 [55] using a principle originally pioneered with insulin [18]. Elements of the D ring of the GA molecule are not of major importance in determining biological activity of GAs [127, 128] and we have exploited this by synthesizing a GA_4 derivative with a thiol-containing addition on the D ring. This compound (GA_4-17-S-$(CH_2)_3$-SH) induces α-amylase in aleurone protoplasts of *A. fatua*, although its biological activity is approximately two orders of magnitude lower than that of GA_4 [55]. The derivative has been coupled, using a hydrophobic spacer arm, to 120 μm diameter beads of Sepharose 6B, such that elements of the GA_4 molecule that are most likely to confer biological activity are exposed, but can extend no further than ca. 1.95 nm from the surface of the Sepharose 6B. GA_4-17-Sepharose beads are not likely to cross the plasma membrane of aleurone cells. However, the exposed regions of the GA_4 molecule will be able to interact with both the surface of the plasma membrane and components within the membrane to a depth of ca. 1.95 nm. GA_4-17-Sepharose will not be able to penetrate the aleurone cell wall and this property has been used to monitor for any free GA_4 that might be released from the GA_4-17-Sepharose. When aleurone layers were co-incubated with aleurone protoplasts and the amounts of α-amylase mRNA induced in each tissue by the GA_4-Sepharose was determined, the impermeant GA_4 was found to induce high level α-amylase gene expression in aleurone protoplasts and only very low levels of α-amylase mRNA in aleurone cells [55]. These observations led us to propose that GA_4 can be perceived at the aleurone plasma membrane and is coupled to the regulation of α-amylase gene expression by an, as yet, undefined signal transduction pathway [55].

Another method of reducing the membrane permeability of GAs has been explored by the synthesis of GA_4-17-sulphonic acid [10]. The pK_a of the sulphonic acid group is 0.6 and, therefore, it will be ionised, and hence membrane-impermeant, at all physiological pH values. Nevertheless, GA_4-17-sulphonic acid is biologically active in aleurone layers and protoplasts of *A. fatua* and has the same activity as a similar derivative that contains a short hydrophobic addition at C-17 in place of the sulphonic acid group [10]. In the absence of evidence for carrier-mediated GA uptake in aleurone cells, the fact that two GA_4 derivatives that differ profoundly in their ability to cross the plasma membrane by passive diffusion have identical activities in aleurone layers and protoplasts again argues for GA perception at the external face of the plasma membrane.

GA microinjection
The theory that GA may be perceived at the aleurone plasma membrane [55] has recently been supported by the results of experiments [38] in which either GA_3 or GA_3 plus ABA were microinjected into single barley aleurone protoplasts embedded in thin films of agarose [51]. The responses were assessed by three criteria: stimulation of vacuolation, secretion of α-amylase into a starch-agarose film and transient GUS expression driven by 1.8 kb of the barley *α-Amy1/6-4* promoter. None of these responses was stimulated when GA_3 was microinjected to internal concentrations of between 1 and 250 μM, while subsequent addition of GA_3 to the external medium stimulated the responses. Similarly, external ABA antagonised the GA_3-stimulated re-

sponses while microinjection of up to 250 μM ABA did not. Microinjection most likely delivered the hormones directly into the cytoplasm and it is clear, therefore, that, when GA and ABA are presented to aleurone protoplasts in this way, they are not active [38].

A substantial body of evidence now supports a plasma membrane location for GA perception in aleurone cells and for ABA perception in both aleurone and stomatal guard cells [1]. The cellular location of GA receptors has not been examined in other tissues. Nevertheless, until receptors for these plant hormones are identified definitively it may be prudent to anticipate mechanisms of plant hormone perception that have evolved to meet the needs of higher plants and which may, therefore, be unique to them, rather than to adopt whole-heartedly preconceptions derived from models of mammalian hormone action. The possibility that there may be more than one class of GA receptor should also be considered. This principle has been established for steroid hormones, since there are now known to be specific steroid-binding sites in neuronal plasma membranes, which include GABA receptors [109].

GA-binding proteins

Since the mid 1970s, with the availability of high specific activity (30–50 Ci/mmol) [^{3}H]GA_1 and [^{3}H]GA_4, GA-binding activity has been sought in a range of plant tissues. Initial studies were based on *in vivo* assays analogous to those used for defining mammalian steroid hormone binding. Subsequently, *in vitro* binding assays were developed and used to define and to partially purify GA-binding activities, particularly in soluble protein fractions prepared from tissues that elongate in response to GAs. More recently, GA affinity chromatography and photoaffinity labelling with high-specific-activity radioiodinated GA derivatives have been developed and used to define specific GA-binding proteins. At the present time, however, there is no well characterised GA-binding activity that can be considered a firm candidate for a GA receptor.

Soluble GA-binding proteins and elongation growth

GA-binding activity has been detected in cytosol from pea epicotyls, cucumber hypocotyls, maize leaf sheaths and mung bean hypocotyls, tissues that show an elongation growth response to GAs. The existence of these soluble proteins that specifically bind biologically active GAs has been interpreted in favour of a steroid-like signalling mechanism for GAs [137]. The possibility that soluble GA receptors mediate the action of this class of plant hormones through a transduction pathway unrelated to a steroid mechanism of action seems to have been ignored.

The first clear demonstration of GA-binding activity came from studies with the highly GA-sensitive epicotyl hook of a dark-grown GA-deficient dwarf mutant of pea [138] and was subsequently characterised further by Keith and Srivastava [79]. In both of these investigations, [^{3}H]GA_1 was supplied to intact tissue. After homogenization, cytosol was recovered and binding activity was monitored in fractions eluting from a Sephadex gel filtration column. [^{3}H]GA_1 was associated with a high-molecular-weight fraction and a 30 kDa fraction. About 50% of the [^{3}H]GA_1 associated with the 30 kDa fraction had been metabolised, by 2β hydroxylation, to GA_8. [^{3}H]GA_1 associated with the high-molecular-weight fraction had not been metabolised. The binding activity in the high-molecular-weight fraction appears to have been due to both soluble and microsomal proteins, although the investigations concentrated on defining the former rather than the latter activity.

In vivo GA binding was reduced by biologically active, but not by inactive, GAs. However, it was not possible to exchange [^{3}H]GA_1 *in vitro* with excess GA_1 GA_3 or GA_8, nor was the binding pH-sensitive. These are not encouraging features as far as the biological relevance of the binding activity is concerned. Nevertheless, this work did spur *in vitro* GA-binding assays with cucumber hypocotyl cytosol [80] and a soluble GA_4-binding activity was demonstrated to be saturable, reversible, pH-sensitive with an optimum of 7.5 and displacable by active, but not inactive, GAs.

Because the [^{3}H]GA_4 dissociated from its binding protein more rapidly than the activity detected in pea epicotyls, a rapid DEAE-cellulose filter-binding assay was devised [81] and used to characterise the binding activity in greater detail. A single class of binding sites with a K_D of about 70 nM, a half life of dissociation of 6–7 min at 0–2 °C and an abundance of about 0.4 pmol per mg soluble protein was described. There was a good correlation between the binding affinity for different GAs and GA derivatives and their biological activity in this tissue [81, 167].

It was recognised that the soluble GA-binding activities were labile and easily lost during extraction. Improved methods of protein extraction combined with either the DEAE filter paper or gel-filtration assays were used to search for GA-binding activity *in vitro* in cytosol from pea epicotyls [90, 92]. The DEAE filter assay revealed a saturable, exchangeable and specific [^{3}H]GA_4 binding activity in both dwarf and tall pea. The K_D for [^{3}H]GA_4 was 130 nM in dwarf pea and 70 nM in tall pea, with the number of binding sites estimated as 0.66 and 0.43 pmol per mg soluble protein in the dwarf and tall, respectively. Specific binding of [^{3}H]GA_1 to pea proteins could not be demonstrated using the DEAE filter binding assay but Lashbrook *et al.* [90] were able to measure some, apparently specific, saturable and pH-sensitive [^{3}H]GA_1 binding to pea cytosol proteins. However, this was against a high background of non-specific binding.

GA-deficient mutants of *Zea mays* are highly responsive to GA_1, which is believed to be the endogenous GA regulating elongation growth in this, and probably other, plant species. Nevertheless, a detailed investigation of [^{3}H]GA_1 binding by leaf sheath cytosol from wild-type, GA-deficient dwarfs and the GA-insensitive *D8* mutant did not reveal any binding activity that was specific for biologically active GAs [82, 83].

Taken together, these observations indicate that specific binding of the endogenous growth-promoting GA_1 is very difficult to demonstrate in either *in vivo* or, particularly, *in vitro* binding assays with soluble proteins extracted from GA-responsive elongating tissues. Under certain conditions of extraction and assay, specific, reversible, saturable and high-affinity binding of [^{3}H]GA_4 can be demonstrated with soluble proteins prepared from some, but by no means all, tissues that show GA-regulated elongation growth. Attempts to purify these soluble GA-binding activities have met with limited success [137]. These proteins might be receptors or enzymes involved in GA metabolism. Evidence for and against a receptor role has been discussed [137, 168]. However, assignment of a definitive role must await purification and identification of these binding activities.

GA affinity chromatography has recently been used to purify GA-binding proteins [103]. A crude protein fraction containing cytosol and other proteins was prepared from mung bean hypocotyls. Non-specific binding proteins were removed by passage through 2,4-D-Sepharose before allowing proteins to bind to a GA_3-Sepharose matrix. Because of difficulties in preparing the affinity matrix, GA_3 was linked to the Sepharose through the carboxyl group on C-6. This is not an ideal choice, considering the importance of this functionality for biological activity. Nevertheless, the decision to use this type of matrix was also based on the observation, in small scale experiments, that a GA-binding activity was recovered from both GA_3-7-Sepharose and the more difficult-to-prepare GA_4-17-Sepharose. GA-binding proteins were recovered by washing the affinity matrix with a high-salt pH 7.6 buffer and purified further by anion-exchange and gel filtration chromatography. GA-binding activity was monitored by a simple ammonium sulphate precipitation assay using [^{3}H]GA_4 [103]. It is, perhaps, unfortunate that the authors chose to use a binding assay that has been so heavily discredited [149]. Nonetheless, a specific [^{3}H]GA_4 binding activity was observed in the initial crude protein fraction which became enriched during subsequent chromatography steps. The native protein was estimated, by gel filtration, to be between 150 and 200 kDa and, on the basis of SDS-PAGE analysis of protein fractions recovered from native PAGE that were shown to bind [^{3}H]GA_4, it was suggested that the protein might be heteroli-

gomeric with subunits of 23 and 35 kDa. Notwithstanding the potential difficulties outlined above, the binding appeared to be specific for GA_4, GA_7 and GA_9. While this binding activity does not appear to have the characteristics expected of a receptor, the technique of GA affinity chromatography appears to have considerable potential for the purification of GA-binding proteins.

GA-binding activity in lettuce hypocotyl cell wall fractions

One other example of binding of the endogenous growth-promoting GA_1 with plant material has come from studies with lettuce hypocotyl. Tissue sections undergo elongation growth when exposed to exogenous biologically active GAs. Of the [^{3}H]GA_1 taken up by hypocotyl sections, some 2–5% was associated with a cell wall-containing fraction. Association of [^{3}H]GA_1 with cell wall material increased with time and was dependent on protein synthesis [139, 140, 141] but could only partly be displaced by prolonged chasing with unlabelled GA_1. The available evidence points strongly to a covalent association between the [^{3}H]GA_1 and components of the cell wall that might include carbohydrates. Studies of the temporal relationship between cell extension and [^{3}H]GA_1 association with cell wall material under conditions permissive and inhibitory for elongation growth indicated that the phenomenon was not simply a consequence of growth [142]. It has been suggested that interaction between GA_1 and cell wall components might interfere with the action of cross-linking enzymes that limit cell wall expansion [142]. This hypothesis has not been tested further.

GA-binding activity in aleurone

GA-regulation of α-amylase gene expression in aleurone cells of members of the Gramineae is, perhaps, the most extensively studied response to this plant hormone. Aleurone cells are a uniform cell type that are highly responsive to GA and, as such, should be a good source of receptors for this plant hormone. GA-binding activity in aleurones was first reported in an *in vitro* binding study with wheat subcellular fractions in which [^{3}H]GA_1 binding was assessed using a centrifugation assay [70]. Non-aqueous subcellular fractionation techniques led to the recovery of a fraction enriched in aleurone grains which bound [^{3}H]GA_1. However, the binding activity was competed for by ABA [70] and this raises some uncertainty about its possible biological relevance. Use of the *in vivo* GA-binding technique was complicated by rapid metabolism of GAs to inactive 2-β-hydroxylated and glycosylated forms. An attempt to define *in vivo* [^{3}H]G_1binding in barley aleurone overcame the problem of metabolism by incubation at 0–1.5 °C [78]. At equilibrium, [^{3}H]GA_1 was present at a higher concentration inside the cells than in the medium and, because no significant metabolism could be detected, this suggested that the [^{3}H]GA_1 had bound to subcellular components. A small proportion of the [^{3}H]GA_1 binding could be competed by a 50-fold molar excess of unlabelled GA_1, but not by the inactive GA_8 [78]. These observations provided evidence of specific GA binding in aleurone cells but the nature of the interaction has not been elaborated any further.

GA photoaffinity labelling

Photoaffinity labelling is a powerful technique for identifying hormone-binding proteins. Photoaffinity probes have been developed for, and used to identify and characterise, a number of proteins that appear to be involved in plant hormone sequestration, transport and action [13, 28, 30, 50, 57, 169].

In order to identify GA-binding proteins that may be involved in GA perception in aleurone, several aryl azido GA photoaffinity reagents have been developed [9, 54]. Initially, a tritiated reagent [^{3}H]GA_4-S-ABA [9] was used to photoaffinity label *A. fatua* aleurone protoplasts but it

was not possible to detect any specific GA-binding proteins with this probe [54]. One shortcoming of this reagent was the long exposure times required for fluorographs. These difficulties were overcome by preparing GA_4 photoaffinity reagents radiolabelled to higher specific activity with ^{125}I. The GA_4 analogue (GA_4-17-yl-1′-(1′thia)-propan-3′-ol-4-azido-5-^{125}iodosalicylate), hereafter referred to as [^{125}I]GA_4-O-ASA, was synthesised and radioiodinated to >120 Ci/mmol [9]. The non-radiolabelled, iodinated GA_4-O-ASA is biologically active, inducing high-level α-amylase gene expression in *A. fatua* aleurone layers and protoplasts. It can be photolysed within seconds on exposure to intense visible light >300 nm [9].

The effectiveness and specificity of [^{125}I]GA_4-O-ASA as a photoaffinity probe for a model GA_4-binding protein has been confirmed by assessing covalent attachment of the reagent to a number of anti-GA monoclonal antibodies [150]. Further modification of the substrate has led to the preparation of a closely related GA_4 analogue GA_4-17-sulphoxyethyl-*p*-azido-salicylate (GA_4-17-SE-*p*-ASA) that can be radioiodinated to a specific activity of greater than 1000 Ci/mmol [56]. In the absence of an established *in vitro* GA-binding activity from aleurone cells, *in vivo* GA photoaffinity labelling has been performed as an extension of the *in vivo* GA-binding assay principle described above under conditions where [^{125}I]GA_4-O-ASA and [^{125}I]GA_4-17-SE-*p*-ASA are inducing α-amylase gene expression and, therefore, are likely to be interacting with a GA receptor. Numerous aleurone polypeptides are labelled by the photoaffinity probes, several approximately in proportion to their abundance [56, 152]. This non-specific labelling may be a feature of the photoaffinity probe that can be improved on by further refinements in its design. Nonetheless, judicious use of non-radiolabelled GAs as competitors allowed specific and non-specific interactions to be discriminated and led to the identification of two GA-binding proteins in aleurone cells of *A. fatua*, a 50 kDa polypeptide recovered in a soluble fraction and a 60 kDa polypeptide recovered in a microsomal membrane-containing fraction [56, 152]. Although the identities of these two GA-binding proteins are not yet known, the fact that they can be identified after SDS-PAGE means that it may be possible to purify and sequence them and thereby gain insight into their possible functions.

Attempts to detect specific GA binding in *in vitro* GA photoaffinity labelling experiments have so far met with only limited success [152], perhaps indicating the labile nature of specific binding observed *in vitro*. Photoactive derivatives of N-substituted phthalimides that have GA-agonist activity are also being developed as photoaffinity probes for GA-binding proteins [144].

Interaction of GAs with liposomes

The permeability of soybean lecithin liposomes to both neutral and charged molecules can be enhanced by GA_3, which lowers the phase transition temperature of the liposomes in a concentration-dependent manner over the range 250 μM to 2.5 mM [164, 165]. However, biologically inactive GA_8 had very similar effects. This is consistent with the suggestion, based on nuclear magnetic resonance studies [166], that the carboxyl group on C-6 associates with the quaternary nitrogen of phospholipid head groups. The effect of GAs on the phase-transition temperature of phospholipid liposomes has also been studied by electron spin resonance and differential scanning calorimetry [112]. A GA_4/GA_7 mixture interacted with the liposome surface and lowered the phase-transition temperature. In contrast to the observations of Wood and Paleg [164, 165], however, GA_8 was found to be inactive. The interaction between $GA_{4/7}$ and the liposome surface was enhanced at low pH presumably through minimising charge repulsion at the carboxyl group on C-6.

Whilst these observations with artificial membranes suggest that interactions between GAs and plant membranes may indeed occur and lead to changes in physicochemical properties, the biological significance of these *in vivo* is questionable. The specificity of the interactions for biologically

active GAs is still controversial and the concentration range over which the responses are observed suggest affinities several orders of magnitude lower than the biological activity of the hormone *in vivo* [105].

Low-temperature preincubation has been reported to increase the sensitivity, measured as the amount of α-amylase synthesised, of aleurone layers from *Rht3* dwarf wheat to GA [131]. Changes in aleurone phospholipids, in particular the head group and acyl content of phosphatidylcholine and phosphatidylethanolamine, were correlated with changes in sensitivity in response to low-temperature treatment and this has been interpreted as indicating that membrane lipids are primary sites of GA_3 perception [131, 132, 133]. Whilst phospholipid composition may well influence the interaction of GAs with membranes [112], the available evidence is at best only correlative and provides no direct evidence for membrane lipids being the site of GA perception. In addition, a major difficulty with the interpretation of the studies of Singh and Paleg [131, 132, 133] is that phospholipid analyses were performed on total aleurone phospholipids. Clearly, as far as GA perception mechanisms are concerned, it would be more pertinent to analyse and quantify changes in the lipid composition of defined membrane fractions.

GA-response mutants

Single gene response mutants provide a means of identifying genes whose products may encode receptors or components of signalling pathways. They can be generated by either chemical mutagenesis, by ionising radiation or by insertional mutagenesis using transposons or *Agrobacterium tumefaciens* T-DNA. The mutated gene can be identified by positional cloning if sufficient markers are nearby, genomic subtraction if it comprises a large deletion, or, in the case of insertional mutants, using specific hybridisation probes. A number of higher-plant genes that encode, or are thought to encode, components of hormone signalling pathways have been identified by these means and others are certain to follow [22, 39, 77, 91, 93, 96].

Molecular genetic analysis of ethylene-signalling mutants has provided evidence that the regulation of growth by this plant hormone may involve a protein kinase cascade. A genetic model based on the epistatic relationships between ethylene-resistant and constitutive response mutants has been defined and molecular cloning has revealed the identity of the products of two genes in this pathway. The first, *ETR1*, encodes a histidine kinase with sequence similarity to two-component regulators, while the second, *CTR1*, encodes a Raf-like serine/threonine kinase [22, 77]. Components of ABA-signalling pathways have also been identified by molecular genetic approaches. A transcriptional activator which appears to interact with transcription factors involved in ABA-regulated gene expression and is confined to seed-specific ABA signalling [39, 93] has been cloned from maize and *Arabidopsis*. A component common to all ABA-signalling pathways has been identified as a novel type of protein serine/threonine phosphatase of the 2C type [91, 96].

A major factor in determining the success of the molecular genetic approach to studying signal transduction is the stringency of the screen used to select the desired mutant. This is based on an anticipated phenotype. For GA-response mutants, two strategies have been adopted, each based almost exclusively on plant stature, and these have led to the selection of two classes of GA-response mutants: (1) GA-insensitive dwarfs or semi-dwarfs and (2) constitutive GA responders (slender mutants). In view of these rather general selection criteria, perhaps it is not surprising that GA-response mutants are probably the most common plant hormone mutants [118].

Dwarf and semi-dwarf GA-insensitive mutants

A range of genetic lesions could be expected to give rise to plants with dwarf phenotypes and reduced sensitivity to GA. The majority of these will have nothing to do with GA perception or

signal transduction. The strategy for identifying putative GA perception or transduction mutants has been to analyse the phenotypes in detail and identify those which very closely resemble GA-deficient mutants in all aspects except sensitivity to exogenous GA. Although extreme response capacity mutants could fall into this class, they may nevertheless help provide insight into later stages in transduction pathways and components of the responses *per se*. The recessive *lka* and *lkb* dwarf GA-insensitive mutants of pea, for example, have been found to have substantially higher cell wall yield thresholds than wild-type plants [118] and may, therefore, be useful tools with which to study the effects of GA on cell wall physicochemical properties during cell growth responses.

Dwarf and semi-dwarf GA-insensitive mutants have been characterised in a number of species [118]. Those that have received most attention are the *D8*, *D9* and *Mpl1* mutants of maize [45, 162], *gai Arabidopsis* [113] and *Rht3* wheat [34]. All are dominant or semi-dominant mutations. Based on the similarity in phenotype to GA-deficient mutants, it has been argued that the most likely candidates for receptor or signal transduction pathway mutants are maize *D8*, *Mpl1* and *Arabidopsis gai*.

Dominant mutations that confer insensitivity to GA might act through one of two mechanisms, depending on the function of the wild-type gene product. In the first mechanism, the wild-type gene product would be a negative regulator of the GA-response pathway and be inactivated by GA. A mutant in which the negative regulator could no longer be inactivated by GA would be a dominant gain-of-function mutant. In the second mechanism [48], the wild-type gene product would be a positive regulator of the response pathway but would function only as a multimer. A mutant in which this positive regulator loses its function would be dominant if the defective protein disrupted or inactivated the multimer necessary for GA action, a phenomenon also referred to as complex poisoning. Dominant mutations conferring insensitivity to other plant hormones have been identified. The *ETR1* and *ABI1* mutants of *Arabidopsis* are currently the best examples, since the gene products for both of these mutants have been identified and the amino acid substitutions conferring the mutant phenotype identified [22, 91, 96]. In neither of these cases is it yet possible to state whether the mutations are gain- or loss-of-function, but this should become clearer after detailed biochemical analyses.

Genetic analysis of the *D8* and *Mpl1* mutants of maize suggest that they are gain-of-function mutations that are largely independent of the wild-type gene products, although they could also result from overproduction or ectopic expression of the wild-type gene product. Clonal analysis of X-ray-induced wild-type somatic sectors in *D8* and *Mpl1* backgrounds indicates that, in certain tissues, the mutations are cell-autonomous [45].

The *gai* mutant of *Arabidopsis* has a phenotype similar in many respects to the *D8* and *Mpl1* mutants of maize and, because it is in a genetically tractible species, efforts have been focused on cloning this gene [113]. *GAI* is located on the top arm of chromosome 1 of *Arabidopsis* at map position 21.8 and has been placed some 0.6 centimorgans (cM) distal to the GA-deficient mutant *GA4* [113]. RFLP mapping of *GAI* has been hampered by difficulties in scoring *gai* segregation in the ecotypes used although *ga4* has been located between markers m219 and g2359 and it seems likely that *gai* will be in this vicinity too [113]. Derivative mutations of *gai* have been generated by γ-ray irradiation and an M1 screening procedure used to select for mutants with a wild-type phenotype. Although this strategy was aimed at generating second site suppressor mutations, segregation analysis of three derivative alleles recovered showed that they are most likely to be intragenic derivative alleles of *gai*. A fourth appears to be a large deletion or rearrangement [113].

It seems most likely that the derivative alleles of *gai* are loss-of-function mutations rather than restoration of wild-type since chance would favour the former. Peng and Harberd [113] have argued that the balance of evidence suggests that *gai* is most likely to be a gain-of-function mutation, although they are cautious to point out that this cannot be concluded until the *gai* and its

derivative alleles are identified. Nevertheless, the observations suggest that the wild-type *GAI* may be dispensible. This might be because a similar protein, perhaps encoded by a related member of a multigene family, can substitute for the GAI product. Clear interpretation of the available data is not yet possible. However, the prospect that *gai* will soon be identified is good since it has been located to an approximately 50 kb region of genomic DNA (N. P. Harberd, personal communication).

Constitutive GA-response mutants

Mutants that have a phenotype similar to the wild-type plant that has been treated repeatedly with GA are referred to as slender mutants. Where it can be demonstrated that this phenotype has not been caused by overproduction of GA, it can be considered to be a GA constitutive response mutant. These are rare by comparison with dwarf and semi-dwarf mutants. The best characterised of this class of mutants are the *cry^{s}la* mutant of *Pisum sativum* [117], *sln1* in barley [32], the *Spy* mutants of *Arabidopsis* [68] and the *procera* mutant of tomato [71]. These mutations are all recessive. It is likely that slender mutants are loss-of-function mutations of a negative regulator, or regulators, of the GA perception-transduction pathway. Furthermore, because slender mutants are pleiotropic they may affect a step in the GA perception-transduction pathway that is common to all responses to GA. This class of mutant, therefore, provides further evidence of conservation in the mechanism of action of GA, in spite of heterogeneity in the classes of response mediated by the hormone. In addition, slender mutants also suggest that at least one element of the GA perception-response pathway, or an unrelated pathway that cross-talks strongly with it, has a repressor-like function.

The combined effect of mutations at two loci, *cry^s* and *la*, in pea produce a slender phenotype with parthenocarpic fruit development. Because the phenotype of plants with a mutation at only one, rather than both, of these loci is wild-type, it may be that the products of these two genes can substitute for one another in the GA perception-transduction pathway [117]. This slender phenotype appears to be independent of endogenous GA since it is unaffected by the presence of an additional mutation *na* that reduces endogenous GAs [117].

Another slender mutant of pea, *sln*, is of interest because it illustrates an unusual way in which a slender phenotype can arise [119]. At the seedling stage *sln* has a phenotype very similar to *cry^{s}la* and is insensitive to early-stage GA biosynthesis inhibitors. However, this mutant is not a GA-independent constitutive responder. The phenotype appears to be caused by abnormally high levels of the inactive GA_{20} in *sln* seed which are converted to active GA_1 in the young seedling. During development of wild-type seeds GA_{20} is catabolised to GA_{29} and GA_{29} catabolite, substrates that cannot be converted to active GAs. Seed of the *sln* mutant, therefore, have an unusually large reserve of potentially active GAs. Because these conversions take place, at least in part, in the maternal tissue of the testa, the *sln* phenotype has an unusual pattern of inheritence [119].

The *sln* mutant of barley is affected in cell growth, flower development and seed reserve mobilisation responses to GA [20, 32, 88]. The phenotype of *sln* barley is unaffected by GA biosynthesis inhibitors [24, 88] suggesting that the GA perception-transduction pathway is constitutively activated in this mutant. Nevertheless, ABA inhibits GA-induction of α-amylase, protease and nuclease in aleurone layers of the mutant, suggesting either that ABA negatively regulates the GA-perception-transduction pathway downstream of *sln* or that it operates through a parallel, but unrelated, pathway. ABA-induction of gene expression is unaffected in the mutant, an mRNA inducible by ABA, and dehydration in roots showed similar response to these treatments in the *sln* mutant and wild type [20].

Three independent recessive mutations with a slender GA-constitutive response phenotype have been generated in *Arabidopsis* by EMS mutagenesis combined with a screening strategy based on

resistance to the GA biosynthesis inhibitor, paclobutrazol [68]. These mutations are at the same locus which has been named *SPINDLY* (*SPY*) and they, like the other slender mutants, affect all responses to GAs ranging from hypocotyl and stem elongation, anther development, flowering and fruit development. The phenotype of double mutants between *spy-1* and the GA-deficient dwarf *gal-2* indicates that *spy-1* suppresses all of the growth and development effects associated with GA deficiency.

Unlike *cry^{s}la* pea and *sln* barley mutants, the phenotype of *spy* mutants is influenced by GA levels. Hypocotyl length in the *spy 1 gal-2* double mutants increases in response to exogenous GA_3 and the dose-response curve parallels that of the single *gal-2* mutant. These observations have been interpreted as indicating that, in a GA-deficient background, *spy-1* and exogenous GA_3 can interact additively [68]. Two models explaining the function of spy have been suggested [68]. In the first, the GA perception-transduction pathway is branched and *spy* constitutively activates one of the branches allowing GA to signal through the other and augment the response. In the second model, the wild-type gene product of *SPY* negatively regulates cross-talk between the GA transduction pathway and an unrelated pathway.

Whether or not the slender mutants all affect the same component of the GA perception-transduction pathway is not entirely clear. The distinction between GA-responsive and GA-insensitive slender phenotypes might be related to the strength of the different alleles as suggested by Jupe *et al.* [74]. However, this may not be the case for *spy* mutants [68].

Relationships between GA-response mutants

In view of the pleiotropic nature of responses of plants to GAs, it is likely that response mutants that are either specific for different tissues or classes of response or that are affected at different points in the perception-transduction chain will be isolated. The dominant GA-insensitive dwarfs and recessive constitutive responders described above are probably affected at different points in the GA perception-transduction pathway. However, since both of these classes of mutant appear to be affected in every response to GA, these mutations must be in elements common to all GA responses throughout the plant.

Evidence of GA-response mutations that are either tissue- or response-specific is very limited. Barley mutants generated by sodium azide treatment were screened for those which had altered GA and ABA sensitivity in a half-seed assay that measured α-amylase production. Several response mutants were isolated, two of which were characterised and found to have reduced sensitivity to GA in the half-seed assay, producing less α-amylase and phosphatase in response to GA_3. They each had normal, or near-normal, growth phenotypes [52]. These mutants have not been characterised in any detail and it is not known where in the perception-transduction-response pathway they are affected.

Response mutants of other plant hormones are restricted to particular tissues and/or types of response. The ABA-insensitive mutants of *Arabidopsis ABI1* and *ABI3* provide a good example of this where the transcriptional activator encoded by *ABI3* is involved in seed-specific gene expression, while the protein phosphatase 2C-like gene product of *ABI1* appears to be involved in all ABA responses. If a larger number of GA–response mutants were generated, it might be possible to position them on both the common and distinct parts of GA perception-transduction pathways involved in the multiple responses of plant cells to GAs. However, there is very little evidence at present that it will be possible to separate cell growth, seed reserve mobilisation and fruit and flower development response categories with specific mutations.

Response regulation of GA biosynthesis

The GA-insensitive dominant or semi-dominant dwarf mutants of *Arabidopsis*, wheat and maize provide an interesting contrast to the positive correlation between GA concentration and growth

observed in wild-type plants where elongation growth is associated with elevated levels of biologically active GAs. In these GA-insensitive mutants an inverse correlation exists and the levels of GA_1 and its precursor GA_{20} are elevated while GA_{19} is reduced [2, 33, 47, 145]. It has been argued that this is evidence that a negative feed back regulation of GA metabolism is defective in the mutants as a consequence of impairment in GA-perception or transduction. This phenomenon is thought to exist also with ethylene [43]. GA-insensitive mutants, such as the recessive *lk*, *lka* and *lkb* pea mutants that are thought to be affected in their response capacity, do not show the same correlation, suggesting that the feedback regulation is not directly influenced by growth rate *per se*. The endogenous GAs in slender barley are consistent with the response regulation theory since GA_1 and GA_{20} are lower than in the wild type, while GA_{19} is higher [24]. Certainly, it appears that one component of the GA perception-transduction pathway impacts on GA biosynthesis, probably through influencing the activity of GA 20 oxidase [2, 47].

Phospholipid metabolism: a lipid-based GA-signalling pathway?

Changes in phospholipid and choline metabolism are among the earliest effects of GA on wheat aleurone cells and provide support, for a lipid-based GA-signalling pathway. Within 2 h of exposure to GA_3, the activities of two enzymes involved in phospholipid biosynthesis, phosphorylcholine-cytidyl transferase and phosphorylcholine-glyceride transferase increase substantially [148]. During the lag phase between GA_3 addition and the induction of α-amylase, there is a marked change in the rate of turnover of *N*-methyl and methylene carbons of the phosphatidyl choline (PC) head groups [49, 148]. In non-GA_3-treated controls, *N*-methyl carbons turn over more rapidly than methylene carbons. GA_3 promotes phospholipid breakdown and changes the pattern of PC metabolism such that the whole choline head group, rather than *N*-methyl carbons, turn over. It has been suggested that this change in PC turnover may be a component of a lipid-based GA signal transduction pathway in aleurone [49, 148]. However, until further supporting evidence for this comes to light, the theory must remain speculative. In fact, GA_3-regulated PC turnover and phospholipid metabolism have also been shown to be associated with the aleurone spherosome fraction and, therefore, might be involved in endomembrane assembly [3, 160].

A further rapid effect of GA on aleurone lipids has been demonstrated by Fernandez and Staehelin [29], who found that GA induces the transfer of lipase activity from a subcellular fraction enriched in aleurone storage protein bodies to lipid bodies. This response is quite rapid, occurring between 45 and 120 min after GA treatment. It is partially overcome by ABA.

Calcium and calmodulin: components of the GA transduction-response pathway?

Many of the environmental and hormonal stimuli that act on plant cells induce changes in cytosolic Ca^{2+} (for recent reviews see [14, 116]). These changes are quite rapid and fall into three basic patterns [14]: (1) large, transient increases, (2) small, steady-state increases or decreases and (3) oscillatory changes with regular or irregular periodicities. GA has been shown to cause small steady-state increases in cytosolic Ca^{2+} in aleurone cells [15, 36]. It is important to consider how GA-induced changes in cytoplasmic Ca^{2+} come about, whether or not this category of cytosolic Ca^{2+} modulation has a signalling function and, if so, on what targets it has an impact.

A sizeable body of evidence indicates that Ca^{2+}, possibly in conjunction with calmodulin or a calmodulin-like protein, appears to be involved in coordinating the response of aleurone cells to GA and ABA [12, 17, 36, 37].

Cytosolic Ca^{2+} in aleurone protoplasts

The cytosolic Ca^{2+} status of barley aleurone protoplasts has been investigated by acid-loading of

indo-1 and fluo-3 combined with fluorescence ratio analysis and confocal microscopy [15, 36]. Cytosolic Ca^{2+} increased from 50 to 150 nM between 4 and 6 h after treating barley aleurone protoplasts with GA. The increase was not uniform across the cytoplasm. Highest levels (700 nM) were observed at the periphery of the protoplasts and may have been associated with cortical ER [36, 37]. The GA-induced increase in cytosolic Ca^{2+} preceded, by several hours, GA-induced synthesis and secretion of α-amylase. When the concentration of Ca^{2+} in the medium bathing aleurone protoplasts was reduced below 1 mM, both the increase in cytosolic Ca^{2+} and α-amylase secretion were reduced, suggesting that Ca^{2+} influx at the aleurone plasma membrane sustains both these processes [36].

ABA overcomes the GA elevation of cytoplasmic Ca^{2+} within 3 h, restoring near basal levels some 2 h before it inhibits GA stimulation of α-amylase secretion [36]. This response of aleurone cells to ABA contrasts with the increase in cytoplasmic Ca^{2+} that ABA causes in stomatal guard cells [126].

There are three mechanisms by which GA (and ABA) might regulate Ca^{2+} influx at the aleurone plasma membrane. Firstly, the GA receptor may be a ligand-gated anion channel, secondly, GA may be perceived by an intracellular receptor which indirectly modulates the activity of a plasma membrane Ca^{2+} channel and, thirdly, a GA receptor at the aleurone plasma membrane [55] may modulate the activity of a plasma membrane Ca^{2+} channel. Plant plasma membrane Ca^{2+}-permeable channels have been characterised [126, 146] and, in stomatal guard cells, can be activated by ABA, leading to the elevation of cytoplasmic Ca^{2+} [126].

Research aimed at identifying, characterising and studying the regulation of Ca^{2+} channels in aleurone plasma membrane may give insight into GA and ABA signalling in this tissue. In addition, the observation that ABA elevates cytosolic Ca^{2+} in stomatal guard cells [126] while reducing it in aleurone cells [36] suggests that some tissue-specific regulation may be uncovered.

While it is clear that GA and ABA coordinately regulate cytoplasmic Ca^{2+} in aleurone cells, leading to small sustained changes in concentration, there have been few attempts to define biochemical targets for such changes. One candidate may be a homologue of the putative Ca^{2+}-modulated protein phosphatase 2C that has been identified as a component in ABA signalling in *Arabidopsis* [91, 96].

Ca^{2+}-calmodulin regulation of ion channel activity

Another target that has been suggested for Ca^{2+} and calmodulin regulation in aleurone cells is the tonoplast slow vacuolar (SV) ion channel. This channel is probably involved in the mobilisation of K^+ from phytate stored in aleurone protein bodies [12]. SV channel activity has been detected by patch-clamp analysis of the membrane (tonoplast) of barley aleurone storage protein bodies [12]. Higher current densities were detected in protein bodies from GA-treated protoplasts compared with ABA or minus GA treatments, although single channel recordings were similar between these treatments. Channel opening was stimulated by Ca^{2+} and Ca^{2+} sensitivity was enhanced by calmodulin. Calmodulin antagonists, W7 and trifluoperazine, reduced channel activity. These observations provide evidence for Ca^{2+}-calmodulin regulation of the activity of this SV channel and this may be related to GA-induced increases in both of these signalling molecules in aleurone cells [36, 37]. However, the sensitivity of the SV channel to Ca^{2+} *in vitro* is outside the range of concentrations of cytoplasmic Ca^{2+} occurring in aleurone protoplasts [36]. Clearly, further investigation is required before Ca^{2+} and calmodulin can be placed with any confidence on a signal transduction pathway linking GA with SV channel activity *in vivo*.

Ca^{2+} transport and protein secretion in aleurone

Certainly one of the fates of cytoplasmic Ca^{2+} in aleurone cells is to join a substantial flux out of the cell via the endomembrane system. α-Amylase is a Ca^{2+}-containing metalloenzyme that requires

at least equimolar bound Ca^{2+} for activity [16]. It can comprise as much as 70% of newly synthesised secreted protein in GA-treated aleurone cells and its flux through the endomembrane system demands maintenance of ER lumenal Ca^{2+} levels in excess of 5 μM [16]. Because cytoplasmic Ca^{2+} in GA-treated aleurone cells is ca. 150 nM, Ca^{2+} has to accumulate into the ER against a concentration gradient. This is achieved by a Ca^{2+}-ATPase located in the ER membrane [16]. Studies of ^{45}Ca transport into barley aleurone microsomal membrane fractions enriched in ER indicate that the transport of Ca^{2+} into ER is some 5-fold higher in GA-treated aleurone, compared with non-GA-treated controls. This is due to GA stimulating the activity of the ER Ca^{2+}-ATPase, an effect which is overcome by ABA [16, 17].

The effects of both GA and ABA on the activity of the ER Ca^{2+}-ATPase are slow, developing over 4 to 8 h, and, therefore, are unlikely to involve a direct action of the hormones on the activity of pre-existing transporters [17]. One mechanism of regulation appears to be stimulation by a calmodulin-like protein which increases in barley aleurone layers treated with GA [37]. Calmodulin stimulates Ca^{2+} uptake into ER membrane vesicles isolated from barley aleurone that has not been treated with GA to levels comparable with ER membranes from GA-treated tissue. In addition, the calmodulin antagonists, W5 and W7, inhibit Ca^{2+} uptake into ER membrane vesicles prepared from GA-treated aleurone [37]. GA also increases the level of another calcium binding protein, BiP, in the ER of aleurone cells and this is likely to be involved in protein folding [72].

Another role suggested for the GA- and ABA-induced changes in cytoplasmic Ca^{2+} in aleurone cells is the regulation of exocytosis, possibly by a mechanism similar to that in mammalian cells [170].

Calcium and cell growth responses to GA

There is no clear evidence at present to support a role for cytoplasmic Ca^{2+} in GA-induced stem elongation. Although a variety of Ca^{2+} channel blockers and antagonists has been shown to inhibit the growth of GA-treated stem segments of *A. sativa*, they did not affect the early growth in response to the hormone, even when the tissue was preincubated with millimolar concentrations of verapamil for 12 h prior to GA addition. Rather, the Ca^{2+} antagonists inhibited sustained growth of the tissue segments. These findings have been interpreted in support of a role for Ca^{2+} movement in maintaining, but not initiating, GA-induced growth [97]. Clearly there are limitations to the deductions that one may make on the basis of treating whole tissues with Ca^{2+} agonists and approaches that monitor cytoplasmic Ca^{2+} during GA-induced stem elongation may cast further light on this issue. It is not known whether cytosolic Ca^{2+} or calmodulin are involved in other plant cell responses to GA.

GA regulation of gene expression

Gene expression and cell growth responses to GA

Auxin can stimulate cell elongation in excised tissue segments in less than 20 to 30 min. In advance of this, there are rapid and specific auxin-induced changes in the transcription of primary response genes [4]. Elongation growth in response to GA is a much slower response, taking place over hours rather than minutes. In GA-deficient mutants of pea (*le*) and maize (*d5*), increases and decreases in the steady-state levels of *in vitro* translatable mRNAs have been reported as early as 30 minutes after applying GA, even though elongation responses were not visible until several hours later [23]. At the present time these are among the fastest responses yet reported to GA. Although the genes encoding these transcripts have not been identified, it is possible that they may be primary response genes, analogous to those identified in auxin-induced elongation growth.

In the GA-deficient *gib-1* mutant of tomato, decreases in the abundance of several translatable mRNAs have been reported, but only after

6 h of GA treatment. Other mRNAs decreased by 24 h [69]. cDNA clones corresponding to three of these mRNAs and showing both sustained and transient decreases in abundance have been isolated [69]. The first gene shown to be upregulated by GA during GA-stimulated stem elongation (*GAST1*) was identified in shoots of the GA-deficient *gib-1* mutant of tomato and encodes a 12.8 kDa polypeptide of unknown function [129]. An increase in *GAST1* mRNA is detectable 2 h after GA treatment and, by 12 h, levels are > 20-fold higher than those in untreated controls. Thereafter, transcript levels decline to untreated levels by 48 h. GA regulation of expression appears to be at both transcriptional and post-transcriptional levels and ABA partially inhibits GA regulation of the gene [129]. In 244 nucleotides upstream of the start of transcription of *GAST1*, there do not appear to be sequences similar to the elements identified in α-*Amy* promoters which are known to be important for high-level GA-regulated expression. The significance of an inverted pyrimidine box sequence in the 3′-untranslated region is not known.

The first genes with a known function to be shown to be modulated by GA during elongation growth were tubulin genes [95]. Tubulin gene expression is enhanced in a concentration-dependent manner during GA_3-induced internode elongation in *A. sativa*. Levels of both α- and β-tubulin transcripts increase 5–6-fold 6 h after GA_3 treatment, a time when GA stimulation of growth is just detectable. Transcript levels continue to increase for up to 24 h. Elongation growth and GA stimulation of tubulin mRNA could be inhibited by ABA and cycloheximide. These observations suggest a close correlation between tubulin gene expression and GA-regulated elongation growth but also indicate that GA regulation of tubulin gene expression requires sustained protein synthesis. Tubulin genes therefore do not have the characteristics of primary response genes directly influenced by GA, suggesting that GA regulation of their expression might be through the hormone inducing the production of a regulatory protein that may be a component of the perception-transduction pathway.

Using a PCR-based subtractive hybridisation technique, Phillips and Huttly [115] have identified two cDNAs that are up-regulated after GA treatment of the GA-deficient *ga1* mutant of *Arabidopsis*. One of these cDNAs encodes tonoplast intrinsic protein (γ-TIP) present in vegetative tissues that is thought to function as a passive water channel. Gamma-TIP mRNA increases substantially between 8 and 24 h after GA treatment, although whether or not this increase is confined to extending tissue is not clear. It has been suggested [115] that an increase in γ-TIP may promote water transfer into the vacuole that could either generate turgor as a driving force for cell expansion or restore turgor lost through GA-induced stress relaxation of the cell wall. The other GA-stimulated cDNA encodes a proline- and glycine-rich protein with a potential secretory signal peptide. GA stimulates the abundance of this transcript between 8 and 24 h after treatment and, based on its expression in wild-type Landsberg *erecta*, it may be restricted to flowers [115].

Gene expression during flower and fruit development

Flower induction

A preliminary report suggests that GA may directly or indirectly regulate the expression of floral homeotic genes [108].

Anther development

GA-application to *gib-1* tomato plants rescues stamen development and gives rise to both increases and decreases in the abundance of a number of *in vitro* translation products [69] Within 8 h of GA application, increases and decreases in specific translation products could be seen. Changes in other translation products were not detected until 24 or 48 h. By comparing the *in-vitro* translation products from stamen and shoot poly(A)$^+$ RNA of GA-treated and untreated plants, it was possible to identify translation products that were modulated by GA and appeared to be specific to stamens. Because some of these altered in abundance before morphologi-

cal changes associated with the rescue of stamen development became apparent, it is possible that they may be involved in GA-regulated stamen development [69]. The cDNA cloning and identification of the genes encoding these translation products has potential to give insight into GA regulation of gene expression during stamen development.

Anthocyanin biosynthesis

The anthers of petunia flowers are a source of GAs that promote the pigmentation and growth of tissues of the corolla [154]. GA induces the anthocyanin production necessary for corolla pigmentation in petunia when applied to emasculated flowers or to isolated petals incubated in the presence of sucrose [154, 156, 157]. Exogenous GA induces the expression of a number of anthocyanin biosynthetic genes. Chalcone synthase (CHS) is normally expressed in corollas. When they are detached from the flower and cultured without GA, CHS mRNA decreases but can be restimulated by GA. Nuclear run-on transcription experiments confirm that this effect is at the level of transcription and has a lag time of some 10 h. The fact that GA regulation of CHS transcription is sensitive to cycloheximide suggests that CHS is not a primary response gene regulated by GA [156, 157].

Other genes of the anthocyanin biosynthesis pathway are regulated by GA in petunia corollas. On the basis of the expression of chalcone isomerase (CHI) promoter-GUS fusions in transgenic petunia flowers, it seems likely that CHI may also be regulated at the level of transcription [156]. Two genes involved in later stages of the anthocyanin biosynthesis pathway, dihydroflavonol 4-reductase [155] and *ant17* [158], are also regulated by GA.

The regulation of expression of anthocyanin biosynthesis genes in petals is complex and appears to be controlled, at least in part, by regulatory genes that encode transcription factors of the basic helix-loop-helix and c-*myb* classes [94]. The possibility that GA may coordinately regulate genes of the anthocyanin biosynthesis pathway in petunia by acting on regulatory genes encoding these types of transcription factors [94, 157] is an attractive concept that should be tested. A perception-transduction-response cascade that has one or more regulatory genes as central components would be an effective mechanism for a single ligand to regulate coordinately the expression of multiple genes in a tissue-specific manner.

GA regulation of gene expression in aleurone

Aleurone cells are an excellent system for studying GA and ABA regulation of gene expression. Genes encoding α-amylase [73], a number of proteases [19, 85, 153, 159], (1-3,1-4)-β-glucanase [163] and *BEG1* (globulin-1) [46] are upregulated by GA and, in all cases, this effect is repressed by ABA. The expression of genes encoding alcohol dehydrogenase and an α-amylase/protease inhibitor is down regulated by GA [73].

The regulation of α-amylase gene expression in aleurone cells of wheat, barley and oat is the most intensively studied effect of this plant hormone on gene expression. Aleurone cells and protoplasts synthesise α-amylase in response to GA and this synthesis is accompanied by an increase in the steady-state levels of α-amylase mRNA [73]. Run-on transcriptions with nuclei isolated from barley and wild-oat aleurone protoplasts have demonstrated that the GA-induced increase in α-amylase mRNA is due primarily to increased transcription of α-amylase genes which can be overcome by ABA [65, 171].

In common with all other GA-responsive genes characterised to date, α-amylase expression increases only after several hours of exposure of aleurone cells to GA. GA induction of α-amylase mRNA is sensitive to cycloheximide and the amino acid analogue aminoethyl-*L*-cysteine [99, 100, 101], suggesting that protein synthesis is required before α-amylase gene transcription can be stimulated. Inhibition of protein synthesis after 2 or 4 h treatment with GA did not substantially inhibit α-amylase mRNA, suggesting that the protein(s) important for GA-regulated α-amylase gene expression had been synthesised during this

period. It is likely that one or more of these proteins may be components of the GA perception-transduction pathway, or may be involved in an unrelated signal transduction pathway that cross-talks strongly with it.

In Gramineae, α-amylase is encoded by a multigene family that gives rise to several classes of isozymes [58, 60]. These genes are not all subject to the same regulation by GA. Some are insensitive to GA and are subject to developmental or tissue-specific regulation. Those which are GA-responsive can differ temporally and quantitatively in their expression, at both mRNA and protein levels. To a large extent this differential regulation of expression can be correlated with groups of α-amylase genes located on particular chromosomes and encoding proteins that can be grouped according to their isoelectric points (pIs) [7, 21, 59, 61, 62, 75].

Transient expression analysis of α-*Amy* promoter-reporter gene constructs in aleurone protoplasts and layers has been used to identify *cis* elements important for high-level GA- and ABA-regulated expression of wheat and barley α-amylase genes. Analysis of the promoter of the wheat low pI α-amylase gene (*α-Amy2/54*) in oat aleurone protoplasts revealed that elements involved in directing GA- and ABA-regulated expression lie within 289 bp upstream of the start of transcription [63]. Similar analysis of a barley high pI *Amy pHV19* gene promoter showed that the main elements important for GA and ABA regulation lie between 174 and 41 bp upstream from the start of transcription [66].

Transient expression analysis has also been used to assess the activity of multimerised individual elements that are known to be highly conserved in α-amylase promoters [58, 135]. Multiple copies of a 21 bp sequence from the barley *α-Amy1/6-4* promoter confer GA-inducible and ABA-repressible expression on a minimal promoter-reporter gene fusion. It has been suggested that a GA-response element (GARE) resides within these 21 bp [135] and the alignment of GA-regulated α-amylase gene promoters [58, 121] reveals a conserved motif UTAACAUANTCYGG (where U = A/G, Y = C/T and N = A/C/T/G) within this region. Sequences similar to this conserved motif are also present in the promoters of a thiol protease gene [159], a carboxypeptidase gene [8] and a gene encoding (1-3,1-4)-β-glucanase isoenzyme II [163], all of which are GA-regulated in aleurone cells. However, the promoter of a cathepsin B-like gene that is GA-regulated in wheat aleurone does not contain the GARE motif and a different conserved element has been shown to be important for GA-regulated expression [19]. The *GAST1* gene which is GA-regulated in tomato shoots does not contain the GARE motif [129].

Evidence of greater complexity in the GA regulation of α-amylase genes has emerged from subsequent studies. A deletion and mutation analysis within the 289 bp region of the wheat *α-Amy2/54* promoter identified at least three regions, one of which probably corresponds to the GARE area, necessary for high-level GA- and ABA-regulated expression. These elements appeared to operate in concert [64]. A similar linker-scanning mutation and deletion analysis of the barley low-pI *α-Amy32b* gene promoter [89] identified the GARE and a putative Opaque-2-binding sequence (O2S) as being essential for expression. A pyrimidine box [58] and a separate motif, TATCCATGCAGTG, were found to be important in determining absolute levels of expression [89]. It has been suggested that GARE cooperates with other *cis* elements, in particular O2S, thereby functioning as a GA-response complex (GARC) in which the pyrimidine box may facilitate interactions between proteins binding to O2S and GARE [89, 121].

Both the GARE and a TATCCAC motif are important for GA- and ABA-regulated expression of the high-pI barley *Amy pHV19* gene promoter. The pyrimidine box was found not to be important for expression and whether or not GARE cooperates with neighbouring *cis* elements is debatable [42, 122]. In view of the expression patterns of high- and low-pI α-amylase genes, differences between their promoters may not be unexpected and this may be evidence of such variation.

Cis elements are assumed to be sites with which

transcription factors interact in a sequence-specific manner. There is now convincing evidence, from gel retardation and DNase 1 footprinting analyses, that aleurone nuclear proteins interact specifically with DNA sequence elements in GA- and ABA-regulated α-amylase promoters [84, 110, 124, 143]. There are good correlations between *cis* elements defined by transient expression analysis and sequence elements shown to bind nuclear proteins. Clear interactions have been demonstrated between nuclear proteins and GARE, O2S, the TATCCAT motif, cAMP- and phorbol ester-like response elements and the pyrimidine box. Some evidence suggests that protein binding to GARE and the TATCCAT motif may be induced by GA [143], although other reports show no evidence for GA-inducible interactions [84, 124].

Future prospects

Evidence that GA can be perceived at the plasma membrane of aleurone cells has led to a reassessment of previous models of GA signal transduction. However, until a definitive identification can be made of GA receptors, their subcellular location is likely to remain controvertial. Recently developed techniques of GA photoaffinity labelling and GA affinity chromatography appear to offer powerful and specific methods for identifying GA-binding proteins, although whether or not they will help identify the elusive GA receptor remains to be seen. The recent successful isolation of high-purity aleurone plasma membrane [151] may help greatly in identifying GA-binding activity at this subcellular site.

It has become clear that Ca^{2+} and calmodulin play important roles in coordinating the responses of aleurone cells to GA and ABA. A better understanding of the temporal and spatial dynamics of cytoplasmic and ER Ca^{2+} may help elucidate the multifunctional role of Ca^{2+} in these cells. It will also be of value to determine whether or not GA modulates cytoplasmic Ca^{2+} in other GA-responsive tissues where Ca^{2+} flux associated with α-amylase secretion is not present. Biochemical approaches may identify specific targets for Ca^{2+} and calmodulin regulation.

It is reasonable to anticipate that genes encoding proteins which bind specifically to GARE and other sequence elements in α-amylase promoters will be identified soon. These may encode transcription factors that are components of the GA perception-transduction-response pathway. They might even be primary response genes regulated by GA. Similar detailed analysis of the promoters of other genes upregulated by GA during each of the three classes of response to this plant hormone may lead to the identification of other *cis* elements and *trans*-acting factors involved in GA-regulated gene expression.

Molecular genetic analysis of GA-response mutants seems well placed to identify genes responsible for insensitivity to GA and it is likely that the first of these to be identified will be the *gai* locus in *Arabidopsis*. A greater range of GA-response mutants is needed, in particular for mutants affected in specific, rather than all, responses to GAs. These may well come from refined screening procedures. We can also anticipate insight into GA signal transduction coming from molecular genetic studies of other plant hormones. Cross-talk between signalling pathways for different plant hormones seems very likely and may involve proteins that play central roles in plant cell signalling.

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