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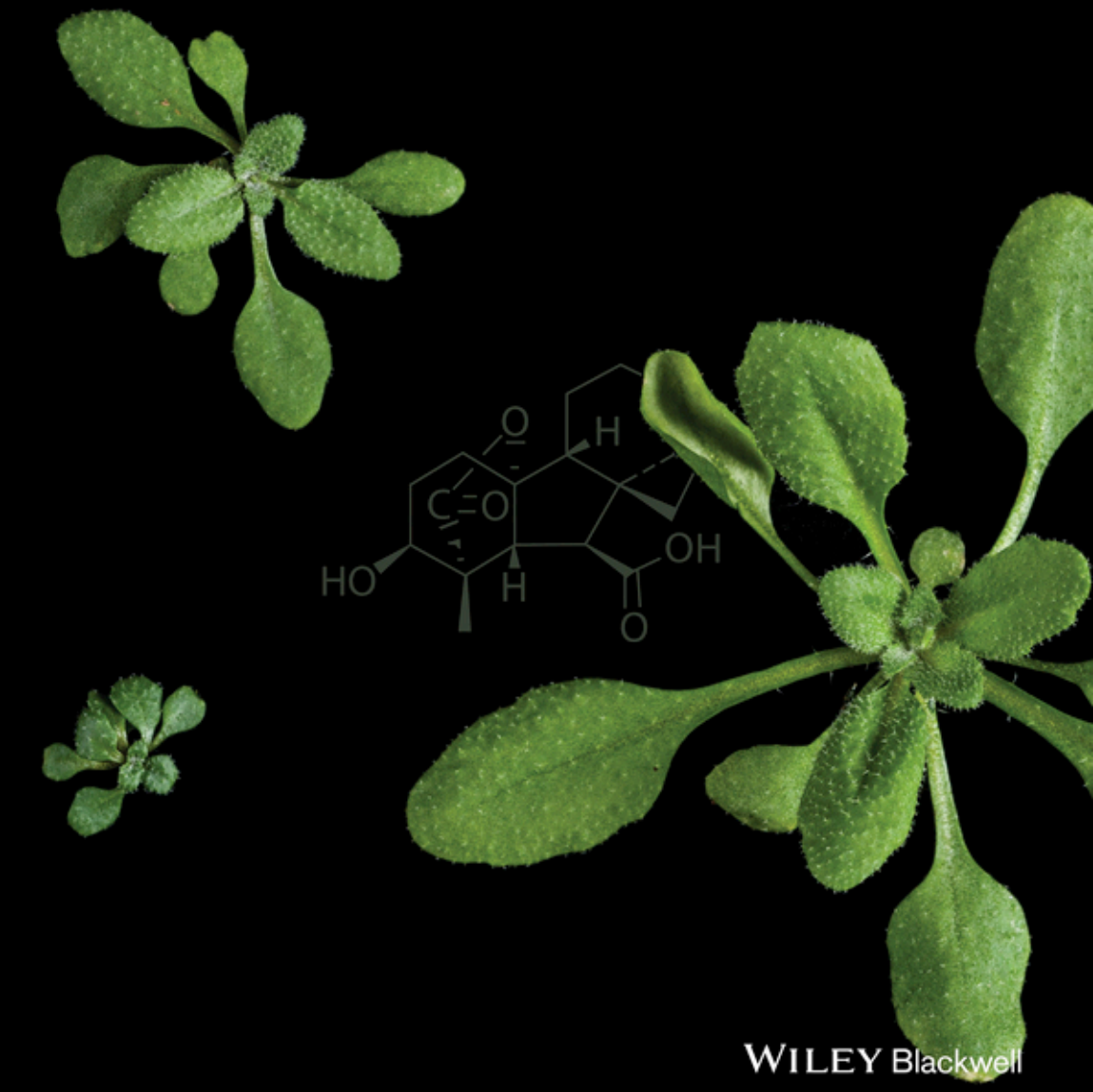
The Gibberellins

Edited by **Peter Hedden** and **Stephen G. Thomas**



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PREFACE

It is now nine years since the publication in 2006 of the Annual Plant Reviews volume on plant hormone signalling, which included a chapter on gibberellin (GA) metabolism and signal transduction. At the time of this publication the GA receptor GID1 had just been discovered, opening up a rich vein of research on GA perception. Since 2006 there have been substantial advances in our understanding of GA signalling and, although there have been several reviews covering aspects of this topic in the intervening years, a volume covering all facets of GA research is now timely. The last volume dedicated to the GAs, which contained the proceedings of a conference in Tokyo to commemorate the retirement of Professor Nobutaka Takahashi, was published as along ago as 1991.

We have included an appendix providing the structures of the 136 chemically characterized GAs. It is noteworthy that it is over 10 years since that last novel GA was identified, although further uncharacterised GAs are present in plants and some may have physiological importance. Due to the very low abundance of GAs in plant tissues, identification of novel compounds has necessitated the synthesis of proposed structures for comparison with the natural metabolites. Regrettably there are now very few laboratories engaged in GA chemistry, making this task increasingly less feasible. The GA research community owes considerable debt to the pioneering chemists, such as Jake MacMillan, who sadly died in 2014, Nobutaka Takahashi and Lewis Mander. In particular, the isotopically labelled GA standards produced by Professor Mander have provided an enormous boost to GA research. It is crucial to the GA field that it continues to receive adequate chemical support.

As described in the following chapters, there have been numerous highlights in GA research in the last nine years. In terms of GA biosynthesis, the cloning of 13-hydroxylases from rice, provided an important piece missing from our understanding of the metabolic pathway. The determination of the X-ray crystal structure of the GID1 receptor and the identification of many of the transcription factors and other proteins that interact with the DELLA GA signalling components are key advances. The establishment of DELLAs as hubs that integrate GA signalling with that of other hormones is of particular note, although the physiological relevance of these observations still needs to be fully explored. These topics will continue to occupy scientists interested in GA research in the coming years, as will the emerging interest in GA transport, which, with the identification of GA transporters and the observed structural specificity of GA movement, is providing evidence to suggest that transport is not dependent solely on membrane diffusion as

previously assumed. Although there have been advances in localising the sites of GA synthesis, catabolism and action, further refinement in analytical methods is required to define these at the cellular level. The development of *in situ* methods for visualising GA, as has been reported for auxin and jasmonate, is a high priority. Such approaches will ensure that GA research remains an active and exciting field in the next nine years and beyond.

Peter Hedden and **Stephen G. Thomas**

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Chapter 1

SIGNAL ACHIEVEMENTS IN GIBBERELLIN RESEARCH: THE SECOND HALF-CENTURY

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Abstract: Chapter 1 briefly recounts the discovery of gibberellins (GAs) as natural products of the fungus *Gibberella fujikuroi* in the early part of the twentieth century, and provides a historical overview of GA research from the late 1950s to the present day. It describes how biosynthetic pathways to GAs in *Gibberella* and higher plants were defined, and how stem length mutants of cereals and legumes were instrumental in establishing which GAs are biologically active and have hormonal function. The chapter presents an overview of the cereal aleurone system in which GA signalling was first studied, and describes how more recent use of *Arabidopsis* and rice led to the characterisation of a GA receptor (GID1) and downstream regulatory proteins (DELLAs). A number of DELLA-interacting proteins are described, illustrating how it is that GA-induced degradation of DELLAs facilitates downstream responses including cell elongation. Other 'classical' GA responses include germination and flowering in some species.

Keywords: Cereal aleurone, DELLA proteins, *Gibberella fujikuroi*, gibberellin biosynthesis, gibberellin receptor, gibberellin signalling, stem length mutants

1.1 Introduction

Gibberellins (GAs), once known only as fungal products, comprise a group of over 136 structurally related compounds that are natural constituents of plants. Just a small number of GAs have intrinsic biological activity, and they regulate many aspects of growth and development throughout the plant life cycle. Other GAs are biosynthetic precursors or inactivation products of the

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bioactive GAs, or may be metabolic by-products with no known function. Commercial-scale microbiological production of gibberellic acid (GA_3) facilitates its use in agriculture, particularly in fruit production, and there are also important uses for synthetic inhibitors of GA biosynthesis that act as dwarfing agents (discussed in Chapter 12).

Gibberellins were first identified in *Gibberella fujikuroi*, which is a fungal pathogen of rice.[1] The 'bakanae' or 'foolish seedling' disease, which has been known to rice farmers in the Orient for at least 200 years, causes supra-optimal elongation of seedlings and reduced yield of grain. At the end of the nineteenth century, Shotaro Hori, a mycologist working at the Imperial Agricultural Experiment Station in Nishigahara, Tokyo, induced these symptoms in healthy rice seedlings by infecting them with the 'bakanae' fungus. More than two decades later, Eiichi Kurosawa, a Japanese scientist working in Taipei, Taiwan, succeeded in producing sterile filtrate from *G. fujikuroi* cultures which, when applied to uninfected rice seedlings, could duplicate the pathological symptoms. The race was then on to identify the chemical substances that were secreted by *Gibberella*, and which caused overgrowth and reduced grain yield of infected seedlings. Phinney, who has documented the early history of GAs, reported the publication of more than 50 articles on the subject between 1927 and 1940 (Phinney, 1983). Teijiro Yabuta, an organic chemist working with Kurosawa, who had moved from Taipei to Nishigahara in 1933, obtained a semi-purified non-crystalline material from culture filtrates, which he termed 'gibberellin'. It could stimulate stem elongation not only in rice, but in several other important crops, including barley, buckwheat and soybean. The material was crystallised two years later (Yabuta and Sumiki, 1938), yielding two biologically active components, which they named gibberellin A and B.

After World War II interest in these growth-promoting factors from *Gibberella* reached the West, and two research groups, one at the Imperial Chemical Industries (ICI) Akers Research Laboratory in Welwyn, UK and the other at the United States Department of Agriculture (USDA) Laboratory in Peoria, Illinois, took on the task of chemical characterisation of the compounds secreted by *Gibberella fujikuroi*. It culminated in the isolation of gibberellic acid by the UK group (Cross, 1954) and gibberellin X by the US group (Stodola *et al.*, 1955). It was soon discovered that gibberellic acid and gibberellin X were the same, and the latter name was dropped. Gibberellic acid (see GA_3, Figure 1.1) was defined as a tetracyclic-dihydroxy-lactonic acid with the molecular formula $C_{19}H_{22}O_6$ (Cross, 1954). A reinvestigation

[1]The fungus has had a succession of names, being identified initially as the Deuteromycete, *Fusarium moniliforme*, prior to the discovery of its perfect (sexual) stage whereupon it was reclassified as the Ascomycete *Gibberella fujikuroi*. Recently the name *Fusarium fujikuroi* has been adopted. Because the fungus has been known predominantly as *Gibberella* for the period of time this history covers, that is the name used throughout this chapter.

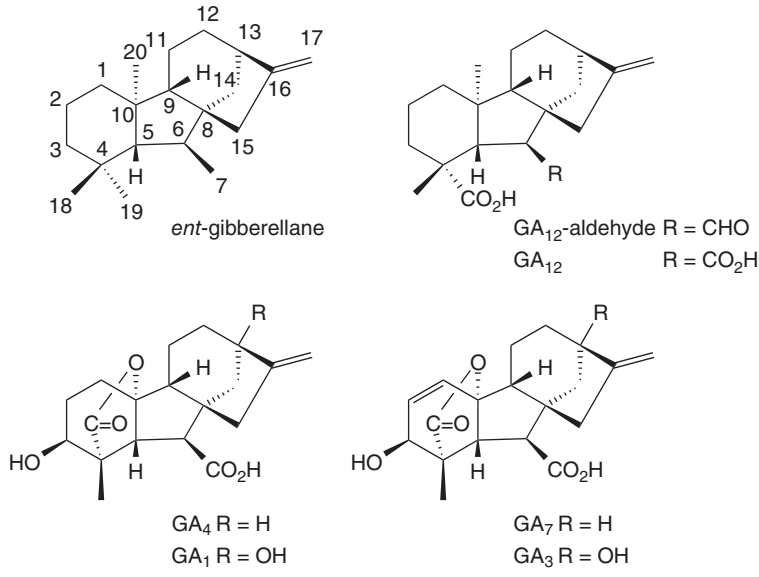

Figure 1.1 The *ent*-gibberellane skeleton shows the carbon atom numbering scheme used for gibberellins. GA_{12}-aldehyde is the first-formed GA in fungal and plant pathways. It is oxidised to the C-7 acid, GA_{12}. C_{20}-GAs, such as GA_{12}, contain the full complement of carbon atoms. They are precursors of C_{19}-GAs in which carbon-20 has been lost by metabolism. GA_1, GA_3, GA_4, and GA_7 are biologically active C_{19}-GAs, each possessing a 3β-hydroxyl group and a γ-lactone.

by Japanese chemists of the gibberellin 'A' sample that had been isolated more than a decade earlier yielded three components, which were termed gibberellins A_1, A_2 and A_3 (Takahashi *et al.*, 1955). An additional GA, GA_4, was isolated from *Gibberella* culture filtrate in 1959. Thus began the nomenclature of this large class of structurally related compounds that has now reached gibberellin A_{136}. The trivial name gibberellin A_x is now commonly abbreviated to GA_x, with GA used as a general abbreviation for gibberellin. GA is often used erroneously to represent gibberellic acid, which is identical to gibberellin A_3 (GA_3). Both names are still in use for this compound. It is the major product of GA biosynthesis in *Gibberella* (discussed in Chapter 5) and is produced commercially for horticultural and agronomic use.

A review of the extensive series of publications from the UK group in the late 1950s and early 1960s summarises the evidence for the structure of GA_3, particularly that of ring A, including the location of the hydroxyl group, the olefinic double bond and the lactone (Cross *et al.*, 1961). The C numbering scheme used at that time has been superseded by that shown on the *ent*-gibberellane skeleton in Figure 1.1. The structural determination of the other fungal GAs that were known at the time, namely GA_1, GA_2, GA_4,

GA_7 and GA_9, was also reviewed, with reference to GA_3. The assignment of stereochemistry to GA_3 was discussed too.

The response of plants to exogenous GA_3 was a topic of intense interest beginning in the mid-1950s. Dwarf and rosette plants were particularly responsive, and many papers appeared in the literature documenting the spectacular internode elongation in, for example, seedlings of dwarf pea (Brian and Hemming, 1955) and maize (Phinney, 1956) (Figure 1.2), and the rapid bolting of non-induced photoperiodic plants such as henbane (*Hyoscyamus*) (Lang, 1956). Almost immediately the search began for endogenous compounds in plants that could mimic the biological effects of applied GA_3. Margaret Radley, following up her work with P.W. Brian, provided bioassay evidence for endogenous growth-stimulating activity in pea seedlings (Radley, 1956). The observation that dwarfism appeared to be associated with GA-deficiency was also documented. However, Phinney, who had produced many different non-allelic dwarf mutants of maize, noted that while most recessive mutants responded to GA_3, two dominant dwarf mutants did not. Three decades later the recessive (responsive) mutants were used to determine metabolic sequences between GAs, while four decades later dominant (non-responsive) mutants were used to investigate GA signalling.

The first definitive characterisation of GA from plants came after the extraction of kilograms of developing bean seeds. It was a wise choice of plant material as immature seeds of both *Phaseolus coccineus* (formerly *multiflorus,* runner bean) and *Ph. vulgaris* (French bean) are rich sources of many GAs compared to vegetative tissue, though the task was still mammoth. Working at the ICI Akers Laboratory, Jake MacMillan and P.J. Suter identified GA_1 (which had been isolated previously from *Gibberella*) from *Ph. coccineus* seeds, obtaining 2 mg of the crystalline GA_1 from 87.3 kg of immature seeds that had been harvested from approx. 2 metric tons of locally grown pods (MacMillan and Suter, 1958). In a prescient comment in the final paragraph to their paper, MacMillan and Suter wrote, 'The occurrence of gibberellin A_1 in higher plants adds new significance to the gibberellins and their growth promoting properties. It leaves little doubt that at least gibberellin A_1 participates directly in the growth regulating system of higher plants.' Indeed, GA_1 is now known to have intrinsic bioactivity and to be the major bioactive GA in most plants studied to date. In subsequent papers, the same research group characterised several additional GAs, namely GA_5, GA_6 and GA_8 from the same extract.

Concurrent work taking place at the University of California at Los Angeles (UCLA) led to the isolation of bean factors I and II from *Ph. vulgaris* seeds. Factor I was shown to be GA_1 and it was equally active on *dwarf-1* and *dwarf-5* mutants of maize, whereas factor II was a new GA with less bioactivity than GA_1 when assayed on *dwarf-1* (West and Phinney, 1959). Their inference that 'the genetically controlled enzymatic block in *dwarf-1* would be between the production of factor II and the active gibberellin' predated by more than 20 years the characterisation of *DWARF-1* as encoding

Figure 1.2 The ability of exogenous GA_1 applied to *dwarf-1* maize seedlings to restore a normal (wild-type) phenotype was one of the earliest demonstrations of the growth-promoting activity of bioactive GAs. Note that GA_1 has no effect on normal (wild-type) seedlings. (After B.O. Phinney. © Sinauer Associates, Inc. 2015.)

a 3β-hydroxylase that is necessary for GA bioactivity (Spray *et al.*, 1984). Bean factor II was shown to be GA_5 (MacMillan *et al.*, 1959).

The continued report of new GAs from *Gibberella* and *Phaseolus* by scientists, many of whom had worked in the Akers Laboratory at ICI or at the University of Tokyo, brought the number to 17 (GA_1–GA_{17}) by 1967. However, the proposal that additional GAs be assigned trivial names based on the plant source (for example *Canavalia* GAs I and II) was anticipated to 'result in complete confusion' by Jake MacMillan and Nobutaka Takahashi,

since the same GA was often present in more than one species. For this reason they proposed assigning 'A numbers' in approximate chronological order of discovery to 'naturally occurring, fully characterised compounds which possess the gibbane skeleton and the appropriate biological properties' (MacMillan and Takahashi, 1968). The provision of infrared and mass spectra was required to ensure that each compound assigned an A number had a unique structure. The allocation of gibberellin A numbers by these organic chemists worked exceedingly well, and was a service to the plant biology community, though the need to prove 'appropriate biological properties' was not strictly enforced. Many of the 136 known GAs[2] do not have biologically activity *per se*, and the class of hormones is defined by chemical structure rather than bioactivity. However, because of the early reliance on bioassay for GA discovery and isolation, the GAs with the highest biological activities (e.g. GA_1, GA_3, GA_4 and GA_7) were among the first to be characterised (see Figure 1.1). Gibberellins contain either 19 or 20 carbon atoms. The C_{20}-GAs contain the full diterpenoid complement of 20 carbon atoms, whereas the C_{19}-GAs have lost one carbon through metabolism.

The remainder of this chapter focuses on the history of gibberellin research from the late 1950s to the present day. Due to the amount and scope of research during this period the review must be selective. Broadly, the chapter describes our acquisition of knowledge of GA biosynthetic pathways, both in *Gibberella* and in flowering plants. The specific pathways are described in detail in Chapters 5 and 2, respectively. The chapter documents our knowledge of the biosynthetic enzymes and the genes that encode them, and our current understanding of their regulation. It describes the discovery of the GA receptor, and the body of information on DELLA proteins that repress GA response (see Chapter 6). The current identification of DELLA-interacting proteins is moving the field forward in exciting ways as we discover the downstream events that mediate GA responses that lead, for example, to seed germination, stem growth, and reproductive growth, which are discussed later in the book. The chapter closes with a brief review of the research that established these physiological responses to GA.

1.2 Gibberellin biosynthesis

The biosynthesis of GAs, which are tetracyclic diterpenes, was studied initially in *Gibberella*. There were many reasons for using the fungus as a model system: it is easy to grow in defined liquid media, substrates can be administered in and products can be extracted from the medium with ease, and

[2]Continuing the UK–Japanese partnership in assigning A numbers, Peter Hedden and Yuji Kamiya took over this responsibility for the plant biology community in the late 1990s.

the levels of GAs in *Gibberella* are several orders of magnitude higher than those in plants. Moreover, the major end product, GA_3, accumulates, facilitating its isolation for determination of site-specific labelling, which can be diagnostic for assigning structure and biosynthetic origin. Although the end products of the fungal and plant pathways are not the same (GA_3 does not occur universally in higher plants, and even in those plants in which it occurs it is a usually minor metabolite), the assumption was made that GA biosynthetic pathways in the fungus and in higher plants would be similar. What we *now* know about the similarities and differences between the fungal and plant pathways is discussed at the end of this section.

Feeds of radiolabelled substrates to *Gibberella* cultures, followed by degradation and analysis of the resulting [^{14}C]GA_3, showed that it is formed from 12 molecules of [^{14}C]acetate or from four molecules of [^{14}C]mevalonic lactone (MVL) (Birch *et al.*, 1958). The pathway proceeds from MVL to isopentenyl diphosphate, the five-carbon building block of all terpenoids, and thence to the linear diterpene geranylgeranyl diphosphate (GGPP). The pathways from GGPP in *Gibberella* are shown in Figure 1.3. The conversion of GGPP to the bicyclic intermediate *ent*-copalyl diphosphate, and the subsequent conversion of this intermediate to tetracyclic *ent*-kaurene was demonstrated in a cell-free system from *Gibberella* (Shechter and West, 1969). The two-stage reaction was shown to be catalysed by *ent*-kaurene synthase A and B (Fall and West, 1971). This terpene cyclase appeared to be a single protein with two separate catalytic activities, since the two cyclisation reactions, from GGPP to CPP, and from CPP to *ent*-kaurene, had different pH optima, metal ion requirements and sensitivities to plant growth retardants (Fall and West, 1971).

Earlier studies (Cross *et al.*, 1964) had shown *ent*-kaurene to be on the pathway to GA_3. The oxidative steps beyond *ent*-kaurene were identified in *Gibberella* concurrently with research on GA biosynthesis in cell-free systems from plants (discussed below). Much of this early work, on both *Gibberella* and plants, was conducted at UCLA in the laboratory of Charles West. *ent*-Kaurenol, *ent*-kaurenal and *ent*-kaurenoic acid were all individually shown to be precursors of GA_3, inferring the sequential oxidation of the CH_3 group at C-19 in *ent*-kaurene to CH_2OH (*ent*-kaurenol), to CHO (*ent*-kaurenal), and to COOH (*ent*-kaurenoic acid) (Figure 1.3). The enzymes catalysing these steps were shown to be microsomal cytochrome-P450-dependent mono-oxygenases.

The steps in the pathway after *ent*-kaurenoic acid constitute a branch-point, with one branch being the committed pathway to GAs, and the other (not shown) being a route to poly-oxygenated kaurenoids that accumulate in the fungus and some plants, and for which there is no known function. The dedicated pathway to GAs requires the contraction of the six-membered B-ring, with extrusion of C-7, giving GA_{12}-aldehyde (see Figure 1.1), which is the first-formed GA in all systems studied. Considerable work on the mechanism of the ring contraction in the fungus and higher plants was conducted.

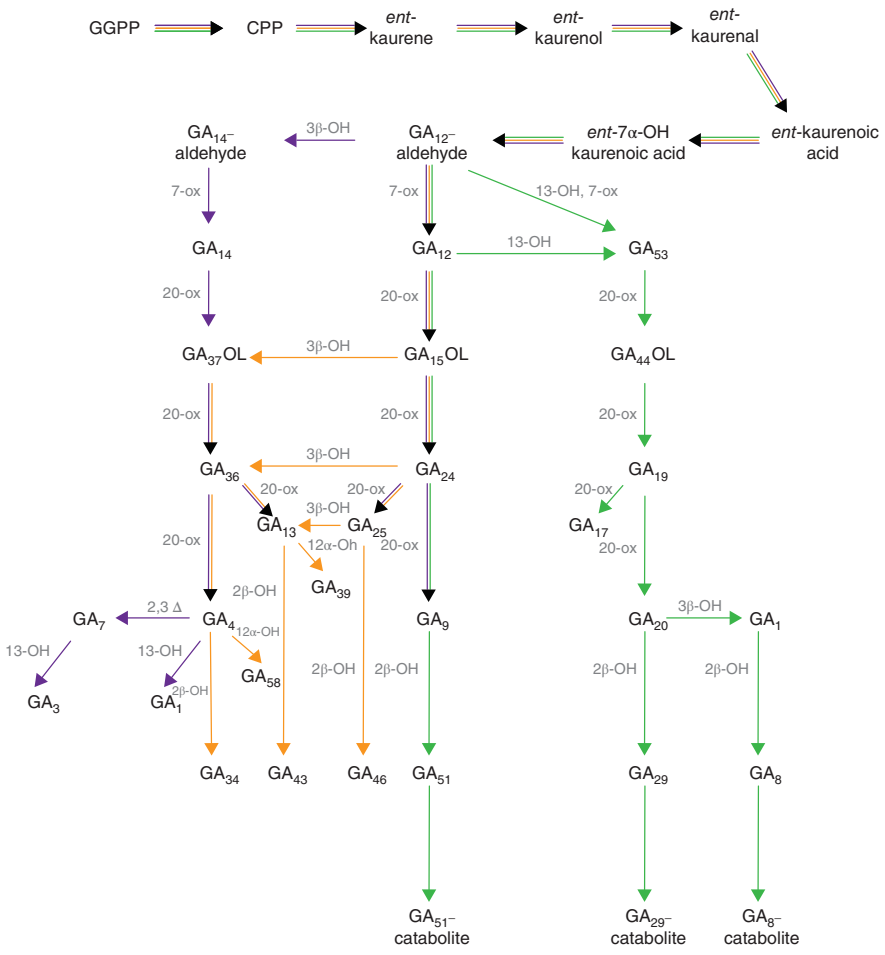

Figure 1.3 Composite scheme showing the conversion of GGPP to the first-formed GA, GA_{12}-aldehyde, and the predominant GA metabolic pathways from GA_{12}-aldehyde in three model systems: *Gibberella* (early 3-hydroxylation pathway, left), pumpkin (late 3-hydroxylation pathway, centre and left), and pea (early 13-hydroxylation pathway, right, and non-hydroxylation pathway, center). Note that GA_1 occurs on both left and right sides of the scheme. GGPP geranylgeranyl diphosphate, CPP copalyl diphosphate, OL open lactone (CH_2OH at C-20). (See insert for colour representation of this figure.)

Potential intermediates between *ent*-kaurenoic acid and GA_{12}-aldehyde, with stereospecific ^{14}C or ^{3}H labeling of atoms in the B ring, were tested. Feeds of labelled *ent*-7α-hydroxykaurenoic acid produced labelled GA_3 in sufficiently high yield (4%) after 2 days to anticipate that it was an intermediate on the GA pathway (Lew and West, 1971). The intermediacy

of *ent*-7α-hydroxykaurenoic acid was subsequently confirmed (Hanson *et al.*, 1972).

Gibberellin A_{12}-aldehyde is on the main pathway to GAs in *Gibberella*, (see Figure 1.3), whereas the C-7 acid, GA_{12}, is not (Bearder *et al.*, 1973). Hydroxylation of GA_{12}-aldehyde at C-3 gives GA_{14}-aldehyde, feeds of which produce 3-hydroxylated C_{19}-GAs (Figure 1.3). Gibberellin A_3, a 3,13-dihydroxylated C_{19}-GA, is the major end product of GA biosynthesis in *Gibberella*, and it accumulates. Geissman had previously obtained evidence from feeds of *ent*-kaurenoic acid that GA_4, the first C_{19}-GA on the pathway, was a precursor of GA_7 (1,2-dehydro-GA_4), and GA_3 (13-OH GA_7) (Geissman *et al.*, 1966). This and all other evidence suggested that 13-hydroxylation occurs late in the pathway in *Gibberella*. Intermediates between GA_{14}-aldehyde and GA_4 did not accumulate in the fungus. In a separate, though minor, pathway in *Gibberella*, the C-7 acid, GA_{12}, is the precursor of non-hydroxylated GAs, including GA_9 (Bearder *et al.*, 1973; Bearder *et al.*, 1975).

The highly vigorous wild-type strain of *Gibberella*, GF-1a, was shown by combined gas chromatography-mass spectrometry (GC-MS) to contain at least 25 diterpenes, including 15 known or putative GAs (MacMillan and Wels, 1974). Gibberellins are not required for the growth of *Gibberella* in culture, although they may facilitate pathogenesis by affecting the host plant. The B1-41a strain of *Gibberella*, which was isolated by Bernard Phinney after UV irradiation of GF-1a, was shown to be essentially GA-deficient and yet its growth and morphology was indistinguishable from that of GF-1a. *ent*-Kaurene oxidation is blocked in B1-41a (Bearder *et al.*, 1974), and the absence of downstream metabolites meant that GA metabolic studies could be conducted without the need for isotopic labelling of substrates.

The ability to identify products in complex mixtures using GC-MS, which was pioneered by the laboratory of Jake MacMillan (Binks *et al.*, 1969), was revolutionary to the field. Individual products, if they were known compounds, could be identified unequivocally, even in complex mixtures, without the need for isolation. In addition, detection of ^{14}C or stable isotopes in the mass spectra of products could prove the biogenic origin of metabolites. Furthermore, mass spectral information of unknown compounds was often very informative for structural determination. For all these reasons, exceedingly rapid progress was made in the mid-1970s defining naturally occurring pathways beyond GA_{12}-aldehyde in *Gibberella* (Bearder *et al.*, 1975).

Concurrent with these early studies using *Gibberella* were concerted efforts to study GA biosynthesis in plants. The plants most frequently used for metabolic work were cucurbits, legumes, and cereals. The major pathways were defined well before *Arabidopsis thaliana* became the model system of choice. Much of the earliest work focused on *in vitro* systems from plants, and was conducted by Charles West's group, which included Jan Graebe. The tissue selected for use was liquid endosperm from seeds of members

of the Cucurbitaceae, notably *Marah macrocarpus* (Californian wild cucumber, previously called *Echinocystis macrocarpa*) and later *Cucurbita maxima* (pumpkin).

For plants, *in vitro* systems, such as those using liquid endosperm, have advantages over *in vivo* studies – substrates can be administered to cell-free systems without concerns about differential uptake, the products can be extracted with ease, incubation conditions can be defined, and individual enzymatic reactions can be studied by including or excluding a particular cofactor, or adding an inhibitor.

Up to 1 mL of gelatinous acellular endosperm can be squeezed from each developing seed of wild cucumber, and after filtration, with perhaps additional purification by dialysis, the preparation is ready for use. Initial studies with *Marah* confirmed the conversion of MVA to *ent*-kaurene, and its sequential oxidation (Graebe *et al.*, 1965). On a historical note, the ease of isolating intermediates from feeds to cell-free systems from *Marah* facilitated the preparation of labelled compounds for subsequent feeds to *Gibberella* (Graebe *et al.*, 1965). Some years later, the ease of feeding derivatives and analogs to GA-deficient cultures of the B1-41a fungal mutant allowed for the preparation of labelled GAs, such as 12- and 13-hydroxylated GAs, for feeding to plant systems (Gaskin *et al.*, 1984).

A cell-free extract from pumpkin endosperm, with which much pioneering work was done by the research group established by Jan Graebe in Göttingen, was the first plant system in which the conversion of MVA to GA_{12}-aldehyde was achieved (Graebe *et al.*, 1972). Feeding of intermediates confirmed the sequence of *ent*-kaurene oxidation described for the fungus, with each conversion shown to be enzymatic. When *ent*-7α-hydroxykaurenoic acid was fed, it was completely converted, giving GA_{12}-aldehyde, GA_{12}, and two unidentified compounds that were later identified as *ent*-kaurenoids. Graebe and Hedden further examined the ring-contraction mechanism, by which the gibbane skeleton in GA_{12}-aldehyde and all other GAs is formed.

Subsequent conversion of GA_{12}-aldehyde to GA_{12}, GA_{15}, GA_{24}, GA_{36} and GA_{37} in the pumpkin system demonstrated oxidation at C-7, C-20, and C-3 was occurring *in vitro* (Graebe *et al.*, 1974a) (Figure 1.3). Feeds of GA_{12} gave GA_{15}, GA_{24}, GA_{36} and GA_{37} too, unlike the fungal system in which GA_{12}-aldehyde and GA_{12} give different products. The 3- and 20-oxidation of both GA_{12}-aldehyde and GA_{12} required different incubation conditions from earlier enzymatic reactions that are catalysed by mono-oxygenases, notably the omission of Mn^{2+}. In a breakthrough the same year the first conversion in a plant system of MVA to a C_{19}-GA, namely GA_4, was achieved (Graebe *et al.*, 1974b). C_{20}-GA products also identified in these incubations were the tricarboxylic acids GA_{13} and its metabolite, GA_{43} (Figure 1.3). This 2β-hydroxylated C_{20}-derivative was diluted by endogenous GA_{43}, underscoring that the metabolic conversions observed *in vitro* reflected those occurring in pumpkin seeds. Subsequently a comprehensive examination by GC-MS of both endosperm and embryo extracts of pumpkin seeds of

several different developmental stages showed over 30 compounds, including eleven GAs, and many poly-hydroxylated *ent*-kaurenoid derivatives (Blechschmidt *et al.*, 1984). In addition to the GAs that had been identified as products in metabolic studies, four new GAs were identified, namely 12α-hydroxylated derivatives of GA_{12}, GA_{14}, GA_{37} and GA_4, the last of which was named GA_{58} (Blechschmidt *et al.*, 1984). Gibberellin A_{58} accumulates more than GA_4. To aid in structural determination of new GAs, *ent*-12α-and 12β-hydroxylated kaurenoic acids were fed to *Gibberella* to obtain reference samples for comparison (Gaskin *et al.*, 1984). These compounds were later obtained as metabolites of GA_{12}-aldehyde in the pumpkin cell-free system, but only when the pH during incubation was between 6 and 7. Above pH 7, hydroxylation at C-12 was not observed, with GA_{12}-aldehyde being converted predominantly to GA_{43} instead (Hedden *et al.*, 1984).

One of the novel features of GA metabolism in pumpkin seeds is that C_{20}-tricarboxylic acids, such as GA_{13} and GA_{43}, accumulate to a much greater extent than in other plants that were also being used for GA metabolic studies, such as pea and corn. In addition, the 13-hydroxylation pathway, which would turn out to be the predominant pathway in many plants is of minor importance in pumpkin (Hedden *et al.*, 1984).

Work with *in vitro* systems from other plants besides pumpkin provided additional useful information. Studies using cell-free systems from pea shoot tips were novel in that they sought to relate *ent*-kaurene biosynthesising activity with seedling phenotype (Coolbaugh *et al.*, 1973), but correlation of enzymatic activity with altered seedling growth in wild-type and dwarf cultivars grown in dark and light gave equivocal results (Ecklund and Moore, 1974). In contrast, cell-free systems from shoots of the *dwarf-5* maize produced less *ent*-kaurene and more *ent*-isokaurene (which would not be a precursor of bioactive GAs) than preparations from wild-type seedlings (Hedden and Phinney, 1979).

The properties and cofactor requirements for GA-metabolizing enzymes in plants were studied most comprehensively by Jan Graebe's research group. Similar to the situation in *Gibberella*, the enzymes catalysing the oxidation of *ent*-kaurene and derivatives are endoplasmic-reticulum-localised cytochrome-P450-dependent mono-oxygenases. So too are the enzymes that catalyse the oxidation of GA_{12}-aldehyde at C-7 and C-13. In contrast, enzymes that catalyse oxidation at C-20, C-3, and C-2 were shown to be soluble 2-oxoglutarate-dependent dioxygenases (2ODDs) (Hedden and Graebe, 1982; Smith and MacMillan, 1984). This is in contrast to the enzymes that oxidise GAs in *Gibberella*, which, like earlier enzymes in the pathway, are also mono-oxygenases.

In vivo metabolic studies with plants began in the early 1970s, and the main focus was on developing seeds, predominantly from legumes. From a historical perspective, the discovery process was different from that with Cucurbits in which, as described previously, work with cell-free systems in the 1970s was predictive of GAs that would later be found as endogenous components.

With pea, for example, analyses of native GAs and *in vivo* metabolic studies in the 1970s were predictive of the pathways that would later be confirmed with cell-free systems.

Work in the MacMillan group on pea seeds began by identifying the major C_{20}- and C_{19}-GAs in immature seeds at different developmental stages (Frydman *et al.*, 1974). *In vivo* metabolic studies were conducted using intact plants, by injecting labelled substrates through the pod wall into the cotyledons of developing seeds. The results of these feeds predicted the presence of two parallel pathways, one with 13-hydroxylation occurring early (at the C_{20}-GA stage), giving GA_{20} as the first C_{19}-GA, and one pathway in which 13-hydroxylation does not occur, giving GA_9 as the first C_{19}-GA (Sponsel and MacMillan, 1977) (see Figure 1.3). The presence of 13-hydroxylated C_{20}-GAs as endogenous components of developing pea seeds supported this contention.

The presence of the early 13-hydroxylation pathway as the major pathway in pea was later confirmed in cell-free systems from developing seeds (Kamiya and Graebe, 1983). Both GA_{12}-aldehyde and GA_{12} could be 13-hydroxylated by a microsomal preparation, yielding GA_{53}. Feeds of GA_{53} to soluble enzymes gave GA_{44}, GA_{19} and GA_{20} (see Figure 1.3). Refeeding all intermediates (GA_{44} was refed in the open lactone form) gave the sequence GA_{53} to GA_{44} to GA_{19} to GA_{20}. GA_{20} was 2β-hydroxylated in preparations from older seeds, giving GA_{29}. Gibberellin A_{12} fed to a soluble enzyme preparation gave non-13-hydroxylated C_{20}-intermediates and GA_9 and GA_{51} (the later step was demonstrated predominantly in preparations from older seeds). Thus the two parallel pathways inferred from *in vivo* studies were demonstrated in entirety *in vitro* (Kamiya and Graebe, 1983) (Figure 1.3).

No evidence of 3-OH was observed in either feeds to maturing pea seeds (10 days from anthesis and older) or in these cell-free systems. Later studies utilising younger fruits of pea showed that 3-hydroxylated C_{19}-GAs (GA_1 and GA_3) do occur transiently in both developing seeds and pericarps shortly after pollination and may well be necessary for the earliest stages of seed development, and for pod elongation (Garcia-Martinez *et al.*, 1991).

In vivo studies of pea seeds also showed the importance of 2β-hydroxylation during the later stages of seed maturation, and the production of novel α, β-unsaturated ketone derivatives called GA-catabolites that accumulated predominantly in the testa (Sponsel, 1983) (Figure 1.3). The accumulation of biologically inactive GA catabolites in pea and in the closely related species *Vicia faba* was seen as an alternative to GA-conjugation, which is observed in other legumes. For example, the multiplicity of free GAs in developing *Ph. vulgaris* seeds, and the accumulation of GA conjugates in mature seeds has been documented (Hiraga *et al.*, 1974). In feeds to older seeds, GAs were conjugated to glucose, either through ether or ester linkages. Evidence for hydrolysis of GA_{20}-glucosyl ether to liberate GA_{20}, which was itself further metabolised to GA_1 when it was fed to maize plants, suggested the conjugate could represent a form for temporary sequestration of GA for later use.

However, conjugates of already inactive GAs would be permanently inactive (Schneider and Schliemann, 1994). These enzymatic reactions in plants that inactivate GAs have not evolved in *Gibberella*, in agreement with the proposition that GAs have no biological activity in the fungus. Mechanisms for GA-inactivation are described in Chapter 3.

Not only did metabolic studies in plants demonstrate that GAs can be inactivated by metabolism, they indicated that many GAs may show bioactivity only because they are converted to an active GA in the plant material used for bioassay. Structure/activity relationships, coupled with metabolic studies, revealed the requirement for certain functional groups for intrinsic activity (Reeve and Crozier, 1974). Bioactive GAs possess 19 rather than 20 carbon atoms, and have a γ-lactone between C-19 and C-10 (Figure 1.1). They possess an exocyclic methylene at C-16, and carboxylic acid at C-6. 3β-Hydroxylation or other functionality at C-3 is required for bioactivity. 13-Hydroxylation neither enhances nor inhibits activity except in certain plants such as members of the Cucurbitaceae and, as shown later, in *Arabidopsis* in which 13-hydroxylated GAs have less activity than their 13-deoxy-counterparts. On the other hand, 2β-hydroxylation (as in GA_8, GA_{29}, GA_{34}, GA_{51}) always reduced bioactivity or the potential to be metabolised to an bioactive GA. Gibberellins with a 1,2 double bond (GA_7 and GA_3) are not inactivated by 2β-hydroxylation. Gibberellin derivatives such as 2,2-dimethyl GA_4 and 2β-methyl GA_4 were synthesised and tested to see whether they would have higher bioactivity than GA_4, since 2β-hydroxylation should not occur for these GAs (Hoad *et al.*, 1981). The results varied by test material, but with bioassays using monocotyledonous plants (e.g. oat first leaf, dwarf rice, and *dwarf-5* maize assays) and with extended duration of testing, the GA derivatives in which 2β-hydroxylation is blocked displayed longer-lasting activity than GA_4.

Extraction of seeds of many different species increased the number of known GAs very substantially during the 1970s and 80s. All GAs had to have confirmed chemical structures before A numbers could be assigned. For some species there was a characteristic pattern of hydroxylation. For example, immature seeds of moonflower, *Caloniction aculeatum* (now *Ipomoea alba*) were shown to contain three GAs that possess 12α-hydroxyl groups, and after structural determination they were assigned the numbers GA_{30}, GA_{31}, and GA_{33} (Murofushi *et al.*, 1988). Developing grain of wheat (*Triticum aestivum*) was shown to contain GAs that are hydroxylated at C-1, two of which were named GA_{60}, and GA_{61} after preparation of authentic reference compounds (Gaskin *et al.*, 1980). Sunflower, *Helianthus annuus*, contains many GAs that are hydroxylated at C-15. After structural determination they were assigned the numbers, GA_{64}, GA_{65}, GA_{66}, GA_{67} and GA_{72} (Hutchison *et al.*, 1988).

The numerous poly-hydroxylated (and thus very polar) GAs that accumulate in developing seeds have little bioactivity is seedling assays, and are not known to have physiological function in seed development. Why such

a diversity of GA structures occurs in maturing seeds, and why they accumulate to very high levels during development and decline during the later stages of maturation, is still something of a mystery.

Continuing improvements in the sensitivity of GC-MS instrumentation were being made over time. The MacMillan group was one of the leaders in this area, with Paul Gaskin assembling a large array of reference spectra of naturally occurring GAs, kaurenoids and synthetic analogs, as the methyl esters and trimethylsilyl ether derivatives. Eventually GC-MS instrumentation had the requisite sensitivity to make comprehensive analysis of GAs in vegetative material feasible. Simultaneously work proceeded on pea and corn seedlings.

Two groups led by geneticists Ian Murfet in Hobart, Tasmania and Bernard Phinney (UCLA) had, over time, been isolating single gene dwarf mutants of pea and corn, respectively (Phinney, 1956; Reid *et al.*, 1983). The early 13-hydroxylation pathway was known to be the major pathway in pea seeds (Kamiya and Graebe, 1983) and all GAs that were identified in maize tassels were 13-hydroxylated (Hedden *et al.*, 1982), thus, feeding studies focused on the metabolism of GA_{20}, which is the first-formed C_{19}-GA in that pathway.

GC-MS analyses of seedlings of GA-responsive dwarf mutants of pea and maize helped to define the enzymatic steps that were blocked by each genetic lesion. Researchers fed labelled GA_{20} to *LE* and *le* pea seedlings (Ingram *et al.*, 1984), and to *DWARF-1* and *dwarf-1* maize seedlings (Spray *et al.*, 1984). Results showed that the *le* mutation of pea and the *dwarf-1* mutation of maize both prevent 3β-hydroxylation, thus blocking the conversion of GA_{20} to GA_1 (Figure 1.3). This is a crucial step – the responses of *le* and *dwarf-1* mutants to exogenous GA application indicated that GA_{20} has no activity *per se*, and the metabolite of GA_1, GA_8, is inactive. Thus, GA_1 must have hormonal function for internode elongation in both species. That the LE/le gene difference defines Mendel's tall and dwarf lines of pea made the discovery particularly exciting (Ingram *et al.*, 1984). Additional work on both pea and maize GA biosynthesis mutants have subsequently revealed the locations in the GA biosynthetic pathway at which other mutations block (Fujioka *et al.*, 1988; Davidson *et al.*, 2003; 2004).

Reviewing these three decades of GA metabolic studies in plants, it became evident that there was a multiplicity of pathways beyond the first-formed GA, GA_{12}-aldehyde, especially in developing seeds, which produced a plethora of GAs with many interesting functional features, but of unknown function. In time, the near universality of the early-13-hydroxylation pathway in vegetative tissue, and the importance of GA_1 as a 'hormone' was substantiated. The comment made by MacMillan and Suter in 1956 that 'the occurrence of gibberellin A_1 in higher plants … leaves little doubt that at least gibberellin A_1 participates directly in the growth regulating system of higher plants' was indeed prescient. The observation that in some plants 13-hydroxylation may reduce biological activity (Magome *et al.*, 2013)

identifies GA_4 as another GA with intrinsic hormonal activity in members of the Cucurbitaceae, *Arabidopsis*, and rice.

The advent of *Arabidopsis thaliana* as a model system from the 1980s moved our knowledge of GA biosynthesis further as it facilitated the study of genes encoding biosynthetic enzymes. The endogenous GAs in *Arabidopsis* were first identified by Jan Zeevaart's research group (Talon *et al.*, 1990). Twenty GAs were identified by GC-MS in shoots of the Landsberg ecotype. The GAs were representative of three pathways, non-hydroxylated, early-3-hydroxylation, and early-13-hydroxylation. In contrast to most plants previously studied, and in fact in contrast to most crop plants studied to date, the early 13-hydroxylation pathway in *Arabidopsis* is a minor pathway. The non-hydroxylation pathway predominates.

A series of GA-responsive dwarf mutants of *Arabidopsis* had been generated by Maarten Koornneef at Wageningen, the Netherlands, in the 1980s (Koornneef and van der Veen, 1980). He named the mutant loci *ga1*, *ga2*, *ga3*, *ga4*, and *ga5* based on epistasis tests. *GA1* was cloned by Sun *et al.* using the *ga1-3* mutant that Koornneef had generated by fast neutron bombardment. Because *ga1-3* has a large deletion they were able to use a novel technique of genomic subtraction to identify the sequence present in the wild-type that was missing from the mutant (Sun *et al.*, 1992). GA1 is a terpene cyclase that catalyses the conversion of GGPP to the bicyclic intermediate *ent*-copalyl-diphosphate (Sun and Kamiya, 1994). To clone GA2, Yamaguchi and co-workers used pumpkin *ent*-kaurene synthase cDNA to isolate a homologous cDNA from *Arabidopsis* that when expressed as a fusion protein in *E. coli* had *ent*-kaurene synthase activity (Yamaguchi *et al.*, 1998). The *ga2-1* mutant contains a truncated protein and could be complemented with the wild-type cDNA, confirming that *GA2* encodes *ent*-kaurene synthase. *ent*-Kaurene oxidase, encoded by *GA3*, was cloned by conventional map-based cloning and random sequencing (Helliwell *et al.*, 1998). Expressing the cDNA in yeast confirmed that the enzyme can catalyse the three sequential steps in the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Helliwell *et al.*, 1999). Intriguingly, although GA1 and GA2 are expressed in chloroplasts, GA3 is localised on the outer face of the chloroplast membrane (Helliwell *et al.*, 2001b), and may direct the catalytic product, *ent*-kaurenoic acid, to the next enzyme in the pathway, *ent*-kaurenoic acid oxidase. This enzyme, originally defined by the *grd5* mutant of barley and the *dwarf-3* mutant of maize, was cloned from barley (Helliwell *et al.*, 2001a). *Arabidopsis* contains two genes encoding *ent*-kaurenoic acid oxidase with overlapping function (Regnault *et al.*, 2014), and this redundancy precluded a mutant phenotype in *Arabidopsis*. Like GA3 *ent*-kaurenoic acid oxidase is a multi-functional cytochrome-P450-dependent mono-oxygenase. It catalyses the three-step oxidation from *ent*-kaurenoic acid to GA_{12}. In *Arabidopsis* this enzyme is localised to the endoplasmic reticulum (Helliwell *et al.*, 2001a).

The mutants *ga1*, *ga2* and *ga3* are extreme dwarfs. As *GA1* and *GA2* are the only genes encoding CPS and KS, respectively, it has been assumed that

these dwarf seedlings are completely GA-deficient, though traces of GAs of unknown origin are apparent. However, *ga1*, *ga2* and *ga3* are all sterile dwarfs that can be rescued by treating with an *ent*-kaurenoid or GA beyond the metabolic block.

Talon *et al*. analysed the GA content of *ga4* and *ga5* mutants, and proposed that *GA4* encodes a 3β-hydroxylase, and that *GA5* encodes a multi-functional GA 20-oxidase responsible for catalysing the formation of C_{19}-GAs (Talon *et al.*, 1990). They also recognised the importance of 3β-hydroxylation, reporting that GA_9 had no biological activity on *ga4* mutant seedlings, and that GA_1 and GA_4 were probably the active hormones. The *GA4* gene was cloned by Chiang *et al.* and *GA5* was cloned by Phillips *et al.* and Xu *et al.*, providing important information on the enzymatic reactions catalysed by the enzymes, their specificity, and their regulation by feedback repression (Chiang *et al.*, 1995; Phillips *et al.*, 1995; Xu *et al.*, 1995). The notable cloning of the first plant GA 20-oxidase had been reported the previous year by Lange *et al.* from pumpkin (Lange *et al.*, 1994). It was shown to be a dioxygenase that could indeed catalyse the multi-step conversion of GA_{12} to a C_{19}-GA.

Several years later the completion of the *Arabidopsis* genome (2000) revealed that the GA 20-, 3- and 2-oxidases are all encoded by small gene families, as described in detail in Chapter 2. Because of redundancy, albeit it partial in some cases, a severely dwarf phenotype only results when mutations exist in multiple members of the GA 20-oxidase or GA 3-oxidase gene families.

In concluding this section on GA metabolism it is worthwhile reflecting on the use of *Gibberella* for initial studies. In many ways it was a wise choice for the practical reasons mentioned earlier. Moreover, it provided a useful model on which to base the *in vitro* and *in vivo* plant studies. But 50 years on, it is now known that GA biosynthesis in *Gibberella* and in plants is not the same. In fact there are many differences. For example, an alternative to the mevalonic acid pathway for producing IPP, namely the methyl erythritol phosphate (MEP) pathway was identified in plant plastids, and although it occurs in some bacteria and algae it does not occur in fungi (Rohmer, 1999). The MEP pathway appears to be the predominant route for the production of IPP to serve as a precursor for GAs in plants, at least in vegetative tissues, though a minor contribution of the MVA pathway cannot be ruled out (Kasahara *et al.*, 2002). Furthermore, over the past two decades, information has been obtained by the group of Bettina Tudzynski on the enzymes that catalyse *ent*-kaurenoid and GA metabolism in *Gibberella* (see Chapter 5). Many of the fungal enzymes have different properties from those encoding similar steps in the pathway in plants, including some fungal enzymes that demonstrate remarkable multi-functionality. Even the mechanism to produce GA_3 from its immediate precursor differs between *Gibberella* and plants (Albone *et al.*, 1990). It is evident from this work that the pathways in *Gibberella* and in plants evolved separately (Bömke and Tudzynski, 2009). The identification of GAs in a small number of other fungi provides evidence there may have

been horizontal gene transfer from one fungus to another, but horizontal gene transfer from *Gibberella* to plants is ruled out by the fundamental differences in the nature of the pathways. Continued study of the fungal pathway, and its regulation, is timely because of the continued commercial production of GA_3 using *Gibberella*.

1.3 Gibberellin signalling

Classically, there have been two major foci for research on GA signal transduction: the cereal aleurone and the stem apex (Paleg, 1965). Germinating cereal grain has been the subject of scientific study for nearly two centuries with a view to enhancing the malting of grain for the brewing industry. It had been known for some time that the presence of the embryo enhanced amylolytic activity in the endosperm, and that barley and malt (germinated grain) contained GA-like biological activity. In 1960 Yomo and Paleg independently showed that pre-incubation of embryo-less half seeds of barley with GA_3 increases the amounts of amylase and reducing sugars released from the endosperm. Historical aspects of this groundbreaking work have been reviewed in detail (Paleg, 1965). In intact grain the embryo supplies the GA for induction of starch breakdown in the endosperm.

The origin of the α-amylase in cereal grains was shown to be the aleurone, the outermost layer of living cells that surrounds the dead, starch-filled cells of the mature endosperm. The synthesis and release of α-amylase by isolated aleurone layers matched that of intact endosperm as long as the incubation buffer contained calcium (Chrispeels and Varner, 1967). Experiments utilising $H_2{}^{18}O$ elegantly demonstrated that essentially all of the α-amylase required for breakdown of stored starch arises by *de novo* synthesis (Filner and Varner, 1967), and Varner and Chandra noted 'it is a delightful nicety that the key to these reserves is kept by the embryo, the only tissue capable of growth' (Varner and Chandra, 1964).

Thus began several decades of productive research on the biochemical mechanism whereby the 'key' (GA) from the embryo induces *de novo* synthesis of several isoforms of α-amylase in the aleurone to 'unlock' (hydrolyse) starch in non-living cells of the endosperm. The advantages of this system for studying GA action are manifold – aleurone layers, which can be readily separated from the rest of the endosperm, provide a population of uniform differentiating cells from which protoplasts can be prepared. The cytology of these cells/protoplasts has been studied in detail, including the effects of GA on the number and appearance of protein storage vacuoles, oleosomes and endomembranes, and eventual programmed cell death (Bethke *et al.*, 1999). Moreover, unlike other GA responses like internode elongation, the GA response in aleurone cells has a well-defined and measurable biochemical end point – the production of α-amylase.

The nature of the GA receptor in aleurone cells is somewhat controversial. Several lines of evidence suggested that it was in the plasma membrane. For example, GA_4 that had been covalently linked to agarose beads to prevent its uptake into oat aleurone protoplasts was still able to induce the synthesis of amylase, though it was inactive on aleurone cells (Hooley *et al.*, 1991). Furthermore, if GA is injected directly into the cytosol of barley aleurone protoplasts it is inactive (Gilroy and Jones, 1994). Despite this convincing evidence, a GA receptor from aleurone plasma membranes has not been identified. The identification of GID1, which is a soluble GA receptor (see later), raised the possibility that there may be two types of GA receptor, one that is plasma-membrane-localised and one that is soluble. Recent convincing evidence that GID1 is the *only* GA receptor in rice (Yano *et al.* 2015) does not preclude the existence of an additional plasma-membrane-localised receptor in barley and oat.

The involvement of second messengers in GA response in aleurone cells has been extensively studied. Applied GA induces both Ca^{2+}-independent and Ca^{2+}-dependent events. The induction of amylase synthesis by GA does not require Ca^{2+}, whereas secretion of the enzyme does (Jones and Carbonell, 1984). In addition, evidence for the involvement of G-proteins, cyclic GMP, and protein phosphorylation is reviewed in detail (Bethke *et al.*, 1997) (Figure 1.4). In the pathway leading to amylase production GA acts primarily by increasing the transcription of amylase genes. The purification of α-*amylase* mRNA, which is produced in relatively large amounts in aleurone cells, enabled the isolation of genomic clones containing both the structural gene for α-amylase and its upstream promoter sequences. The partial deletion of known sequences of bases from α-*amylase* promoters indicates that sequences conferring GA responsiveness, termed GA response elements (GREs), are 200–300 base pairs upstream of the transcription start site. Identical GREs were found to occur in all cereal α-*amylase* promoters so far examined, and their presence was shown to be essential for the induction of α-*amylase* gene transcription by GA.

The sequence of the GRE (TAACAAA) in the α-*amylase* gene promoter resembles a motif in the binding site for MYB transcription factors. *GAMYB* mRNA increases in aleurone cells as early as 1 hour after GA treatment, preceding the increase in α-*amylase* mRNA by several hours (see Figure 1.4). These and other data discussed in Chapter 6 are consistent with GAMYB regulating α-*amylase* gene expression (Gubler *et al.*, 1995). Cycloheximide has no effect on the production of *GAMYB* mRNA, indicating that protein synthesis is not required for *GAMYB* expression, and that *GAMYB* can therefore be defined as a primary or early response gene. In contrast, the α-*amylase* gene is a secondary or late response gene.

Turning to the second focus of research on GA signalling, namely that on stem apices and internode elongation, single gene dwarf mutants whose internode growth was *not* correlated with endogenous GA levels were crucial to gaining insight into GA signal transduction. It had been known for some

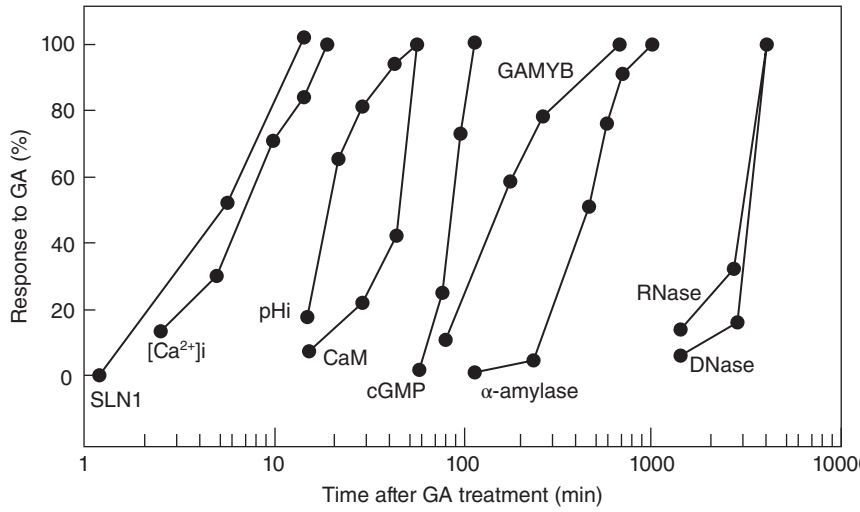

Figure 1.4 Following the addition of bioactive GA to barley aleurone protoplasts, a multiple-component signalling pathway is initiated. CaM calmodulin. (Sun and Gubler, 2004. Reproduced with permission from Annual Reviews.)

time that some GA non-responsive, semi-dominant, dwarf mutants of maize (*Dwarf-8*), wheat (*Reduced height*, *Rht*), and *Arabidopsis* (*gai-1*) accumulated high levels of endogenous GAs and yet were still dwarf. In addition, other stem length mutants e.g. barley (*sln*) and pea (*la cry*) were characteristically taller than their respective wild-type seedlings. These so-called 'slender' mutants were resistant to inhibitors of GA biosynthesis, and continued to have a slender phenotype even if they were lacking endogenous GAs. The characterisation of these two types of mutants, in which (a) the GA response was irretrievably repressed, or (b) it was constitutively expressed, defined genes that were involved in the GA signal transduction in stem growth, and spurred an exciting phase of GA research.

Work on the GA-insensitive dwarf mutants of *Arabidopsis* utilised, at first, the semi-dominant dwarf *gai-1* mutant (Koornneef *et al.*, 1985). Cloning of *GAI,* together with a gene referred to as *GRS* (*GAI Related Sequence*) determined that these genes encode putative transcription factors each with a nuclear localisation sequence. A deletion of 17 amino acids in the N-terminal region of GAI (gai-1) gave a semi-dominant GA-resistant dwarf phenotype (Peng *et al.*, 1997). The deletion included a five-amino-acid motif, DELLA, though the significance of this motif was not immediately recognised. Peng *et al.* concluded that GAI is a repressor of GA responses, and that GA can release the repression by the wild-type protein, but not that imposed by the gain-of-function mutation *gai-1*. Intriguingly, other mutant alleles of *GAI*, rather than giving gain-of-function phenotypes like *gai-1*, gave loss-of-function phenotypes such that mutant plants appeared wild-type.

Silverstone *et al.* independently identified a loss-of-function mutation which they called *rga* (*repressor of ga1-3*), which could partially rescue the semi-dwarf phenotype of the GA-deficient mutant *ga1-3* (Silverstone *et al.*, 1997). Cloning the gene showed that RGA was 82% identical at the amino acid level to GAI, and that *RGA* was, in fact, identical to *GRS* (Peng *et al.*, 1997; Silverstone *et al.*, 1998). Significantly, it was shown that RGA-GFP fusion protein localised to the nucleus, and was degraded in the presence of GA_3 (Dill *et al.*, 2001; Dill and Sun, 2001). An *rga-Δ17* mutation, which encoded a protein missing the same 17 amino acids as those missing from gai-1, was generated to determine whether this mutation would lead to constitutive repression. GFP-(rga-Δ17), under the control of the endogenous RGA promoter, was not degraded by GA, as measured both by confocal microscopy of root tip cells and by immunoblot analysis using anti-GFP antibodies, demonstrating the importance of the DELLA domain for GA-induced proteolysis. The effect of bioactive GA_4, which is the major bioactive GA in *Arabidopsis*, on degradation of the wild-type RGA protein is comparatively rapid, being visible in plants that have a GA-deficient (*ga1-3*) background in less than 30 minutes (Dill *et al.*, 2001). Generation of a triple mutant line containing null alleles at both *RGA* and *GAI* loci (*rga-24* and *gai-t6*) along with *ga1-3* could completely rescue the GA-deficient phenotype of *ga1-3* (Dill and Sun, 2001). Thus it was confirmed that the ground state in wild-type individuals is one of growth repression caused by GAI and RGA. Repression can be relieved by bioactive GA, but GA is not required for some aspects of growth in the absence of GAI and RGA.

Shortly after the cloning of *GAI* and *RGA*, it established that *DWARF-8* in maize and *Rht-1* in wheat are their functional orthologues (Peng *et al.*, 1999). Semi-dominant, gain-of-function mutations *dwarf-8*, *Rht-B1b*, and *Rht-D1b*, gave GA-insensitive dwarf phenotypes, and were all shown to have deletions in the N-terminus regions of the respective proteins. The observation that orthologous proteins regulate GA response in monocots and dicots underscored the importance and potential universality of these regulatory proteins. Moreover, the *Rht-B1b* and *Rht-D1b* mutations were those selected in wheat to give the short-stemmed, lodging-resistant, high-yielding strains introduced as part of the Green Revolution almost 50 years earlier demonstrating the immensely valuable agronomic benefits that can be attained by modulating the activity of these regulatory proteins (Peng *et al.*, 1999). Semi-dwarf varieties of wheat containing the *Rht-B1b* and *Rht-D1b* mutations, when grown with fertiliser and irrigation, have been credited with saving billions of lives. Norman Borlaug, one of the scientists most closely involved in the breeding program, received a Nobel Peace Prize in 1970, and on March 25 2014, the 100th anniversary of his birth, a statue of Borlaug was unveiled in the US Capitol.

GAI, RGA, DWARF-8 and RHT1 belong to the plant-specific GRAS family of putative transcriptional regulators, named after the first three to be discovered GAI, RGA and SCARECROW (SCR). They all contain a GRAS

domain at the carboxy-terminus to which is ascribed the transcriptional regulatory function. Three additional homologues to GAI and RGA in *Arabidopsis*, RGA-LIKE 1 (RGL1), RGL2, and RGL3 have been identified. The five homologues have some overlapping and some unique functions (Lee *et al.*, 2002; Cheng *et al.*, 2004; Tyler *et al.*, 2004). The N-terminal regions of all five have highly conserved DELLA and VHYNP motifs, which are required for GA-induced proteolysis (see later). The importance of the DELLA motif has led to the N-terminal region being referred to as the DELLA domain, and also gives the name to this entire sub-family of GRAS proteins. In the GRAS domain there are leucine heptad repeats (LHR), which were anticipated to be a site of protein/protein interaction, together with the nuclear localisation signal.

Unlike *Arabidopsis*, rice and barley were shown to each have a single DELLA protein, SLENDER1 (SLR1) in rice and SLENDER1 (SLN1) in barley (Ikeda *et al.*, 2001; Chandler *et al.*, 2002). The genes encoding these proteins were initially defined by loss-of-function mutations that caused the plants to have taller than wild-type phenotypes even in the absence of GA, underscoring the contention that GA signalling was constitutive in these slender mutants because they were lacking a repressor of GA response (see Figure 1.5). In contrast, a dominant gain-of-function mutation, *Sln1d*, in barley and a deletion of 17 amino acid in the DELLA domain of SLR1 in rice gave dwarf phenotypes. The contrast between the slender and dwarf phenotypes of loss- and gain-of-function mutations, respectively, was particularly striking in these cereals (Figure 1.5). In contrast, in *Arabidopsis*, in which there is considerable redundancy between the five DELLA proteins, a slender phenotype is not evident if only one DELLA is deleted.

Work on signalling in cereal aleurone and on stem growth converged after the observation that embryo-less half seeds of the loss-of-function *slr1* and *sln1* mutants produced amylase in the absence of exogenous GA, and that aleurone cells from the dominant dwarf mutants were far less responsive to GA than wild-type. Furthermore, GA treatment of aleurone cells from wild-type barley caused the reduction in SLN levels within 5 minutes of treatment, almost 2 hours prior to the buildup of GAMYB (Gubler *et al.*, 2002). Figure 1.4 provides an integrated view of aleurone response (Sun and Gubler, 2004). Thus not only do DELLA proteins appear to be universal, but more and more information was appearing to link them to GA signalling in multiple response pathways.

The next piece in the puzzle of GA signal transduction fell into place when two genes defined by gain-of-function mutations in *Arabidopsis* (*sly1*) and rice (*gid2*) were cloned (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003). These mutations gave GA-insensitive semi-dwarf phenotypes, and defined genes that encode F-box sub-units that are each part of an SCF E3-ubiquitin ligase complex. Poly-ubiquitination of DELLA proteins and their proteolysis by the SCF $^{SLY1/GID2}$-proteasome pathway in *Arabidopsis*/rice relieves growth repression caused by DELLA proteins. These proteins cannot be degraded in *sly1* and

Figure 1.5 Two-week old wild-type (WT) and two mutants of barley demonstrating the opposite effects on growth of two different mutations in the same gene, *SLN1*. Left, the *slnc* loss-of-function mutation confers a slender, GA-constitutive phenotype. Right, the gain-of-function *sln1d* mutation gives a dominant dwarf phenotype. The mutations are in different domains in the single DELLA protein in barley. (From Chandler *et al.*, 2002, courtesy of P. M. Chandler.)

gid2 mutants, keeping the repression of growth in place. However, why the DELLA domain is so important had to await the characterisation of the GA receptor. This breakthrough came shortly after when a soluble protein for rice that had all the hallmarks of a GA-receptor was cloned (Ueguchi-Tanaka *et al.*, 2005).

Rice contains just a single DELLA protein, and it also contains just a single gene encoding a GA receptor (Ueguchi-Tanaka *et al.*, 2005). Cloning of GID1

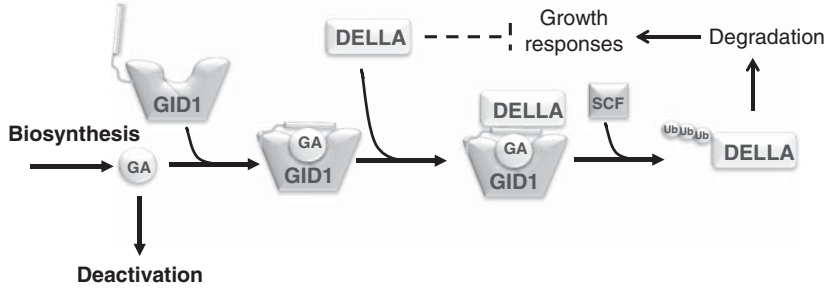

Figure 1.6 Overview of GA signalling. Bioactive GA binds the GID1 receptor bringing about an allosteric change that allows a DELLA protein that represses GA responses to bind to the GID1-GA complex. The DELLA protein then undergoes an allosteric change that allows for its polyubiquitination by the SCF complex. It can then be degraded by the 26S proteasome, relieving repression, and facilitating GA responses.

showed it to have close homology to a family of hormone-sensitive lipases (HSLs). The binding of radiolabelled GA_4 (the 16,17 diol was used) to the wild-type GID1 protein was rapid and saturable, and could be competed out by non-labelled GA equally rapidly. Moreover there was low to no binding of inactive GAs to the wild-type protein, nor was there binding of bioactive GAs to the mutant protein.

Genetic tests placed SLR1 and GID1 on the same pathway, with GID1 being upstream of SLR1 and essential for the GA_3-induced proteolysis of SLR1. SLR1 is resistant to GA-induced proteolysis in the *gid1* mutant. Moreover, in yeast two-hybrid assays GID1 and SLR1 were shown to interact, but *only* in the presence of GA_3 (Ueguchi-Tanaka *et al.*, 2005). GA-dependent binding of GID1 and SLR was later confirmed *in planta* (Ueguchi-Tanaka *et al.*, 2007). The GA signalling pathway is outlined in Figure 1.6, and described in detail in later chapters.

Analysis of the *Arabidopsis* genome revealed that it contains three orthologues of *GID1* (Ueguchi-Tanaka *et al.*, 2005), and these have been termed *GID1a*, *GID1b*, and *GID1c* (Nakajima *et al.*, 2006). Mutation in any one of these homologues does not give a discernible phenotype (Griffiths *et al.*, 2006), explaining perhaps why identification of the GA receptor eluded *Arabidopsis* researchers. Double mutants show reduced growth and fertility, but phenotypes differ somewhat depending on which two of the three homologues are knocked out, whereas triple mutants are extremely severe, sterile, non-GA-responding dwarfs, indicating that the GID1 homologues are the only receptors in *Arabidopsis* (Griffiths *et al.*, 2006). This work is discussed in further detail in Chapter 6.

Binding of the *Arabidopsis* receptors to DELLA proteins was demonstrated by several workers (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006; Willige *et al.*, 2007), but the actual mechanism of GA-GID1-DELLA interaction was best defined when the crystal structures of the rice GID1 receptor protein plus

GA, and an *Arabidopsis* GID1a plus GA plus RGA complex were resolved (Murase *et al.*, 2008; Shimada *et al.*, 2008). GID1 proteins from *Arabidopsis* and rice both have a cleft into which bioactive GA binds, bringing about an allosteric change in the protein so that an N-terminal extension closes over the cleft to completely enclose the GA. Defined interactions of specific amino acids within the cleft to bioactive GA were consistent with the structural features of GAs that are known to be necessary for bioactivity (Murase *et al.*, 2008; Shimada *et al.*, 2008).

For GID1 in rice, and GID1a and GID1c in *Arabidopsis*, the closing of this N-terminal extension over a bioactive GA is absolutely required before any of the DELLA proteins can bind to the receptor. The DELLA/VHYNP motifs within the N-terminal domain of the DELLA proteins interact with hydrophobic amino acids on the upper surface of the GID1 'lid'. In contrast, the GID1b lid can partially close in the absence of GA within the cleft, allowing some binding of DELLA even in the absence of GA.

The binding of DELLA proteins to the receptor brings about an allosteric change in the GRAS domain of those proteins, that facilitates them binding to the F-box components of, for example, SCF^{SLY1} in *Arabidopsis* and SCF^{GID2} in rice, resulting in ubiquitination and proteolysis. Thus the degradation of DELLA proteins is a crucial step in transducing a GA signal. Anything that prevents this process occurring is likely to block GA signalling. The absence of the DELLA motif in the *gai-1*, *rga-*Δ*17*, and *sln1d* mutants prevents the binding of these proteins to the receptor, precluding the conformational change in their GRAS domains that is required for them to be substrates for SLY1 and GID2. Thus these mutants are dwarf, and the repression by mutated proteins cannot be relieved by application of GA.

In certain circumstances it is possible to discern an unusual scenario: that of GA signalling in the absence of DELLA protein degradation. This was encountered first by scientists working with the *sly1* mutant, in which DELLA proteins are not targeted for proteolysis (Ariizumi and Steber, 2007; Ariizumi *et al.*, 2008) and is discussed in detail in Chapter 6.

Since DELLA proteins have no recognisable DNA-binding domain it had been postulated for some time that they would bring about transcriptional regulation through protein–protein interaction. Several microarray investigations conducted within the past decade have been important for recognising DELLA-interacting proteins. For example, Zentella, working with 8-day-old seedlings of the *ga1-3* mutant, looked at alterations in gene expression as a consequence of applied GA_4 or of inducing *rga-*Δ*17* expression (Zentella *et al.*, 2007). Expression of 14 genes was down-regulated by GA and up-regulated by the DELLA protein. Several targets of GA and DELLA were genes involved in GA homeostasis, including those encoding the GA 3- and 20-oxidases and GID1a and c. Other genes encoded bHLH, MYB and WRKY transcription factors. Supporting the notion that DELLA proteins are involved in regulating hormone crosstalk, XERICO, an inducer of abscisic acid biosynthesis, was down-regulated by GA and up-regulated

by DELLA (Zentella *et al.*, 2007). More detailed discussion of GA perception and the early events in signal transduction are described in Chapter 6.

Gibberellin research has entered an exciting phase over the past few years with the characterisation of several DELLA-interacting proteins, clarifying how DELLAs can mediate so many fundamental changes in growth and development. This is an extremely active area of research at the present time, which is discussed in Chapter 7. For example, DELLA proteins interact with phytochrome interacting factors (PIFs). These are bHLH transcription factors that can mediate the transition from skotomorphogenesis to photomorphogenesis (de Lucas *et al.*, 2008; Feng *et al.*, 2008). In dark-grown seedlings, PIFs bind to promoter sequences to activate transcription of genes encoding expansins and other factors that promote cell elongation and hypocotyl growth. If DELLA proteins are abundant (for example in light) they can bind to PIFs to prevent the transcription of their target genes. In the presence of bioactive GA DELLA proteins are degraded and cannot bind PIFs, so that PIFs activate transcription and hypocotyls elongate. Thus the classic GA response of hypocotyl elongation in light-grown *Arabidopsis* seedlings occurs because GAs disrupt the DELLA–PIF interaction, thereby releasing PIFs to activate transcription of genes whose products induce cell elongation.

In another example, in *Arabidopsis* hypocotyls the DELLA protein, GAI, interacts with prefoldin5 (PFD5) which is one of the sub-units of a chaperone protein involved in α/β-tubulin dimerisation (Locascio *et al.*, 2013). Tubulin dimerisation facilitates the assembly of microtubules whose orientation in the cortical cytoplasm will, in turn, direct the orientation of cellulose microfibrils in cell walls. When PFD5 is bound to GAI, the chaperone–GAI complex is localised in the nucleus and is inactive. In the presence of bioactive GA_4 the DELLA protein is degraded, allowing for cytoplasmic localisation of the chaperone. The now active cytoplasmic chaperone facilitates the assembly of tubulin dimers into microtubules and their orientation in a transverse direction. This is turn directs the laying down of cellulose microfibrils in a transverse orientation, which is conducive to cell elongation (Locascio *et al.*, 2013).

Many other examples of DELLA-interacting proteins have been described recently. This research provides an avenue for further investigating downstream events that will define the biochemical mechanisms for GA responses.

1.4 Physiological responses to gibberellins

Gibberellins are active in regulating growth and development throughout the entire life cycle. Sometimes they act alone, more often they act in concert with other hormones, either synergistically or in an antagonistic manner. The action of GA in particular target cells is a result of an intricate series of events such as hormone biosynthesis, transport, presence and accessibility of

the receptor, occurrence of DELLA proteins and interacting protein partners, and the machinery to degrade DELLA proteins. Often environmental factors such as presence or absence of light, light quality and duration, or temperature can impinge on any part of parts of this sequence of events.

Gibberellins break dormancy in seeds, especially those that have a light or cold requirement for germination. They can speed up germination of grain by promoting the hydrolysis of reserves, and can aid seedling establishment by promoting hypocotyl and internode growth. Gibberellins are important for shortening the juvenile phase of some species, and they induce flowering in certain species by transducing the effect of the appropriate temperature or photoperiod. They are necessary for pollen formation, pollen tube growth, and fruit and seed development. These processes and phenomena are considered in detail in several subsequent chapters.

As mentioned earlier in this chapter, the clearly defined biochemical events in the cereal aleurone system make it an ideal system for studying mechanism of GA action. In most other instances the responses were described in morphological terms, and it is only comparatively recently that they have been 'dissected' to reveal the biochemical events that are responsible for the macroscopic changes in plant size or form.

Historically, the physiological effects of GAs were recognised even before the first GAs had been identified as natural components of plants. Stowe and Yamaki reviewed the effects of GA (obtained from fungal cultures) when applied to 80 different species, leading them to attest that the action of GAs corresponds to that of naturally occurring compounds in higher plants (Stowe and Yamaki, 1957). As a way of distinguishing GAs from the already well-characterised auxin, they defined GAs as a class of compounds that causes internode elongation when applied to certain intact genetically dwarfed plants, with the elongation of monocot leaves as a supporting definition. Although, as described earlier in this chapter, it was the chemical structure not the biological activity that became the defining feature of GAs, internode elongation remains one of the most notable effects.

Stowe and Yamaki inferred that the action of GAs in shoot elongation, leaf expansion, growth of dwarfs, parthenocarpy, bolting of long-day plants, or reversal of light-inhibition involved the 'removal of certain limitations on cell elongation'. Citing the possibility of suppression of an inhibitor as a way to remove the 'normal limitation' Stowe and Yamaki predated by several decades the recognition of the GA-induced proteolysis of the DELLA class of transcriptional regulators as the way to remove the limitation to cell elongation. The suggestion that GAs exerted their growth-promoting effects by enhancing the levels of auxin received considerable attention, but the multiplicity of responses that were unique to GAs ensured that these compounds were duly recognised as a second distinct class of hormones.

Internode elongation is the basis for many GA bioassays by which 'GA-like substances' (i.e. biologically-active compound(s) that had not yet been chemically characterised) were first recognised and later 'quantified'

in plant extracts in order to discern their potential roles in growth and development (Phinney and West, 1960). Correlation between the level of GA-like substances in extracts and plant growth were, in some instances, comparatively easy to obtain. For example, the amount of GA-like material in extracts of single gene dwarf mutants of corn was half (or less) of that in their wild-type seedling counterparts. *Hyocyamus niger* (black henbane) plants that had received inductive long days (LDs), and exhibited the earliest stages of bolting and had microscopic flower primordia, contained more GA-like activity than extracts of non-induced plants (Lang, 1960). The judicious use of GA biosynthesis inhibitors and the demonstration that further application of GA can normalise inhibitor-treated plants (Zeevaart, 1964) supported the contention that GAs are naturally occurring regulators of processes such as stem growth and flowering.

When the steady-state levels of GA-like substances in plant extracts did not match the amount of growth, scientists postulated that the rate of GA turnover may be more important than the static size of the GA pool. At the time, a lack of information about GA pathways of biosynthesis and degradation, and the paucity of labelled substrates precluded many metabolic studies. However, an estimate of the dynamic GA status within plants could be obtained by allowing GA-like material to diffuse from plant parts into agar gel over several hours or days (Jones and Phillips, 1964). Using a combination of diffusate and tissue extraction it was shown that not all GA-like substances were mobile, in some cases the non-diffusible GA-like substances appeared to be precursors of the mobile substances and external conditions could influence GA metabolism.

In due course, once many more GAs had been characterised, the pathways for GA biosynthesis and deactivation had been defined, and the methods for isotopically labelling GAs had been developed, it became feasible to study GA occurrence, biosynthesis, and degradation more directly. Evidence quickly accumulated that plants contain many different GAs, not just two or three observed as zones of bioactivity after separation by thin layer chromatography. The scientific inquiry that established that it is the 3β-hydroxylated C_{19}-GAs that have intrinsic biological activity was described in detail earlier in this chapter. In addition, specific metabolic steps could be altered by inductive photoperiods. For example, in the long-day plant (LDP) spinach, GA_1 is the bioactive GA that causes stem growth in LDs. The C_{20}-GA, GA_{53}, is a substrate for two competing enzymes, a GA 20-oxidase that is upregulated in LDs and can lead to elevated levels of bioactive GA_1 (Gilmour *et al.*, 1986; Lee and Zeevaart, 2007) or a GA2-oxidase that converts it to GA_{97} in reaction that is predominant in SDs (Lee and Zeevaart, 2005). In a similar way, low temperatures are also inductive for flowering in some species. For example, vernalisation of *Thalaspi arvense* (field pennycress) leads to elevated levels of bioactive GA and flowering, though in this instance the inductive treatment enhances an earlier step in GA biosynthesis, namely the oxidation of *ent*-kaurene (Hazebroek *et al.*, 1993).

The effect of GA on flowering is particularly complex. Not only has there been intense academic interest in the subject for decades, but the ability to manipulate flowering by GAs, inhibitors of GA biosynthesis, or environmental factors has profound practical applications in agronomy and horticulture (see Chapters 12 and 13). The flowering responses of many types of plants have been tested over the years (Pharis and King, 1985; King and Evans, 2003 and see Chapter 11). Although for a brief period of time the notion was considered that bioactive GA might be the flower-inducing substance 'florigen' that travelled from leaf to apex, this idea quickly lost favour when it became evident that the positive effects of GA on flowering were not universal. Moreover, there was no consensus on whether GA induced bolting *and* flowering in LDPs, or whether flowering was a consequence of bolting.

After decades of intense research we now have a profound understanding of the regulation of flowering. The transcriptional regulator CONSTANS (CO) accumulates in the light in companion cells of leaves in inductive conditions, leading to the production of a phloem-mobile signal FLOWERING LOCUS T (FT) (or its orthologue, Hd3a in rice) (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). The sequence of events transduced by the arrival of FT or Hd3a at the stem apex is considered in Chapter 11. FT/Hd3 are phosphatidylethanolamine-binding proteins that are transcriptional regulators themselves, and their production in leaves, transport in the phloem and their action in the apex fulfill the criteria laid down long ago for 'florigen'. GA acts downstream of FT, and activates *LFY*, one of the floral meristem identity genes (Blazquez *et al.*, 1998).

In the grass, *Lolium temulentum*, detailed studies have provided evidence for a florigen-like role for GA_5. King *et al.* traced the movement of GA_5 from induced leaves to apices after a single long day, and demonstrated its arrival at the stem apex prior to the appearance of floral primordia (King *et al.*, 2006). This interesting scenario not only indicates a novel situation with regard to a florigenic role for a GA in *Lolium*, it reveals that different bioactive GAs can have unique roles – thus in *Lolium* GA_5 (but not GA_4) is florigenic, whereas GA_4 (but not GA_5) induces stem elongation. This is thought not to be due to inherent differences in the biochemical functions of GA_4 and GA_5, but to differences in their susceptibility to deactivation. In *Arabidopsis* GA_4 is the primary bioactive GA both for *LFY* transcription and stem elongation (Eriksson *et al.*, 2006).

Application of GA to woody gymnosperms alters reproductive behavior differently from that in woody angiosperms. In conifers, GA_3 can promote strobilus formation in members of the Cupressaceae and Taxodiaceae, whereas less polar GAs such as GA_4 are more effective in members of the Pinaceae (Pharis and King, 1985). In contrast, GAs tend to inhibit flowering in woody angiosperms such as apple. Research has revealed that these effects of exogenous application reflect the GA status of these plants, and GAs and GA biosynthesis inhibitors are used commercially in forestry and fruit-growing industries.

The mechanism by which GAs could stimulate stem elongation has been a topic of sustained inquiry, since it is one of the most notable manifestations of GA response. From the earliest studies it was evident that GAs promoted *cell* elongation, though whether cell elongation alone was sufficient to account for observed increases in plant height was not clear. Experiments with gamma-irradiated wheat seedlings, in which cell division cannot occur, showed a normal response to GA by cell elongation alone. In contrast GA-induced bolting of rosette plants was shown to involve mitotic activity in the sub-apical meristem to provide a source of cells for subsequent elongation (Sachs, 1965).

Defining the biochemical and physical factors that facilitate irreversible cell elongation has helped to distinguish the action of GA from that of auxin, which also causes cell elongation. GA was found to have little effect on turgor pressure, but instead affected the wall-yielding properties of the cell wall (Cosgrove and Sovonick-Dunford, 1989). The anisotropic growth that occurs in cells of pea epicotyls in response to both GAs and auxin was compared. Both hormones enhance the rate of relaxation, but only GAs affect the value of the yield threshold. The requirement of GA for pea pollen tube growth (Singh *et al.*, 2002) suggests that GAs are also important in regulating tip growth in certain types of cells.

Although 50 years ago GAs were initially considered to exert their biological effects through increasing auxin levels, half a century of research has revealed the unique and vital roles of GAs in plant growth and development. However, as we understand more fully the molecular events that underlie the changes in plant size and form, and especially the impact of the external environment, we are aware that integration of signalling pathways plays a vital role. This aspect is considered in more detail in Chapter 8.

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