1

Molecular and Genetic Approaches to the Study of Plant Hormone Action

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ABBREVIATIONS

ABA	abscisic acid
ABRE	ABA response element
ACC	1-aminocyclopropane-1-carboxylate
BA	benzyladenine
bZip	basic-leucine zipper
CaMV	cauliflower mosaic virus
CAT	chloramphenicol acetyl-transferase
EMBP-1	*Em* binding protein 1
EMSA	electrophoretic mobility shift assay

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GA	gibberellic acid
GUS	β-glucuronidase
IAA	indole-3-acetic acid
LUC	luciferase
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SAUR	small auxin up-regulated
YAC	yeast artificial chromosome

I. INTRODUCTION

A small number of plant hormones have been shown to influence every aspect of plant growth and development. Tremendous progress has been made in this century in identification of plant hormones, discovery of their effects on plants, and elucidation of their chemical structures. For some of the plant hormones considerable knowledge about their biosynthetic pathways has been obtained. However, we still know very little about the molecular mechanism of plant hormone action. The specifics of how plant cells perceive hormonal substances and translate those signals into a particular response are still largely unclear. Understanding how plant hormones work will require investigating the cellular and molecular details of plant hormone responses. In this review we outline the molecular and genetic approaches that are currently being used to analyze these processes. A more general review on approaches to plant hormone studies was previously presented by Zucconi and Bukovac (1989).

The most wide spread paradigm for the mechanism of plant hormone action is derived from well-characterized animal hormone models. It is expected that there will be a hormone receptor, that binding to the receptor will initiate a signal transduction sequence, and that one target of signal transduction will be gene expression (Libbenga and Mennés 1987; King 1988):

Hormone —> receptor —> signal transduction —> gene expression —> Effect on plant growth and development.

This model is a reasonable starting point for investigation of plant hormone action and much of the past and current work in this area has been designed to confirm elements of this model.

The molecular and genetic approaches we will discuss include strategies for addressing various steps of the hormone action model. In

Section II, methods for modifying hormone levels in plants using gene transfer techniques are described. Plant biology is fortunate in having relatively easy methods for insertion of foreign genes into plants and these methods have been used to alter plant hormone levels by transformation with hormone biosynthesis genes. This experimental approach is a sophisticated extension of traditional hormone application experiments and has provided significant new information about how hormone concentration affects plant growth and development. The biochemical characterization of plant hormone receptors will be covered in Section III. How hormone-regulated genes are cloned and how these genes can be used to identify DNA regulatory sequences and transcription factors that mediate the hormone response will be described in Section IV using an abscisic acid regulated gene as an example. Finally, the use of genetics coupled with molecular biology to study plant hormone action will be reviewed in Section V. The genetic approach has proven itself to be a productive way of discovering components of hormone signal transduction. In each section, selected results will be used to illustrate the current status of these investigations although this review is not meant to be inclusive.

II. EXPRESSION OF HORMONE BIOSYNTHESIS AND METABOLISM GENES IN TRANSGENIC PLANTS

A number of genes that encode enzymes involved in hormone biosynthesis or metabolism have been isolated from plants and bacteria. These genes have been used to study the role of hormones in plant development by transferring them into plants and manipulating their expression patterns. One type of experiment that has been performed with these transgenic plants is to express a hormone biosynthesis or metabolism gene that originated from bacteria to cause overproduction of a hormone, degradation of endogenous hormone or production of the hormone at improper times during development. The advantages of such experiments over externally applying hormones are that hormone levels can be elevated throughout the life of the plant, and that hormone uptake is not a variable. Using various promoters with different expression patterns it is also possible to increase hormone biosynthesis selectively in specific tissues or at specific times during development.

Most of the hormone biosynthesis and metabolism genes that have been used in transgenic plant experiments have come from bacteria, most notably from *Agrobacterium tumefaciens*. *A. tumefaciens* causes plant tissues to grow in a hormone autonomous manner. This transformation of plant cells occurs because *A. tumefaciens* transfers to the plant genome

genes encoding enzymes that produce auxin and cytokinin (reviewed by Klee et al. 1987a, b). Although these hormone biosynthesis genes are bacterial in origin, they are functional in plants and the enzymes use substrates that are ubiquitous in plant cells. Auxin biosynthesis requires two genes: *iaaM* (*tms1*) and *iaaH* (*tms2*), which encode tryptophan monooxygenase and indoleacetamide hydrolase, respectively. Together, these enzymes synthesize indoleacetic acid from tryptophan. Indoleacetamide is the intermediate. Cytokinin biosynthesis is carried out by the product of the *ipt* (*tmr*) gene, which encodes an isopentenyl transferase. An isopentenyl group is attached to 5'-adenosine monophosphate to form the cytokinin isopentenyl adenosine. The availability of plant hormone biosynthesis genes from *Agrobacterium tumefaciens* fueled a series of experiments by several laboratories to express these genes in transgenic plants in order to study the effects of overproduction of auxin and cytokinin.

A second kind of experiment involving transgenic plants has involved suppression of an endogenous hormone biosynthesis gene using antisense technology. An artificial gene is introduced that produces an mRNA complementary to the native message. Expression of the complementary (antisense) strand of specific mRNAs has been shown to reduce expression of the gene (e.g., Gray et al. 1992). Antisense suppression is believed to work by formation of an antisense RNA : mRNA hybrid (Green et al. 1986). Therefore, if a gene is a member of a large family with many sequence differences, it is possible that expression of some family members will not be suppressed. To be effective, the antisense mRNA must be produced at a high level and it must be produced at the right time during development. Many antisense experiments in plants have used the CaMV 35S promoter, which is expressed strongly in many cell types. So far, of all the plant hormones, only ethylene biosynthesis genes have been isolated from plants and so these experiments have been limited to suppression of ethylene biosynthesis (see below).

A. Cytokinins

Constitutive overproduction of the *ipt* gene resulted in plants with extreme phenotypes. Transgenic petunias and tobacco overproducing cytokinins had almost no root production (Klee et al. 1987a; Smigocki and Owens 1989). They lacked apical dominance and were very green. These are effects that had been previously associated with cytokinins by physiological experiments.

Several groups tried to control *ipt* expression in transgenic plants with heat-shock inducible promoters from maize, soybean, or Drosophila

(Schmulling et al. 1989; Medford et al. 1989; Smart et al. 1991; Smigocki 1991). In each case, nonheat shocked plants displayed evidence of excess cytokinins, which was due to leaky expression of the heat-shock promoter. The plants had reduced apical dominance, reduced stem and leaf area, underdeveloped roots, and delayed leaf senescence. Heat-shock caused large increases in cytokinin levels, but did not alter plant development in every case (Medford et al. 1989). When heat-shock did cause visible effects, those effects were exaggerations of the nonheat shock phenotype: increase in green pigments, delayed leaf senescence, and further reduced apical dominance (Smigocki 1991; Smart et al. 1991). Probably the variation in results was due to differences in promoters and heat-shock treatments. Although cytokinins have been implicated from tissue culture experiments to be involved in organogenesis, these results do not supply evidence that cytokinins alter the plant's differentiation pattern. Rather, cytokinins just affected growth of already formed organs (Medford et al. 1989).

In contrast, more dramatic effects on plant development were obtained when the *ipt* gene was expressed in tobacco plants that were somatic genetic mosaics (Estruch et al. 1991). These plants had viviparous leaves, meaning that buds were formed on the surface or edges of the leaves. To generate these plants, the maize transposon *Ac* was inserted into the untranslated leader sequence of a 35S-*ipt* gene. The *ipt* gene was thus inactivated unless the *Ac* element jumped out, reactivating expression of the 35S-*ipt* gene in random cells and their progeny. Viviparous leaves were produced on plants in which *Ac* excision took place late in development, creating small patches of cytokinin producing cells. Increased levels of cytokinins were measured in the altered leaves, but not in normal parts of the leaves. When the *ipt* gene was placed under the control of the auxin-regulated, tissue-specific SAUR (small auxin up-regulated) promoter similar effects on plant development were obtained (Li et al. 1992); for example, adventitious shoots formed on petioles and leaf veins. These results support the notion that cytokinins are able to change the developmental program of plant cells. They also reinforce the idea that it is the local differences in amounts of cytokinins rather than absolute concentration that determines the effect of cytokinins on cellular processes.

B. Auxin

Transgenic petunias, which overproduced auxins, displayed traits that have been associated with auxin or ethylene exposure (Klee et al. 1987a, b): increased apical dominance, leaf curling, increased vasculature, and parthenocarpic fruit development. In order to determine which of these

effects was due to auxin, and which to auxin-induced ethylene, auxin-overproducing plants were crossed to transgenic plants with reduced levels of ethylene and to ethylene-insensitive mutants (Romano et al. 1993). The results showed that increased apical dominance was an effect of auxin alone. Several lines of physiological experiments had suggested a role for ethylene in mediating apical dominance although there were contradictory reports about whether ethylene suppressed or promoted outgrowth of lateral buds (Cline 1991). Ethylene was found to be responsible for part of the inhibition of stem elongation that occurs in transgenic tobacco plants that overproduce auxins while auxin alone was responsible for the leaf curling (Romano et al. 1993).

Auxin levels were reduced in transgenic plants by expression of the indoleacetic acid-lysine synthetase gene from *Pseudomonas syringae*, subspecies *savastanoi* (Romano et al. 1991). Measurement of IAA levels in the plants showed that endogenous IAA was reduced 2.5- to 19-fold. Interestingly, the phenotype of these transgenic plants was similar to that of transgenic plants with excess cytokinins. The plants had reduced apical dominance, reduced root development, and inhibited vascular differentiation. These experiments provide further support for the hypothesis that it is relative amounts of cytokinins and auxins rather than their absolute levels that regulate plant development (Skoog and Miller 1957). Measurement of both cytokinin and auxin levels in plants with altered cytokinin and auxin production is still necessary to strengthen this hypothesis.

C. Ethylene

The pathway for ethylene biosynthesis in plants is known, and genes encoding the key enzymes in the pathway have been cloned. 1-Aminocyclopropane-1-carboxylic acid (ACC) is the precursor for ethylene. It is synthesized from S-adenosylmethionine by the enzyme ACC synthase, which was originally purified and cloned from zucchini and tomato (Bleecker et al. 1986; Sato and Theologis 1989; Van der Straeten et al. 1990; Rottmann et al. 1991; Sato et al. 1991). ACC is converted to ethylene by ACC oxidase (also known as ethylene forming enzyme), which was first cloned from tomato (Hamilton et al. 1991). These genes have been manipulated in transgenic plants in experiments designed to control tomato fruit ripening by reducing ethylene production.

A number of laboratories have sought to suppress ethylene production by expressing antisense copies of ACC synthase or ACC oxidase (Hamilton et al. 1990; Oeller et al. 1991). Suppression of either gene results in reduced ethylene synthesis in tomato plants and delay or inhibition of

fruit ripening. These experiments have helped clarify ethylene's role in controlling fruit ripening. Because ACC synthase and ACC oxidase are members of gene families, only the genes expressed in the fruit are known to have been inhibited by the antisense experiments. Therefore, further analysis needs to be carried out before conclusions about ethylene's role in vegetative plant development can be derived from these studies.

Another approach has been used to reduce ethylene levels in transgenic plants. A gene encoding an enzyme that degrades ACC, ACC deaminase, was cloned from a soil bacterium, *Pseudomonas* sp., and expressed in tomato plants under the control of the 35S promoter (Klee et al. 1991). Ethylene levels in transgenic tomato plants expressing this gene were reduced in both fruit and leaves. Ripening of fruit from these plants was also delayed, but the transgenic plants did not display obvious abnormalities in vegetative stages of plant development. These experiments support the conclusion drawn from ethylene insensitive mutants: Ethylene does not play an important role in vegetative plant development. Ethylene may only be important for certain processes such as fruit ripening, seed germination, and response to plant stress. Further experiments with transgenic plants will help elucidate ethylene's role in plant growth and development.

III. HORMONE RECEPTOR BIOCHEMISTRY

By analogy with animal hormone responses, it has been proposed that the first step in plant hormone signal transduction is the interaction of the hormone with a receptor protein (Palme et al. 1992). If so, then the interaction between the hormone and its receptor should be highly specific, of high affinity, saturable, reversible, and result in a biological response. In recent years, technological advances have resulted in rapid progress in the identification of plant hormone binding proteins (i.e., putative receptors). Proteins that bind to each of the classes of plant hormones have been reported (reviewed by Libbenga and Mennes 1987), but by far the most progress has been made with auxin receptors, which will be the focus of this review. More detailed reviews of auxin binding proteins have been presented (Jones and Prasad 1992; Palme et al. 1992). A more general review of various hormone binding studies was presented by Libbenga and Mennes (1987).

Several different putative auxin receptors have been described, including a 22-kilodalton (kDa) protein from maize (Lobler and Klambt 1985; Shimomura et al. 1986; Napier et al. 1988; Jones and Venis 1989; Palme et al. 1992), a 43-kDa protein from zucchini (Hicks

et al. 1989) and maize (Jones and Venis 1989) and a 65-kDa protein in a diverse array of plant species (Prasad and Jones 1991). Several independent and innovative approaches were used in these studies, which will be briefly described.

The tritiated IAA analog (5-azidoindole-3-acetic acid, 5-N_3IAA) retains biological activity and can be used to specifically cross link proteins to which it binds (Jones and Venis 1989). Photolysis of the protein-N_3IAA complex with UV light results in labeling of the associated proteins, which can then be visualized after gel electrophoresis by autoradiography. Specificity of binding can be demonstrated using control proteins such as lysozyme which is labeled about 20-fold less efficiently. Using this approach, specific auxin binding proteins of 22-, 24-, and 43-kDa molecular weights were identified in maize membrane protein extracts (Jones and Venis 1989). Several other approaches have identified a 22-kDa auxin binding protein in maize extracts including affinity chromatography (Shimomura et al. 1986), immunoaffinity chromatography (Lobler and Klambt 1985), and traditional chromatography (Napier et al. 1988). The photoaffinity IAA analog has been used to identify binding proteins in *Hyoscyamus muticus* cells revealing two polypeptides of 31- and 24-kDa with high auxin binding specificity (MacDonald et al. 1991).

Anti-idiotypic antibodies were used to identify a putative auxin receptor of 65 kDa (Prasad and Jones 1991). In this approach, antibodies that bind auxin were produced and used as antigens to produce a second series of antibodies. This approach relies on the theory that the anti-IAA antibody binding site is complementary to IAA in molecular topology and therefore the second anti-anti-IAA antibody (anti-idiotypic) binding site should be complementary to the first antibody and thus resemble, in molecular shape, IAA. This IAA shaped protein should in turn, be recognized and bound by IAA binding proteins. This approach had previously been successfully used in studies of animal receptor proteins. The specificity of binding to the 65-kDa protein was demonstrated, and conservation of this protein between a number of diverse plant species was confirmed. Immunoblotting of protein extracts from soybean seedling sections showed that the protein was found along the entire length of the hypocotyl, but that the hook region contained relatively less reacting protein. Subcellular localization revealed that the 65-kDa auxin binding protein was localized in the nucleus, supporting the exciting possibility that this protein may act at the level of gene transcription.

The identification of these proteins has led to the isolation of genes encoding the 22-kDa protein from maize and from *Arabidopsis* (Palme et al. 1992). The availability of these genes will make possible a detailed structure-function analysis, which should lead to the molecular details

of their action in cells. The use of these genes in transgenic plant analysis should further reveal the in vivo function of the proteins they encode and provide valuable insights into the physiological role they play in auxin perception.

One of the generalized conclusions that can be made from this work is that multiple auxin binding proteins are found within plant cells and that they can be localized to the plasma membrane, endoplasmic reticulum, the nucleus or they may be soluble. That any of the identified proteins actually acts as a receptor in mediating auxin effects has not yet been shown, but it is possible that multiple auxin receptors exist, perhaps within the same cell. This model has important implications as to the multiple modes of auxin action in plant cells.

IV. CONTROL OF GENE EXPRESSION BY PLANT HORMONES

Many of the physiological effects of plant hormones are brought about by alterations in gene expression patterns. Presumably the polypeptide products these genes encode are involved in the responses induced by the particular hormone. Each of the classes of plant hormones have been shown to induce the expression of specific gene sets. The expression levels of hormonally regulated promoters are thought to be controlled via interactions with nuclear DNA binding proteins (*transcription factors*). The study of hormone responsive gene promoters and the transcription factors they interact with will provide insight into the molecules and mechanisms involved in the signal transduction pathways leading from the hormone to alterations in gene expression patterns. Modern molecular biological techniques provide a powerful array of approaches for these investigations. To illustrate the methods used in this type of research, we will focus on one of the most advanced systems, the abscisic acid (ABA) inducible wheat *Em* gene (recently reviewed by Quatrano et al. 1993).

A. Abscisic Acid Regulated Genes

A large number of genes which are induced by ABA have been identified (for example, Yamaguchi-Shinozaki 1989; Anderberg and Walker-Simmons 1992; Goday et al. 1988; reviewed by Skriver and Mundy 1990; and see below). ABA is known to have particular relevance during seed maturation and during osmotic stress responses. ABA prevents wheat embryos from germinating and promotes maturation when immature

embryos are cultured in vitro (Quatrano et al. 1993). To investigate whether protein synthesis patterns in developing wheat embryos are altered by treatment with ABA, excised embryos were cultured with radiolabeled ^{35}S methionine (Williamson et al. 1985). Gel electrophoresis of the resulting polypeptides revealed a distinctly different set of proteins in ABA treated embryos. In order to isolate genes encoding the proteins induced by ABA, differential cDNA cloning was performed (Williamson and Quatrano 1988). Cellular mRNA was isolated from ABA-treated and ABA-untreated embryos, and a cDNA library was constructed from mRNA isolated from the treated sample. This library was propagated in *E. coli* and contained a large number of different cDNA sequences, each representative of a particular message present in the ABA-treated embryos. RNA from both samples was then radiolabeled and used as hybridization probes to screen the library. Clones, which reacted strongly to mRNA from ABA-treated embryos, but did not react with mRNA isolated from untreated embryos represented cDNA sequences expressed only in the ABA-treated embryos and were thus isolated as putative ABA-regulated genes. The nucleotide sequences of several of these cDNA clones were determined (Litts et al. 1987). One of these clones (designated *Em* for Early Methionine labeled) was shown to encode a small hydrophilic protein. This cDNA clone was used as a hybridization probe to isolate DNA fragments containing the *Em* gene from a genomic library (Litts et al. 1991). The isolation of the wheat *Em* gene provided the DNA sequences necessary to map the promoter elements within the *Em* gene, which were involved in the ABA response (Marcotte et al. 1988, 1989; Guiltinan et al. 1990).

The DNA sequences involved in the expression of a given gene are called *promoter* and *enhancer elements* (reviewed by Maniatis et al. 1987). Generally, these sequences reside in the DNA flanking the coding region of a gene, 5' to the transcription start site. Short DNA sequences (approximately 10 base pairs) contribute specificity to the pattern of expression exhibited by a given gene. Multiple elements reside in most promoters, and it is the combined effects of these elements that determines the expression pattern of each gene. These DNA sequences are sites for binding by various protein factors, and it is the resulting DNA-protein complex that determines the transcriptional activity of that gene. The combination of promoter elements (*cis-* regulatory elements) and their associated DNA binding proteins (transcription factors) constitutes a molecular switching system, which can regulate the temporal, quantitative, and spatial pattern of gene expression.

In order to functionally define *cis-* regulatory elements, a series of experiments is performed in which DNA sequences suspected of pro-

moter activity are either deleted or mutated. The altered promoters can then be fused to a coding sequence fragment encoding a "reporter" enzyme, which is readily assayed, making the measurement of promoter activity more rapid and sensitive. Commonly used reporter genes include ß-glucuronidase (GUS), (Gallagher 1992); luciferase (LUC), (Ow et al. 1987); and chloramphenicol acetyl-transferase (CAT) (Hererra-Estrella et al. 1983). The resulting promoter–reporter "gene fusion" is then introduced into living cells, and its transcriptional activity measured as a function of reporter protein activity.

Gene expression assays are performed in either a transient system (over a period of several days), or by stably introducing the DNA into the plant chromosome and regenerating transgenic plants (reviewed by Gasser and Fraley 1990). Transient expression assays consist of a method to introduce DNA into cells or tissues and measurement of resulting gene expression levels. The main advantage of a transient assay is the short period of time needed to perform an experiment and the large number of replications which can be performed. DNA can be introduced into cells using a number of approaches including particle bombardment (the "gene gun"), electroporation of tissues or protoplasts, and polyethylene glycol treatment of protoplasts. Stable transformation of reporter genes involves introduction of DNA into a cell, incorporation of the DNA into the plant chromosome, and regeneration of "transgenic" plants from the transformed cell. Progeny from transgenic plants can be produced and tested for the activity of the introduced gene. The main advantage of transgenic plant analysis is that various tissue and cellular types can be assayed during the course of development, and the introduced gene is in a chromosomal location. The growing evidence for chromosomal influences on gene expression patterns makes this an important consideration (Allen et al. 1993).

In the case of the wheat *Em* gene, the promoter region consisting of several hundred bases of DNA from the upstream region was fused to the *uid* gene, which encodes GUS (Marcotte et al. 1988). The DNA was introduced into protoplasts derived from rice tissue cultured cells by precipitation with polyethylene glycol. The cells were then cultured for several days in the presence or absence of ABA after which the amount of GUS protein was quantified by a rapid and sensitive fluorometric assay. The amount of GUS activity was shown to be increased by 10- to 20-fold in the cells treated with ABA, indicating that the DNA sequences necessary for ABA responsiveness were located within the DNA fragment tested. Progressive deletion of DNA sequences from this fragment showed that when the region spanning -168 to -106 upstream of the mRNA initiation site was removed, nearly all ABA responsiveness was

abolished. When a 76-bp fragment containing this sequence was then inserted into the promoter of a non-ABA regulated gene (the cauliflower mosaic virus 35S gene, CaMV 35S) it conferred ABA responsiveness on that promoter in the rice transient assay. This result directly implicated that particular sequence in being involved in the ABA induced transcriptional response of the wheat *Em* gene, and thus it has been called an ABRE for ABA Responsive Element. Further gene fusion and mutagenesis experiments have supported these conclusions (Guiltinan et al. 1990; Quatrano et al. 1993). ABREs from other plant genes were subsequently identified and shown to be similar to the wheat ABRE (Mundy and Chua 1988; Skriver and Mundy 1990; Vilardell et al. 1990; Pla et al. 1993).

In numerous precedents from prokaryotic and eukaryotic systems, DNA binding proteins (transcription factors) bind to promoter regulatory elements such as the ABRE (Maniatis et al. 1987). In order to search for protein(s), which might interact with the ABRE and which thus may be integral to the ABA signal transduction mechanisms, DNA binding protein analysis was performed (Guiltinan et al. 1990). One of the most important methods used to study DNA binding proteins is the Electrophoretic Mobility Shift Assay (EMSA). In this method, radiolabeled, double-stranded DNA fragments from a promoter under study (i.e., the ABRE) are incubated with nuclear protein extracts and the resulting complex is electrophoresed on a nondenaturing gel matrix. DNA-protein complexes are then visualized by autoradiography as bands with reduced mobility compared to the free DNA alone. The specificity of DNA binding activity is then assessed by including in the reaction excess unlabeled DNA competitor fragments containing either the DNA sequence under study or a different, nonrelated control DNA sequence. If a particular DNA-protein complex is the result of sequence-specific DNA binding activity, the specific sequence will compete for binding to the protein and will result in reduction in the amount of radiolabeled DNA fragment in the complex. The nonrelated DNA competitor should not compete efficiently for the DNA binding protein and therefore not alter the autoradiographic pattern observed.

The wheat ABRE DNA sequence described above was used in EMSA experiments using extracts isolated from rice embryos and from rice tissue cultured cells grown with and without ABA (Guiltinan et al. 1990). Specific DNA binding activity was detected in all of the extracts, and interestingly, the amount of DNA binding activity was greater in extracts, from the ABA-treated cells. To determine the specificity of binding, a mutated DNA sequence, which differed from the ABRE by only two bases, was tested as a competitor. As expected, it did not compete for binding to the ABRE binding factor(s). Additionally, the same two base

pair mutations abolished the ABA inducibility in the transient assay system described above. These results and others strongly implicated the DNA binding activity as being involved in the ABA transcriptional response. In order to learn more about the DNA binding protein(s) involved, isolation of the gene(s) encoding them was undertaken.

Several approaches can be used to attempt to characterize nuclear transcription factors including biochemical and molecular cloning strategies. While biochemical purification of such proteins has been successfully performed (Kadonaga and Tjian 1986), this method may require massive amounts of tissues and high purification efficiencies due to the relative low abundance of most transcription factors. Once purified, however, it is relatively straightforward to determine the amino acid sequence of a portion of the protein. From this information a degenerate DNA sequence can be deduced that encodes the sequenced peptide. This DNA sequence can then be synthesized and used as a hybridization probe to screen cDNA or genomic libraries to isolate the gene which encodes the purified protein.

Another approach for isolating genes encoding DNA binding proteins involves the screening of cDNA expression libraries. This type of library is designed for high level expression of random protein sequences encoded in the mRNA isolated from the tissue under study in *E. coli*. Such libraries can be screened using a radiolabeled, double-stranded DNA fragment containing a hormone response element under conditions favoring DNA-protein interactions. Clones producing proteins capable of binding to the DNA fragments are visualized by autoradiography and isolated. Such a screen was performed using the wheat ABRE sequences and a cDNA expression library produced from ABA treated wheat embryo RNA (Guiltinan et al. 1990). Two clones were isolated, which exhibited DNA binding activities identical to those in the wheat and rice nuclear extracts. The nucleotide sequences of the two clones were determined and comparison to known sequences in the Genbank database revealed that they encode a protein with characteristics of a known class of transcription factors called the *basic-leucine zipper* (bZip) family (Guiltinan et al. 1990). This protein has been given the name EmBP-1 for *Em* Binding Protein 1. bZip family proteins were first identified in yeast and human cells where they play important roles in controlling gene expression in these systems (Struhl 1989). While we do not understand exactly how any of the bZip proteins function in controlling gene expression, many of the molecular details of how they work are beginning to emerge. This class of proteins has at least three functional domains: the leucine zipper, responsible for efficient dimerization between two bZip monomers; the basic domain, responsible for DNA

recognition and binding; and the transcriptional activation domain, necessary for the interaction of the protein with the transcriptional machinery resulting in increased levels of gene expression. We are now attempting to understand the functional domains of EmBP-1 and to identify the way in which they are involved in ABA responsiveness.

Similar approaches for isolating hormone responsive genes and their associated *cis-* and *trans-* acting factors are in progress for each of the major plant hormone classes. Genes responsive to each of the hormones have already been isolated. With the exception of cytokinin regulated genes, DNA sequences involved in their response have been identified and in some cases have been shown to interact with nuclear factors. A brief, noninclusive summary of these studies is presented as follows.

B. Cytokinins

The effects of cytokinins of gene expression have been previously reviewed (Chen 1989). Genes regulated by cytokinin include the chlorophyll a/b binding protein (Flores and Tobin 1988), a tobacco stress response gene pLS216 (Dominov et al. 1992), ribosomal protein genes and several other unknown soybean genes (Crowell et al. 1990), the early nodulin gene from *Sesbania rostrata* (Dehio and de Bruijn 1992), the tobacco ß-1,3-glucanase gene (Mohnen et al. 1985), two genes encoding proteins of unknown function from apple (Watillon et al. 1991), and plant defense genes in tobacco including extensin, chitinase, and pathogenesis related protein-1 (Memelink et al. 1987). To date, DNA sequences specifying cytokinin responsiveness have not been identified.

C. Auxin

A large number of auxin regulated genes have been identified (Hagen et al. 1984, Theologis et al. 1985, Key et al. 1986, Alliotte et al. 1989; Ainley et al. 1988; reviewed by Key 1989; McClure et al. 1989; Takahashi et al. 1989; An et al. 1990; Conner et al. 1990; Guilfoyle 1990; Van der Zaal et al. (1991); Sundas et al. 1992). DNA sequences important in auxin responsiveness have begun to be characterized (Takahashi et al. 1990).

D. Ethylene

A large number of ethylene responsive genes have been identified including genes from carnation (petal senescence, Lawton et al. 1990; Woodson et al. 1992), tomato (fruit ripening, Lincoln et al. 1987), and tobacco (Broglie et al. 1986). In several of these genes, DNA sequences

necessary for the ethylene response have been isolated and DNA binding proteins which interact with these sequences detected (Deikman and Fischer 1988; Broglie et al. 1989; Cordes et al. 1989; Deikman et al. 1992; Montgomery et al. 1993).

E. Gibberellic Acid

The α-amylase genes of cereal grains have been shown to be regulated by GA and DNA sequences and nuclear factors involved in this control have been identified (Khursheed and Rogers 1988; and Huang et al. 1990; Skriver et al. 1991; Huttly et al. 1992; Lanahan et al. 1992; Rushton et al. 1992). Interestingly, the observation that GA and ABA play opposing roles in cereal grain development is supported by the discovery that the GA induction of the α-amylase genes can be inhibited by ABA (Jacobson and Chandler 1985; Lanahan et al. 1992). This is an interesting example of interactions of two hormones at the level of gene expression.

V. GENETIC ANALYSIS OF HORMONE RESPONSE

Genetic analysis has proven to be a powerful tool for studying complex developmental processes. The most well-developed example for higher plants is flower development. Analysis of *Arabidopsis thaliana* and *Antirhinum majus* mutants has allowed modeling of flower development (Coen and Meyerowitz 1991). Currently, genes identified by flower mutants are being cloned (e.g., Coen et al. 1990; Yanofsky et al. 1990; Jack et al. 1992; Weigel et al. 1992) and these molecular studies will permit verification and elaboration of the models developed with classical genetics. Methods for cloning genes identified by mutant phenotypes will be discussed below. These techniques have recently been successfully applied to the study of plant hormone mutants, and we will summarize some of the recent results of these efforts.

While much important genetic work has been accomplished in species such as maize, tomato, pea, and barley, *Arabidopsis thaliana* figures prominently in many of the recent genetic studies. The reason for the large increase in use of this small crucifer is that *Arabidopsis* has many advantages for genetic work (reviewed by Redei 1975; Meyerowitz and Pruitt 1985; Estelle and Somerville 1986). The small size of the plant permits screening of large numbers of individuals in a small space. The relatively short generation time of as little as six weeks allows examination of many generations per year. *Arabidopsis* is self-fertile so that genetic lines can be easily maintained. The plant can also be cross-fertilized, and

each plant produces a large number of seed. Of great importance for molecular cloning experiments is the small size of the *Arabidopsis* genome, containing only 70,000 kb of DNA, the smallest plant genome known. Additionally, there is very little repetitive DNA present in the *Arabidopsis* genome. These traits greatly simplify efforts to clone genes by techniques such as chromosome walking, described below.

A. Identification and Characterization of Plant Hormone Mutants

In plant hormone research, genetics has been useful in contributing to our understanding of the roles of GA, ABA, auxin, ethylene, and cytokinins in plant development (Phinney 1984; Reid 1986, 1987, 1990; Estelle and Somerville 1987; Bleecker et al. 1988; King 1988; Guzman and Ecker 1990; Lincoln et al. 1990; Wilson et al. 1990; Wang 1994). Two types of plant hormone mutants have been studied: mutants that have altered levels of a hormone, and mutants that are defective in their response to a hormone.

Mutants with altered hormone levels can arise by defects in either hormone biosynthesis or metabolism. Hormone biosynthesis mutants can generally be corrected by application of exogenous hormone. This class of mutants has been useful in augmenting physiological data concerning the roles of particular hormones in plant development. For instance, GA mutants provided convincing data that GA_1 is the active gibberellin in controlling elongation in maize, peas, and rice (Phinney 1984). Hormone biosynthesis mutants are valuable for identifying the steps involved in hormone biosynthesis. GA-deficient mutants have already proven their worth in helping elucidate the complicated GA biosynthetic pathway (Reid 1990). For cytokinins and auxins, however, no hormone biosynthesis mutants have been identified in higher plants.

For studying the mechanism of hormone action, hormone response mutants are of the greatest value. The concept of sensitivity to plant hormones has been discussed and elaborated on by Firn (1986). He points out that the term "sensitivity" is vague when applied to plant hormones and suggests some specific terms to describe different changes in plant hormone response. These terms are (1) "receptivity" to describe the number of receptors, (2) "affinity" to describe the affinity of the receptor, (3) "response capacity" to describe changes in signal transduction and response, and (4) "uptake efficiency." Analysis of plant response to hormone concentration should be useful to help distinguish between mutations in these various steps of hormone response (Firn 1986).

A number of natural and induced mutants have been isolated that affect response to a particular plant hormone. Identification of plant

hormone mutants depends on our understanding of the role of the hormone in plant development and has often relied on hormone bioassays that were developed early in study of the hormone (Wang, in press). For example, ethylene response mutants were identified by looking for plants that failed to exhibit the "triple response" in the presence of exogenous ethylene (Guzman and Ecker 1990). Mutants insensitive to gibberellic acid (GA) have been identified on the basis of their dwarf nature (Reid 1986). Abscisic acid mutants are identified on the basis of precocious seed germination or tendency to wilt (Koornneef 1986).

Selection for hormone insensitive or resistant mutants has been carried out by growing plants in the presence of elevated, toxic levels of the hormone and screening for plants which display resistance. Mutants resistant to ethylene (Bleecker et al. 1988; Guzman and Ecker 1990), abscisic acid (Koornneef 1986), auxins (Maher and Martindale 1980; Mirza and Maher 1985; Muller et al. 1985; Estelle and Somerville 1987; Wilson et al. 1990), and cytokinins (Jullien et al. 1992; Su and Howell 1992) have been obtained in this manner. However, this approach can be problematic for several reasons. Mutants that are cross-resistant to more than one hormone have been obtained with this method in a number of instances. In some cases this result has been explained by the hypothesis that different hormones share a common signal transduction pathway, or that they share elements of a pathway (Wilson et al. 1990). It is also possible, however, that these mutants are altered in a way that permits them to escape the toxic effects of high levels of exogenous hormone (Wang, in press). One example of such a case was seen with a putative cytokinin-resistant *Nicotiana plumbaginifolia* mutant identified on the basis of ability to germinate on high levels of benzylaminopurine (Blonstein et al. 1991). These plants were resistant to auxin as well, and it was found that the plants were deficient in ABA biosynthesis (Parry et al. 1991). It has been suggested that because the plants were able to germinate more rapidly than wild-type, they were able to outgrow the toxic hormone. However, other mutants appear to be specifically resistant to one hormone, and it is likely that these mutations alter uptake, perception or signal transduction of that hormone.

B. Cloning Genes Identified by Mutations

Once a mutation has been characterized and determined to be involved in plant hormone response, it is valuable to clone the mutated gene so that its gene product can be studied at the molecular level and its role in hormone action can be elucidated. Three techniques have been used in plants to clone genes identified by mutant phenotype: gene tagging, genomic subtraction, and chromosome walking.

Gene tagging is the simplest of these methods technically, but it requires that the mutation be caused by insertion of a known DNA sequence. Once such a mutation is obtained it is a relatively straightforward matter to isolate the DNA sequences surrounding the insertion by molecular cloning. The endogenous transposons *Activator* (*Ac*) and Robertson's *Mutator* (*Mu*) in maize and *tam* in *Antirhinum* have been used to tag genes in these species. Use of these transposable elements for gene tagging was recently reviewed (Gierl and Saedler 1992; Walbot 1992). Several groups are developing *Ac* tagging systems for use in dicotyledonous species such as tomato and *Arabidopsis* (Walbot 1992), and the tagging of a petunia flower color gene with *Ac* was recently reported (Chuck et al. 1993). Another type of DNA sequence that has been successfully used for insertional mutagenesis is the T-DNA from *Agrobacterium tumefaciens* (Feldmann 1991; Koncz et al. 1992). A number of genes important for plant hormone response have been cloned by transposon or T-DNA tagging including a maize gene involved in ABA responsiveness (McCarty et al. 1989) and a gene necessary for ethylene responsiveness (Kieber et al. 1993).

The second method for cloning mutagenized genes, *genomic subtraction*, can be employed if the mutation is the result of a deletion (Straus and Ausubel 1990; Sun et al. 1992). This procedure identifies sequences present in wild-type but absent in the mutant. DNA is isolated from homozygous mutant plants and is hybridized to DNA from wild-type plants. Sequences in common between the two DNA samples are removed. Several cycles of hybridization and subtraction are required to enrich for the target sequences sufficiently to allow identification of the gene responsible for the mutant phenotype. This technique is feasible only with relatively small genomes, but was demonstrated to work with *Arabidopsis*, by cloning a GA biosynthesis gene using a deletion that spanned 5 kb (Sun et al. 1992). In theory, smaller deletions could be used for this procedure (Straus and Ausubel 1990). One limitation of the procedure is that it can only be used if the homozygous deletion mutant is viable. Ionizing radiation, such as γ- or x-rays, causes deletions of the appropriate size at an acceptable frequency (Shirley et al. 1992). However, ionizing radiation can also cause gross chromosome rearrangments that do not involve deletions. For this reason, it is useful to generate multiple alleles of the gene to be cloned.

Chromosome walking involves cloning a gene by using information of genetic map position. This technique is laborious and time consuming, but it is the only method for cloning genes mutated by chemical means, since mutagenic chemicals such as EMS cause only single-base changes in DNA, which do not allow detection by methods that physically compare wild-type and mutant DNA. In the first step of chromosome

walking, the gene of interest is mapped relative to restriction fragment length polymorphism (RFLP) markers (Chang et al. 1988; Nam et al. 1989; Hauge et al. 1993). It has been estimated that about 80% of the *Arabidopsis* genome should be within 300 kb of the published RFLP markers (Meyerowitz 1992), and this distance can be covered in a manageable number of cosmid or yeast artificial chromosome (YAC) clones. Another set of markers, random amplified polymorphic DNAs (RAPDs), have more recently been developed and they greatly increase the density of markers (Reiter et al. 1992). YAC libraries (Guzman and Ecker 1988; Ward and Jen 1990; Grill and Somerville 1991) have been constructed and are being ordered into overlapping cloned DNA fragments spanning the entire *Arabidopsis* genome. Completion of this physical map will permit gene cloning by simply determining the map position of the gene and then retrieving the proper clone from the YAC library (Matallana et al. 1992). In addition to the genome work accomplished with *Arabidopsis*, progress is being made with some important crop species. High-density RFLP maps have been constructed for tomato, potato, and rice (McCouch et al. 1988; Ronald et al. 1992; Tanksley et al. 1992), and a tomato YAC library suitable for chromosome walking has been established (Martin et al. 1992). The first published report of a gene isolated in *Arabidopsis* by positional cloning involved cloning a gene required for response to ABA, *ABI3* (Giraudat et al. 1992).

Verification that the correct gene has been cloned by any of these methods can be carried out by a complementation test. When mutant plants are transformed with the wild-type gene, the wild-type phenotype should be restored. In addition, comparison of the DNA sequences of the wild-type and mutant genes should reveal the specific genetic defect.

C. Recent Results

Some recent results from molecular genetic analysis of plant hormone action are summarized below to demonstrate the exciting progress that has been made in the last few years using this approach to identify components of hormone signal transduction.

1. Ethylene. Identification of ethylene response mutants in *Arabidopsis* has relied on the classical "triple response" of plants to ethylene first described in peas by Neljubow (1901). Plants that fail to exhibit the triple response in the presence of exogenous ethylene include at least 3 loci: *etr* (equivalent to *ein1*), *ein 2* and *ein 3* (Bleecker et al. 1988; Guzman and Ecker 1990; Kieber et al. 1993; Guzman and Ecker 1990). An additional ethylene response mutant, *ctr1*, shows a triple response constitutively in the absence of exogenous ethylene (Kieber et al. 1993). Double-mutant

studies have permitted ordering some of these genes in a signal transduction pathway. These mutants do not represent all of the genes in the ethylene signal transduction pathway, because additional ethylene-response mutants have recently been found, although their description is not yet published (Kieber et al. 1993).

The gene corresponding to the *ctr1* mutation was cloned using a T-DNA mutagenized allele (Kieber et al. 1993). This gene has strong homology to the Raf family of protein kinases. The current model is that *CTR1* is a negative regulator of a multistep signal transduction pathway, which acts by phosphorylation of another protein in a cascade (Kieber et al. 1993). The cloning of this and other ethylene-response genes will permit the testing of this model.

2. Abscisic Acid. Mutants that are insensitive to ABA have been studied in several plant species (Koornneef 1986), but progress at the molecular level has been most notable in maize and *Arabidopsis*. Nine loci have been identified that control vivipary in maize, but *Vp1* is the only locus that has reduced sensitivity to ABA. *Vp1*, but not the other *Vp* loci, also controls accumulation of anthocyanin pigments in the aleurone layer of the seed. The *Vp1* gene was cloned by transposon tagging with a *Mu* element, and was shown to be expressed specifically in the embryo and endosperm tissues of the developing seed (McCarty et al. 1989). *Vp1* encodes a 73,335 dalton protein with no homology to known proteins (McCarty et al. 1991). The *Vp1* protein was able to transactivate the ABA-responsive *Em* promoter (see above) in maize protoplasts. The protein contains an acidic domain, which is functionally interchangeable with a known transcriptional activation domain. It is possible that *Vp1* activates ABA-responsive genes directly or it is possible that *Vp1* potentiates the ABA response in maturing seed tissues. McCarty et al. (1991) favor the second hypothesis because of the timing of *Vp1* and *Em* mRNA accumulation in maturing seeds and *Vp1*'s involvement in regulating anthocyanin production.

In *Arabidopsis*, ABA insensitive mutants (*abi -1, 2*, and *3*) were selected based on the ability of seeds to germinate in the presence of inhibitory levels of ABA (Koornneef et al. 1984). The *abi* mutants have phenotypes similar to ABA-deficient (*aba*) mutants. The seeds have reduced dormancy and *abi1* and *abi2* are more prone to wilting (Koornneef 1986). However, the mutants are not sensitive to exogenous ABA. The *ABI3* gene has been isolated by positional cloning (Giraudat et al. 1992). The ABI3 protein contains a putative nuclear targeting sequence and domains with features typical of transcription factors. The current hypothesis is that the ABI3 protein participates in ABA-regulated gene

expression during seed development (Giraudat et al. 1992). The predicted ABI3 protein has discrete regions of high similarity to the maize Vp1 protein (Giraudat et al. 1992). The phenotypes of the *abi3* and *vp1* mutants suggest similarities and differences in their functions, but whether they are functional counterparts or just related genes is unresolved. There should be exciting new developments in this area in the near future.

3. Gibberellic Acid. A semidominant *Arabidopsis* mutation that confers insensitivity to GA, *gai*, results in plants that are dwarfed, and that have reduced apical dominance (Koornneef et al. 1985). This phenotype is similar to that of GA-sensitive dwarf mutants, but *gai* mutants fail to respond to exogenous GA. This mutant accumulates biologically active GAs to levels greater than wild-type (Talon et al. 1990). Similar semidominant mutants have been identified in maize, wheat, and Brassica (Fujioka et al. 1988; Harberd and Freeling 1989; Zanewich et al. 1991; Stoddart 1984). Progress in cloning the *gai* locus has included mapping the gene to RFLP markers and generation of derivative alleles using γ-irradiation (Peng and Harberd 1993). At least one of the derivative alleles contains a large deletion or rearrangement and may be useful for cloning using a technique such as genomic subtraction (Sun et al. 1992).

4. Auxin. Several laboratories have studied mutants resistant to elevated levels of auxins (Maher and Martindale 1980; Mirza and Maher 1985; Muller et al. 1985; Estelle and Somerville 1987; Lincoln et al. 1990; Wilson et al. 1990). In *Arabidopsis*, four distinct loci have been identified, which confer resistance to auxins: *aux1*, *axr1*, *axr2*, and *dwf* (Maher and Martindale 1980; Estelle and Somerville 1987; Wilson et al. 1990; Mirza and Maher 1985). Two of the mutations, *axr2* and *dwf*, are dominant. Gravitropism is affected in roots of each of the mutants, but in *axr2* and *dwf* mutants shoots are affected as well.

The *AXR1* gene was cloned by chromosome walking and was found to encode a protein with significant homology to ubiquitin-activating enzyme (E1; Leyser et al. 1993). Since the *AXR1* protein is highly diverged from previously characterized E1 proteins, and since it lacks a critical cysteine residue, it is possible that *AXR1* has a function other than ubiquitin-activation. Alternatively, the *AXR1* protein may belong to a new class of enzymes in the ubiquitin pathway. Ubiquination of some vertebrate membrane receptors may have a regulatory function. The intriguing hypothesis that the auxin receptor could be regulated by ubiquination was suggested (Leyser et al. 1993).

A different approach was used to tag genes involved in auxin action in tissue culture (Hayashi et al. 1992). A T-DNA tagging vector with the potential to produce dominant mutations was transferred into protoplasts. The vector included multiple transcriptional enhancers, derived from the cauliflower mosaic virus (CaMV) 35S RNA promoter, located near the right border sequence. When this T-DNA is inserted into the plant genome, plant genes flanking the T-DNA insert become overexpressed. Plant protoplasts, which normally require exogenously supplied auxin for growth, were transformed with this vector and then calli, which could grow in the absence of auxin were selected. Plants were regenerated from the transgenic, auxin-independent calli. A gene was identified with this method that, when constitutively expressed in plants, allows protoplasts to grow in the absence of exogenous auxin. This gene encodes a highly basic protein, but its function is unknown. Interestingly, plants overexpressing this gene have normal morphology and are fertile. Further analysis of the function of this gene in plants should reveal its role in auxin action. This approach of creating dominant tagged mutations has potential for identifying genes involved in other processes that can be selected in tissue culture.

5. Cytokinins. Higher plant mutants, which are insensitive to elevated levels of cytokinins have been identified in *Arabidopsis* (Su and Howell 1992) and *Nicotiana plumbaginifolia* (Jullien et al. 1992). The *Arabidopsis ckr* locus was identified by screening for seedlings in which root growth was not as severely inhibited by elevated concentrations of the synthetic cytokinin benzyladenine (BA). In the absence of exogenous cytokinins the plants do not have a severe phenotype and have only subtle differences in appearance from wild-type. The mutants are not resistant to auxins and so may be specific cytokinin response mutants. The weak phenotype may result from a leaky or organ-specific defect.

Screening for *Nicotiana plumbaginifolia* plants resistant to elevated levels of zeatin has resulted in identification of three *zea* loci (Jullien et al. 1992). The mutant plants grew well at zeatin concentrations that were severely inhibitory to wildtype plants. Preliminary results indicated that the absorption and metabolism of tritiated BA is normal in the mutants and so they are believed to affect cytokinin sensitivity.

Cytokinins affect many light-regulated processes in plants such as chloroplast development and expression of photosynthesis genes (Horgan 1984; Flores and Tobin 1988; Lu et al. 1990). *Arabidopsis* mutants, which are de-etiolated when grown in the dark (*det* mutants), are also abnormal in having delayed senescence (Chory et al. 1991) and reduced apical dominance (Chory and Peto 1990; Chory et al. 1991), two characteristics associated with cytokinins. Alterations in cytokinin

sensitivity or biosynthesis is being explored as the basis for the *det* mutant phenotype (Chory 1992).

Considering the multiplicity of developmental responses that plants have to each of the plant hormones it is likely that plant hormone signal transduction is a complex process and that many genes are involved in the process for each hormone. The experience of researchers interested in ethylene has been that continued searches for additional mutants has been rewarded with identification of novel mutations (Bleecker et al. 1988; Guzman and Ecker 1990; Van der Straeten et al. 1991; Kieber et al. 1993). Identification of multiple hormone-resistant mutants will be necessary to fully understand hormone action in plants.

VI. CONCLUSIONS

A detailed understanding of the molecular mechanisms by which any of the plant hormones control gene expression is, to date, elusive and will await further experimentation and integration of results from a large number of studies. Recently, the development of powerful methods such as those described above have enabled new experimental strategies to be used to characterize some of the molecular components of hormone response mechanisms and to reveal details of the effects hormones play in plant growth and development. The use of transgenic plant technology has revealed some of the nuances of hormone effects as yet not detected in more traditional exogenous application and excision studies. Sensitive biochemical and molecular techniques have been used to identify putative hormone receptor molecules, and in several cases, genes encoding these have been isolated. Promoter elements and transcription factors implicated in hormone mediated gene expression have been identified for nearly every class of hormone. The development of *Arabidopsis* as a model system for molecular genetic approaches has already yielded a number of very interesting mutants, which will provide a means to further dissect hormone signal transduction pathways. Rapid progress can be expected in the next decade as even more powerful technology and innovative experimental strategies are developed.

It has become clear from both classical studies and from the studies presented in this review that the various plant hormones interact in their physiological effects in complex and sometimes surprising ways. One of the most interesting questions that may be answered in the near future is how the various hormones interact at the level of the gene. Do composite promoter elements exist that are responsive to more than one hormone independently or do combinations of hormones act through separate, integrating transduction pathways? Other important questions involve the

ideas of multiple versus single receptors and transduction pathways, how signal transduction pathways may branch to result in multiple effects and, finally, how independent signal transduction pathways might merge, utilizing a common molecular intermediate for one or several transduction steps. Clearly, the new technologies available to the plant hormone biologist will be powerful tools in answering these and many other questions.

Finally, in addition to the molecular approaches described above, we must return to traditional biochemical and physiological techniques to elucidate the functional roles the hormonally induced proteins play in the physiological changes that occur in response to the various hormones. While the function of a number of such proteins are already known, the majority are not understood. Only when we have this knowledge will we have a clear picture of the roles hormones play in plant growth and development from the signaling mechanisms they stimulate to the physiological processes they alter.

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