Annu. Rev. Phytopathol. 1997. 35:45–66

THE IMPACT OF TI-PLASMID-DERIVED GENE VECTORS ON THE STUDY OF THE MECHANISM OF ACTION OF PHYTOHORMONES

R. Walden, B. Reiss,C. Koncz, and J. Schell
Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Cologne, Germany; e-mail: Walden@mpiz-koeln.mpg.de

KEY WORDS: *Agrobacterium*, T-DNA, plant transformation vectors, phytohormones

ABSTRACT

The molecular basis of tumor formation on dicotyledonous plants by *Agrobacterium* relies on the transfer to the plant cell of a unique segment of bacterial DNA, the T-DNA. The T-DNA contains genes that are active in the plant cell and encode hormone biosynthetic enzymes, or proteins that deregulate the cell's response to phytohormones. Study of this process has yielded not only knowledge of how alterations in phytohormone homeostasis can affect plant cell growth, but also has provided the essential tools to study phytohormone signaling in transgenic plants. Furthermore, T-DNA insertion into the plant genome forms the basis of gene tagging, a versatile method for isolating genes involved in phytohormone signal transduction and action.

INTRODUCTION

The study of the molecular basis of tumor initiation on dicotyledonous plants by the soil bacterium *Agrobacterium tumefaciens* has formed one of the foundations of plant hormone research. In the first instance, comparison of the growth characteristics of tumors with those of untransformed tissues confirmed the importance of phytohormones in tissue culture to promote growth of plant cells in vitro as well as triggering differing patterns of organogenesis. Subsequently, study of the genes encoded by *Agrobacterium* responsible for tumor

0066-4286/97/0901-0045$08.00

formation provided the first examples of genes encoding enzymes involved in the biosynthesis of the phytohormones, auxin and cytokinin. More recently, vectors based on the T-DNA have been developed for use in plant transformation mediated by *Agrobacterium* to study the effects of expression of genes encoding either phytohormone biosynthetic enzymes, or genes whose products affect the response of plant cells to phytohormones. Moreover, *Agrobacterium*-based vectors have been further developed as gene tags for use in insertional mutagenesis. These vectors can be used not only to create novel mutants changed in their response to phytohormones, but also to isolate the corresponding genes. In this review, rather than detailing the processes of *Agrobacterium*-mediated transformation or exhaustively describing the molecular basis of phytohormone action, we focus on how plant transformation vectors based on *Agrobacterium* have been applied to phytohormone research, describe some strategies adopted to investigate phytohormone action via transformation, and outline several lessons that have been learned.

T-DNA Transfer: Key to Studying the Molecular Basis of Phytohormone Action

Though many details of the transfer of *Agrobacterium* T-DNA to plant cell are unresolved, the major facets of the process have been identified (6, 13, 45, 49, 80, 118, 120). Relevant aspects of the process are as follows.

1. The transferred DNA (T-DNA) of the Ti plasmid is flanked by two 25-bp imperfect repeats and is transferred to plant cells by a mechanism resembling bacterial conjugation (15, 66);

2. The *vir* region of the Ti plasmid encodes the proteins required by *Agrobacterium* both for sensing the wounded plant cell and for transferring the T-DNA to the plant genome (118);

3. T-DNA is transferred to the plant cell as a single-stranded intermediate (110, 117) and inserts stably into the nuclear genome, preferably into transcribed sequences (60) by a method resembling illegitimate recombination (33, 74);

4. Following integration of the T-DNA into the genome, the T-DNA encoded genes become active, modify the normal phytohormone biosynthetic pathways (see later), and produce novel conjugates of organic acids and amino acids, or sugars called opines. Opines are metabolized exclusively by the infecting *Agrobacterium*.

Elements of this intriguing example of interkingdom DNA transfer have been used on the one hand to study the effects of the expression of genes affecting

the homeostasis of auxins and cytokinins in plant cells, and on the other, to develop transformation vectors for use to transfer foreign DNA to plants and to act as a gene tag.

Agrobacterium-Based Transformation Vectors

The Ti plasmid of *Agrobacterium* has been modified in various ways to use as a "gene ferry" for the transformation of plants. With the initial T-DNA–based transformation vectors, the cointegrative vectors, the genes to be introduced into the plant genome were first cloned into intermediate vectors. This step allowed manipulation of DNA in *Escherichia coli.* The intermediate vector was then transferred to *Agrobacterium*, where it was integrated within the left and right borders of the Ti-plasmid–derived cointegrative vector by homologous recombination between homologous sequences shared by both vectors. In the resulting cointegrate, most of the T-DNA was replaced by foreign DNA. Cointegrative vectors lack most, if not at all, of the tumor-inducing genes from the T-DNA, thus transformant plants carrying such constructs had a normal appearance. The prototype of these vectors is pGV3850 (119). In this vector, sequences between the left and right border, except the nopaline synthase gene, were replaced by pBR322. Homologous recombination with a pBR322-based intermediate vector resulted in integration of the gene of interest in the T-DNA of the Ti plasmid. A further development was the creation "split end vectors" (SEV) (27). Here, one T-DNA border is located on the Ti plasmid and the other is on the intermediate vector. After recombination, both recombined segments define the T-DNA and form one contiguous sequence on the Ti plasmid.

Cointegrative vectors have proven very useful, but they are difficult to manipulate and the products resulting from homologous recombination arise at low frequencies. However, the main advantage of this type of vector is its unique stability. Because cointegrative vectors are Ti plasmid derived, they are normally relatively stable in *Agrobacterium.*

A recent variant on a theme of cointegrative vectors is a "super-binary" vector based on a plasmid that allows the independent co-transformation of two T-DNAs to the plant cell (59). First a plasmid was constructed containing a bacterial selectable marker and origin of replication; the *vir*B, C, and G genes and a T-DNA containing a plant-specific hygromycin resistance gene. A second plasmid contains a region of homology to the first, a further bacterial antibiotic gene, and a second T-DNA containing the GUS marker gene (47a). Recombination between the two plasmids results in a plasmid containing 2 T-DNAs. When *Agrobacterium* with such a plasmid was used to transform tobacco and rice, up to 47% of the transformants contained both T-DNAs. At least half of these contained the T-DNAs at separate sites, raising the possibility of subsequently creating transgenics that lack antibiotic marker genes by genetic

segregation. This allows the creation of transgenic tissue in which unwanted foreign sequences, such as antibiotic resistance genes, may be removed.

Binary vectors are a further development of T-DNA based vectors. These vectors, now very popular, are currently used almost exclusively. Binary vector technology is based on the observation that the T-DNA 25-bp borders are all that are required to transfer the T-DNA into the plant genome, provided that the transfer capability from the *vir* region is supplied in *trans* (43). The prototype of a binary vector consists of replicons and selectable markers active in *Agrobacterium* and, for cloning purposes, in *E. coli,* and the left and right border regions of T-DNA between which the gene(s) of interest to be integrated into the plant are inserted. A wide variety of differing binary vectors are available (3, 8, 11, 17, 56, 58, 61, 63, 86, 91). In general, however, the T-DNA usually contains a marker active in plant cells that can be used to select the transformants. Many vectors contain polylinkers to ease cloning, or additional selectable markers or reporter genes to screen transformants, as well as expression cassettes to measure differing levels of gene expression. Several selectable marker genes have been used for selection in *Agrobacterium* or *E. coli.* The most popular selectable markers are resistance to kanamycin and ampicillin. Binary vectors differ in the use of the replicons active in *Agrobacterium.* Many first-generation binary vectors use the RK2 replicon (e.g. pBIN19; 11) or minimized derivatives of it (e.g. pPCV vectors; 63). Due to the limited functionality of mini RK2 plasmids in *Agrobacterium*, the vectors based on this replicon are inherently unstable and require selection to ensure the presence of plasmids in *Agrobacterium*. More recently developed binary vectors use replicons ensuring stability in *Agrobacterium* (17), derived, for example, from the *Pseudomonas* plasmid pVS1, or from the Ri plasmids of *Agrobacterium rhizogenes* (14). Such vectors are stably maintained over many generations in the absence of selection. Binary vectors are generally used in an *Agrobacterium* background containing a helper plasmid, that is disarmed Ti-plasmid in which the T-DNA region was deleted, or rendered nonfunctional. However, wild-type Ti plasmids also may be used (3). Binary vectors are introduced into *Agrobacterium* strains by direct DNA transfer [electroporation (116), or the freeze-thaw transformation method (44)], or by conjugation (19). The great advantage of binary vectors over cointegrative vectors lies in their improved ease in cloning and handling and their flexibility (i.e. free choice of *Agrobacterium* strains and helper plasmids). For example, BIBAC vectors have recently been developed to transfer large fragments of DNA ($>$150 kb) to the plant genome (36). The older type of binary vectors are relatively unstable and tend to become rearranged. However, this disadvantage is largely overcome with vectors using stable origins of replication. In routine plant transformation work with easily transformable plant species, plasmid instability usually does not present a major obstacle inasmuch

as the integration frequencies obtained with *Agrobacterium*-based methods are generally high and the loss of a few copies of vectors during the transformation process is relatively unimportant. For special applications or when plant species are more difficult to transform, however, the instability of the vectors may be significant. Nonetheless, binary vectors in *Agrobacterium* have been used to successfully transform rice (41), cassava (67), and maize (47)—plants traditionally thought to be "untransformable" by *Agrobacterium*.

Agrobacterium-Based Plant Gene Tagging Vectors

By the very nature of its insertion into the plant genome, the T-DNA, in addition to being considered as a transformation vector, can also be thought of as an insertional mutagen. Indeed, up to 30% of all T-DNA inserts disrupt host genes upon insertion into the genome (60). Thus, T-DNA can be considered as a gene tag: a known sequence that inserts into the genome, which subsequently allows reisolation of flanking plant DNA via hybridization, inverse PCR, or plasmid rescue (37). Traditionally, gene tagging in plants has been carried out using transposable elements in either homologous, or heterologous hosts (5, 114). More recently, however, T-DNA tagging in various forms has been employed to create mutations, including targeted phenotypes, and to isolate the affected genes (7, 23, 39, 62). To date, most success has been achieved in *Arabidopsis*, however, T-DNA tagging is also feasible in other species (see below).

T-DNA–based vectors have been used in many ways to induce mutations by gene tagging in plants. If integration occurs into a gene, or a transcriptional unit, the function will be destroyed and the transformant will become a heterozygous mutant. Since most mutations are recessive, the phenotype will become apparent only after self-fertilization in the next generation when homozygous plants are produced.

In the simplest tagging strategies, basic transformation vectors carrying the NPT II selectable marker gene were used to construct libraries, or populations of transformants, containing genes inactivated by T-DNA insertions (23). In more elaborate strategies, the tagging vector contains a reporter gene close to one of the T-DNA borders such that expression of this gene is directed by plant genomic sequences after integration. These strategies can lead to tagging of promoter or enhancer sequences, a process referred to as trapping (7, 62, 106, 111). Reporter genes used for this purpose were NPT II (4, 60, 108), GUS (7, 51, 106, 112), luciferase (48, 60), and more recently, green fluorescent proteins (GFP) (38). The obvious advantages of the promoter/enhancer trapping approach are that genes with particular expression patterns can be identified and that fusion with an active gene guarantees insertional inactivation of a gene, even in the absence of an obvious phenotype. In many cases, the tagging vectors contain a pBR322 replicon and the ampicillin selectable marker gene that is active in *E. coli* as

part of the T-DNA. This feature allows a relatively easy isolation of the plant DNA sequences flanking the T-DNA after integration by cutting the genomic DNA of the mutant with an enzyme cleaving outside of the T-DNA, religating and transforming bacteria.

Activation tagging is a different approach of T-DNA tagging. Instead of creating mutants by insertional inactivation, activation tagging produces a gain-of-function phenotype by deregulated expression of the tagged genes. Obvious advantages of this approach are that it can be used in polyploid species, that the mutation is dominant, and that more subtle changes can be generated than by gene disruption. Two different activation tagging strategies have been used: enhancement of expression and ectopic expression. In the first case, the activating vector contains enhancer sequences close to one T-DNA border (39). These enhancer sequences are expected to act over relatively long distances and to boost the expression from endogenous promoters, which would act weakly under normal conditions. In the second, an entire constitutive promoter is located at one of the T-DNA borders in an outward-pointing orientation (87). After integration, the tagged genes are directly under transcriptional control of this promoter and are ectopically expressed, depending on the promoter used for the tagging. Obviously, this approach more radically overrides the expression pattern of the tagged genes, but may not be effective since many promoters only work at limited distances. Clearly, both approaches have their advantages and both seem to work equally well in that plant genes have been isolated successfully by both gene activation tagging methods (28, 39, 88).

Transformation Strategies

Transformation strategies depend on the stable introduction of foreign DNA stably into the genome of a cell, followed by the regeneration of that cell into a whole plant. Two basic tissue culture methods for *Agrobacterium*-mediated transformation are cocultivation with tissue explants or protoplasts. Infection of tissue explants is generally a simple, reproducible, and reliable method for obtaining transgenic plants (46). Ease of handling makes this the method of choice when the aim is to produce a few transformants. The type of tissue used for infection depends greatly on the plant species to be transformed. For example, for tobacco and petunia the preferred tissue is leaf disks (46), whereas infection of root explants gives demonstrably better results with *Arabidopsis thaliana* (34).

Gene tagging experiments require the production of large numbers of individual transformants. One example is *Agrobacterium* protoplast cocultivation, where tobacco mesophyll protoplasts are incubated with excess *Agrobacterium* prior to callus induction (18, 70). The high transformation frequencies and the simplicity with which large numbers of individuals can be handled with this

method allow millions of transformants to be generated with relative ease. Another development for the mass transformation of *Arabidopsis* is cocultivation with single cells or small cell aggregates derived from suspension cultures (72). A major disadvantage of transformation procedures relying on tissue culture, however, is the generation of somaclonal variation, i.e. the generation of genomic alterations that may lead to phenotypic mutations. This problem is especially troublesome after regeneration of transgenic plants from protoplasts. Thus methods of transformation that avoid the need for tissue culture have gained in importance.

In planta transformation, i.e. the creation of transgenic plants in the absence of tissue culture, holds the promise of creating plants without the risk of somaclonal effects. However, to date this has only proved feasible with *Arabidopsis.* Initially this method involved inbibing seeds in a suspension of *Agrobacterium*. The resultant plants were selfed and transformants were selected from germinating progeny (24). This approach was used to create the first populations of T-DNA–tagged *Arabidopsis* lines (23, 26). Important in this method were the culture conditions adopted and the use of an *Agrobacterium* containing a cointegrative vector, presumably because of its stability. Although this approach has been valuable in creating T-DNA–tagged plant populations, a large proportion of all mutations seen are not T-DNA tagged. The reason is not clear, though abortive T-DNA insertion causing a "footprint" is the most likely explanation. More recently, vacuum infiltration transformation of *Arabidopsis* has been widely adopted to create transgenics (7); flowering plants are placed in a suspension of *Agrobacterium* and subjected to a vacuum. The *Agrobacterium* enters the plant and probably transforms the egg cells, or their progenitors, with the result that the transformants can be screened by germination of selfed progeny. This technique appears to be generally applicable in creating transgenic *Arabidopsis*, and with scaling up it is feasible to create T-DNA–tagged populations. It is important, however, to use a transformation vector with an origin of replication allowing stability of the transformation vector in the bacterium.

Knock-Out Insertional Mutagenesis

T-DNA tagging has been used effectively in *Arabidopsis.* Several novel functions involved in the biosynthesis and signaling pathways of plant hormones have been uncovered by using T-DNA tags. These include an amino acid permease encoded by the *AUX1* gene regulating auxin-dependent root growth (10), the *RCN* gene–encoded protein phosphatase 2A subunit affecting auxin transport (31), as well as the *CTR1* constitutive triple ethylene response mutation in a gene coding for a Raf-kinase homolog (52), and the hookless ethylene mutant defining an N-acetyltransferase gene (65). Moreover, a T-DNA tag in the

GA4 gene has identified a hydroxylase in the gibberellin biosynthesis (16), and T-DNA tagging of the *CPD* locus coding for the CYP90 hydroxylase provided proof for an essential role played by steroid hormones in plants (107).

Activation Tagging

Activation tagging relies on the deregulated expression of genes flanking the T-DNA insertion. The dominant mutation obtained allows selection for a predetermined phenotype. To date, this approach has been used to create tobacco protoplast lines able to grow in the absence of auxin (39); cytokinin (77), or in the presence of an inhibitor of polyamine biosynthesis (28); as well as a mutant callus of *Catherostigma plantagineum* changed in response to dessication (29); and *Arabidopsis* callus able to form shoots in the absence of cytokinins (50). The genes tagged include axi1, which affects gene expression (115); cyi1, a small peptide growth factor (77) and cki1, a two-component receptor kinase (50); and factors involved in the regulation of seed maturation (88).

Use of Plant Transformation Vectors to Study Phytohormone Action

Studies of plants engineered to express genes involved in hormonal action/response can effectively be divided into two types: (*a*) those where the function of the gene product is known and the interest lies in the effects of its expression in intact plant tissue; (*b*) those where the function of the gene is unknown and the goal is to elucidate the potential function of the gene product. Although transgenic approaches have shed considerable light on phytohormone action, the results obtained are often inconclusive. Since the function of individual genes in phytohormone synthesis/perception has been adequately covered in recent reviews (42, 53, 57), we concentrate instead on the experimental strategies that have been adopted when using transgenic material to study the effects of the expression of these genes.

Genes Encoding Proteins Involved in Phytohormone Biosynthesis or Perception

Studies of the molecular basis of phytohormone action/response in plants are based firmly on studies involving phytopathogenic, symbiotic, or free-living bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* (78). These bacteria encode genes that induce disorganized growth on infected plant tissue by either modifying the active levels of auxins and/or cytokinins in the cell, or by deregulating different responses to phytohormones. In addition, the *rol* genes have been isolated from *Agrobacterium rhizogenes* (98, 104). These genes, which play a role in inducing root formation in infected tissue, are thought to be involved also in modulating phytohormone action. The genes

encoded by bacteria were the first to be characterized affect the response of plant cells to phytohormones, and they have been studied in most detail. The results to date can be summarized as follows.

1. Expression of phytohormone biosynthetic genes results in phytohormone auxotrophy;

2. Auxin can be synthesized from tryptophan, via indoleacetamide, though this does not appear to be the pathway used by plants; and

3. Expression of differing oncogenes in callus can lead to differential organogenesis, i.e. root, or shoot induction.

More recently, plant genes involved either in phytohormone biosynthesis/action have been cloned, generally by biochemical analysis, or, as previously described, increasingly by mutant characterization.

Constitutive Overexpression of Genes

With the genes involved in phytohormone biosynthesis/action in hand, routinely the next step is to link them to a promoter directing constitutive overexpression, introduce them into a novel plant host, and study the effect of the expression of the transgene. Normally, the 35S RNA promoter from cauliflower mosaic virus (CaMV) is used. This promoter and elements thereof are relatively well characterized and are generally found to be active in most cell types (9).

The earliest experiments investigating the overexpression of hormone biosynthetic genes involved linking *iaaH* and *iaaM* to the 35S RNA and 19S RNA promoters of CaMV, respectively, and transferring them to petunia (55). *iaaM* and *iaaH* are encoded by the T-DNA of *A. tumefaciens* and convert tryptophan to indoleacetamide (IAM) and IAM to indole-3-acetic acid (IAA), respectively (96, 109). Overexpression of *iaaH* has no apparent morphogenic effect on transgenic plants, though explants converted the IAM analog napthalene acetamide (NAM) to NAA. Overexpression of *iaaM* resulted in up to a tenfold excess of IAA and changes in phenotypes consistent with overproduction of auxin and ethylene. As noted above, plants apparently do not synthesize IAA from tryptophan via indoleacetamide. Thus, the increase in IAA from the action of *iaaM* most likely results from the action of a nonspecific aminohydrolase. In addition, the lack of effect seen with *iaaH* probably is a consequence of there being little, or no, substrate indoleacetamide available for conversion.

Manipulation of internal levels of active auxins in transgenics has been elegantly demonstrated by Klee and co-workers in their investigations of the effects of overexpression of *iaaL*. This bacterial gene encodes a IAA-lysine synthetase (89), which conjugates active IAA to lysine and produces an inactive conjugate. When introduced into plants and linked to the 35S RNA promoter, expression

of *iaaL* results in up to a 19-fold reduction in free IAA (92) and produces a plant with reduced apical dominance, reduced rooting, and inhibition of vascular differentiation. Interestingly, these plants appear to attempt to overcome the decrease in active auxin by increases in auxin synthesis (92). Similar results were found in transgenic potato, where reduced internodal length and petiole epinasty were observed (103). The phenotypic effects observed with *iaaL* overexpression can be reversed by crossing with plants overexpressing *iaaM* (92).

A key enzyme in cytokinin biosynthesis, *ipt*, has been isolated both from *A. tumefaciens* and *Pseudomonas* species. It catalyzes the condensation of adenosine monophosphate and isopentyl pyrophosphate to form the cytokinin isopentyl adenosine monophosphate (IPA) (2). Overexpression of the *ipt* in transgenic tissue results in plantlets with reduced stature, smaller curled leaves, and lack of apical dominance. Generally, shooting is enhanced from disorganized callus because of increases of endogenous cytokinins (101). However, a common trait is the inability to form roots, and thus transgenics have been difficult to study. In potato, grafting of tissue overexpressing *ipt* is possible (84), which indicates that increased cytokinin is limited to tissue where it is synthesized. Overexpression of *ipt* in tobacco can lead to auxin-independent growth of tissue from explants despite there being no increase in endogenous auxin levels (12). This intriguing result suggests that increased endogenous levels of cytokinins can offset auxin requirement.

The studies with transgenic tissue overexpressing *ipt* have served to confirm observations obtained by external application of cytokinins to plant tissues. In contrast, studies of the effects of overexpression of the *rol* genes of *Agrobacterium rhizogenes* have raised more questions concerning *rol* gene action than they have answered.

The *rolA*, *B*, and *C* are encoded by the T-DNA of *A. rhizogenes* (98, 104). Independent expression of each results in the induction of root growth when bacteria containing them are inoculated onto test plants. Linking the *rol* genes to the 35S RNA promoter and transferring them to tobacco results in the plants displaying more pronounced phenotypes than those seen when the genes are linked to their own promoters: *rolA*, dwarfing, leaf wrinkling, delayed flowering, and flower malformation; *rolB*, altered leaf and flower heterostyle, increased formation of adventitious roots or stems, and leaf necrosis; *rolC*, reduced apical dominance, pale lanceloate leaves, and small male sterile flowers (102). How the action of these genes result in the differing phenotypes is still a matter of debate. Measuring auxin and cytokinin levels in *rolA* overexpressing plants does not reveal significant change, though the auxin/cytokinin balance seems functionally altered in favor of cytokinin (95). Interestingly, the effects of *rolA* overexpression correlate with the accumulation of conjugates between polyamines and hydroxycinnamic acids (105). Overexpression of *rolB* in plants produces

phenotypes indicative of increases in auxin activity. However, the transgenes do not appear to have an effect on the free pool of IAA nor on its metabolism, which suggests that the effect is indirect (81, 95). It may be relevant that isolated cells from *rolB* overexpressing plants are more sensitive to auxin (73) and grow in an auxin-independent manner (115). Recently, it has been shown that *E. coli* overexpressing *rolB* exhibits increased tyrosine phosphatase activity (25). Plants overexpressing *rolC* have changes in both cytokinin and gibberelin levels (82, 95).

The molecular basis of ethylene biosynthesis and action has received much attention (85). The key enzymes of ethylene biosynthesis: aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase have been isolated and characterized (35, 93). Downregulation of ethylene accumulation has been achieved by engineering the overexpression of ACC deaminase (54). This enzyme, which is not encoded by plants, has been isolated from *Pseudomonas*, and it degrades ACC to alpha-ketobutyric acid (54, 97). Ethylene production in fruit from homozygous ACC deaminase plants was reduced up to 97% during ripening. Moreover, fruit attached to the plant showed a reduced rate of softening and a delay in abscission.

Controlling Transgene Expression

In contrast to constitutive overexpression of a transgene, controlled expression provides the opportunity to specifically induce expression in a predetermined tissue and follow the effects of transcript accumulation and increases of enzyme activity. A more precise view may thus be obtained of the effect of expression of a particular gene. Moreover, controlled expression overcomes the concern over the potentially detrimental and nonspecific effect of high levels of transgene expression during the process of transformation and regeneration, or that it might produce a phenotype that hinders further analysis of the tissue, for instance, sterility. Several approaches have been described to control transgene expression, with varying levels of success.

Heat Induction of Expression

Because overexpression of *ipt* results in reduced root growth and thus makes it difficult to study the effects of its expression in whole plants, several groups have linked the gene to heat shock promoters to study the effects of induction of the gene in transgenic tissue (1, 75, 94, 99, 100). Success has been mixed. Linking the *ipt* gene to the maize *hsp70* promoter and transferring it to *Arabidopis* resulted in plants with background (i.e. uninduced) expression of *ipt* that produced a threefold increase in zeatin-riboside and a sevenfold increase in zeatin monophosphate (75). The resulting plants were smaller than nontransformed and displayed reduced apical dominance, leaf size, and root growth.

Heat induction of the gene increased levels of zeatin 30-fold, and zeatin riboside and monophosphate were increased 50- and 20-fold, respectively, but the phenotypic changes were not enhanced. This suggests that the response of plants to cytokinins is already saturated at relatively low levels of the phytohormone. Similar results have been reported for tobacco transformed with a soybean heat shock promoter linked to the *ipt* gene (99). Once again, leaky expression of *ipt* resulted in changed phenotypes in the absence of induction, though the induction of *ipt* expression resulted in delayed senescence. Probably a tighter control of expression of the *ipt* gene appeared to result when the gene was linked to a heat shock promoter from *Drosophila* (94). In this case, uninduced transformants were phenotypically normal. Following heat treatment, callus was able to grow in vitro in the absence of exogenously applied cytokinin, and regenerated plants displayed the phenotypes typical of cytokinin overproduction.

Tissue-Specific Expression of Transgenes

Several reports have described the linkage of *ipt* to promoters displaying tissue, or organ-specific expression. Li and coworkers (68) linked *ipt* to a bidirectional SAUR promoter. The soybean SAUR promoter is expressed in the epidermal and cortical cells of elongating hypocotyls and epicotyls. The bidirectional promoters were linked in one direction to GUS, to monitor expression, in the other direction to *ipt.* Resultant plants expressing GUS displayed many of the phenotypes expected from *ipt* expression. These phenotypes apparently are due to the direct action of cytokinins as well as the mobilization of plant nutrients to tissues rich in cytokinins.

An interesting example of controlling expression, and at the same time demonstrating that cytokinin is an important controlling factor in senescence, involved linking the *ipt* gene to a senescence-specific promoter from *Arabidopsis* and transferring it to tobacco (30). Expression of the *ipt* gene is apparently tightly controlled in these plants. The effects of *ipt* action were only observed at the onset of senescence when the promoter is activated. The increase in cytokinin content prevents further senescence. This in turn attenuates expression from the promoter and prevents overproduction of cytokinin. The overall effect is that senescence is significantly delayed in these plants.

In a similar experiment, the *ipt* gene has been linked to a fruit-specific promoter and transferred to tomato (69). Transgenic plants appeared normal until fruit maturation and ripening. Fruit had islands of green pericarp tissue against a background of red tissue. In fruit, cytokinin levels were 10–100 times higher. Intriguingly, although no *ipt* mRNA could be detected in mature leaf tissue, cytokinin levels were fourfold higher than normal and were apparently sufficiently high to induce pathogen-responsive gene expression.

Control by Transposon Excision

To overcome the difficulties of the effect of background transgene expression a system has been developed that makes expression dependent on transposon excision. Here the *ipt* gene is linked to the 35S RNA promoter and *Ac*, the maize autonomous transposable element, is inserted into the nontranslated leader sequence. The presence of the *Ac* element inhibits expression. The whole cassette was transferred to tobacco. Somatic excision of *Ac* results in a mosaic plant where regions of cells express *ipt* in a background of nonexpressing regions (21). Mosaic plants had reduced apical dominance and extensions at the leaf blade. These extensions contained higher levels of cytokinins. In addition, the tips of the leaves formed adventitious buds, most probably resulting from the accumulation of cytokinins. The buds were either vegetative or floral. In the latter case, increased cytokinins correlate with a decrease of the steady-state levels of floral homeotic genes (20). Such vivipary demonstrates the ability of high levels of cytokinins to trigger reprogramming of development from apparently terminally developed tissue.

The Tet Repressor System

This system relies on two components: constitutive expression of the *tet* repressor isolated from the bacterial transposon *Tn*10, linked to the 35S RNA promoter, and the test gene linked to the 35S RNA promoter, which is interrupted by two *tet* operator sequences (32). Under normal circumstances, when both components of the system are present in the same cell, the tet repressor binds to its operator, and inhibits expression of the test gene. In the presence of tetracycline the repressor is removed, allowing expression. The combination of repressor expression cassette and the test gene construct can be produced by crossing two lines, each carrying the individual constructs, or by transforming the test construct directly into a line already containing the repressor cassette.

The feasibility of the *tet* system has been demonstrated by using the *rolB* gene as a test construct in tobacco (90). In the absence of tetracycline, the plants were phenotypically normal, but following induction, high levels of *rolB* transcript were found and the induced tissues displayed characteristic *rolB* phenotypes: necrotic and wrinkled leaves, stunted growth, and lack of a floral meristem. These phenotypes could be reverted to normal upon removal of the tetracycline. This experiment is of interest because it shows that the *rolB* phenotype is reversible and that the effect of *rolB* expression is apparently limited to the tissues in which the gene is expressed.

In other studies where the *tet* repressor system is used to control the expression of the *ipt* gene, increases in cytokinin levels are accompanied by increases in cytokinin oxidase activity (79). Thus, cytokinin oxidase activity may be substrate induced and this may contribute to cytokinin homeostasis. Similarly,

the *tet* repressor system has been used to study the controlled expression of *rolC* (22). Upon induction of the gene the plants developed all the symptoms of the *rolC* syndrome. After release of induction, plants grew similarly to normal, indicating that the *rolC* product is permanently required for the establishment of the *rolC* phenotype.

Further examples of the work of the *tet* system are provided by studies to assess the role of polyamines in plant growth and development. The polyamine putrescine is produced in plants by the action of either ornithine decarboxylase (ODC) or arginine decarboxylase (ADC). Putrescine in turn is converted to spermidine and spermine as a consequence of the action of S-adenosylmethionine decarboxylase (SAMDC). Kumar and coworkers linked the potato SAMDC gene to the *tet* repressor system and studied the effects of its induction in potato leaf explants. A sixfold increase in SAMDC transcript level was observed following tetracycline induction along with increases in levels of both SAMDC activity and levels of the polyamines spermidine, spermine, and putrescine. These results add weight to the notion that the *tet* system can be used to manipulate a biochemical pathway, but because it is carried out in leaf disks, it unfortunately does not shed light on what may occur in the whole plant. Difficulties in the uptake of tetracycline in the hydroponic system used to feed the potato plants have limited investigation in the whole plant.

More success with the *tet* repressor system to study polyamine biosynthesis has been achieved in tobacco containing the oat ADC gene (71). After whole plant induction, both ADC activities and putrescine levels increased, and these increases correlated with morphological effects such as leaf wrinkling, reduced root growth, and a delay in flowering time. Though these results do not definitively link polyamines to an active role in these processes in normal development, they do provide evidence that manipulating internal levels of polyamines can affect plant growth.

Promoter Tagging

The effect of a gene modifying the response of the cell to phytohormones is likely to differ depending on cell type and age (113). This differing effect may be checked by linking the gene under investigation to promoters directing expression in differing tissues and studying the effects of expression in transformed plants. One imaginative way of achieving this is to link a promoterless gene, in this case *ipt,* to the right border of the T-DNA and carry out promoter tagging (40). In this example, some of the 85 differing transgenic lines studied contained concentrations of cytokinins, expressed as zeatin riboside equivalents that increased up to sevenfold. To differing degrees, the plants displayed traits indicative of increased levels of endogenous cytokinins: reduced stem and root growth, reduced apical dominance and leaf surface, as

well as retarded leaf senescence. There was apparently no correlation between the severity of the *ipt* phenotypes observed and mRNA levels in the individual transgenics. Thus other factors, possibly the capacity of differing cells to achieve cytokinin functional homeostasis (e.g. by conjugation), may play a role. In addition, cytokinin-induced alterations were observed in otherwise normal plants. This finding suggests that increases in levels of active cytokinins may remain localized in the plants.

Antisense Expression

The aim of experiments involving antisense expression is, in contrast to overexpression, to downregulate expression of test genes. The test gene is cloned in a reverse orientation to a strong promoter, again usually the CaMV 35S RNA promoter, and transferred to a plant. A number of transformants usually need to be screened to observe an antisense effect. The mechanism by which antisense repression acts remains a matter of debate (76), but it is possible to recover plants displaying antisense effects to a varying level, which can help the interpretation of the experiment.

The effect of antisense inhibition of a hormonal response has been demonstrated most graphically in experiments involving tomato ripening. Ethylene plays a central role in fruit ripening (85) and its two-step biosynthetic pathway eases molecular manipulation. Use of antisense technology to identify a gene product by reverse genetics is well illustrated by the case of pTOM13 (35). This clone was isolated from a library constructed using RNA from ripening tomato, but its identity initially was unknown. Tomato plants containing pTOM13 cloned in antisense were inhibited in ethylene production, and in homozygous plants ACC oxidase activity was reduced by 93%. This result provided the necessary clue that pTOM13 in fact encoded ACC oxidase. The dramatic effect of inhibiting ethylene production by antisense technology is also demonstrated by the effect of antisense expression of ACC synthase (83). ACC synthase is the rate limiting step in ethylene biosynthesis and the enzyme itself has a relatively short half life (25 min), which makes it an ideal target for antisense technology. In 34 tomato transformants, 3 showed marked inhibition of ethylene biosynthesis and fruit ripening. Fruits from the plants in air never turn red or soft, nor do they develop an aroma. This inhibition can be reversed by the application of ethylene. Apart from the biotechnological significance of these experiments, the results show that ethylene controls the climateric rise of respiration and the biochemical changes such as fruit softening, ripening, and aroma development that are associated with it.

Antisense expression has also been used to investigate the effect of reducing polyamine biosynthesis by downregulating SAMDC activity (64). Transgenic potato expressing SAMDC in antisense display a variety of novel traits

including stunted growth, shorter internodes, increased branching, and smaller leaves. The plants contained lower levels of all polyamines. Interestingly, the plant displaying the largest decrease in mRNA levels, enzyme activity, and polyamines had a 46-fold decrease in ethylene evolution compared with wild-type. Ethylene production and polyamine biosynthesis share S-adenosyl methionine as a precursor, thus this observation lends support to the notion of a competitive interaction between ethylene and polyamine biosynthesis. Though the regenerated plants studied in this case shared some traits indicative of ethylene overproduction, there are differences suggesting that they result from the combined effect of depleted polyamine levels and elevated levels of ethylene.

CONCLUSIONS

The study of the molecular basis of tumor formation by the T-DNA of *Agrobacterium* has provided a unique opportunity to study the molecular basis of phytohormone action. In the first instance, the T-DNA itself yielded a variety of genes involved either in the synthesis of, or in the modulation of the response of the plant cell to, phytohormones. Furthermore, study of the mechanism of T-DNA transfer has provided the necessary molecular tools to create transformation vectors not only to carry foreign DNA into plant cells to assess the effect of expression of a specific transgene, but also to isolate plant genes playing a role in phytohormone action by gene tagging.

From the study of bacterial genes involved in modifying the plants' response to phytohormones one might have thought that having access to the bacterial genes would have allowed the isolation of plant homologs via hybridization. This has not been the case. Presumably, either the biochemical pathways encoded by the bacteria are not represented in plants (as say, in the case of *iaaH* and *iaaM*), or homologies are not sufficient to detect plant genes. Nevertheless, studies of the T-DNA genes have yielded several important points: (*a*) expressed as transgenes, bacterial genes can modify the internal content of active phytohormones; (*b*) conjugation/conjugate hydrolysis, as previously proposed, is important in modifying active phytohormone levels in growing plants; (*c*) manipulation of expression of transgenes can radically affect plant morphology and produce novel phenotypes of agronomic relevance. However, many gaps in our knowledge remain. What, for example, is the function of the *rol* genes? Despite the availability of the genes and the dramatic phenotypes produced when they are expressed as transgenes, debate still surrounds their function. This is a cautionary tale to all who assume that gene sequence and detailed biochemical analysis can explain the function of a gene product.

One advantage, it is suggested, of studying the effects of the expression of genes involved in phytohormone action/response, compared with external

phytohormone application, is the absence of problems associated with phytohormone uptake. This supposition is, of course, true, although transgene expression also has its limitations: It is often hard to judge in which cells transgene expression occurs; and it is difficult to accurately assess the amount of enzymatic activity and product in whole tissues. Moreover, the promoters used are likely to be controlled in a positive or negative manner by endogenous phytohormone levels. In general, the phenotypes observed in transgenics overexpressing a specific gene modifying phytohormone response resemble what might be predicted by external phytohormone action.

A persistent limitation in the study of the effects of transgene expression is controlling precisely the location and levels of transgene expression. As we have seen, success with precisely controlling transgene expression has been limited. Most progress has perhaps been made with the *tet* repressor system. As demonstrated with the use of *rolB*, *rolC*, SAMDC, and *ipt*, the system allows the induction of expression, accompanied by increases in transcript level and enzyme activity, followed by scoring for phenotypic effect.

T-DNA tagging, either by gene knock-out or by gene activation, is a versatile means of gene isolation limited only by our imagination in devising a selection scheme. However, having the gene in hand does not necessarily explain function. In such cases, function can only be discerned by an integrated approach involving biochemistry, physiology, and genetics. Thus, despite great progress in phytohormone research, particularly with Ti-plasmid-derived vectors, much remains to be done and exciting opportunities abound.

Literature Cited

1. Ainley WM, McNeil KJ, Hill JW, Lingle WL, Simpson RB, et al. 1993. Regulatable endogenous production of cytokinins up to "toxic" levels in transgenic plants and plant tissues. *Plant Mol. Biol.* 22:13–24
2. Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Nat. Acad. Sci. USA* 81:5994–98
3. An G, Ebert P, Mitra A, Ha SB. 1988. Binary vectors. In *Plant Molecular Biology Manual*, ed. SB Gelvin, RA Schilperoort, 1:A3:1–19. Dordrecht: Kluwer
4. André D, Colau D, Schell J, Van Montagu M, Hernalsteens J-P. 1986. Gene tagging in plants by a T-DNA insertion that generates APH(3′)II plant gene fusions. *Mol. Gen. Genet.* 204:512–18
5. Baker B, Schell J, Lörz H, Federoff N. 1986. Transposition of the maize controlling element Activator in tobacco. *Proc. Natl. Acad. Sci. USA* 83:4844–48
6. Baron C, Zambryski PC. 1995. Notes from the underground: highlights from plant microbe interactions. *TIBTECH* 13:356–62
7. Bechtold N, Ellis J, Pelletier G. 1993. In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. Paris* 316:1194–99
8. Becker D, Kemper E, Schell J, Masterson R. 1992. New plant binary vectors with selectable markers located proximal

to the left T-DNA border. *Plant Mol. Biol.* 20:1195–97
9. Benfey PN, Chua N-H. 1990. The cauliflower mosaic virus promoter: combinatorial regulation of transcription in plants. *Science* 250:959–66
10. Bennett MJ, Marchant A, Green HG, May ST, Ward SP, et al. 1996. *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. *Science* 273:948–50
11. Bevan M. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* 12:8711–21
12. Binns AN, Labiola J, Black RC. 1987. Initiation of auxin autonomy in *Nicotiana glutinosa* cells by cytokinin-biosynthesis gene from *Agrobacterium tumefaciens. Planta* 171:539–48
13. Binns AN, Thomashow MF. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. *Annu. Rev. Microbiol.* 42:575–606
14. Bouchez D, Camilleri C, Caboche M. 1993. A binary vector based on Basta resistance for in planta transformation of *Arabidopsis thaliana. C. R. Acad. Sci. Paris* 316:1188–93
15. Bundock P, Dendulkras A, Beijersbergen A, Hooykaas PJJ. 1995. Transkingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae. EMBO J.* 14:3206–14
16. Chiang H-H, Hwang I, Goodman H. 1995. Isolation of the *Arabidopsis GA4* locus. *Plant Cell* 7:195–201
16a. Davies PJ, ed. 1995. *Plant Hormones.* Dordrecht: Kluwer
17. Deblaere R, Reynaerts A, Höfte H, Hernalsteens J-P, Leemans J, et al. 1987. Vectors for cloning in plant cells. *Methods Enzymol.* 153:277–92
18. Depicker AG, Herman L, Jacobs A, Schell J, Van Montagu M. 1987. Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium* plant cell interaction. *Mol. Gen. Genet.* 201:477–84
19. Ditta G, Stanfield S, Corbin D, Helinski D. 1981. Broad host range cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. USA* 77:7347–51
20. Estruch JJ, Granell A, Hansen G, Prinsen E, Redig P, et al. 1993. Floral development and expression of floral homeotic genes are influenced by cytokinins. *Plant J.* 4:379–84
21. Estruch JJ, Prinsen E, Fladung M, Schulze SC, Van Onckelen, et al. 1991. Viviparous leaves produced by somatic activation of an inactive cytokinin synthesizing gene. *Science* 254:1364–67
22. Faiss M, Strnad M, Redig P, Dolezal K, Hanus J, et al. 1996. Chemically induced expression of the *rolC*-encoded ß-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: *rolC* does not hydrolyze endogenous cytokinin glucosides in planta. *Plant J.* 10:33–46
23. Feldmann KA. 1991. T-DNA insertional mutagenesis in *Arabidopsis*: mutational spectrum. *Plant J.* 1:71–82
24. Feldmann KA, Marks MD. 1987. *Agrobacterium* mediated transformation of germinating seeds of *Arabidopsis thaliana*—a non-tissue culture approach. *Mol. Gen. Genet.* 208:1–9
25. Filippini F, Rossi V, Marin O, Trovato M, Downey PM, et al. 1996. The *rolB* plant oncogene is a tyrosine phosphatase. *Nature* 379:499–500
26. Forsthoefel NR, Wu Y, Schultz B, Bennet MJ, Feldmann KA. 1992. T-DNA insertion mutagenesis in *Arabidopsis*: prospects and perspectives. *Aust. J. Plant Physiol.* 19:353–66
27. Fraley RT, Rogers SG, Horsch RB, Eichholtz DA, Flick JS, et al. 1985. The SEV system: a new disarmed Ti plasmid vector system for plant transformation. *Bio/Technology* 3:629–35
28. Fritze K, Czaja I, Walden R. 1995. T-DNA tagging of genes influencing polyamine metabolism: isolation of mutant plant lines and rescue of DNA promoting growth in the presence of a polyamine biosynthetic inhibitor. *Plant J.* 7:261–71
29. Furini A, Bartels D, Salamini F. 1995. T-DNA tagging of a gene inducing desiccation tolerance in *Craterostigma plantagineum.* In *Current Issues in Plant Molecular and Cellular Biology,* ed. M Terzi, R Cella, A Falavigna, pp. 513–18. Dordrecht: Kluwer
30. Gan S, Amasino R. 1995. Inhibition of senescence by autoregulated production of cytokinin. *Science* 270:1986–88
31. Garbers C, Delong A, Deruere J, Bernasconi P, Söll D. 1996. A mutation in a protein phosphatase 2A regulatory subunit A affects auxin transport in *Arabidopsis. EMBO J.* 15:2115–24
32. Gatz C, Frohberg C, Wendenburg R. 1992. Stringent repression and homogeneous de-repression by tetraccline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant J.* 2:397–404
33. Gheysen G, Villarroel R, Van Montagu M. 1991. Illegitimate recombination in

plants—a model for T-DNA integration. *Genes Dev.* 5:287–97
34. Grevelding C, Fantes V, Kemper E, Schell J, Masterson R. 1993. Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. *Plant Mol. Biol.* 23:847–60
35. Hamilton AJ, Lycett GW, Grierson D. 1990. Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature* 346:284–87
36. Hamilton CM, Frary A, Lewis C, Tanksley SD. 1996. Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proc. Natl. Acad. Sci. USA* 93:9975–79
37. Hanahan D, Lane D, Lipsich L, Wigle M, Botchan M. 1980. Characterisitics of an SV40-plasmid recombinant and its movement into and out of the genome of a murine cell. *Cell* 21:127–39
38. Hasseloff J, Siemerling K, Hodge S, Golbik R, Prasher D. 1996. The green fluorescent protein gene must be modified for use as a vital marker in *Arabidopsis thaliana. Abstr. Int. Conf. Arabidopsis Res., 7th, Norwich, England*
39. Hayashi H, Czaja I, Lubenow H, Schell J, Walden R. 1992. Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. *Science* 258:1350–53
40. Hewelt A, Prinsen E, Schell J, Van Onckelen H, Schmülling T. 1994. Promoter tagging with a promoterless *ipt* gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. *Plant J.* 6:879–91
41. Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6:271–82
42. Hobbie L, Timpte C, Estelle M. 1994. Molecular genetics of auxin and cytokinin. *Plant Mol. Biol.* 26:1499–519
43. Hoekema A, Hirsch PR, Hooykaas PJ, Schilperoort RA. 1983. A binary plant vector strategy based on the separation of the vir and T-region of *Agrobacteria*. *Nature* 303:79–81
44. Holsters M, de Waele D, Depicker A, Messens E, van Montagu M, et al. 1978. Transfection and transformation of *Agrobacterium tumefaciens. Mol. Gen. Genet.* 163:181–87
45. Hooykaas PJJ. 1989. Transformation of plant cells via *Agrobacterium. Plant Mol. Biol.* 13:327–36
46. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, et al. 1985. A simple and general method for transferring genes into plants. *Science* 227:1229–31
47. Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, et al. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens. Nat. Biotechnol.* 14:745–50
47a. Jefferson RA, Kavanaugh TA, Bevan MW. 1987. GUS-fusions beta glucoronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6:3901–7
48. Jianmg C, Langridge WHR, Szalay AA. 1993. Isolation of plant promoters by insertional activation of a promoterless bacterial luciferase gene. *Int. Symp. Biolumin. Chemilumin., 7th, Banff, Alberta, Canada*
49. Kado CI. 1991. Molecular mechanisms of crown gall tumorigenesis. *Crit. Rev. Plant Sci.* 10:1–32
50. Kakimoto T. 1996. CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274:982–85
51. Kertbundit S, De Greve H, Deboeck F, Van Montagu M, Hernalsteens J-P. 1991. In vivo random ß-glucuronidase gene fusions in *Arabidopsis* thaliana. *Proc. Natl. Acad. Sci. USA* 88:5212–16
52. Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR. 1993. The ethylene response pathway in *Arabidopsis thaliana* is negatively regulated by *CTR1*, a predicted member of the Raf familiy of protein kinases. *Cell* 72:427–41
53. Klee HJ, Estelle M. 1991. Molecular genetic approaches to plant hormone biology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:529–51
54. Klee HJ, Hayford MB, Kretzmer KA, Barry GF, Kishore GM. 1991. Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell* 3:1187–93
55. Klee HJ, Horsch RB, Hinchee MA, Hein MB, Hoffman NL. 1987. The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. *Genes Dev.* 1:86–96
56. Klee HJ, Horsch R, Rogers S. 1987. *Agrobacterium* mediated transformation and its further applications to plant biology. *Annu. Rev. Plant Physiol.* 38:467–86
57. Klee HJ, Lanahan MB. 1995. Transgenic plants in hormone biology. See Ref. 16a, pp. 340–53

58. Klee HJ, Yanofsky MF, Nester EW. 1985. Vectors for transformation of higher plants. *Bio/Technology* 3:637–42
59. Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant J.* 10:165–74
60. Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Körber H, et al. 1989. High-frequency T-DNA-mediated gene tagging in plants. *Proc. Natl. Acad. Sci. USA* 86:8467–71
61. Koncz C, Martini N, Szabados L, Hrouda M, Bachmair A, et al. 1994. Specialised vectors for gene tagging and expression studies. In *Plant Molecular Biology Manual*, ed. SB Gelvin, RA Schilperoort, B2:1–22. Dordrecht/Boston/London: Kluwer
62. Koncz C, Németh K, Rédei GP, Schell J. 1992. T-DNA insertional mutagenesis in *Arabidopsis. Plant Mol. Biol.* 20:963–76
63. Koncz C, Schell J. 1986. The promoter of Tl-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. *Mol. Gen. Genet.* 204:383–96
64. Kumar A, Taylor M, Mad Arif SA, Davies H, 1996. Potato plants expressing antisense and sense S adenosylmethionine decarboxylase SAMDC transgenes show altered levels of polyamines and ethylene: antisense plants display abnormal phenotypes. *Plant J.* 9:147–58
65. Lehman A, Blach R, Ecker J. 1996. Hookless 1, an ethylene response gene is required for differential cell elongation in the *Arabidopsis* hypocotyl. *Cell* 85:183–94
66. Lessl M, Lanka E. 1994. Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. *Cell* 77:321–24
67. Li H-Q, Sautter C, Potrykus I, Puonti-Kaerlas. 1996. Genetic transformation of cassava (*Manihot esculenta Crantz*). *Nat. Biotechnol.* 14: 736–40
68. Li Y, Hagen G, Guilfoyle TJ. 1992. Altered morphology of transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. *Dev. Biol.* 153:386–95
69. Martineau B, Houck CM, Sheehy RE, Hiatt WR. 1994. Fruit-specific expression of the *A. tumefaciens* isopentenyl transferase gene in tomato: effects on fruit ripening and defense-related gene expression in leaves. *Plant J.* 5:11–19
70. Marton L, Wullems GJ, Molendijk, L, Schilperoort RA. 1979. In vitro transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens. Nature* 277:129–31
71. Masgrau C, Altabella T, Farrás R, Flores D, Thompson AJ, et al. 1997 Inducible overexpression of oat arginine decarboxylase in transgeninc tobacco plants. *Plant J.* In press
72. Mathur J, Koncz C, Szabados L. 1995. A simple method for isolation, liquid culture, transformation and regeneration of *Arabidopsis thaliana* protoplasts. *Plant Cell Rep.* 14:221–26
73. Maurel C, Barbier Brygoo H, Spena A, Tempé J, Guern J. 1991. Single rol genes from *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum. Plant Physiol.* 97:212–16
74. Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, et al. 1991. T-DNA integration: a mode of illegitimate recombination. *EMBO J.* 10:697–704
75. Medford JI, Horgan R, El-Sawi Z, Klee HJ. 1989. Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *Plant Cell* 1:403–13
76. Meyer D, ed. 1995. Gene silencing in higher plants and related phenomena in other eukaryotes, *Curr. Topics Microbiol. Immunol.* 197
77. Miklashevichs E, Czaja I, Cordeiro A, Prinsen E, Schell J, et al. 1997. T-DNA tagging reveals a novel cDNA triggering cytokinin and auxin independent protoplast division. *Plant J.* In press
78. Morris RO. 1995. Genes specifying auxin and cytokinin biosynthesis in prokaryotes. See Ref. 16a, pp. 318–39
79. Motyka V, Faiss M, Strnad M, Kaminek M, Schmülling T. 1996. Changes in cytokinin content and cytokinin oxidase activity in response to derepression of *ipt* gene transcription in transgenic tobacco calli and plants. *Plant Physiol.* 112:1035–43
80. Nester EW, Grodon MP, Amasino R, Yanofsky MF. 1984. Crown gall: a molecular and physiological analysis. *Annu. Rev. Plant Physiol.* 35:387–413
81. Nilsson O, Crozier A, Schmülling T, Sandberg G, Ollson O. 1993. Indole-3-acetic acid homeostasis in transgenic tobacco plants expressing the *Agrobacterium rhizogenes rolB* gene. *Plant J.* 3:681–89

82. Nilsson O, Moritz T, Imbault N, Sandberg G, Ollson O. 1993. Hormonal characterisation of transgenic plants expressing the rolC gene of *Agrobacterium rhizogenes* T_L-DNA. *Plant Physiol.* 102:363–71
83. Oeller W, Min-Wong L, Taylor LP, Pike DA, Theologis A. 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* 254:437–39
84. Ooms G, Lenton JR. 1985. T-DNA genes to study plant development: precocious tuberisation and enhanced cytokinins in *A. tumefaciens* transformed potato. *Plant Mol. Biol.* 5:205–12
85. Picton S, Gray JE, Grierson D. 1995. Ethylene genes and fruit ripening. See Ref. 16a, pp. 372–94
86. Reiss B, Koncz C, Moore I, Schell J. 1994. A family of binary gene vectors with low inter-transformant variation. *Plant Physiol. (Life Sci. Adv.)* 13:143–49
87. Reiss B, Koncz C, Plum C, Schell J. 1989. T-DNA mutagenesis and the identification of genes involved in the regulation of tobacco seed maturation. *J. Biol. Chem. Suppl. 13C*
88. Reiss B, Suter-Crazzolara C, Balsalobre JM, Schell J. 1994. Genes identified by activation tagging involved in the regulation of seed maturation. *Abstr. Int. Congr. Plant Mol. Biol., 4th, Amsterdam*
89. Roberto FF, Klee H, White F, Nordeen R, Kosuge T. 1990. Expression and fine structure of the gene encoding N^{α}-(indole-3-acetyl)-L-lysine synthetase from *Pseudomonas savastoni*. *Proc. Natl. Acad. Sci. USA* 87:5797–801
90. Röder FT, Schmülling T, Gatz C. 1994. Efficiency of the tetracycline dependent gene expression sytsem: complete suppression and efficient induction of the *rolB* phenotype in transgenic plants. *Mol. Gen. Genet.* 243:32–38
91. Rogers SG, Klee HJ, Horsch RB, Fraley RT. 1987 Improved vectors for plant transformation: expression cassette vectors and new selectable markers. *Methods Enzymol.* 153:253–77
92. Romano CP, Hein MB, Klee HJ. 1991. Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthetase gene of *Pseudomonas savastonoi*. *Genes Dev.* 5:438–46
93. Sato T, Theologis A. 1989. Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene synthesis in plants. *Proc. Natl. Acad. Sci. USA.* 86:6621–25
94. Schmülling T, Beinsburger S, De Greef J, Schell J, Van Onckelen H, et al. 1989. Construction of a heat inducible chimeric gene to increase the cytokinin content in transgenic plant tissue. *FEBS Lett.* 249:401–6
95. Schmülling T, Fladung M, Grossman K, Schell J. 1993. Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. *Plant J.* 3:371–82
96. Schröder G, Waffenschmidt S, Weiler EW, Schröder J. 1984. The T-region of Ti plasmid codes for an enzyme synthesising indole-3-acetic acid. *Eur J. Biochem.* 138:387–91
97. Sheehy R, Homma M, Yamada M, Saski T, Matineau B, et al. 1991. Isolation, sequence and expression in *E.coli* of the *Pseudomonas* sp. strain ACP gene encoding 1-aminocyclopropane-1-carboxlyate deaminase. *J. Bacteriol.* 173:5260–62
98. Sinkar VP, Pythoud F, White FF, Nester EW, Gordon MP. 1987. *rolA* locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. *Genes Dev.* 2:688–97
99. Smart CM, Scofield SR, Bevan MW, Dyer TA. 1991. Delayed leaf senescence in tobacco plants transformed with tmr, a gene for cytokinin production in *Agrobacterium. Plant Cell* 3:647–56
100. Smigocki AC. 1991. Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. *Plant Mol. Biol.* 16:105–15
101. Smigocki AC, Owens LD. 1988. Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. *Proc. Natl. Acad. Sci. USA.* 85:5131–35
102. Spena A, Estruch JJ, Schell J. 1992. On microbes and plants: new insights in phytohormone research. *Curr. Opin. Biotechnol.* 3:159–63
103. Spena A, Prinsen E, Fladung M, Schulze SC, Van Onckelen H. 1991. The indoleacetic acid-lysine synthase gene of *Pseudomonas syringae* susp. *savastanoi* induces developmental alterations in transgenic tobacco and potato plants. *Mol. Gen. Genet.* 227:205–12
104. Spena A, Schmülling T, Koncz C, Schell J. 1987. Independent and synergistic activity of *rolA, B* and *C* loci in stimulating abnormal growth in plants. *EMBO J.* 6:3891–99
105. Sun L-Y, Monneuse M-O, Martin-Tanguy J, Tepfer D. 1991. Changes in flowering and the accumulation of polyamines and hydroxycinnamic acid-polyamine conjugates in tobacco plants transformed by

the *rolA* locus from the Ri TL-DNA of *Agrobacterium rhizogenes*. *Plant Sci.* 80:145–56
106. Sundaresan V, Springer P, Volpe T, Haward S, Jones JDG, et al. 1995. Patterns of gene-action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* 9:1797–810
107. Szekeres M, Németh K, Koncz-Kálmán Z, Mathur J, Kauschmann A, et al. 1996. Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. *Cell* 85:171–82
108. Teeri TH, Herrera-Estrella L, Depicker A, Van Montagu M, Palva T. 1986. Identification of plant promoters by T-DNA mediated transcriptional fusions to the nptII gene. *EMBO J.* 5:1755–60
109. Thomashow LS, Reeves S, Thomashow MF. 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. *Proc. Natl. Acad. Sci. USA* 81:5071–75
110. Tinland B, Hohn B, Puchta H. 1994. *Agrobacterium tumefaciens* transfers single-stranded transferred DNA (T-DNA) into the plant cell nucleus. *Proc. Natl. Acad. Sci. USA* 91:8000–4
111. Topping JF, Lindsey K. 1995. Insertional mutagenesis and promoter trapping in plants for the isolation of genes and the study of development. *Transgen. Res.* 4:291–305
112. Topping JF, Wei W, Lindsey K. 1991. Functional tagging of regulatory elements in the plant genome. *Development* 112:1009–19
113. Trewavas AJ, Cleland RE. 1983. Is plant development regulated by changes in the concentrations of growth substances or by changes in the sensitivity to growth substances? *Trends Biochem. Sci.* 8:354–57
114. Walbot V. 1992. Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:49–82
115. Walden R, Hayashi H, Lubenow H, Czaja I, Schell J. 1994. Auxin inducibility and developmental expression of *axi 1*: a gene directing auxin independent growth in tobacco protoplasts. *EMBO J.* 13:4729–36
116. Wirth R, Friesenegger A, Fiedler S. 1989. Transformation of various species of gram-negative bacteria belonging to 11 different genera by electroporation. *Mol. Gen. Genet.* 216:175–84
117. Yusibov VM, Streck TR, Gupta V, Gelvin SB. 1994. Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells. *Proc. Natl. Acad. Sci. USA* 91:2994–98
118. Zambryski PC. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Mol. Biol.* 43:465–90
119. Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M, et al. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* 2:2143–50
120. Zambryski P, Tempé J, Schell J. 1989. Transfer and function of T-DNA genes from Agrobacterium Ti and Ri plasmids in plants. *Cell* 56:193–201

Annual Review of Phytopathology
Volume 35, 1997

CONTENTS