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Genes involved in the control of growth and differentiation in plants*

(Recombinant DNA; totipotent; phytohormones; auxins; cytokinins; Ti-plasmids; T-DNA tagging)

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SUMMARY

The mechanisms underlying totipotency, the unique ability of isolated plant cells to regenerate into plants, offer developmental biology a unique challenge. While it has been recognised for some time that phytohormones, such as auxin and cytokinin, play a role in this process by inducing a variety of growth patterns in both isolated cells, unorganised callus and intact plants, the molecular basis of their action remains unknown. The molecular and biochemical analysis of the novel interaction between tumour-inducing soil bacteria and the wounded plant has provided a valuable insight into how plants respond to phytohormones. During tumour formation, the bacteria transfer to the genome of the host plant a variety of genes which either short circuit the normal pathways of accumulation of phytohormones or modify how the plant cell responds to them. In parallel to these studies, we have been investigating plant genes involved directly or indirectly in the mechanism of phytohormone action. Auxin-binding proteins (putative receptors) have been localised in various cellular locations and the genes encoding them are currently undergoing analysis. Recently, a novel form of T-DNA has been devised by which mutant plant cell lines can be generated which grow in culture in the absence of exogenously applied auxin. The tagged genes, which are in effect plant cellular proto-oncogenes, are likely to shed more light on how auxin serves to regulate growth and development.

INTRODUCTION

Development in plants is characterized by the fact that the fate of most cells is not irreversibly fixed at some relatively early stage during the development of the embryo. Cell differentiation and cell fate determination in most plants are ongoing processes throughout development. As a consequence, many organs consist of somatic cells that are potentially 'totipotent'. In plants, cell division and differentiation are regulated by simple molecules, such as ethylene, sugars, oligosaccharides and metabolites of tryptophan, phenylalanine, adenine, carotenoids, etc. Some of these growth factors have been called 'phytohormones', because they are known to be transported from sites of synthesis to sites of action. While recognised as playing an essential role in plant growth and development the molecular action of phytohormones remains unknown. Our interest in the molecular dissection of a developmental process unique to plants sprang initially from our studies on an intriguing interaction between soil bacteria and plants. This system provided us not only with a view of how phytohormones

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Abbreviations: *A.*, *Agrobacterium*; axi, auxin independent; βGlu, β-glucosidase(s); *E.*, *Escherichia*; [^{3}H]N_3IAA; 5′-azido-[7-^{3}H]indole-3-acetic acid; IAA, indole-3-acetic acid; *iaaH*, gene(s) encoding tryptophan-2-monooxygenase; *iaaM*, gene(s) encoding indole-3-acetamide hydrolase; ILA, indole-lactic acid; 9-iP, isopentenyladenosine-5-monophosphate; *ipt*, gene(s) encoding isopentenyl transferase; kb, kilobase(s) or 1000 bp; p60, 60-kDa protein; pm60, isoform of p60; Ri, root inducing; *rol*, gene(s) encoding root loci; T-DNA, transfer-DNA; Ti, tumour inducing; wt, wild type.

can influence plant development but also the tools with which plant genes involved in this process might be isolated.

PHYTOHORMONES, GROWTH AND DIFFERENTIATION

(a) Deregulated synthesis of plant growth factors leads to dedifferentiation and tumorous growth

Many bacteria and fungi living in association with plants synthesize, modify, catabolise and excrete similar compounds that are ultimately recognized as growth factors by plants. Auxins (Trp and Phe derivatives) and cytokinins (adenine/adenosine derivatives) represent some of the most important classes of plant growth regulators. Well-known plant pathogens, such as *Pseudomonas*, *Xanthomonas* and *Agrobacterium*, induce plant cell proliferation resulting in development of neoplasmic galls. The growth of *Pseudomonas* and *Xanthomonas* galls is strictly dependent on bacterial synthesis and excretion of the auxin and cytokinin type of compounds. In contrast, tumorous plant cells induced by *Agrobacterium* are capable of proliferation in bacterium-free axenic cultures. These tumorous plant cells are stably transformed by an interspecies conjugational transfer from *Agrobacterium* to plant cells of a Ti-plasmid segment, the T-DNA, that is stably integrated into the plant nuclear genome (for review see Binns and Thomashow, 1988; Zambryski et al., 1989; Kado, 1991). This demonstrates a natural form of 'genetic engineering' which has evolved in certain soil bacteria. T-DNA transfer occurs by a mechanism which appears to be derived originally from bacterial conjugation. In this case a bacterial cell and a plant cell are partners in a conjugation process. The T-DNA encodes genes that are expressed in plants. T-DNAs of different Ti-plasmids carry a set of conserved genes that are required for the maintenance of undifferentiated growth of tumorous plant cells. The T-DNA genes *iaaM* (tryptophan-2-monooxygenase), *iaaH* (indole-3-acetamide hydrolase) and *ipt* (isopentenyl transferase) encode, respectively, the synthesis of auxin (IAA, indole-3-acetic acid) and cytokinin (9-iP, isopentenyladenosine-5-monophosphate), the production of which triggers continuous proliferation of transformed cells.

Undifferentiated cell growth in untransformed plants in tissue culture requires precise balancing of the concentration ratio between auxin and cytokinin. It has been rewarding to study the structure and function of prokaryotic genes that allow bacteria to specifically interfere with the 'normal' development of their eukaryotic hosts. It was our assumption that the mechanisms by which these prokaryotic genes control plant growth and development would mimic the mechanisms normally active in the plants themselves. Hence, operationally these prokaryotic genes could be regarded as 'oncogenes'.

(b) The activity of growth factors can be modulated by the synthesis of specific antagonists

The T-DNA segments of *A. tumefaciens* strains not only harbour the *iaaM*, *iaaH* and *ipt* genes coding for enzymes catalysing the synthesis of auxins and cytokinins, but in addition these T-DNAs carry genes, such as gene *5*, the function of which is to modulate the activity of the growth factors produced by the major oncogenes.

Indeed, gene *5* was shown to be responsible for the synthesis, in transformed plant cells, of an auxin analog: indole-lactic acid (ILA). Transgenic tobacco plants expressing gene *5* produce ILA and develop without readily observable morphological alterations. Their seedlings, however, tolerate levels of exogenously applied auxins that are toxic to isogenic non-transgenic tobacco seedlings. This protection against toxic levels of the growth hormone auxin might well result from the observation that indole-lactic acid (ILA) competes with active auxins such as indole-acetic acid (IAA) for binding to proteins that probably act as auxin receptors. T-DNAs therefore not only introduce genes in plant cells forcing them to synthesise growth factors in a deregulated fashion but also introduce a linked gene (gene *5*) coding for the synthesis of a growth factor antagonist. The expression of these prokaryotic 'pathogenesis genes' in the transformed host cells is fine tuned, since the promoter of gene *5* becomes active in the presence of high levels of auxins but is repressed in the presence of both auxin and its antagonist ILA. In this manner ILA can autoregulate its own synthesis (Körber et al., 1991). This autoregulation may well be the direct consequence of the antagonistic activity of ILA on auxin 'receptors'. Indeed, since the gene *5* promoter is activated by auxin, its activity should be negatively regulated by an antagonist of auxin.

(c) Plant growth factors can not only act extracellularly after transport to target cells but also intracellularly in a cell specific fashion, by activation of intra-cellular pools of inactive conjugates

Agrobacterium rhizogenes is a pathogen that induces the formation of adventitious roots (called 'hairy roots') on a number of plant organs that would not otherwise make such roots. Also, in this case, the abnormal growth was shown to be due to the transfer and expression in plant cells of a set of genes carried on a transferable T-DNA fragment harboured by a plasmid (Ri-plasmid) in *A. rhizogenes*.

The prokaryotic genes responsible for the abnormal growth were called *rol* (for *ro*ot *l*ocus) (White et al., 1985).

It has been demonstrated that the *rolB* gene, in combination with either the *rolC* gene or the *rolA* gene, was sufficient to induce root growth in several plants and that these genes acted in a cell specific fashion. Indeed, only cells containing these *rol* genes were able to grow as transformed roots (Schmülling et al., 1988). In fact it was found that the *rolC* gene codes for an enzyme that releases active cytokinins from inactive intracellular cytokinin glucosides (Estruch et al., 1991a), whereas *rolB* was shown to code for an enzyme capable of hydrolysing indoxyl-glucosides, somehow affecting the function, or levels, of free auxins in transformed plant cells (Estruch et al., 1991b). These observations not only demonstrated the correctness of the hypothesis proposed by Cohen and Bandurski (1982), that conjugation and release of free phytohormones from conjugates can modulate the intracellular levels of active growth substances, but also that such effects must be cell-specific, since this modulation in cellular phytohormone levels did not appear to affect neighbouring untransformed cells. In general plant cells apparently have a variety of mechanisms allowing them to internally regulate the activity of phytohormones.

(d) Isolation of a plant gene coding for β-glucosidase (βGlu) activity capable of hydrolysing phytohormone conjugates

As a strategy to investigate plant proteins involved in the action of auxin, we have analysed proteins which bind auxin, assuming that binding of auxin is a prerequisite of a functional link between the two. Protein extracts from maize coleoptiles grown in the dark were photoaffinity labelled with 5′-azido-[7-^{3}H]indole-3-acetic acid ([^{3}H]N_3IAA). A 60-kDa protein, termed p60, was thus identified. This protein was initially detected in the post-ribosomal supernatant, indicating that it might be present in the cytosol of the intact cells. p60 was also detected in protein extracts prepared after solubilization of microsomal fractions. In both cases, labelling of p60 was strong and no other protein present in the extract was labelled. To demonstrate the specificity of photoaffinity labelling of p60, competition studies were performed using various unlabelled auxin analogues. Physiologically active natural and synthetic auxins significantly reduced the incorporation of [^{3}H]N_3IAA into p60. Compounds specific for the indole ring such as L-tryptophan, or compounds specific for the aromatic ring system, or radical scavengers such as *p*-aminobenzoic acid, did not compete the labelling of p60.

To study the presence of the p60 in different organs of the maize seedling, we isolated crude microsomal fractions from coleoptiles (including the node and the primary leaf), from the mesocotyl and from roots. Photoaffinity labelling of the corresponding protein extracts showed that p60 was mainly present in the coleoptile fraction. In addition we found an isoform of p60 (pm60) in plasma membranes.

Microsequencing studies were performed with purified p60 after proteolytic digestion. The primary amino acid sequence of p60 revealed similarities to βGlu. p60 was therefore analysed for βGlu activity. It was found that a fraction containing p60 indeed exhibited βGlu activity towards general βGlu substrates (e.g., *p*-nitrophenyl-glucopyranoside or 6-bromo-2-naphthyl-β-D-glucopyranoside/fast blue BB for activity staining in native polyacrylamide gels). Specific staining of the p60 with 6-bromo-2-naphthyl-β-D-glucopyranoside/fast blue BB demonstrated that p60 has β-D-glucoside glucohydrolase activity (EC 3.2.1.21). To define the substrate specificity of p60, we tested different compounds that are commonly cleaved by a wide variety of glucosidases. In contrast to other βGlu enzymes which hydrolyse a broad range of substrates, a distinct pattern of substrate specificity was found for p60.

The results of photoaffinity labelling discussed above support the view that the p60 can bind auxins. In this context, it is important to know whether the presence of auxins has any effect on the βGlu activity of p60. We found that both IAA and 1-naphthylacetic acid, as well as the auxin transport inhibitor naphthylphthalamic acid, inhibit p60 associated βGlu activity in a competitive manner. In contrast, the presence of non-functional auxin analogues such as L-tryptophan or 5-hydroxy-IAA, or aromatic compounds such as benzoic acid, had no effect on the βGlu activity of p60. These results suggest that IAA and related compounds are aglycones which can bind to the active site of p60. Further experiments demonstrated that p60 readily hydrolysed indoxyl-*O*-glucoside, a synthetic compound structurally related to the natural auxin conjugate indole-3-acetyl-β-D-glucose. This activity appears to be highly specific, since p60 is not able to hydrolyse other IAA conjugates like IAA-*myo*-inositol or IAA-aspartate. Present data suggest that p60 might be involved in vivo in the hydrolysis of glucosidic phytohormone conjugates.

Extensive amino acid sequence analysis of this protein allowed the construction of several synthetic oligodeoxynucleotide probes which were used to isolate a cDNA clone coding for a protein related to p60. The cDNA, named Zmp60.1, corresponded to a mRNA with a 3′-poly(A)$^+$ sequence with a single open reading frame. When the Zmp60.1 primary sequence was compared with other amino acid sequences available in protein data bases, similarities were observed to other βGlu enzymes from archaebacteria, eubacteria and eukaryotes. Amino

acid sequence motifs showing similarity to the rolC protein from *Agrobacterium rhizogenes* were also found. The motifs shared between the rolC protein and Zmp60.1 pointed to the possibility that the two proteins could share common substrates. Transgenic tobacco plants stably expressing this gene could consequently have altered phytohormone ratios and exhibit abnormal growth.

(e) Control of plant development by phytohormone-specific βGlu

Maize kernels are a rich source for phytohormone conjugates, compounds which accumulate in the endosperm during seed maturation and which are mobilized to other parts of the seedling during germination. Hydrolysis and transport of conjugates from the endosperm to the shoot and to the root could be of importance for controlling maize seedling development. Protein p60 could play a pivotal role in the germination process by controlling the release of free cytokinin. To test whether Zmp60.1 expression was able to influence plant growth, tobacco protoplasts were transiently transformed with Zmp60.1. These protoplasts acquired the ability to use exogenous cytokinin glucosides to initiate division. Further immunocytochemical analysis of maize seedling roots localized Zmp60.1 to meristematic cells, suggesting that Zmp60.1 is a glucosidase capable of supplying the developing embryo with biological active cytokinins (our unpublished observation).

One of the most attractive features of a model explaining plant growth control is based on the action of βGlu. Particularly attractive is the simplicity by which developmental adaption to environmental cues could be provided. Auxin and cytokinin conjugates have been found to be broadly distributed in plants. The activity of phytohormone-specific βGlu could easily be regulated by environmental as well as by endogenous factors. Thus, phytohormone-specific βGlu might be a link between environmental stimuli and the activation of phytohormones in precise locations of the plant. Although these ideas are far from being proven, they open a promising area of research in plant development. We hope that future investigations will contribute to define precisely the importance of phytohormone-specific βGlu in the control of developmental processes in plants.

(f) Use of gene tagging to identify genes involved in phytohormone perception/regulation

In order to test whether or not plants normally make use of mechanisms similar to those exploited by soil bacteria in tumour formation, we initiated a search for plant cell mutants that would be able to grow and differentiate in the absence of extracellularly supplied auxins. In order to rapidly identify and clone genes involved in conveying auxin-independent growth, specially designed T-DNA vectors were used to activate and tag genes that are normally silent in freshly isolated protoplasts in the absence of auxins (Hayashi et al., 1992). Because the inserted tag is designed to stimulate the transcription of genes, the mutants are dominant. In addition, the tagging insert contained sequences allowing the tagged plant DNA to be readily recovered in *E. coli* as a plasmid.

A number of different classes of dominant auxin-independent mutants were thus obtained. Calli from these mutants grow well in the absence of extracellularly supplied auxins but can be regenerated to form fertile plants. Protoplasts derived from the leaves from these regenerated plants were shown to be able to form calli on media devoid of auxins.

One of the regenerated plant lines, *axi159*, has been studied in detail (Hayashi et al., 1992). Line *axi159* contains a single insert of T-DNA and displays no obvious phenotypic changes. The ability of protoplasts isolated from *axi159* to grow in culture in the absence of auxin genetically co-segregates with the T-DNA insert. The T-DNA in *axi159* is located on a single 17.5-kb *Eco*RI fragment of genomic DNA and this has been rescued from the plant genome as plasmid pHH159. Once transfected into SR1 protoplasts, pHH159 confers the ability to the protoplasts to grow in vitro in the absence of auxin indicating that it contains the DNA responsible for this characteristic and allowing deletion analysis to define the position of the gene sequence which carries this out. Using this sequence as a hybridisation probe we isolated a full length cDNA from *axi159* leaves corresponding to the region in question, which upon subcloning into an expression vector and reintroduction into protoplasts directed growth of callus in the absence of auxin. Comparison of the sequence of the cDNA and the genomic sequence indicates that the gene responsible for producing auxin-independent growth upon overexpression, *axi1*, is approximately 4000 bp long and contains nine introns. Sequence comparison with data bases reveals no obvious homology with previously characterised proteins.

Currently studies are underway to investigate how the overexpression of *axi1* might lead to callus formation in the absence of auxin. Preliminary Northern analysis indicates that the expression of the wt allele of *axi1* in freshly isolated protoplasts requires auxin. Our experiments at the moment are aimed at determining the location of the *axi1* gene product in the cell and its enzyme activity.

(g) Conclusions

By analogy with other systems it may come as no surprise that the study of tumorigenesis in plants has shed

light on how plant cells control growth and development. What has been a surprise, however, is that differing levels of control have been revealed. Not only do plants synthesise phytohormones at distinct sites and transport them to other regions of the plant but also the individual plant cell, in an autonomous manner, can regulate its own internal active levels of phytohormones. This complexity may account, at least in part, for totipotency which in turn may be a consequence of the sessile life style of the plant.

While a combination of biochemical and genetic approaches have yielded a glimpse of the molecular action of phytohormones our understanding is far from complete. Major challenges remain. For example, how does morphologically unorganised callus initiate the formation of determined growth by shoot, root meristems and how do phytohormones interact with environmental stimuli such as light or gravity.

In the past the genetic tools generated from the study of the intriguing interaction between plant cells and pathogenic bacteria have provided a valuable means in addressing the question of the action of phytohormones. It is not unreasonable to suspect that this will continue to be the case in the future.

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