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Molecular approaches to study plant hormone signalling

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1. Introduction

Both molecular genetic and molecular biology approaches have contributed significantly to plant hormone research. Especially the use of *Arabidopsis thaliana* as model plant has accelerated the research in this field tremendously. Major breakthroughs in the analysis of phytohormone action have come from the analysis of *Arabidopsis* hormone biosynthesis and response mutants. Molecular cloning of the mutant genes has led to the identification of enzymes involved in hormone biosynthesis (see other relevant chapters) and components in hormone signalling pathways. In addition, different molecular biology approaches, as well as the current world-wide *Arabidopsis* genome sequencing project have uncovered the sequences of many genes, whose functions in plant hormone signalling have subsequently been determined by reverse genetics techniques.

In chapters 18–23 the signal transduction pathways of the major plant hormones are reviewed. This chapter provides an overview of the methodology that has led to the current knowledge on plant hormone signalling. Where possible examples are taken from the research on gibberellins (GAs) or the recently discovered brassinosteroids (BRs), as the signalling of these hormones is not covered by the other chapters and recent findings shed new light on the action of these phytohormones.

2. The mutant approach

Hormone response mutants have been identified in several crop plant species such as pea, tobacco, tomato, barley, maize and wheat. In some cases the mutations led to crop improvement, as with the GA-insensitive mutant of wheat, *Rht*, which has provided the genetic basis for the high yielding, semi-dwarf wheat varieties [1]. However, most of these mutants have not been very informative in elucidating the mechanism of hormone signalling, since it has not yet been possible to characterize the genotypic variation at the molecular level. In contrast, response mutants from the model plant *Arabidopsis thaliana* have been a very efficient source of information. The small genome (100 Mb), the availability of a high density genetic map and an efficient transformation system have allowed for the identification and characterization of numerous hormone response mutants in a relatively short period of time. Moreover, the small surface area needed to test a large

number of plant lines and the relatively high seed production per plant allow large scale mutagenesis experiments to be performed.

The first arabidopsis mutants were obtained through treatment of seeds with ionizing radiation. Later, the high efficiency of the chemical mutagens ethylmethane sulfonate (EMS) was demonstrated for arabidopsis [2]. EMS mutagenesis is now widely used for arabidopsis genetics as it results in base pair changes which can lead to a very broad spectrum of mutations per gene.

The development of transformation technology for plants, and in particular for arabidopsis, opened new possibilities for creating mutants. On one hand this allowed the introduction of genes, often from bacterial origin, to alter the endogenous hormone levels or the sensitivity of the plant cell to hormones. On the other hand, the introduced DNA served as the mutagens itself by knocking out functional genes via insertional mutagenesis.

The system most widely used for DNA transfer to arabidopsis is based on the natural ability of the soil bacterium *Agrobacterium tumefaciens* to transfer a DNA segment, the transferred-DNA (T-DNA), to plant cells (Fig. 1). In nature this system is used by agrobacterium to induce tumours on the host plant, which provide the bacterium with the proper environment and nutrients for survival. The T-DNA is a single stranded DNA copy from the T-region on the large tumour inducing (Ti-) plasmid of agrobacterium. The T-region contains several genes that can be expressed in plant cells and which encode proteins involved in phytohormone biosynthesis, metabolism and signalling. The T-region is bordered by two imperfect direct repeats, which are recognized by the transfer machinery and are the only DNA sequences of the T-region that are essential for the DNA transfer. A second region on the Ti-plasmid, the virulence region, contains several genes

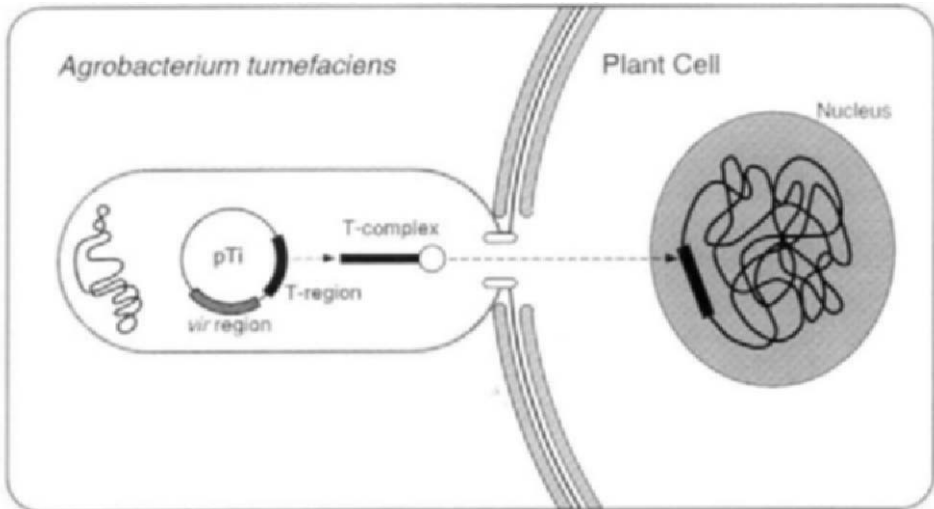


Fig. 1. A widely used method for DNA transfer to plants makes use of the natural DNA transfer system of *Agrobacterium tumefaciens*. Virulence genes located on the Ti plasmid (*vir*-region) are part of the machinery by which the T-DNA-protein complex (T-complex) is transferred from agrobacterium to the plant cell nucleus. Here the T-DNA is inserted into the plant genome.

(*vir*) that are also essential for DNA transfer. A number of *Vir* proteins are involved in the generation of the T-DNA, while others allow for the transport of the T-DNA to the plant cell nucleus where it is incorporated into the chromosomal DNA. The discovery that the T-region and the *vir*-region can be physically separated led to the development of the binary vector system. In this system the T-region is moved from the Ti plasmid to a smaller binary plasmid that allows for manipulation of the T-region in *E. coli*. Thus any DNA sequence cloned between border repeats on a binary vector can be transferred to plants by agrobacterium [3].

One of the most widely used protocols for agrobacterium-mediated DNA transfer to arabidopsis relies on the co-cultivation of arabidopsis roots with agrobacteria followed by selection and regeneration of shoots from the transgenic cells [4]. Although the transformation efficiencies obtained with this protocol have been optimized [5], the regeneration of transgenic lines is a laborious and time consuming process which hampers large scale transformation experiments. Protocols that are more suited for these experiments and that have resulted in the creation of large populations of T-DNA transformed lines rely on the infiltration of arabidopsis seed or immature flower buds with agrobacterium [6,7]. The infiltration of immature flower buds is now routinely used and can give up to 2% transgenic seedlings in the progeny of the infiltrated parent plants, depending on the protocol and agrobacterium strain used [8] (personal observations). Although the molecular analysis of these transgenic lines has not yet been published in great detail, it is reported that greater than 50% of transgenic lines contain single locus T-DNA insertions [9]. The T-DNA insertions seem to be derived from independent transformation events of the female gametophyte [8,10].

T-DNA transfer has also allowed other insertional mutagens, such as the maize *Ac/Ds* or *En/Spm-I/dSpm* transposable elements [11–14], to be introduced into arabidopsis [15,16]. After their introduction, the transposable elements are allowed to propagate resulting in several collections of plants containing multiple transposon inserts at different locations in the plant genome. Apart from being a source of mutants for specific screens, these collections are now a valuable source for reverse genetics approaches (see below).

Which type of mutagens to use is totally depending on the objectives of the research:

- (1) With EMS treatment the largest collection of mutants with the most diverse and subtle mutations can be created. This gives a good indication of the frequency of occurrence of a specific type of mutant. However, EMS induces single base pair changes and identification of the mutated gene can only occur through mapped based cloning. Although now successfully applied to several mutants [17–19], the method relies on close linkage of the mutation with genetic markers (RFLP, AFLP, CAPS or visible markers) [20] or on the availability and the quality of the physical mapping data in the genomic region [21]. When such prerequisites are not available, map based cloning becomes a time consuming procedure. With the current effort to sequence the entire arabidopsis genome the physical map is rapidly expanding and these limitations are gradually being overcome.
- (2) Ionizing radiation induces DNA deletions. Although it is not as efficient and subtle a mutagens as EMS, ionizing radiation mutagenesis has generated unique hormone response mutants that can not be obtained through EMS mutagenesis [22,23].

Moreover, the mutated gene can be identified through the procedure of genomic subtraction [24], which is based on cycles of hybridization between wildtype and mutant DNA. Although genomic subtraction has been successfully used on a few occasions [25,26], it has not been widely applied, possibly because of the technical difficulties.

- (3) Chromosomal positions for which a physical map is not yet available require that the insertional mutagenesis approach be taken. The advantage of this type of mutagenesis is that the mutator DNA itself serves as a tag for the insertion site, which then allows for relatively rapid identification of the mutated gene. Because of this property, insertional mutagenesis is also referred to as gene tagging. Additional mutations may arise during the process of transformation or transposition and because of this the phenotype observed may not always be caused by the insertional mutagens but rather by a second site mutation. The relatively low percentage of linkage between the inserted DNA element and the mutant phenotype is one of the major drawbacks to the use of insertional mutagenesis [6,27,28]. Furthermore, if the second mutation causing the phenotype is closely linked to the inserted DNA element, it may be difficult to distinguish between the two. In this case, the use of transposable elements provides an advantage over T-DNA, in that re-excision of the transposon can lead to reversion of the mutant phenotype. However, since the transposon may leave a deletion or duplication of a few base pairs at the insertion site (footprint), one has to keep in mind that excision does not necessarily have to lead to reversion [29,30].

In most cases the mutation will be limited to a simple gene knock-out by insertion of the mutator DNA (Fig. 2a). Other mutation-types require modification of the mutator DNA. This has not been a problem when using agrobacterium T-DNA, since any DNA sequence lying between the T-DNA border repeats will be efficiently transferred to plant cells. In addition to the insertional construct, two other types of constructs can be distinguished. The first is the promoter or enhancer trap construct (Fig. 2b). In this case a reporter gene lacking its own promoter is positioned close to one of the border repeats, so that expression of the reporter relies on the presence of a plant promoter or enhancer at the insertion site [31,32]. When inserted in the correct orientation in a plant gene, the reporter gene can monitor the expression of the mutated gene. The other type of construct is the so-called activator construct, in which a strong plant promoter or enhancer is located in an outward facing direction next to the T-DNA border repeat (Fig. 2c). Insertion of such a construct into a plant gene may, apart from a knock-out, also result in over-expression of this gene in sense or anti-sense orientation, thereby causing a dominant mutation. This type of construct has been successfully applied in arabidopsis to create hormone response and developmental mutants [27,33]. In addition to T-DNA it was also found that *Ac/Ds* transposable elements could be converted into promoter-/enhancer traps [34] or promoter-out transposons [35], without interfering with the transposition capacity of the elements. The enhancer trap strategy was also tested using *En/Spm-I/dSpm* transposable elements, but changes in the transposon sequence were found to result in reduced excision frequencies [36].

One of the useful characteristics of transposons is that they preferentially jump to linked sites on the chromosome [29,37–39]. To make use of this characteristic, transgenic lines

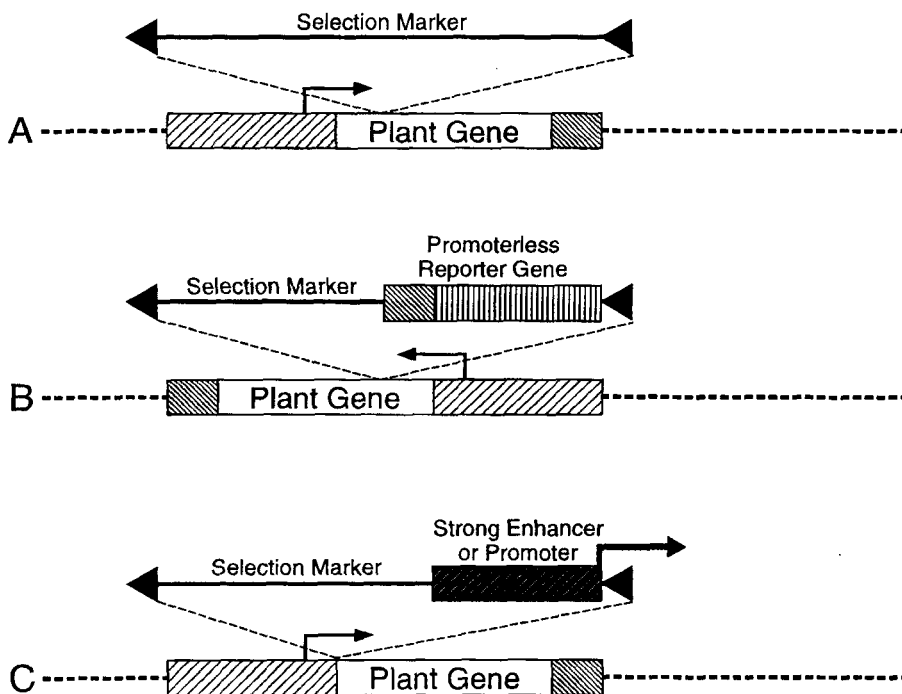


Fig. 2. Three different construct-types are used for T-DNA- or transposon-mediated insertional mutagenesis in plants. A. The knock-out construct. B. The promoter-trap construct. C. The activator construct. The solid triangles indicate either the T-DNA border repeats or the sequence repeats at the ends of a transposable element.

have been created that contain transposons at different loci throughout the arabidopsis genome. These transposon-containing loci can then thus be used as donor sites for the targeted insertional mutagenesis of a gene that maps close to this region [28].

From the previous discussion it is clear that the creation of mutants in arabidopsis is not the rate limiting step in plant hormone research. More crucial is the problem of how to combine a mutagenesis method with a specific selection scheme for mutants that are impaired in their response to a plant hormone. The different strategies that have been used and their effectiveness in helping to elucidate the plant hormone signalling pathways are presented and discussed below.

2.1. Mutants that are insensitive or resistant to plant hormones

One of the first screening procedures for hormone response mutants was to select mutants for their insensitivity or resistance to high concentrations of a given hormone. The screen itself is easy to perform and only requires growing the mutagenized population under a positive selection pressure. Insensitive mutants have been identified for most hormones.

The dominant ethylene resistance mutant *etr1* was isolated by the inability of etiolated seedlings to show the ethylene-induced triple response. Map based cloning of the *ETR1* gene has led to the identification of the probable ethylene receptor [40]. In contrast selection for auxin resistant mutants has resulted in the identification of more pleiotropic mutants, most of which are also resistant to other plant hormones. Three of these mutants, *axr1*, *aux1* and *axr3*, have been characterized at the molecular level (see chapter 18); *axr1* and *axr3* through mapped based cloning [19,41] and *aux1* through T-DNA tagging [42]. The *AXR3* gene encodes a putative transcription factor which acts at the end of a signal transduction chain [41].

Auxin and cytokinin are essential for obtaining regeneration in tissue culture, and one way to identify response mutants for these hormones is to select for mutants that are impaired in regeneration in the presence of these hormones. A screen for temperature sensitive arabidopsis EMS mutants that are defective in shoot regeneration resulted in the isolation of three recessive *srd* mutants from a total of 2700 M3 lines [43]. Unfortunately, molecular analysis of the mutants has not been performed and thus it is not yet clear whether the mutants are impaired in hormone perception or in cell cycle initiation or progression [44].

As with auxin, both GAs and BRs are involved in cell elongation. One semi dominant GA insensitive mutant *gai* was identified from a collection of X-ray treated seeds as a leaky dwarf mutant that did not show height increase upon application of bioactive GAs [22]. The *GAI* gene was recently cloned through the identification of loss-of-function alleles of the mutant *gai* gene, one of which was obtained by targeted insertion of a *Ds* transposable element [23]. *GAI* encodes a nuclear localized protein that belongs to the new VHIID domain family of proteins, members of which show characteristics of transcriptional co-activators and include the SCR protein which is involved in cortex/endodermis differentiation [45]. Sequence analysis of the mutant *gai* gene showed that it encodes a protein which differs from that of the wildtype by a deletion of 17 amino acid residues at the *N*-terminal part. These residues are most likely involved in perception of the GA signal which then inhibits the action of GAI as repressor of elongation growth. The fact that a knock-out mutation in the mutant *gai* gene can partially suppress the dwarf phenotype of the *gai* mutant, suggests that the function of GAI is redundant. This was confirmed by the isolation of a homologous cDNA encoding the GAI-related sequence protein, GRS.

The recessive brassinosteroid insensitive mutant *bri1* was characterized as being specifically insensitive to brassinosteroid-mediated inhibition of root elongation [46]. Nineteen other brassinosteroid insensitive mutants were identified by two other groups as dwarf plants that could not be restored to wild type stature by exogenous application of brassinosteroid [47,48]. All of the mutations turned out to be located in the *BRI1* gene. *BRI1* was identified through map based cloning and encodes a putative leucine-rich repeat receptor kinase [47]. The finding of a putative plasma membrane bound steroid receptor in plants is remarkable, since the steroid hormone receptors that have originally been identified in animals are soluble proteins that shuttle between the cytoplasm and the nucleus. As an explanation for the fact that all *bri1* mutants map to the same locus, the authors suggest that BRI could be the only component in BR signalling or that downstream components may be redundant. Another explanation could be that the

mutations in the *BRI1* gene were specifically selected by the type of screen or that mutations in other genes resulted in a lethal phenotype and were therefore not detected.

2.2. Hormone (independent) phenotypes

A very informative class of hormone response mutants has been identified based on their ability to phenocopy wild type plants grown in the presence of exogenously applied hormone. The phenocopy can be caused by up-regulation of hormone biosynthesis and in these cases biosynthesis-up mutants can be distinguished by their sensitivity to chemicals that block hormone biosynthesis or perception. For example, the constitutive triple response of the ethylene overproducing mutant *eto1* can be inhibited by both aminoethoxyvinylglycine (AVG, inhibitor of biosynthesis) or silver (inhibitor of ethylene binding). In contrast, the *ctr1* mutant still shows a constitutive triple response in the presence of these inhibitors. The *CTR1* gene was cloned through a T-DNA insertion allele and encodes a negative regulator of ethylene response [49] (see also chapter 21). Gibberellin independent mutants were initially isolated based on their capability to germinate on paclobutrazol, an inhibitor of the biosynthesis of active GAs. After discarding the mutants that showed a wilted phenotype caused by a defect in the biosynthesis or perception of abscisic acid (ABA), the remaining mutants were screened for resistance to the dwarfing effects of paclobutrazol. Three recessive mutants were selected that are allelic for a single locus, *SPINDLY* [50]. The mutants show a basal level of GA signal transduction which results in phenotypic characteristics such as longer hypocotyls, early flowering and increased stem elongation. At the same time they are still able to respond to exogenously applied GA. The more recent identification of a T-DNA insertion allele at the *SPINDLY* locus has allowed for the cloning of the corresponding gene. *SPY* encodes a tetratricopeptide repeat (TPR) containing protein. TPR containing proteins are often found in protein complexes for which functions such as transcriptional repression and protein kinase inhibition have been proposed. The TPR is a 34 amino acid repeated sequence motif that has been proposed to mediate protein-protein interactions [51]. *SPY* shows sequence similarity to both the N-terminal TPR domain as well as the C-terminal catalytic domain of O-linked N-acetylglucosamine transferases (OGTs) from rat, human and *C. elegans*. OGTs are thought to regulate signal transduction by competing for phosphorylation sites on regulatory proteins, such as kinases and transcription factors, through O-GlcNAcylation at the serine and threonine residues. Recently, a *SPINDLY* homolog (*HvSPY*) was isolated from barley. Expression studies in aleurone cells indicate that *HvSPY* has a dual function; it acts as a negative regulator of the GA-induced α -amylase promoter and as a positive regulator of the ABA-induced dehydrin promoter [52].

Analogous to the screen for mutants defective in regeneration described above, arabidopsis mutants have been selected that show regeneration of shoots from callus in the absence of cytokinins. A striking detail of this approach was that the mutants were identified from 50.000 hypocotyl derived calli transformed by an activator T-DNA construct. Five mutant calli were obtained that turned green, showed rapid proliferation and produced shoots in the absence of cytokinin. In four of the dominant mutants the T-DNA was inserted upstream of the *CKII* gene, resulting in up-regulation of expression of

this gene. As with ETR1, CKI1 shows similarity to the receptors of the bacterial two-component regulators and may therefore act as a cytokinin receptor [33]. These results show that, although laborious, this approach can lead to a breakthrough in the study of hormone signalling.

2.3. Suppressors of existing mutants

One way to add to the collection of hormone response mutants is to isolate mutants through their capacity to suppress the phenotype of existing hormone response mutants. For GAs this approach has been most productive in terms of the identification of signal transduction components. Some of the genes have also been identified through the approaches discussed in 2.1 and 2.2.

Both intragenic as well as extragenic suppressors have been obtained for the mutant *gai* gene. The intragenic suppressors were used to clone the *GAI* gene as described above. Extragenic suppressors of *gai* were selected by screening for tall individuals among EMS mutagenised M2 plants derived from a *gai* homozygous line. This resulted in the isolation of several *spy* alleles, confirming the observation that *spy* is epistatic to *gai* in the *spy, gai* double mutant [51,53]. In addition a dominant mutation in the *GAR2* gene partially suppressed *gai* [53]. Unfortunately, the role of *GAR2* in GA signalling has not yet been investigated. A similar screen for *gai* suppressors resulted in the isolation of one recessive mutation in the *GAS1* gene [54]. The *gas1* mutation partially suppresses the *gai* mutation, but it has no obvious effects on plant development in the wild type background. Instead of making the plants more sensitive to GA, the *gas1* mutation makes the plant partially GA growth independent.

Suppressors have also been obtained for the GA biosynthetic mutant *gal-3*. Suppressors of the dwarf and male sterile phenotype of *gal-3* were selected from the M2 population of EMS treated *gal-3* seeds. The isolation of intragenic suppressors was avoided by using the *gal-3* allele which contains a 5 kbp deletion at the *GAI* locus. The selected mutants were all recessive and comprised 17 alleles of a new locus, *RGA*, and 10 alleles of the previously identified *SPY* locus [55]. The isolation of 10 additional alleles from a fast-neutron-mutagenized population of *gal-3* mutants allowed for cloning of the *RGA* gene through genomic subtraction [26]. *RGA* turned out to be identical to the *GAI*-related sequence *GRS* (see above). Both *RGA* and *GAI* are thus members of the VHIID-regulatory family of proteins. The mutant alleles of the corresponding genes indicate that both proteins act as repressors on the GA signalling pathway. This suggests that GA modulates plant growth by de-repression of more general (auxin?) signalling pathways. Both *GAI* and *RGA* represent two GA responsive repressor branches, of which the *GAI* branch acts through *SPY*, possibly by *O*-GlcNAcylation of regulatory proteins in the general signalling pathway. Moreover, no *gai* null allele was identified in the *gai* suppressor screen, corroborating the hypothesis that *GAI* and *RGA* have different, but overlapping functions.

2.4. Hormone responsive promoters as tools

Plant hormones are known to exert their effects on plant development by altering the expression of specific genes. A wide range of hormone responsive genes have been

identified, initially by differential screening of cDNA libraries [56,57], and more recently by PCR-based techniques such as subtractive hybridization [58] and differential display [59], and by screening of promoter trap lines (Offringa et al., unpublished results). Genes that are rapidly up-regulated after hormone treatment have been the subject of extensive studies. The up-regulation of such primary response genes is independent of *de novo* protein biosynthesis, indicating that they are direct targets of hormone signal transduction. For some primary hormone responsive genes it has been found that the gene product itself is a component of the hormone signalling pathway. The *IAA17* gene was initially identified in arabidopsis as a homolog of the auxin-responsive *Ps-IAA4/5* gene [60], but later turned out to be identical to the *AXR3* gene [41]. Recently, two primary cytokinin inducible genes (*IBC6* and *IBC7*) were identified from etiolated arabidopsis seedlings through differential display. Remarkably, both genes encode proteins that are homologous to the response regulators of the bacterial two-component system [61]. The two-component histidine kinase CKI was recently identified as a putative sensor in cytokinin signalling [33]. It will be interesting to see whether *IBC6* and *IBC7* act down-stream of CKI in cytokinin signal transduction.

Hormone responsive elements have been identified in the promoter regions of several primary hormone responsive genes. These elements have subsequently been used to identify the transcription factors that bind to these elements by approaches that are discussed in section 3.2 and 3.3 of this chapter. Here we focus on a genetic approach in arabidopsis which allows for the identification of the more upstream components of the signalling pathway which are responsible for the hormonal regulation of gene expression. This approach is based on the introduction of a hormone responsive reporter construct into arabidopsis. This construct contains the hormone responsive promoter fused to a reporter gene, and in some cases to an antibiotic resistance gene (Fig. 3). A transgenic line is selected that is homozygous for a single copy insertion of the construct and shows the proper expression of the promoter. Seeds of this line are mutagenized, germinated and the M1 plants are allowed to self-pollinate. The resulting M2 seeds can be used to screen for mutants that either do not show hormone dependent expression or show hormone independent expression from the promoter. Using an antibiotic resistance gene construct it is possible to select mutants with hormone independent activity of the promoter by their increased resistance to the antibiotic. The reporter gene can subsequently be used to identify those mutants in which the up-regulation is caused by an *in trans* mutation versus a mutation in the promoter linked to the antibiotic resistance gene. This novel genetic approach has been applied in studies of auxin and abscisic acid signalling.

Several primary auxin response genes have been isolated from arabidopsis, using homologous genes isolated previously from other species as probes (see chapter 19). The auxin inducible promoters from these original plant species have been used in screens for auxin response mutants in arabidopsis.

One promoter was from the *Nt103* gene, which was identified from tobacco cell suspensions and which encodes a glutathione-S-transferase [62–64]. The full length promoter was fused to the *gusA* reporter gene coding for β -glucuronidase and to the *nptII* gene which provides resistance to kanamycin (Fig. 3). A T-DNA construct containing both promoter-gene fusions was introduced into arabidopsis and mutants with enhanced auxin-independent expression of the promoter were selected through their increased resistance

to kanamycin. Several mutants were obtained that also showed up-regulation of the *gusA* reporter gene. Three of these mutants also showed up-regulation of the endogenous *At103-1a* gene, whereas the expression of another auxin-regulated gene, *SAUR-AC1*, was not affected [65]. Whether the *gst*-up-regulated (*gup*) mutants are indeed affected in an auxin signal transduction pathway awaits further investigation.

The *Nt103* gene is not specifically induced by auxin, but can also respond to treatments with elicitors or stress related compounds such as salicylic acid and heavy metals [64,66].

Transform roots

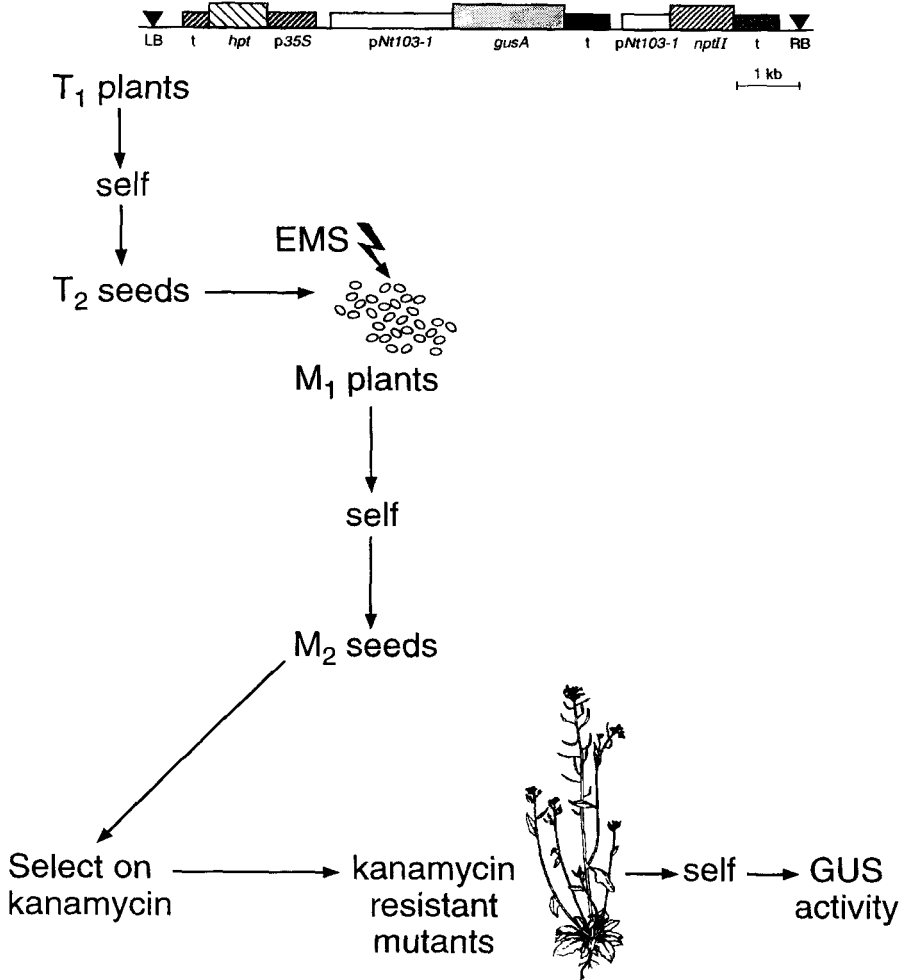


Fig. 3. EMS mutagenesis and selection procedure for *Nt103* promoter-up-regulation mutants in *Arabidopsis thaliana* [65]. RB/LB, right/left T-DNA border repeat. t, transcription termination sequence. *p35S*, constitutive plant promoter.

Two genes that are specifically induced by auxin are the *GH3* gene from soybean and the *PS-IAA4/5* gene that was originally identified in pea. The promoter regions of these genes have been analysed in great detail and the auxin responsive elements (AuxREs) have been identified [67–69]. For each promoter the AuxREs were placed upstream of a minimal plant promoter. The resulting chimeric promoter was shown to give auxin inducible expression in arabidopsis when fused to a reporter gene. The GH3-AuxRE promoter was fused to both the hygromycin resistance *hpt* gene and the *gusA* reporter gene and eight independent hygromycin resistant arabidopsis EMS mutants could be selected that also showed elevated levels of GUS expression [70,71]. As with the *Nt103* promoter, the GH3-AuxRE promoter could be used for selection, since the promoter is not active in seedlings that have not been treated with auxin. This was not the case for the chimeric promoter containing the AuxREs of the *Ps-IAA4/5* gene. Therefore, this promoter was only fused to the *gusA* reporter gene and EMS mutants were screened for constitutive GUS expression in the root using non-lethal histochemical staining conditions. Two altered auxin gene expression mutants (*age*) were obtained, one of which also showed up-regulation of *IAA4/5* homologs in arabidopsis [72]. Although these mutants were identified using the specific AuxREs, future research will reveal whether or not they are actually altered in auxin signalling.

Interestingly, auxin response mutants that show altered regulation of auxin responsive genes have been identified through other screening procedures, such as resistance to high auxin concentrations. For example, the semi-dominant mutations in the *AXR3* gene causes ectopic expression of the auxin responsive *SAUR-AC1* gene [73]. This suggests that the transcription factor-like *AXR3* protein [41] is involved in regulation of *SAUR-AC1* gene expression. Moreover, the *SAUR-AC1* gene can not be induced by auxin in the severe mutant allele of *AXR1*, *axr1-12*. In contrast, the *AUX1* gene product, which is thought to be an auxin import carrier, is clearly not involved in the regulation of the expression of this gene [74].

Several ABA responsive genes were initially identified as osmotic or cold stress-responsive genes, since both stresses increase the level of the phytohormone abscisic acid [75]. The cold and drought responsive gene *RD29A* was believed to be up-regulated via both an ABA-dependent and an ABA-independent pathway, since two elements were identified in the promoter of this gene, one being the ABA-responsive element (ABRE) and the other the dehydration-responsive element (DRE) [76,77]. In an elegant approach aimed at elucidating the two signalling pathways, this promoter was fused to the firefly luciferase coding sequence (*LUC*) and the resulting osmotic stress responsive (OR) reporter gene was introduced into arabidopsis. *LUC* activity was detected by *in vivo* luminescence imaging after spraying with the substrate luciferin. A transgenic line that showed high *LUC* activity only under osmotic or cold stress was used for EMS mutagenesis. By screening for *LUC* activity in the M2 seedlings a large number of *cos* (constitutive expression of OR gene) *los* (low expression of OR gene) and *hos* (high expression of OR gene) mutants were obtained. These mutants could not simply be classified in two groups based on the two pathways. Instead, 14 groups were needed to classify the mutants based on their response to stress and ABA. These results indicate a complex network of cross-talk between the cold stress, osmotic stress and the ABA signalling pathway [78]. Although informative with respect to the interaction between

signalling pathways, future research is needed to prove that at least some mutants are actually altered in ABA signalling.

From the above mentioned examples it cannot yet be concluded that the use of hormone responsive promoters will result in new breakthroughs in hormone signal transduction research, mainly because the isolated mutants are still in need of further characterization. Nonetheless, it seems that this type of approach can lead to the identification of mutants that do show altered regulation of both hormone responsive reporters and endogenous hormone responsive genes. In two of the approaches mutants were selected through their resistance to an antibiotic, but in both cases the hormone responsive selectable marker was not expressed in uninduced wildtype seedlings. For promoters that do give expression in uninduced wildtype seedlings, the alternative is to select mutants by a visual reporter. In this respect the use of the green fluorescent protein encoding gene (*gfp*) from the jellyfish *Aequorea victoria* as a reporter [79] may have advantages over the *gusA* and *luc* genes, since detection of expression does not interfere with viability and is independent on the exogenous application of a substrate. Several improved version of the *gfp* gene are now available that give reliable and detectable expression in plants when fused to relatively strong plant promoters [80,81]. However, for plant promoters that only give weak expression, further improvement of the *gfp* reporter system is required (Offringa et al., unpublished observations).

3. Other approaches

Clearly the mutant approach has led to the identification of many new components involved in hormone signalling. Nonetheless, several plant signal transduction components have been identified and characterized using other, complementary approaches. These will be described below.

3.1. Identification through homology

Signal transduction research in bacterial, yeast and animal systems has always been far ahead of that in plants and has therefore provided an excellent source of information on different signal transduction mechanisms. Many plant researchers have used the genes identified in these systems to search for homologous components in plants. Using low-stringency hybridization or PCR strategies based on the conserved sequences in the genes, homologs of protein kinases [82–86], protein phosphatases [87–89], small GTP binding proteins [90,91] and heterotrimeric G-proteins [92] have been identified in plants. For some of the kinases a role in hormone signalling has been suggested [93,94] but the evidence for such an involvement awaits further experimentation.

The homology based screening has been less successful for other signalling components. One reason for this may be that plants possess signalling pathways that do not exist or have not yet been described in animal or yeast systems, as is the case for the putative membrane bound steroid receptor, *BRI1*. Another explanation may be that the similarity between plant genes and those from other organisms is not sufficiently high to allow detection by hybridization or PCR. For example, it was only after the cloning of the

ETR1 gene through a molecular genetic approach [40] that other genes encoding homologous sensor histidine kinases involved in ethylene perception, such as *NEVER RIPE* from tomato and *ERS* and *ETR2* from arabidopsis, were identified [95–97]. Recently five genes encoding homologs of the response regulators of the bacterial two-component system were identified in arabidopsis [98]. The putative response regulators were identified by designing oligonucleotide PCR primers based on ESTs (expressed sequence tags, which are partial sequences of randomly picked cDNAs) in the sequence databases. Interestingly, two of the clones are identical to *IBC6* and *IBC7*, which have recently been described as cytokinin primary response genes [61]. The increasing number of ESTs and the arabidopsis genome sequencing data will allow to find signal transduction components by computer comparison. A striking example of such an approach is the EST-based cloning of a cDNA encoding a putative seven transmembrane domain, G protein coupled receptor, *GCR1*. *GCR1* shows remarkable sequence identity with the *Dictyostelium* cAMP receptors, which suggests that *GCR1* senses adenosine nucleotide residues. This, combined with the fact that antisense *GCR1* expressing plants show a decreased sensitivity to the cytokinin benzyl-amino purine, suggests a functional role for *GCR1* in cytokinin signal transduction [99].

3.2. Identification of transcription factors mediating the hormone response

As described in Section 2.3 of this chapter, hormone responsive genes have been one of the first targets for hormone signal transduction studies. Approaches that use the promoters of these genes as tools to select for response mutants have only recently been developed. Initially, careful dissection of the promoters led to the identification of the responsive elements. These elements were subsequently used to identify binding proteins through gel retardation and to clone the corresponding genes via protein purification [100] or SouthWestern screening of cDNA expression libraries. Using the latter approach a tobacco cDNA expression library was screened for proteins that bind to the ethylene responsive element (ERE) from the pathogenesis-related (*PR*) genes. These genes are part of the defense response of plants to pathogen attack. In addition to being induced by the defense-related hormone salicylic acid, their expression is also upregulated by ethylene. Four ERE binding proteins (EREBPs) have been identified [101]. The dramatic increase in *EREBP* mRNA levels shortly after ethylene treatment suggests that the EREBPs are the primary targets for ethylene signalling and that they subsequently regulate the secondary response genes such as the *PR* genes.

3.3. Yeast as a tool to study plant signal transduction components

New information about phytohormone signalling has also come from using yeast (*Saccharomyces cerevisiae*) as a research tool, despite the fact that unicellular eukaryotes such as yeast do not respond to plant hormones. Yeast has been used for the further characterization of signal transduction components that were initially identified through homology based screenings. The cyclin dependent kinases and some of the plant phosphatases have been tested for their functionality by expression of the specific cDNA in yeast strains with a mutation in the corresponding yeast gene and subsequently scoring

for functional complementation [83,84,102,103]. This approach is limited to the availability of the appropriate yeast mutant strains and has not been used for proteins involved in phytohormone signalling.

Expression of the putative ethylene receptor, ETR1, in yeast has been used to provide evidence that ETR1 can bind ethylene [104]. Yeast by itself does not show detectable ethylene binding, but a yeast strain expressing ETR1 on its plasma membrane displays saturable binding of [¹⁴C]ethylene. Interestingly, when the mutant form of ETR1 (*etr1-1*) is expressed, the yeast cells do not show detectable ethylene binding. This explains the ethylene insensitive phenotype of the *etr1-1* mutant.

Beside the functional analysis, specific selection techniques have been developed in yeast to identify transcription factors that bind to hormone responsive elements in plant promoters or interacting partners of known components in phytohormone signalling. These techniques are based on the fact that transcription factors consist of two separate domains, a DNA binding domain (DNA-BD) and a transcription activation domain (AD).

The first technique, referred to as the one-hybrid system (Fig. 4A), has been successfully applied to the AuxRE from the *GH3* promoter [105] (see also Chapter 19). Four tandem copies of the AuxRE were placed upstream of a minimal promoter sequence and this artificial promoter sequence was fused to both the histidine biosynthesis gene, *HIS3*, and the β -galactosidase encoding gene *lacZ*. A yeast strain which was *HIS3* deficient but contained both AuxRE-marker genes stably integrated into its genome was transformed with a vector containing fusions between random arabidopsis cDNAs and the yeast GAL4 transcriptional activator domain. A plant cDNA that encodes a protein that binds to the AuxRE will be able to induce *HIS* and *lacZ* expression via the activation domain of the GAL4 transcriptional activator. Yeast cells expressing the protein-GAL4 fusion can be selected through their *HIS* autotrophy and β -galactosidase activity. Of the 1.2×10^8 transformed cells screened, 500 *HIS* autotroph colonies were obtained, five of which also showed *LacZ* activity. All five cDNAs encoded the same Auxin Response Factor 1 (ARF1), none of which was fused in frame with the GAL4 activator domain. Both *in vivo* assays in yeast and carrot protoplasts as well as *in vitro* gel mobility-shift assays confirmed that ARF1 is a nuclear protein that specifically binds the AuxRE and is responsible for auxin-inducibility of gene expression. The protein contains a *N*-terminal DNA binding domain with homology to the binding domain found in transcriptional activators involved in ABA signal transduction. A $\beta\alpha\alpha$ -motif is located at the C-terminus. This motif is also present in proteins belonging to the Aux/IAA family.

A second method, the yeast two-hybrid system, is used to identify interactions between two known proteins or to screen for interacting partners of a known protein (Fig. 4B). The selection in the two-hybrid systems works in the same way as for the one-hybrid system, except that the *HIS3* and/or *LacZ* marker genes are now cloned behind a yeast promoter containing the yeast GAL1 upstream activating sequence (UAS). The cDNA encoding the target protein is cloned into a yeast vector behind a yeast promoter so that a translational fusion is obtained between the GAL4 DNA binding domain and the target protein. The cDNA to be tested is translationally fused to the activator domain of GAL4 and cloned into a second vector behind a yeast promoter. Cotransformation of both vectors into a histidine auxotroph yeast strain containing the UAS-marker(s) should only lead to *HIS*⁺

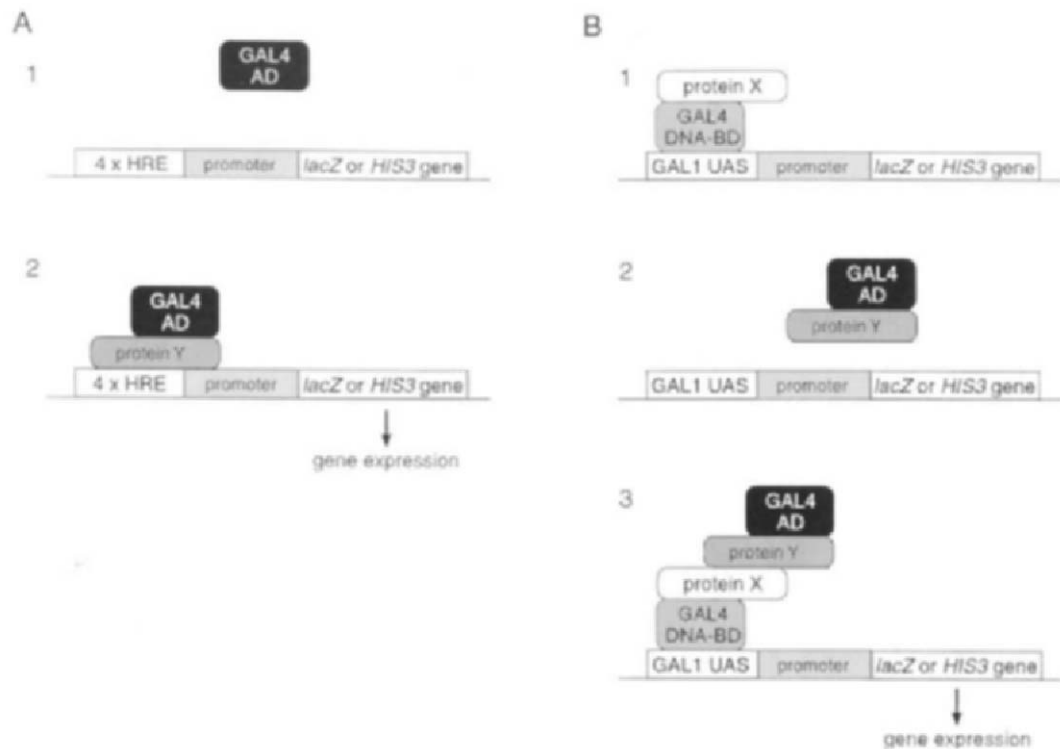


Fig. 4. Genetic selection for plant signal transduction components in yeast. A. The yeast one-hybrid system. The activator domain (AD) of GAL4 (a transcription factor from yeast) alone is not able to bind to the hormone responsive elements placed in tandem (4xHRE) upstream of a minimal yeast promoter (1). Expression of the marker genes is only expected when the GAL4-AD is fused to a plant transcription factor containing the proper DNA binding domain (protein Y) (2). B. The yeast two-hybrid system is used to demonstrate interactions between known components in phytohormone signal transduction or to identify proteins (Y) that interact with a known signal transduction component (protein X). Protein X is fused to the DNA binding domain of GAL4, (GAL4 DNA-BD) which specifically recognizes the upstream activating sequence (UAS) from the GAL1 promoter. The GAL1 UAS is placed upstream of a minimal yeast promoter. Protein Y is fused to the activation domain of GAL4 (GAL4 AD). An interaction between protein X and protein Y brings the DNA binding domain in proximity of the activator domain and allows expression of the marker genes to be initiated.

and/or LacZ⁺ yeast colonies when the GAL4 DNA binding domain and the GAL4 activator domain are brought in close proximity through interaction between target and test protein. Using the two-hybrid system, it was shown that the $\beta\alpha$ -motif present in ARF1, and in members of the Aux/IAA family, mediates interactions between these proteins [60,70].

The one- and two-hybrid systems in yeast are widely used in signal transduction research. Still, one has to be aware that these systems readily lead to artifacts, mostly due to the presence of endogenous interacting proteins in yeast. For putative transcription factors identified through the one-hybrid screen, the *in vivo* and *in vitro* tests mentioned for ARF1 are indispensable. Two-hybrid interactions should be further examined by biochemical assays with purified proteins and the biological significance should be confirmed by *in vivo* analyses.

4. Conclusion

Molecular approaches have contributed considerably to our current understanding of phytohormone action and signal transduction. Putative receptors have been identified for ethylene, cytokinin and brassinosteroids, whereas for hormones such as auxin and GAs components acting more downstream in signal transduction pathways have been identified. However, many steps in phytohormone signalling are still unclear and also the cross-talk between hormones is a field that has hardly been touched.

The mutant approach with the model plant *Arabidopsis thaliana* has led to major breakthroughs. However, for future research it can be expected that some hormone signalling components will not be identified in this way due to the lethality of mutations in the corresponding genes. This may be the reason why no upstream signal transduction components (receptor or kinase) have been isolated for the plant hormone auxin. In part, lethality of the mutation is determined by the mutagenesis technique used and the mutant screen applied. EMS mutagenesis and the promoter-out gene tagging strategy have proven to result in non-lethal mutations in genes that are likely to encode central components in phytohormone signalling. Moreover, the use of existing (response) mutants as starting material in a screen for novel plant hormone response mutants has also proven to be very useful. It is possible that a specific combination of mutagenesis approach and mutant selection strategy will allow for the isolation of components upstream in auxin signal transduction.

The alternative approaches are likely to gain in importance. The arabidopsis genome sequencing project clearly allows for homology based identification of signalling components. Their involvement in phytohormone signal transduction can subsequently be tested through the common reverse genetics approaches such as sense or anti-sense over-expression or via selection of knock-out mutants from collections of transposon or T-DNA insertion lines. In the near future, on locus modification of a gene via gene targeting may become a feasible strategy in plants. Gene targeting allows for the introduction of specific mutations into genes which is more sophisticated than insertional mutagenesis and which overcomes position effects observed with over-expression approaches. Unfortunately, at present gene targeting is not sufficiently efficient for general use in plants [106–108].

Once components in phytohormone signalling have been identified and characterized, these can be used to screen for upstream or downstream interacting factors in the yeast two-hybrid system.

With the current pace in the plant hormone signal transduction research, in the next few years exciting new discoveries in this field can be expected. Elucidation of hormone signal transduction pathways in plants will most likely reveal the existence of new signalling mechanisms that are unique to plants.

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