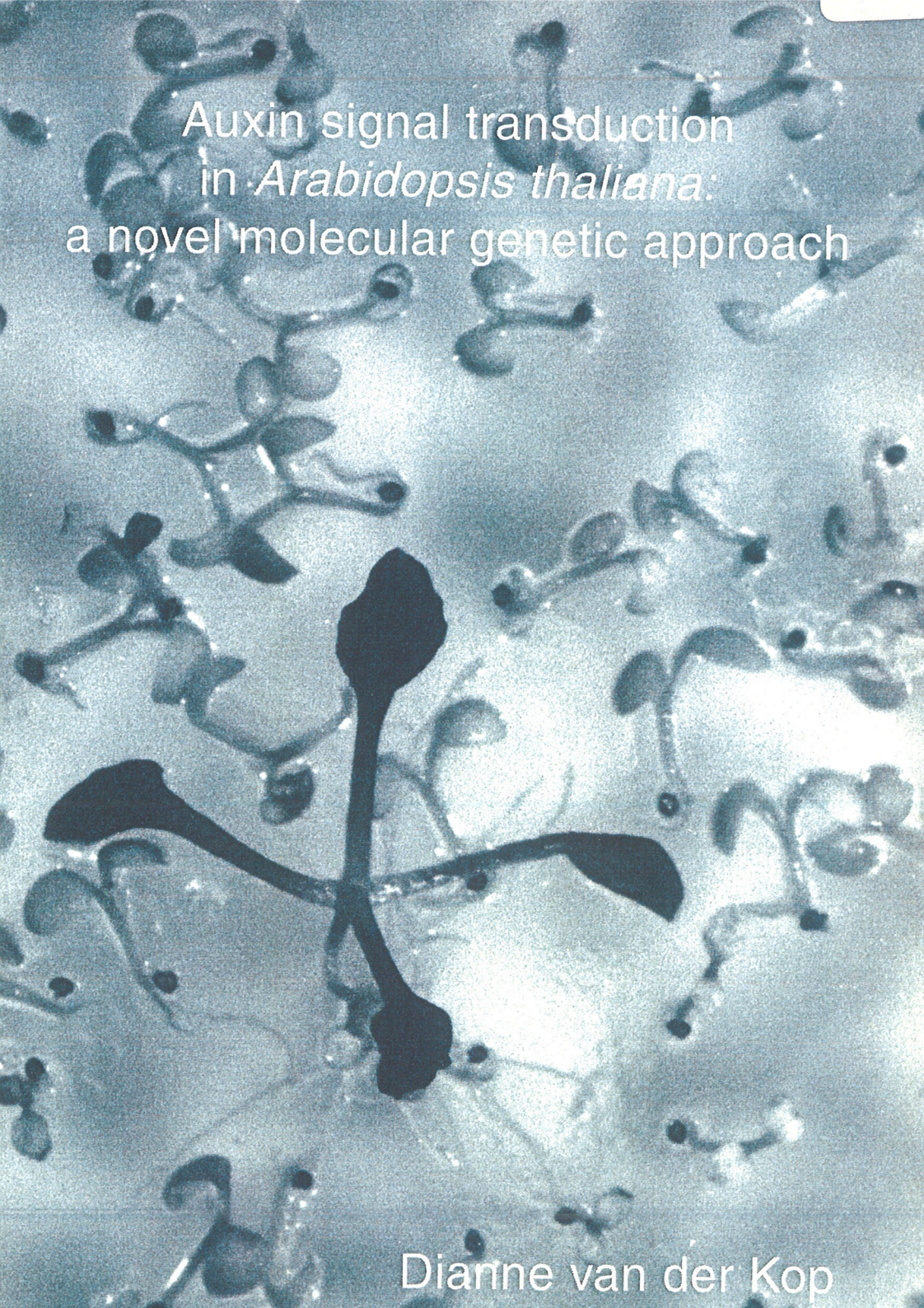

Auxin signal transduction in *Arabidopsis thaliana*: a novel molecular genetic approach

Dianne van der Kop

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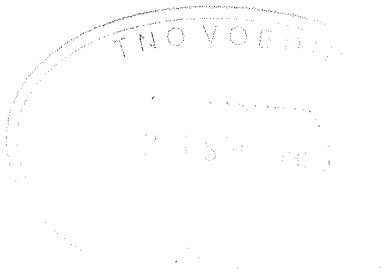

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Auxin signal transduction in *Arabidopsis thaliana*: a novel molecular genetic approach

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Aan mijn ouders

Voor Edward

Contents

CHAPTER 1

General introduction

By a committee of the American Society of Plant Physiologists plant hormones were defined as: "regulators produced by plants, which in low concentrations regulate plant physiological processes. Hormones usually move within the plant from a site of production to a site of action" (1954).

By this definition the group of classical plant hormones comprehend auxins, gibberellins, cytokinins, abscisic acid and ethylene. Also other substances like brassinosteroids (Mandava, 1988), jasmonic acid (Slaswick, 1992), salicylic acid (Raskin, 1992) and oligosaccharins (Tran Thanh Van *et al.*, 1985) were more recently found to be plant hormones.

Auxin, the first plant hormone to be discovered, regulates various aspects of plant growth and development including processes like cell elongation, cell division, vascular differentiation, apical dominance and root formation. In plants different naturally occurring auxins have been discovered. Next to indole-3-acetic acid (IAA) also other auxins like indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA) are present. Synthetic auxins like 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphtylacetic acid (1-NAA) are preferably used in research because they are more stable than the natural auxins.

Auxin signal transduction

Despite the fundamental influence of auxin on almost all aspects of plant development and extensive research, little is known about the molecular mode of action of auxin. In analogy with other eukaryotic systems, it is assumed that phytohormones activate cellular signal transduction systems after binding to specific receptors. Eventually this will lead to a change in gene expression.

To elucidate the signal transduction pathway(s) of auxin different strategies have been explored. Studies have been directed towards the isolation of auxin-binding proteins and auxin-regulated genes. Mutants with resistance for external auxin have been characterized. Transgenic plants with altered auxin levels due

to the insertion of genes for auxin production in their genome have been isolated and become increasingly important in the study of the action of auxin in plants. Results obtained with the different biochemical, genetic and molecular approaches will be discussed in this chapter.

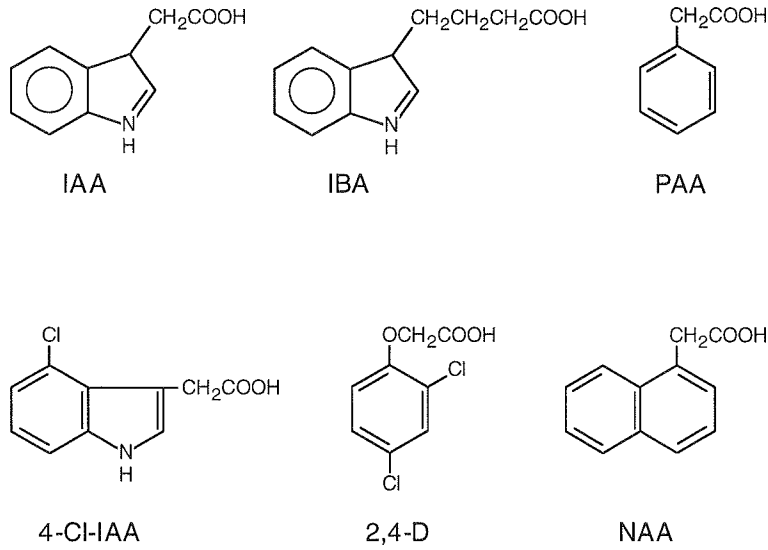

Figure 1. Structures of the naturally occurring auxins indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), phenylacetic acid (PAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA). and of the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphtylacetic acid (1-NAA).

Auxin-binding proteins

From studies on signal transduction pathways in lower and higher eukaryotes it is known that hormones may bind to soluble or membrane-associated receptor proteins. As a result of this binding, biochemical events may be initiated leading to the physiological responses known to be caused by the hormone. For this reason researchers have focused on the isolation of proteins that bind auxin in order to isolate receptor proteins involved in auxin signal transduction.

Auxin-binding activities were first demonstrated in maize in the 1970s (reviewed by Venis, 1977, Rubery, 1981). Later the "major" protein responsible for this binding, Zm-ERabp1 or ABP1, was purified and characterized (Löbler & Klämbt, 1985, Shimomura *et al.*, 1986). Several cDNA clones (Hesse *et al.*, 1989, Inohara *et al.*, 1989, Tillmann *et al.*, 1989, Lazarus *et al.*, 1991) and

genomic clones (Yu & Lazarus, 1991, Schwob *et al.*, 1993) have been isolated for this protein, indicating that five different, but closely related ABP-1 proteins exist in maize. Also other plant species including tobacco, strawberry and arabidopsis contain proteins that are closely related to maize ABP (Shimomura, Palme *et al.*, 1992). The deduced amino acid sequence of ABP-1 revealed that this protein has a hydrophobic NH_2-terminal sequence of 38 residues that may be a signal peptide for translocation of the protein across the membrane of the endoplasmic reticulum. The mature protein is composed of 163 residues and has a molecular weight of 18.3 kDa (Inohara *et al.*, 1989). The native protein is probably active as a dimer (Löbler & Klämbt, 1985, Shimomura *et al.*, 1986). The C-terminal KDEL tail is typical for proteins retained in the lumen of the endoplasmic reticulum (ER). Although the major portion of ABP1 probably exists within the lumen of the ER, there are indications that fractions of the protein are also present at the plasma membrane. Barbier-Brygoo *et al.* (1989, 1991) found that antibodies raised against Zm-ERabp1 caused a specific inhibition of auxin-induced electrical responses at the plasma membrane of tobacco protoplasts. By patch clamp techniques, it was found that these antibodies could inhibit an outwardly directed, auxin-dependent current (Rück *et al.*, 1993). Reversely, antibodies directed against a synthetic protein representing the putative auxin-binding site of ABP1 were able to evoke the same effects as auxin in protoplasts (Venis *et al.*, 1992, Rück *et al.*, 1993). The presence of ABP1 at the plasma membrane was recently confirmed by Diekmann *et al.* (1995) using silverenhanced immunogold labelling. Using photoaffinity labelling and microsequence analysis, the auxin binding site of ABP was pinpointed to a peptide fragment from Ile^{130} to Leu^{145} that was shown to bind auxin at a high specific activity (Brown & Jones, 1994)

By means of photoaffinity labelling other auxin-binding proteins have been detected as well. Hicks *et al.* (1989a) identified two 40/42-kDa auxin-binding proteins from zucchini in this way. These were located at the plasma membrane and might form part of an auxin uptake carrier. Also proteins of 22 and 24 kDa (Jones & Venis, 1989) and later of 23, 58 and 60 kDa were detected using ^3H labelled azido-IAA in maize (Campos *et al.*, 1992, Feldwisch *et al.*, 1992). The 23 kDa protein turned out to be associated with the membrane fraction (Feldwisch *et al.*, 1992) while the 60 kDa protein was present in the cytosol (Campos *et al.*, 1992). After determination of the amino acid sequence the protein was found to be a β-glucosidase with some homology to the *rolC* gene product. It could release active cytokinin from inactive conjugates, suggesting that this protein could play a role in developmental processes by controlling the

release of free cytokinin (Brzobohaty *et al.*, 1993). Other auxin-binding proteins that were isolated using photoaffinity labelling and subsequent characterization also turned out to have enzymatic functions. Among these were a basic form of β1,3-glucanase and a glutathione *S*-transferase in *Hyoscyamus muticus* (MacDonald *et al.*, 1991, Bilang *et al.*, 1993) and another glutathione *S*-transferase identified in arabidopsis (Zettl *et al.*, 1994).

Auxin-binding proteins that may function in the nucleus have also been isolated. There are indications that addition of IAA to the proteins called ABP-I and ABP-II, causes qualitative changes in polymerase selectivity during reinitiation, possibly by physically interacting with RNA polymerase II (Sakai *et al.*, 1992a,b).
Also using anti-idiotype antibodies two ABPs were detected that were probably located in the nucleus (Prasad & Jones, 1991). One of these ABPs was recently identified as a glutathione *S*-transferase (Jones & Prasad, 1994).

Auxin-regulated genes
Rapid alterations in gene expression have been observed after the addition of auxin to different tissues (reviewed by Guilfoyle, 1986, Hagen, 1989, Key, 1989). A number of auxin-regulated genes and cDNA clones have thus been identified in different plant species. They were induced within 15-30 minutes after the addition of auxin. The protein synthesis inhibitor cyclohexamide did not hamper this induction, suggesting that the accumulation of RNA represents a primary effect of auxin on transcription. Auxin-induced genes and/or cDNAs were isolated from *Nicotiana* cell-suspension cultures (Takahashi *et al.*, 1989, 1991, van der Zaal *et al.*, 1991, Dominov *et al.*, 1992, Ishida *et al.*, 1993), soybean hypocotyl (Walker & Key, 1982, Hagen *et al.*, 1984, McClure & Guilfoyle, 1987), pea epicotyl (Theologis *et al.*, 1985), mung bean epicotyl (Yamamoto *et al.*, 1992a,b) and arabidopsis seedlings (Conner *et al.*, 1990). Also genes encoded by the T-DNAs of *Agrobacterium tumefaciens* and *A. rhizogenes* have been reported to be regulated by auxin (An *et al.*, 1990, Maurel *et al.*, 1990, Korber *et al.*, 1991, Kim *et al.*, 1993).

While the genes mentioned thus far were up-regulated by auxin, also some genes that are down-regulated by auxin have been isolated from soybean (Baulcombe & Key, 1980, Datta *et al.*, 1993), strawberry (Reddy & Poovaiah, 1990) and *Catharanthus roseus* (Goddijn *et al.*, 1992, Pasquali *et al.*, 1992).

Based on their sequence similarity the auxin-inducible genes can be divided in three groups. The first group consists of genes homologous to the SAUR genes.

These genes have been isolated from soybean (McClure & Guilfoyle, 1987), arabidopsis (Gil *et al.*, 1994) and mung bean (Yamamoto *et al.*, 1992a). The RNA is expressed in hypocotyl tissue just prior to auxin-induced cell elongation (McClure & Guilfoyle, 1987). A correlation between SAUR gene expression and cell elongation has also been observed during the gravitropic response. The transcript is visible in cells that are going to elongate in response to gravity and can redistribute rapidly upon gravistimulation (McClure & Guilfoyle, 1989). The appearance of the SAURs before auxin-induced cell elongation suggests that they may contribute to this process (McClure *et al.*, 1989). The SAURs disappear rapidly from cells that are not targeted for elongation indicating that SAURs represent a class of unstable mRNAs (McClure & Guilfoyle, 1989). The mRNA instability is caused by DST elements present in the 3' untranslated regions of the genes (Newman *et al.*, 1993). The function of the SAURs is still unknown.

A second group of auxin-inducible genes that, like the SAUR genes, is specifically induced by auxin contains genes homologous to the soybean *Aux28* and *Aux22* genes (Ainley *et al.*, 1988). The arabidopsis genes *AtAux2-11* and *AtAux2-27* (Conner *et al.*, 1990) and *IAA1* and *IAA2* (Abel *et al.*, 1994), the pea genes *PS-IAA4/5* and *PS-IAA6* (Oeller *et al.*, 1993), the soybean gene *GH1* (Guilfoyle *et al.*, 1993) and the mung bean cDNA clones ARG3 and ARG4 (Yamamoto *et al.*, 1992b) also belong to this group. The genes are induced within 5-10 minutes after the addition of auxin or protein synthesis inhibitors (Theologis *et al.*, 1985). The proteins encoded by the genes are unstable and localized in the nucleus (Abel *et al.*, 1994). Because of the presence of a ß*αα* motif similar to the ß-sheet DNA binding domain of the Arc family of prokaryotic repressors, these proteins were suggested to act as activators or repressors of the expression of genes mediating the auxin response (Abel *et al.*, 1994).

The third class of auxin-inducible genes contains genes which encode proteins with homology to the soybean heat shock protein GmHSP26-A (Czarnecka *et al.*, 1988). The *parA/Nt114* gene (van der Zaal *et al.*, 1987, Takahashi *et al.*, 1990, Dominov *et al.*, 1992), the *parC/Nt107* gene (van der Zaal *et al.*, 1987, Takahashi & Nagata, 1992a) and the *parB* gene (Takahashi & Nagata, 1992b) represent three subfamilies of auxin-inducible genes in tobacco. The proteins encoded by the genes of these subfamilies have 63% to 68% homology to each other. The *Nt103* genes represent a fourth subfamily that encode proteins that share 40% to 45% homology with the proteins of the other subfamilies (Droog, 1995). The *GH2/4* gene of soybean (Hagen *et al.*, 1988) also belongs to this third class of auxin-inducible genes. The genes in this class

are not specifically induced by auxin. Also other compounds like salicylic acid, cytokinin and abscisic acid are able to induce some of the genes (Dominov *et al.*, 1992, Boot *et al.*, 1993). The *GmHSP26-A* gene of soybean was isolated as being heat-shock inducible. It could however also be induced by heavy metals, abscisic acid, ethylene and 2,4-D (Czarnecka *et al.*, 1988). Another gene with homology to the genes of the third group was the potato gene *prp1* (Taylor *et al.*, 1990). This gene was isolated as a pathogenesis related protein inducible by fungal elicitors. Proteins encoded by some of the genes of the third group were recently found to have GST activity *in vitro* (Takahashi & Nagata, 1992a, Droog *et al.*, 1993).

Some of the auxin-induced genes can not be classified in one of the groups mentioned. These genes include the *SbRP1* and *GH3* genes from soybean (Hong *et al.*, 1987, Hagen *et al.*, 1991) and the *dbp* gene from *Arabidopsis thaliana* (Alliotte *et al.*, 1989).

Auxin is involved in various processes and acts in different developmental stages and tissues. Next to the different classes of auxin-inducible genes that can be distinguished, the idea arises that more auxin signal transduction pathways exist. Further study on auxin-inducible genes will hopefully elucidate on this hypothesis.

Auxin-responsive elements

The level of mRNA in the plant is dictated by the rate of synthesis and the rate of degradation. With the identification of sequence elements in the promoters of the auxin-inducible genes and the isolation of the proteins binding to these sequences, one hopes to learn more of the factors that determine the rate of synthesis of the mRNA and thus on the auxin signal transduction pathways. By comparing the promoter regions of different auxin-inducible genes and using a number of different experimental techniques, several Auxin-Responsive Elements (AuxRE) have been identified. Because auxin influences the expression of a variety of genes that are involved in many different processes, it is not surprising that a number of different types of AuxREs are present in plant genes. Multiple AuxREs in one promoter can function in combination with each other or independently. They can also function to incrementally increase the auxin inducibility of the promoter. The AuxREs isolated thus far share little or no resemblance to each other. Only for a limited number of elements identified it was really shown that the element conferred auxin-inducibility.

In the promoter of the pea gene *PS-IAA4/5* two domains were identified that

were necessary and sufficient for auxin-inducibility. Domain A contained an auxin-responsive element while domain B contained an enhancer-like element affecting both the auxin inducibility and the constitutive expression of the gene (Ballas *et al.*, 1993). One motif in domain A turned out to be present in many other auxin-regulated genes as well (Oeller *et al.*, 1993).

The NDE region of the soybean SAUR genes was found to be required for auxin-inducibility (McClure *et al.*, 1989). One sequence within this region was also found to be required for auxin-inducibility of the *GH3* promoter (Liu *et al.*, 1994).

The *as-1/ocs* elements of different T-DNA and viral promoters were recently found to mediate auxin inducibility (Kim *et al.*, 1994, Liu & Lam, 1994, Qin *et al.*, 1994). These elements could not only mediate induction by auxin but also by salicylic acid (Qin *et al.*, 1994, Zhang & Singh, 1994). The elements were also found to be responsible for the auxin-triggered expression in the promoters of several genes in the third group of auxin-inducible genes (Droog *et al.*, 1995).

Other possible auxin-responsive elements have been identified in the *Agrobacterium tumefaciens* T-DNA *gene 5* (Korber *et al.*, 1991) and in the soybean gene *GmAux28* (Nagao *et al.*, 1993).

Auxin biosynthesis and the manipulation of auxin levels by bacterial genes

Auxin biosynthesis

The presence of auxin in certain tissues of the plant is due to different pathways that work next to each other (reviewed by Bandurski *et al.*, 1995). For a long time auxin was thought to be converted from tryptophan. Recently also other pathways for the production of IAA have been reported but the exact pathway for the production of IAA by such a route is not yet known. In plants auxin is present both as free acid and in conjugated forms. In most tissues the conjugated forms predominate. They are enzymatically hydrolysed to release the free acid. Also transport of auxin leads to a redistribution of auxin within the plant. Oxidative catabolism and conversion of free acid in a conjugative form result in a loss of auxin activity.

Bacterial genes

By introducing bacterial genes into the plant it is possible to manipulate the level

of active auxin in the plant. The advantage of this technique over the external application of auxin is that the level of auxin can be measured and regulated in a tissue or organ specific manner. The studies provided considerable insight in developmental and physiological processes in which auxin is involved.

The level of free IAA in a plant could be decreased by the expression of the bacterial *iaaL* gene from *Pseudomonas savastanoi*. This gene encodes the enzyme IAA-lysine synthase, that converts IAA to IAA-lysine, a compound with reduced auxin activity (Glass & Kosuge, 1986, Roberto *et al.*, 1990). Potato (Spena *et al.*, 1991), tobacco (Spena *et al.*, 1991, Romano *et al.*, 1991) and *Arabidopsis thaliana* (Estelle & Klee, 1994) have been transformed using this gene. While the level of IAA-lysine increased, the level of free IAA decreased in these plants. The plants expressing the *iaaL* gene displayed a decrease in apical dominance, inhibition of rooting and leaf epinasty due to petiole bending. Also a decrease in vascular development was reported. Due to the unequal development of vascular tissue and other tissues in the leaf, also leaf wrinkling was observed (Romano *et al.*, 1991). These phenotypic changes were in line with the expected effects of reduced IAA levels.

The *iaaM* and *iaaH* genes of *Agrobacterium tumefaciens* code for enzymes that catalyze IAA synthesis from tryptophan (Schroder *et al.*, 1984, Thomashow *et al.*, 1984, van Onckelen *et al.*, 1986). Transgenic plants expressing the *iaaM* gene or both the *iaaH* and *iaaM* genes contained increased levels of IAA (Klee *et al.*, 1987, Sitbon *et al.*, 1991, 1992a, 1992b, 1993). Also the level of conjugated IAA was increased in these plants. The auxin overproducing plants generally displayed increased apical dominance, adventitious root formation, increased vascular tissue formation, epinastic leaf growth and decreased internode elongation. These phenotypes were in general opposite to the phenotypes observed in *iaaL* plants with decreased auxin levels. Part of the phenotype could however be due to an increase in the level of ethylene, because ethylene is known to be formed when auxin levels are increased. Experiments performed by Romano *et al.* (1993), using the *iaaM* gene to increase the auxin level and the bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCase) gene to avoid an increase in the level of ethylene, showed that auxin overproduction was responsible for the increased apical dominance and leaf epinasty, and that ethylene was partially responsible for the reduction in stature of the *iaaM* overproducing plants. Similar results were obtained with ethylene insensitive mutants in which the *iaaM* gene was overexpressed (Romano *et al.*, 1993). Evidence for the hypothesis that many physiological responses are controlled by ratios of hormone concentrations comes from experiments

performed by Klee & Estelle (1991), who showed that some effects of auxin overproduction can be overcome by overproduction or by exogenous application of cytokinin.

Also other bacterial genes like the *rol* genes of *Agrobacterium rhizogenes* alter hormone physiology and thereby affect plant growth and development. The mode of action of these genes is however more complicated and not completely solved.

The effects of overexpression of the above described genes in the transgenic plants shed light on the processes in which auxin is involved and the phenotype caused by the alteration of auxin levels. Plants seem capable of regulating the levels of free IAA and can partially compensate for the removal of IAA (Spena *et al.*, 1991, Romano *et al.*, 1991, Estelle & Klee, 1994). These results are very useful in the isolation of mutants involved in the mode of action of auxin.

Auxin mutants

A number of groups isolated mutants in order to gain insight into the mode of action of auxin. Different plant species have been used to isolate mutants with altered auxin responses. Also auxin synthesis and transport mutants have been described. Interesting mutants will be discussed in this chapter. Especially the mutants of *Arabidopsis thaliana* are discussed to some detail.

Auxin-response mutants

Most of the auxin-response mutants isolated sofar are mutants resistant to auxin. The first auxin resistant mutants were isolated in cultured tobacco cells (Chaleff & Parsons, 1978). Later, other auxin resistant mutants were isolated from different plant species. Some of these mutants were characterized to some detail. The *rac* mutant was isolated from mutagenized mesophyll protoplasts of *Nicotiana tabacum*. The mutant protoplasts were less sensitive to auxin. They required more auxin to achieve the same level of hyperpolarization as wild type protoplasts (Ephrilikhine *et al.*, 1987). Pelese *et al.* (1989) found that the level of IAA, abscisic acid (ABA) and isopentenyladenine was increased in the root tip of the mutant.

The auxin insensitive tomato mutant *dgt* had diagravitropic shoot growth, abnormal vascular tissue, altered leaf morphology and no lateral root branching (Zobel, 1973). The auxin level in this mutant was not altered. Using

photoaffinity labelling, it was found that the mutant lacked an auxin binding-protein in membrane fractions of stems. In roots however this auxin-binding protein was still present (Hicks *et al.*, 1989b).

A screening for small kernel size in maize revealed several mutants with altered IAA levels. In two mutants the kernel size was restored after the application of external auxin (Torti *et al.*, 1986, Lur & Setter, 1993). In *Lemna* a spontaneous mutant was regenerated from tissue culture with 1.5 times larger cells. The IAA level of this mutant turned out to be higher than in wild-type at several stages of development (Slovin & Cohen, 1988). For barley and pea auxin resistant mutants have also been described. They were isolated because of their agravitropic root growth (Tagliani *et al.*, 1986, Eason *et al.*, 1987).

Two auxin sensitive mutants have been isolated from *Nicotiana plumbaginifolia* (De Souza & King, 1991). The mutant seedlings had mildly epinastic leaves, a short root, increased root branching and lacked root hairs. The mutants were more sensitive to auxin, ethylene, 1-aminocyclopropane-1-carboxylate (ACC) and L-tryptophan but not to D-tryptophan, 6-benzyladenine or abscisic acid.

An interesting auxin independent line was isolated after transformation of *Nicotiana tabacum* protoplasts with a T-DNA derived vector containing multiple enhancer sites near the right border. This caused a dominant mutation resulting in overexpression of the gene adjacent to the T-DNA. This gene was isolated and found to confer auxin-independent growth upon re-introduction into tobacco protoplasts. The gene encoded a highly basic protein with unknown function (Hayashi *et al.*, 1992).

Auxin response mutants in arabidopsis

In arabidopsis a number of auxin response mutants have been isolated. Most of the mutants were isolated because of their resistance to the synthetic auxin 2,4-D. More recently mutants were isolated in screenings for NAA resistance.

The mutant *aux1* was first isolated by Maher & Martindale (1980). The mutant has agravitropic roots and increased elongation of roots accompanied by a reduced elongation of hypocotyls. The starch-containing amyloplasts in the roots of the *aux1* mutant, thought to be important for gravity detection, were found to sediment more slowly in the mutant than in wild-type (Olsen *et al.*, 1984). It is however not known if this effect alone can account for the gravitropic defect. The recessive *aux1* mutation caused resistance to auxin, ethylene and cytokinin (Pickett *et al.*, 1990, Hobbie & Estelle, 1994).

The dominant *dwf* mutant is resistant to auxin. The mutant has a dwarf phenotype when heterozygous and is lethal when homozygous. In the early seedling stage the seedlings have long single branched roots that lack hairs and short hypocotyls. Both roots and hypocotyls are agravitropic. Root hair formation could be restored by the addition of auxin (Mirza & Maher, 1987).

One of the best studied auxin mutants is the *axr1* mutant (Estelle & Somerville, 1987). The phenotype of the whole plant is affected by the mutation. The mutant has reduced elongation of stem, stamen and hypocotyl, reduced apical dominance, leaf wrinkling, increased root elongation and decreased root gravitropism and root branching. Also the vascular bundles of the stems are less well differentiated than in the wild-type (Lincoln *et al.*, 1990). The mutant is resistant to auxin, cytokinin and ethylene. The *AXR1* gene was the first gene to be isolated using map-based cloning. It encodes a protein related to the ubiquitin- activating enzyme E1 (Leyser *et al.*, 1993). It is however not thought to have E1 activity because the protein is much smaller than E1 proteins and it lacks a cysteine residue known to be essential for E1 activity.

The dominant *axr2* mutant also has a dwarf phenotype. It displays defects in growth orientation of both shoot and root. The mutant plants lack root hairs (Wilson *et al.*, 1990). A reduction in cell elongation is thought to be responsible for this dwarf phenotype (Timpte *et al.*, 1992). This is in contrast to the *axr1* mutant in which a reduction in cell number was responsible for the reduction in stature (Lincoln *et al.*, 1990). The *axr2* mutant is resistant to auxin, ethylene and abscisic acid. The accumulation of the auxin inducible *SAUR-AC1* gene transcript was found to be almost completely blocked in the *axr2* mutant compared to wild-type (Gil *et al.*, 1994). Since SAUR expression is highly correlated with the effects of auxin on cell elongation, the strong effect of *axr2* on both elongation and SAUR gene expression lends support to the possibility that the growth defect in the *axr2* mutant is the result of a defect in auxin action (Gil *et al.*, 1994).

The *axr3* mutant is less sensitive to auxin. Mutant plants produce a single unbranched inflorescence while the wild-type produces two to five inflorescences and the *axr1* mutant produces highly branched inflorescences. The roots grow from the hypocotyl and are agravitropic, twisted and lack root hairs. This phenotype was explained by the assumption that the mutant has a constitutive auxin response and is therefore not able to respond to exogenous auxin (Hobbie & Estelle, 1994).

The *axr4* mutant is also defective in root gravitropism. Apart from a slight curling of the leaf along their length, the mutant has no phenotype. The mutant

is highly resistant to auxin (Hobbie & Estelle, 1995)

Since the auxin resistant mutants isolated were impaired in their gravitropism, the mutants *agr1, agr2* and *agr3* that were selected as being agravitropic were reversely tested for their auxin response. The recessive *agr3* mutant was indeed more sensitive to auxin over a narrow concentration range (Maher & Bell, 1990)

Table 1. Auxin resistant mutants of *Arabidopsis thaliana*..

mutant	genetics	chromosome location	literature
aux1	recessive	2	Maher & Martindale, 1980
dwf	semi-dominant	3	Mirza & Maher, 1987
axr1	recessive	1	Estelle & Somerville, 1987
axr2	dominant	3	Wilson *et al.*, 1990
axr3	semi-dominant	1	Hobbie & Estelle, 1994
axr4	recessive	1	Hobbie & Estelle, 1995
agr3	recessive	nd	Maher & Bell, 1990

nd = not determined

Auxin transport mutants

The phenotype of the *pin1* mutant of arabidopsis can be mimicked by the addition of the auxin polar transport inhibitors 9-hydroxyfluorene-9-carboxylic acid and N-(1-naphthyl)phthalamic acid to wild types. Auxin polar transport is affected in the *pin* mutants (Okada *et al.*, 1991). The *PIN1* gene may encode a protein important in the auxin transport system. It could however also be that the auxin transport defect is a secondary effect of an alteration in the meristem or inflorescence structure. Because Estelle & Klee (1994) found no reversion to the wild-type phenotype after introduction of the *iaaM* or *iaaL* genes into *pin1* mutants, they argue that the auxin transport defect is indeed due to a secondary effect. However, since the introduction of the genes caused overproduction of auxin in all tissues, auxin would still be overproduced in the meristem if the mutation caused a defect in auxin transport. A reversion to the wild-type phenotype was thus not likely to occur.

Other auxin transport mutants have been isolated and their characterization is in progress (Hobbie & Estelle, 1994).

Arabidopsis thaliana as a model flowering plant species

Already in 1943 Laibach described the benefits of the plant *Arabidopsis thaliana* as a model plant for research purposes. However, until recently only a limited group of researchers used the plant for their studies. This changed quickly when more scientists discovered the favourable features of arabidopsis.

Arabidopsis has a small genome size, comprising about 100 000 kb of DNA. This is 6 times the genome size of *Saccharomyces cerevisiae* but only half of the size of the genome of *Drosophila melanogaster*. The genomes of other higher plants are usually much larger. Also the percentage of methylation of the arabidopsis genome is rather low compared to that of other plant species (Leutwiler *et al.*, 1984). Despite the small genome size, arabidopsis has all characteristics of a higher plant. As a consequence the percentage of repetitive DNA in the genome should be low compared to other plant species. By reassociation kinetic measurements it was shown that the percentage of highly repetitive DNA was about 10 % (Leutwiler *et al.*, 1984). The percentage of moderately repetitive DNA was estimated to be less than 27% of the cellular genome (Meyerowitz & Pruitt, 1985). For this reason many genes in arabidopsis are thought to be single copy genes or members of only small gene families. This however is not true for all genes. The β-tubulin gene family of arabidopsis is the biggest ever found in plant or animal species (Snustad *et al.*, 1992).
One of the other advantages of arabidopsis is the short life cycle and small stature. Plants can easily be grown in tissue culture and a lot of plants can be grown on a small area. One plant produces many small seeds that contain a limited number of cells. These properties make the plant extremely useful for classical mutagenesis experiments using physical or chemical mutagens.

The isolation of mutants in arabidopsis
The mutagen ethylmethane sulfonate (EMS) causes point mutations. Genes corresponding to the mutation have to be isolated by using positional cloning. The small genome size and the presence of only little repetitive DNA make it possible to do this in arabidopsis. This has been achieved already for some mutants of which genes corresponding to the mutation have been cloned recently (Giraudat *et al.*, 1992, Leyser *et al.*, 1993). Physical mutagenesis using ionizing radiation like X-rays, mainly causes deletions. The gene corresponding to the deletion can be isolated using a genomic subtraction technique. This technique has been used successfully in arabidopsis to clone the *GA1* gene (Sun *et al.*, 1992).

Although different techniques have been developed to facilitate the isolation of the genes corresponding to the mutations generated by classical mutagenesis, the procedures are still rather laborious. For this reason other techniques have been developed to isolate mutants and their corresponding genes. Insertional mutagenesis using T-DNA vectors or transposons have been successfully used. The first gene corresponding to a mutation, caused by an insertion of a transposon, was cloned recently from *Arabidopsis* (Aarts *et al.*, 1993). T-DNA tagged genes have been cloned from different mutants using the T-DNA of *Agrobacterium tumefaciens* as a tag (Herman & Marks, 1989, Koncz *et al.*, 1990, Yanofsky *et al.*, 1990). With the rapidly growing number of mutants generated by T-DNA insertion, this technique is becoming increasingly important. The generation of large numbers of arabidopsis transformants is however time consuming and the number of T-DNA tagged lines is still limited. Also the percentage of mutations linked to the DNA is low. Conventional methods to generate mutants are therefore still used and the genes corresponding to the mutation have to be isolated using map based cloning. Different techniques to improve transformation and facilitate map based cloning have been developed in arabidopsis.

Transformation of arabidopsis

One of the prerequisites for a model plant species is the existence of an efficient transformation protocol. Due to the limited tissue culture expertise, arabidopsis was considered to be recalcitrant to *Agrobacterium* transformation for some time. Transformation of protoplasts via direct gene transfer was first achieved (Damm *et al.*, 1989). Later also transformation protocols using *Agrobacterium tumefaciens* were developed. Lloyd *et al.* (1986) developed a method to transform leaf discs. This protocol was optimized by Schmidt & Willmitzer (1988). The protocol most widely used at this moment is the protocol developed by Valvekens *et al.* (1988) using root explants for transformation. The tissue culture protocols are time consuming and generate a lot of somaclonal variation. These problems might be overcome by using *in planta* transformation. Feldmann & Marks (1987) developed a seed transformation protocol. Though they were able to produce many transgenic lines, now used for T-DNA tagging of mutants, the transformation frequency was rather low and not reproducible. Also the percentage of mutations linked to the T-DNA turned out to be rather low. Recently another method to transform arabidopsis *in planta* was described. This protocol is based on vacuum infiltration of a suspension of *Agrobacterium* cells

into arabidopsis plants (Bechtold *et al.*, 1993). The method has the potential to become very important for the generation of large collections of T-DNA tagged lines.

Map based cloning

The relatively simple genome of arabidopsis simplifies the cloning of genes that have been identified by mutational analysis. To facilitate the cloning, genetic maps have been constructed. The genome of arabidopsis consists of 5 chromosomes (n=5). The first genetic map with positions of various mutations mapped on the chromosomes was published by Koornneef *et al.* (1983).

With the advances made in molecular biology new techniques became available that were applied to generate maps. Two maps were constructed containing Restriction Fragment Length Polymorphism (RFLP) markers (Chang *et al.*, 1988, Nam *et al.*, 1989). Later maps with markers based on PCR techniques, like the Random Amplified Polymorphic DNA (RAPD) and Cleaved Amplified Polymorphic Sequence (CAPS) markers became available (Reiter *et al.*, 1992, Konieczny & Ausubel, 1993). An other technique using microsatellites (SSPLs) is not commonly used but has the potential to become important in the future (Bell, arabidopsis newsgroup, 1994). In 1993 the RFLP maps and the visible marker map were integrated, generating a map containing 125 classical markers and 306 RFLP markers (Hauge *et al.*, 1993).

The first mapping set was developed by Koornneef *et al.* (1986) who constructed a tester stock with at least one visible marker on each chromosome arm. Later Fabri & Schäffner (1994) selected RFLP markers that detect segregating RFLP bands after *EcoR*I restriction enzyme digestion. In this way they composed a RFLP mapping set (ARMS) containing 13 markers that uniformly cover the five arabidopsis chromosomes. A mapping set has also been composed for CAPS markers (Konieczny & Ausubel, 1993). These mapping sets greatly facilitate the positioning of a mutation on the genome.

Next to the generation of maps also the existence of genomic libraries will facilitate the isolation of mutated genes. Overlapping cosmid libraries and yeast artificial chromosomes (YACs) have been constructed (Ward & Jen, 1990, Grill & Somerville, 1991, Hauge *et al.*, 1991). The YACs are positioned on the genetic map using the RFLP markers. YAC clones are then linked in walking experiments. Different laboratories focus on different parts of chromosomes (Schmidt & Dean, 1993). The map position of a gene identified by a mutation can be determined by finding linkage between the mutation and markers. After

the map position of a mutated gene has been determined, the YAC clones containing the marker can be isolated. Different strategies have been used to isolate the gene using the YACs (Chang *et al.*, 1993, Leyser *et al.*, 1993).

Recombinant inbred lines have been developed to determine the map position of genes of which the sequence is already known (Lister & Dean, 1993). In this way one hopes to get insight in the way the genome is organized.

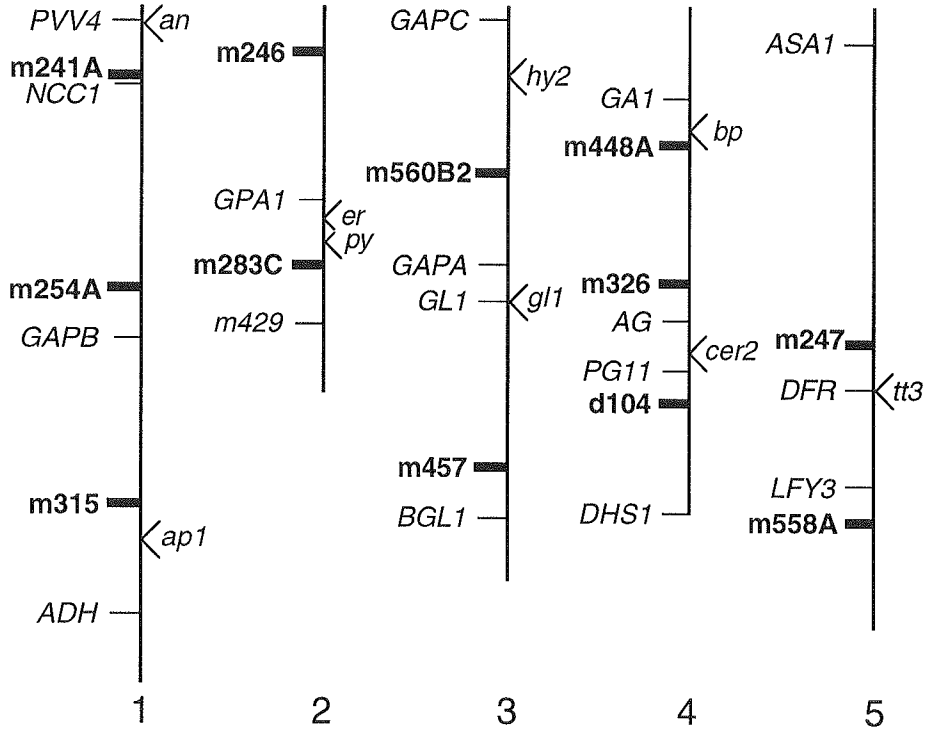

Figure 2. Chromosomal location of markers (according to Lister and Dean, arabidopsis newsgroup, 1994). The RFLP markers of the ARMS marker set (▬)(Fabri & Schäffner, 1994) and the CAPS markers (—) (Konieczny & Ausubel, 1993) are indicated at the left side of each chromosome arm. On the right side of the chromosome the nine phenotypic traits displayed by the tester line W100 (<) (Koornneef *et al.*, 1986) are depicted.

The outline of this thesis

The aim of this thesis was to solve parts of the puzzle of auxin signal transduction. We chose to do this by studying auxin-inducible genes and using

these in a novel genetic approach. In this way we will be able to identify mutations involved in auxin signal transduction without selecting for morphological alterations of the phenotype. We hope to identify components that are important in the auxin signal transduction pathway leading to the expression of these auxin-inducible genes. As an auxin-inducible gene we chose the *Nt103-1* gene of tobacco. Also arabidopsis genes homologous to this tobacco gene are isolated, allowing the use of arabidopsis genetics to analyze the function of the *Nt103* gene family. **Chapter 2** reports on the isolation of arabidopsis genes homologous to one gene of this family, the tobacco auxin-inducible *Nt103-1* gene. The sequences of the promoters and coding regions were compared to each other and to other auxin-inducible genes. The expression patterns of the isolated genes were determined and compared to the expression patterns of the homologous tobacco genes. **Chapter 3** deals with a cDNA corresponding to one of the isolated genes that is sequenced and overexpressed in *E. coli* to check if the protein has, like its tobacco homologs, glutathione *S*-transferase activity. Also transgenic plants are made that overexpressed the gene or in which the gene was repressed. The phenotype and the auxin sensitivity of these plants is investigated. **Chapter 4** describes the introduction of a construct containing the tobacco auxin-inducible *Nt103-1* gene fused to the *β*-glucuronidase (*gusA*) reporter gene into arabidopsis. The expression is studied in the presence and absence of external auxin. The results of this analysis convinced us that we could use this promoter in a mutagenesis experiment as reported in **chapter 5**. In this chapter the construction of the transgenic lines for the mutagenesis experiment is described as well as a study of their expression profiles. The mutagenesis and selection are also described in this chapter. In **chapter 6** some of the selected mutants are characterized in more detail and the map position of two mutants is determined. Finally in **chapter 7** we summarize and discuss the results.

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References

Nomenclature of chemical plant regulators. (1954) Plant Physiol 29: 307

Aarts MGM, Dirkse WG, Stiekema WJ, Pereira A (1993) Transposon tagging of a male sterility gene in arabidopsis. Nature 363: 715-717

Abel S, Oeller PW, Theologis A (1994) Early auxin-induced genes encode short-lived nuclear proteins. Proc Natl Acad Sci *USA* 91: 326-330

Ainley WM, Walker JC, Nagao RT, Key JL (1988) Sequence and characterization of two auxin-regulated genes from soybean. J Biol Chem 263: 10658-10666

Alliotte T, C Tiré, Engler G, Peleman J, Caplan A, van Montagu M, Inzé D (1989) An auxin-regulated gene of *Arabidopsis thaliana* encodes a DNA-binding protein. Plant Physiol 89: 743-752

An G, Costa MA, Ha S-B (1990) Nopaline synthase promoter is wound inducible and auxin inducible. Plant Cell 2: 225-233

Ballas N, Wong L-M, Theologis A (1993) Identification of the auxin-responsive element *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA45*, of pea (*Pisum sativum*). J Mol Biol 233: 580-596

Bandurski, RS, Cohen, JD, Slovin JP, Reinecke DM (1995) Auxin biosynthesis and metabolism. In: Plant hormones. Davies PJ (ed) Kluwer Academic Publishers, the Netherlands: 39-65

Barbier-Brygoo H, Ephritikhine G, Klämbt D, Ghislain M, Guern J (1989) Functional evidence for an auxin receptor at the plasmalemma of tobacco mesophyll protoplasts. Proc Natl Acad Sci *USA* 86: 891-895

Barbier-Brygoo H, Ephritikhine G, Klämbt D, Maurel C, Palme K, Schell J, Guern J (1991) Perception of the auxin signal at the plasma membrane of tobacco mesophyll protoplasts. Plant J 1: 83-93

Baulcombe DC, Key JL (1980) Polyadenylated RNA sequences which are reduced in concentration following auxin treatment of soybean hypocotyls. J Biol Chem 255: 8907-8913

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by filtration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris, Life Sciences 316: 1194-1199

Bell CJ, Maher EP (1990) Mutants of *Arabidopsis thaliana* with abnormal gravitropic responses. Mol Gen Genet 220: 289-293

Bilang J, MacDonald H, King PJ, Sturm A (1993) A soluble auxin-binding protein from *Hyoscyamus muticus* is a glutathione *S*-transferase. Plant Physiol 102: 29-34

Boot KJM, van der Zaal BJ, Velterop J, Quint A, Mennes AM, Hooykaas PJJ, Libbenga KR (1993) Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell-suspension cultures. Plant Physiol 102: 513-520

Brown JC, Jones AM (1994) Mapping the auxin-binding site of auxin-binding protein 1.

The Journal of Biological Chemistry 269: 21136-21140

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, (1993) Release of active cytokinin by a ß-glucosidase localized to the maize root meristem. Science 262: 1051-1054

Campos N, Bako L, Feldwisch J, Schell J, Palme K (1992) A protein from maize labeled with azido-IAA has novel ß-glucosidase activity. Plant J 2: 675-684

Chaleff RS, Parsons MF (1978) Direct selection in vitro for herbicide- resistant mutants of *Nicotiana tabacum*. Proc Natl Acad Sci *USA* 75: 5104-5107

Chang C, Bowman JL, DeJohn AW, Lander ES, Meyerowitz EM (1988) Restriction length polymorphism linkage map for *Arabidopsis thaliana*. Proc Natl Acad Sci *USA* 85: 6856-6860

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene *ETR1*: Similarity of product to two-component regulators. Science 262: 539-544

Conner TW, Goekjian VH, LaFayette PR, Key JL (1990) Structure and expression of two auxin-inducible genes from arabidopsis. Plant Mol Biol 15: 623-632

Czarnecka E, Nagao RT, Key JL, Gurley WB (1988) Characterization of *Gmhsp26-A*, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol Cell Biol 8: 1113-1122

Damm B, Schmidt R, Willmitzer L (1989) Efficient transformation of *Arabidopsis thaliana* using direct gene transfer to protoplasts. Mol Gen Genet 217: 6-12

Datta N, LaFayette PR, Kroner PA, Nagao RT, Key JL (1993) Isolation and characterization of three families of auxin down-regulated cDNA clones. Plant Mol Biol 21: 859-869

DeSouza L, King PJ (1991) Mutants of *Nicotiana plumbaginifolia* with increased sensitivity to auxin. Mol Gen Genet 231: 65-75

Diekmann W, Venis MA, Robinson DG (1995) Auxins induce clustering of the auxin-binding protein at the surface of maize coleoptile protoplasts. Proc Natl Acad Sci *USA* 92: 3425-3429

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. The Plant Cell 4: 451-461

Droog FNJ, Hooykaas PJJ, Libbenga KR, van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferases and one member indeed shows *in vitro* GST activity. Plant Mol Biol 21: 965-972

Droog FNJ, Spek A, van der Kooy A, de Ruyter A, Hoge H, Libbenga KR, Hooykaas PJJ, van der Zaal EJ (1995) Promoter analysis of the auxin-regulated tobacco glutathione *S*-transferase genes *Nt103-1* and *Nt103-35*. Plant Mol Biol *in press*

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD Thesis, Leiden University, 165 pp

Eason WR, Hall G, Wang TL (1987) *In vitro* analysis of gravitropic mutants of pea. Journal of Plant Physiol 131: 201-213

Ephritikhine G, Barbier-Brygoo H, Muller J-F, Guern J (1987) Auxin effect on the transmembrane potential difference of wild-type and mutant tobacco protoplasts exhibiting a differential sensitivity to auxin. Plant Physiol 83: 801-804

Estelle MA, Klee H (1994) Auxin and cytokinin in arabidopsis. In: arabidopsis. Meyerowitz E, Somerville C (ed) Cold Spring Harbour Press, New York, in press

Estelle MA, Somerville C (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genet 206: 200-206

Fabri CO, Schäffner AR (1994) An *Arabidopsis thaliana* RFLP mapping set to localize mutations to chromosomal regions. The Plant Journal 5: 149-156

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

Feldwisch J, Zettl R, Hesse F, Schell J, Palme K (1992) An auxin-binding protein is localized to the plasma membrane of maize coleoptile cells: identification by photoaffinity labeling and purification of a 23-kDa polypeptide. Proc Natl Acad Sci *USA* 89: 475-479

Gil P, Liu Y, Orbovic V, Verkamp E, Poff KL, Green PJ (1994) Characterization of the auxin-inducible *SAUR-AC1* gene for use as a molecular genetic tool in arabidopsis. Plant Physiol 104: 777-784

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell 4: 1251-1261

Glass NL, Kosuge T (1986) Cloning of the gene for indoleacetic acid-lysine synthase from *Pseudomonas syringae* subsp. *savastanoi*. Journal of Bacteriology 166: 598-603

Goddijn OJM, de Kam RJ, Zannetti A, Schilperoort RA, Hoge HC (1992) Auxin rapidly down-regulates transcription of the tryptophan decarboxylase gene from *Catharanthus roseus*. Plant Mol Biol 18: 1113-1120

Grill E, Somerville C (1991) Construction and characterization of a yeast artificial chromosome library of arabidopsis which is suitable for chromosome walking. Mol Gen Genet 226: 484-490

Guilfoyle TJ (1986) Auxin-regulated gene expression in higher plants. CRC Crit Rev Plant Sciences 4: 247-276

Guilfoyle TJ, Hagen G, Li Y, Ulmasov T, Liu Z, Strabala T, Gee M (1993) Auxin-regulated transcription. Aust J Plant Physiol 20: 489-502

Hagen G, Kleinschmidt A, Guilfoyle TJ (1984) Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta 162: 147-153

Hagen G, Uhrhammer N, Guilfoyle TJ (1988) Regulation of expression of an auxin-induced soybean sequence by cadmium. The Journal of Biological Chemistry 263: 6442-6446

Hagen G (1989) Molecular approaches to understanding auxin action. New Biol 1: 19-23

Hagen G, Martin G, Li Y, Guilfoyle TJ (1991) Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. Plant Mol Biol 17: 567-579

Hauge BM, Hanley S, Giraudat J, Goodman H (1991) Mapping the arabidopsis genome. In: Molecular biology of plant development. Jenkins G, Schuch W (ed), Cambridge University Press, Cambridge pp 45-56

Hauge BM, Hanley SM, Cartinhour S, Cherry JM, Goodman HM, Koornneef M, Stam P, Chang C, Kempin S, Medrano L, Meyerowitz EM (1993) An integrated genetic/ RFLP map of the *Arabidopsis thaliana* genome. The Plant Journal 3: 745-754

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: 1350-1353

Herman PL, Marks MD (1989) Trichome development in *Arabidopsis thaliana*. II Isolation and complementation of the *GLABROUS1* gene. Plant Cell 1: 1051-1055

Hesse T, Feldswisch J, Balshüsemann D, Bauw G, Puype M, Vandekerckhove J, Löbler M, Klämbt D, Schell J, Palme K (1989) Molecular cloning and structural analysis of a gene from *Zea mays* (L.) coding for a putative receptor for the plant hormone auxin. EMBO J 8: 2453-2461

Hicks GR, Rayle DL, Jones AM, Lomax TL (1989a) Specific photoaffinity labeling of two plasma membrane polypeptides with an azido auxin. Proc Natl Acad Sci *USA* 86: 4948-4952

Hicks GR, Rayle DL, Lomax TL (1989b) The *Diageotropica* mutant of tomato lacks high specific activity auxin binding sites. Science 245: 52-54

Hobbie L, Estelle M (1994) Genetic approaches to auxin action. Plant, Cell and Environment 17: 525-540

Hobbie L, Estelle M (1995) The axr4 auxin-resistant mutant of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. The Plant Journal 7: 211-220

Hong JC, Nagao RT, Key JL (1987) Characterization and sequence analysis of a developmentally regulated putative cell wall protein gene isolated from soybean JBC 262: 8367-8376

Inohara N, Shimomura S, Fukui T, Futai M (1989) Auxin-binding protein located in the endoplasmic reticulum of maize shoots: Molecular cloning and complete primary structure. Proc Natl Acad Sci *USA* 86: 3564-3568

Ishida S, Takahashi Y, Nagata T (1993) Isolation of cDNA of an auxin- regulated gene encoding a G protein ß subunit-like protein from tobacco BY-2 cells. Proc Natl Acad Sci *USA* 90: 11152-11156

Jones AM, Venis MA (1989) Photoaffinity labelling of auxin-binding proteins in maize. Proc Natl Acad Sci *USA* 86: 6153-6156

Jones AM, Prasad PV (1994) 4th international Congress of Plant Molecular Biology. Abstracts #900

Key JL (1989) Modulation of gene expression by auxin. BioEssays 11: 52-58

Kim S-R, Kim Y, An G (1993) Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (*nos*) promoter. Plant Physiol 103: 97-103

Kim Y, Buckley K, Costa MA, An G (1994) A 20 nucleotide upstream element is essential for the nopaline synthase (*nos*) promoter activity. Plant Mol Biol 24: 105-117

Klee HJ, Horsch RB, Hinchee MA, Hein MB, Hoffmann NL (1987) The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Genes and Development 1: 86-96

Klee H, Estelle M (1991) Molecular approaches to plant hormone biology. Annual Review of Plant Physiol and Plant Mol Biol 42: 529-551

Koncz C, Mayerhofer R, Koncz-Kalman Z, Nawrath C, Reiss B, Rédei GP, Schell J (1990) Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J 9: 1337-1346

Konieczny A, Ausubel FM (1993) A procedure for mapping arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. The Plant Journal 4: 403-410

Koornneef M, van Eden J, Hanhart CJ, Stam P, Braaksma FJ, Feenstra WJ (1983) Linkage map of *Arabidopsis thaliana*. The Journal of Heredity 74: 265-272

Koornneef M, Hanhart CJ, van Loenen-Martinet, van der Veen JH (1986) A marker line, that allows the detection of linkage on all arabidopsis chromosomes. Arab info service: 46-50

Korber H, Strizhov N, Staiger D, Feldwisch J, Olsson O, Sanberg G, Palme K, Schell J, Koncz C (1991) T-DNA gene 5 of *Agrobacterium* modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants. EMBO J 10: 3983-3991

Laibach F (1943) *Arabidopsis thaliana* (L.) Heynh. als objekt für genetische und physiologische untersuchungen. Bot Archiv 44: 439-455

Lazarus CM, Napier RM, Yu L-X, Lynas C, Venis MA (1991) Auxin binding protein-antibodies and genes. In: Jenkins GI, Schuch W (eds): Molecular Biology of Plant Development. Cambridge: Company of Biologists Ltd, pp. 129-148

Leutwiller LS, Hough-Evans BR, Meyerowitz EM (1984) The DNA of *Arabidopsis thaliana*. Mol Gen Genet 194: 15-23

Leyser HMO, Lincoln CA, Timpte C, Lammer D, Turner J, Estelle M (1993) Arabidopsis auxin-resistance gene *axr1* encodes a protein related to ubiquitin-activating enzyme E1. Nature 364: 161-164

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr1* mutants of arabidopsis. The Plant Cell 2: 1071-1080

Lister C, Dean C (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. The Plant Journal 4: 745-750

Liu X, Lam E (1994) Two binding sites for plant transcription factor ASF-1 can respond

to auxin treatments in transgenic tobacco. J Biol Chem 269: 668-675

Liu Z-B, Ulmasov T, Shi X, Hagen G, Guilfoyle TJ (1994) Soybean *GH3* promoter contains multiple auxin-inducible elements. The Plant Cell 6: 645-657

Lloyd AM, Barnason AR, Rogers SG, Byrne MC, Frayey RT, Horsch RB (1986) Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. Science 234: 464-467

Löbler M, Klämbt D (1985) Auxin-binding proteins of corn (*Zea mays* L.) I. Purification by immunological methods and characterization. J Biol Chem 260: 9848-9853

Lur H-S, Setter TL (1993) Endosperm development of maize *Defective Kernel (dek)* mutants. Auxin and cytokinin levels. Annals of botany 72: 1-6

MacDonald H, Jones AM, King PJ (1991) Photoaffinity labeling of soluble auxin-binding proteins. J Biol Chem 266: 7393-7399

Maher EP, Martindale SJB (1980) Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. Biochem Genet 18:1041-1053

Maher EP, Bell CJ (1990) Abnormal responses to gravity and auxin in mutants of *Arabidopsis thaliana*. Plant Science 66: 131-138

Mandava NB (1988) Plant growth-promoting brassinosteroids. Ann Rev Plant Mol Biol 39: 23-52

Maurel C, Brevet J, Barbier-Brygoo H, Guern J, Tempe J (1990) Auxin regulates the promoter of the root-inducing *rolB* gene of *Agrobacterium rhizogenes* in transgenic tobacco. Mol Gen Genet 223: 58-64

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol Biol 9: 611-623

McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ (1989) Transcription, organisation and sequence of an auxin-regulated gene cluster in soybean. The Plant Cell 1: 229-239

McClure BA, Guilfoyle T (1989) Rapid Redistribution of auxin-regulated RNAs during gravitropism. Science 243: 91-93

Meyerowitz EM, Pruitt RE (1985) *Arabidopsis thaliana* and plant molecular genetics. Science 229: 1214-1218

Mirza JI, Maher EP (1987) Physiological characteristics of two auxin- resistant mutants of *Arabidopsis thaliana*, *aux-2* and *Dwf*. Plant Growth Regulation 5: 41-49

Nagao RT, Goekjian VH, Hong JC, Key JL (1993) Identification of protein-binding DNA sequences in an auxin -regulated gene of soybean. Plant Mol Biol 21: 1147-1162

Nam H-G, Giraudat J, den Boer B, Moonan F, Loos WDB, Hauge BM, Goodman H (1989) Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana*. Plant Cell 1: 699-705

Newman TC, Ohme-Takagi M, Taylor CB, Green PJ (1993) DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. The Plant Cell 5: 701-714

Oeller PW, Keller JA, Parks JE, Silbert JE, Theologis A (1993) Structural

characterization of the early indoleacetic acid-inducible genes, *PS-IAA45* and *PS-IAA6*, of pea (*Pisum sativum* L.). J Mol Biol 233: 789-798

Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y (1991) Requirement of the auxin polar transport system in early stages of arabidopsis floral bud formation. The Plant Cell 3: 677-684

Olsen GM, Mirza JI, Maher EP, Iversen T-H (1984) Ultrastructure and movements of cell organelles in the root cap of agravitropic mutants and normal seedlings of *Arabidopsis thaliana*. Physiol Plant 60: 523-531

Onckelen van,H, Prinsen E, Inzé D, Rudelsheim P, van Lijsebettens M, Follin M, Schell A, DeGreef J(1986) *Agrobacterium* T-DNA gene I codes for tryptophan-2-mono oxygenase activity in tobacco crown gall cells. FEBS Letters 198: 357-360

Palme K, Hesse T, Campos N, Garbers C, Yanofsky MF, Schell J (1992) Molecular analysis of an auxin binding protein gene located on chromosome 4 of arabidopsis. Plant Cell 4: 193-201

Pasquali G, Goddijn OJM, de Waal A, Verpoorte R, Schilperoort RA, Hoge HC, Memelink J (1992) Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. Plant Mol Biol 18: 1121-1131

Pelese F, Megnegneau B, Sotta B, Sossountzov L, Caboche M, Miginiac E (1989) Hormonal characterization of a nonrooting naphthaleneacetic acid tolerant tobacco mutant by an immunoenzymic method. Plant Physiol 89: 86-92

Pickett FB, Wilson AK, Estelle M (1990) The *aux1* mutation of arabidopsis confers both auxin and ethylene resistance. Plant Physiol 94: 1462-1466

Prasad PV, Jones AM (1991) A putative receptor for the plant growth hormone auxin identified and characterized by anti-idiotypic antibodies. Proc Natl Acad Sci *USA* 88: 5479-5483

Qin X-F, Holuigue L, Horvath DM, Chua N-H (1994) Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus *as-1* element. The Plant Cell 6: 863-874

Raskin I (1992) Salicylate, a new plant hormone. Plant Physiol 99: 799-803

Reddy ASN, Poovaiah BW (1990) Molecular cloning and sequencing of a cDNA for an auxin-repressed mRNA: correlation between fruit growth and repression of the auxin-regulated gene. Plant Mol Biol 14: 127-136

Reiter RS, Williams JGK, Feldmann KA, Rafalski JA, Tingey SV, Scolnik PA (1992) Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. PNAS 89: 1477-1481

Roberto FF, Klee H, White F, Nordeen R, Kosuge R (1990) Expression and fine structure of the gene encoding N-(indole-3-acetyl)-L-lysine synthase from *Pseudomonas savastanoi*. Proc Natl Acad Sci *USA* 87: 5797-5801

Romano CP, Hein MB, Klee HJ (1991) Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthase gene of *Pseudomonas savastanoi*. Genes and development 5: 438-446

Romano CP, Cooper ML, Klee HJ (1993) Uncoupling auxin and ethylene effects in transgenic tobacco and arabidopsis plants. The Plant Cell 5: 181-189

Rubery PH (1981) Auxin receptors. Annu Rev Plant Physiol 32: 569-596

Rück A, Palme K, Venis MA, Napier RM, Felle HH (1993) Patch-clamp analysis establishes a role for auxin binding protein in the auxin stimulation of plasma membrane current in *Zea mays* protoplasts. The Plant Journal 4: 41-46

Sakai S (1992a) Regulatory functions of soluble auxin-binding proteins. Int Rev Cytol 135: 239-267

Sakai S, Kikuchi M, Nakajima N (1992b) Interaction between auxin-binding protein-I and RNA polymerase II. Biosci Biotech Biochem 56: 1225-1229

Schmidt R, Willmitzer L (1988) High efficiency *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. Plant Cell Reports 7: 583-586

Schmidt R, Dean C (1993) Towards the construction of an overlapping YAC library of the *Arabidopsis thaliana* genome. BioEssays 15: 63-68

Schwob E, Choi S-Y, Simmons C, Migliaccio F, Ilag L, Hesse T, Palme K, Söll D (1993) Molecular analysis of three maize 22 kDa auxin-binding protein genes- transient promoter expression and regulatory regions. Plant J 4: 423-432

Schroder G, Waffenschmidt S, Weiler EW, Schroder J (1984) The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. European J Biochem 138: 387-391

Shimomura S, Sotobayashi S, Futai M, Fukui T (1986) Purification and properties of an auxin-binding protein from maize shoot membranes. J Biochem 99: 1513-1524

Shimomura S, Iiu W, Inohara N, Watanabe S, Futai M (1993) Structure of the gene for an auxin-binding protein and a gene for 7SL RNA from *Arabidopsis thaliana*. Plant Cell Physiol 34: 633-637

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992a) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. Plant Physiol 99: 1062-1069

Sitbon F, Little CHA, Olsson O, Sandberg G (1992b) Correlation between the expression of T-DNA IAA biosynthetic genes from developmentally regulated promoters and the distribution of IAA in different organs of transgenic tobacco. Physiol Plant 85: 679-688

Sitbon F, Ostin A, Sundberg B, Olsson O, Sandberg G (1993) Conjugation of indole-3-acetic acid (IAA) in wild-type and IAA-overproducing transgenic tobacco plants, and identification of the main conjugates by frit-fast atom bombardment liquid chromatography-mass spectrometry. Plant Physiol 101: 313-320

Slaswick PE (1992) Jasmonate, genes and fragant signals. Plant Physiol 99: 804-807

Slovin JP, Cohen JD (1988) Levels of indole-3-acetic acid in *Lemna gibba* G-3 and in a large *Lemna* mutant regenerated from tissue culture. Plant Physiol 86: 522-526

Snustad DP, Haas NA, Kopczak SD, Silflow CD (1992) The small genome of arabidopsis contains at least nine expressed Beta-tubulin genes. Plant Cell 4: 549-556

Spena A, Prinsen E, Fladung M, Schulze SC, van Onckelen H (1991) The indoleacetic acid-lysine synthase gene of *Pseudomonas syringae* subsp. *savastanoi* induces developmental alterations in transgenic tobacco and potato plants. Mol Gen Genet 227: 205-212

Sun T-p, Goodman HM, Ausubel FM (1992) Cloning the arabidopsis *GA1* locus by genomic subtraction. The Plant Cell 4: 119-128

Tagliani L, Nissen S, Blake TK (1986) Comparison of growth, exogenous auxin sensitivity, and endogenous indole-3-acetic acid content in roots of *Hordeum vulgare* L. and an agravitropic mutant. Biochem Genet 24: 839-848

Takahashi Y, H Kuroda, T Tanaka, Y Machida, I Takebe, T Nagata (1989) Isolation of an auxin-regulated gene cDNA expressed during the transition from G0 to S phase in tobacco mesophyll protoplasts. Proc Natl Acad Sci *USA* 86: 9279-9283

Takahashi Y, Niwa Y, Machida Y, Nagata T (1990) Location of the cis-acting auxin-response region in the promoter of the *par* gene from tobacco mesophyll protoplasts. Proc Natl Acad Sci *USA* 87: 8013-8016

Takahashi Y, Kusaba M, Hiraoka Y, Nagata T (1991) Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. Plant J 1: 327-332

Takahashi Y, Nagata T (1992a) *parB*: an auxin-regulated gene encoding glutathione *S*-transferase. Proc Natl Acad Sci *USA* 89: 56-59

Takahashi Y, Nagata T (1992b) Differential expression of an auxin-regulated gene, *parC*, and a novel related gene, C7, from tobacco mesophyll protoplasts in response to external stimuli and plant tissues. Plant Cell Physiol 33: 779-787

Taylor JL, Fritzemeier K-H, Häuser I, E Kombrink, F Rohwer, M Schröder, G Strittmatter, K Hahlbrock (1990) Structural analysis and activiation by fungal infection of a gene encoding a pathogenesis-related protein in potato. Molecular Plant-Microbe Interactions 3: 72-77

Theologis A, Huynh TV, Avis RW (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J Mol Biol 183: 53-68

Theologis A (1986) Rapid gene regulation by auxin. Ann Rev Plant Physiol 37: 407-438

Thomashow LS, Reeves S, Thomashow MF (1984) Crown gall oncogenesis: evidence that a T-DNA gene from *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyses synthesis of indole-3-acetic acid. Proc Natl Acad Sci *USA* 81: 5071-5075

Tillmann U, Viola G, Kayser B, Siemester G, Hesse T, al et (1989) cDNA clones of the auxin-binding protein from corn coleoptiles (*Zea mays*): isolation and characterization by immunological methods. EMBO J 8: 2463-2467

Timpte CS, Wilson AK, Estelle M (1992) Effects of the *axr2* mutation of arabidopsis on the cell shape in hypocotyl and inflorescence. Planta 188: 271-278

Torti G, Manzocchi L, Salamini F (1986) Free and bound indole-acetic acid is low in the endosperm of the maize mutant defective endosperm-B18. Theor Appl Genet 72:

602-605

Tran Thanh Van K, Toubart P, Cousson A, Darvill AG, Gollin DJ, Chelf P, Albersheim P (1985) Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. Nature 314: 615-617

Valvekens D, van Montagu M, van Lijsebettens M (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci *USA* 85: 5536-5540

Venis MA (1977) Affinity labels for auxin binding sites in corn coleoptile membranes. Planta 134: 145-149

Venis MA, Napier RM, Barbier-Brygoo H, Maurel C, Perrot-Reichenmann C, Guern J (1992) Antibodies to a peptide from the maize auxin-binding protein have auxin agonist activity. Proc Natl Acad Sci *USA* 89: 7208-7212

Walker JC, Key JL (1982) Isolation of cloned cDNAs to auxin-responsive poly(A) RNAs of elongating soybean hypocotyl. Proc Natl Acad Sci *USA* 79: 7185-7189

Ward ER, Jen GC (1990) Isolation from single copy sequence clones from a yeast artificial chromosome library of random sheared *Arabidopsis thaliana* DNA. Plant Mol Biol 14: 561-568

Wilson AK, Pickett FB, Turner JC, Estelle M (1990) A dominant mutation in arabidopsis confers resistance to auxin, ethylene and abscisic acid. Mol Gen Genet 222: 377-383

Yamamoto KT, Mori H, Imaseki H (1992a) cDNA cloning of indole-3-acetic acid-regulated genes: *Aux22* and *SAUR* from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol 33: 93-97

Yamamoto KT, Mori H, Imaseki H (1992b) Novel mRNA sequences induced by indole-3-acetic acid in sections of elongating hypocotyl of mung bean (*Vigna radiata*). Plant Cell Physiol 33: 13-20

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM (1990) The protein encoded by the arabidopsis homeotic gene *agamous* resembles transcription factors. Nature 346: 35-39

van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998

Yu L-X, Lazarus CM (1991) Structure and sequence of an auxin-binding protein from maize (*Zea Mays* L.). Plant mol Biol 16: 925-930

Zettl R, Schell J, Palme K (1994) Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azido-[7-^{3}H]indole-3-acetic acid; Identification of a glutathione *S*-transferase. Proc Natl Acad Sci *USA* 91: 689-693

Zhang B, Singh KB (1994) *ocs*-element promoter sequences are activated by auxin and salicylic acid in arabidopsis. Proc Natl Acad Sci *USA* 91: 2507-2511

Zobel RW (1973) Some physiological characteristics of the ethylene-requiring tomato mutant *diageotropica*. Plant Physiol 52: 385-389

CHAPTER 2

Isolation and characterization of genes of *Arabidopsis thaliana* homologous to the auxin-inducible *Nt103-1* gene

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Summary

In order to isolate genes homologous to the auxin-inducible *Nt103-1* gene of tobacco, we screened a genomic library of *Arabidopsis thaliana*. We isolated a λ clone containing two tandemly arranged genes, *At103-1a* and *At103-1b*. Nucleotide sequencing revealed that the coding regions of the genes were highly homologous, while the promoter regions of the genes did not contain homologous sequences.

The *At103-1a* gene was constitutively expressed in the green parts of seedlings. In this property the expression of the arabidopsis gene differed from the expression of the corresponding tobacco gene. An *I* box present in the promoter of the *At103-1a* gene probably accounted for the expression in green tissues. Like the tobacco gene, the *At103-1a* gene could be induced by auxin in roots. In the promoter of the *At103-1a* gene an *as-1* like element was present that was similar to the sequence responsible for auxin-inducible expression of the homologous tobacco gene. The *At103-1b* gene was weakly expressed in leaves and roots and could not be induced in the tissues tested. In the promoter region of this gene no *as-1* or *I* box like elements were present.

Introduction

The plant hormone auxin is responsible for a large number of physiological effects and developmental responses in growing plants. The effects of auxin on the cellular differentiation in plants are likely to be due, at least in part, to changes in the pattern of gene expression. Several auxin-regulated genes have

been identified and cloned (Walker & Key, 1982, Hagen *et al.*, 1984, Hagen & Guilfoyle, 1985, Theologis *et al.*, 1985, McClure & Guilfoyle, 1987, van der Zaal *et al.*, 1987, Ainley *et al.*, 1988, Conner *et al.*, 1990, Franco *et al.*, 1990, Yamamoto *et al.*, 1992). Recently it was found that at least some of these genes, that encode proteins with glutathione *S*-transferase (GST) activity (Droog *et al.*, 1993, 1995, Takahashi & Nagata, 1992), can also be induced by other hormones, heavy metals or environmental stress (Hagen *et al.*, 1988, Taylor *et al.*, 1990, Takahashi *et al.*, 1991, van der Zaal *et al.*, 1991, Dominov *et al.*, 1992, Boot *et al.*, 1993).

The regulation of genes by auxin acts via *cis*-acting elements within the promoter region of the genes. One way to elucidate the mode of action of auxin is therefore to determine sequences in the 5' flanking regions of auxin-inducible genes that are important in auxin-inducibility. This has been done by comparing promoter regions of different genes. Thus regions have been identified that were strongly conserved in promoters of different genes (Ainley *et al.*, 1988, McClure *et al.*, 1989, Conner *et al.*, 1990, Hagen *et al.*, 1991, Oeller *et al.*, 1993). Also promoter deletion analysis, gain-of-function experiments with a minimal promoter and DNase footprinting have revealed sequences important in auxin induction (An *et al.*, 1990, Ballas *et al.*, 1993, Kim *et al.*, 1994, Korber *et al.*, 1991, Liu & Lam, 1994, Liu *et al.*, 1994, Nagao *et al.*, 1993). Thus it has been found that one promoter can contain multiple auxin-responsive elements (AuxREs) (Liu *et al.*, 1994).

The auxin-inducible glutathione *S*-transferase genes studied by our laboratory were isolated from a 2,4-dichlorophenoxyacetic acid (2,4-D) dependent cell-suspension culture of tobacco (van der Zaal *et al.*, 1987). Recently an *as-1* like sequence turned out to be present in the promoter regions important in auxin-inducibility in these genes (Droog *et al.*, 1995). Now we describe the isolation of genes homologous to one of these tobacco genes (*Nt103-1*) from arabidopsis. One of the arabidopsis genes turned out to be inducible by auxins in roots, but unlike the tobacco homolog constitutively expressed in the green parts of seedlings. The presence of homologous genes in arabidopsis is indicative for the importance of this gene in plants. The isolation of the *At103-1* genes in arabidopsis will allow the use of arabidopsis genetics to analyze the function of this conserved gene family.

Results

Screening of a genomic library

In our laboratory several related auxin-inducible *gst* genes of tobacco have been isolated and characterized (Boot *et al.*, 1993, Droog, 1995, van der Zaal *et al.*, 1987, 1991). Now we used the coding region of one of these genes, the *Nt103-1* gene, represented by cDNA clone pCNT103 (van der Zaal *et al.*, 1991), as a probe to fish for homologous genes from arabidopsis.

After screening a genomic library of arabidopsis DNA at low stringency with pCNT103, six recombinant phages were isolated. Phage DNA was isolated and digested with restriction enzymes. In three phages a 1 kb *Hind*III fragment hybridized with the pCNT103 probe, while in three other phages this was a 2 kb *Hind*III fragment. One phage containing the 1 kb fragment was designated λ103-1, and one containing the 2 kb fragment was designated λ103-2. Because mRNA hybridizing to the 2 kb *Hind*III fragment was not induced by auxin (data not shown), this λ clone was not characterized in detail.

The 1 kb *Hind*III fragment of λ103-1 was subcloned in pBlueScript SK$^+$, giving rise to the plasmid pSK103. This plasmid was used as a probe in DNA hybridization experiments. Southern blot analysis at high stringency on arabidopsis genomic DNA digested with *Bam*HI, *Eco*RI or *Hind*III revealed strongly hybridizing fragments of 11 kb, 6 kb and 1 kb, respectively. Also a weakly hybridizing fragment was present in the lanes, indicating that probably a second related gene was present in the genome. The gene that hybridized strongly to the pSK103 probe was designated *At103-1a* while the gene that hybridized weakly to pSK103 was designated *At103-1b*. Digestion of the genomic DNA with *Xho*I revealed two strongly and two weakly hybridizing bands, indicating that internal *Xho*I restriction sites were present in the genes (Figure 1A).

We repeated the Southern blot analysis using digested λ103-1 phage DNA (Figure 1A) and the 1 kb *Hind*III fragment of pSK103 as a probe. Fragments of 1 kb (*Hind*III), 6 kb (*Eco*RI) and 1.3 and 2.3 kb (*Xho*I) were detected. These corresponded in size to the genomic fragments that hybridized strongly to the same probe.

The genomic clone λ103-2 did not cross-hybridize to pSK103 indicating that the bands of *At103-1b* that hybridized weakly to pSK103 in the genomic DNA did not correspond to the gene present in λ103-2 (results not shown). Apparently the gene located on λ103-2 was isolated by virtue of its homology to the tobacco pCNT103 sequence, but was nevertheless clearly different from the

At103-1a and *At103-1b* genes.

Further analysis of the λ103-1 phage revealed that next to the coding region of the *At103-1a* gene, the promoter and part of the coding region of the *At103-1b* gene were also located on this phage clone. A restriction map of the phage was constructed (Figure 1C). The *Cla*I/*Hind*III fragment of the *At103-1b* gene was subcloned in pBlueScript SK$^+$ giving rise to the plasmid pSK307. This plasmid was used as a probe in a Southern blot analysis on genomic and λ103-1 phage DNA (Figure 1B). A fragment of 7 kb (*Bam*HI), 5 kb (*Eco*RI) and 2.5 kb (*Hind*III) hybridized strongly to pSK307. The sizes of these fragments corresponded to the sizes of the fragments that hybridized weakly to pSK103 (Figure 1A). Reversely the fragments that hybridized weakly to pSK307 corresponded in size to the fragments that hybridized strongly to pSK103. It was thus very likely that the homologous gene located on the λclone corresponded to the *At103-1b* gene. The 5 kb *Eco*RI and the 2.5 kb *Hind*III fragments that hybridized in the genomic DNA could not be detected in λ103-1 DNA. Smaller fragments of 0.9 kb (*Eco*RI) and 1 kb (*Hind*III) however could be detected, indicating that only a part of the *At103-1b* gene was located on λ103-1.

Gene structure and organisation

The 1 kb *Hind*III fragment of λ103-1 was sequenced to obtain information on the coding region of the *At103-1a* gene. The promoter region of this gene was cloned via IPCR and then sequenced. The sequence we obtained from the *At103-1a* gene starts at a *Xmn*I site present 376 bp upstream of the ATG initiation codon, and ends at a *Hind*III site 238 bp downstream of the stop codon. The coding region of the gene is interrupted by an 118 bp intron starting at position 319 downstream of the ATG. The intron splice sequences were predicted based on the arabidopsis Splice Site Consensus Table (Mike Cherry, Arabidopsis News group, 1992). The location of the splice site was confirmed after sequencing the cDNA corresponding to the *At103-1a* gene (Chapter 3). The position of the intron was identical in arabidopsis and tobacco. The exons harbour an open reading frame specifying a protein of 224 amino acids. The protein was 67% similar and 48% identical to the tobacco NT103-1 protein. The homology to other related auxin-inducible tobacco proteins of the Nt107 and Nt114 families, was considerable lower (37% and 39% identity) than to the NT103-1 protein.

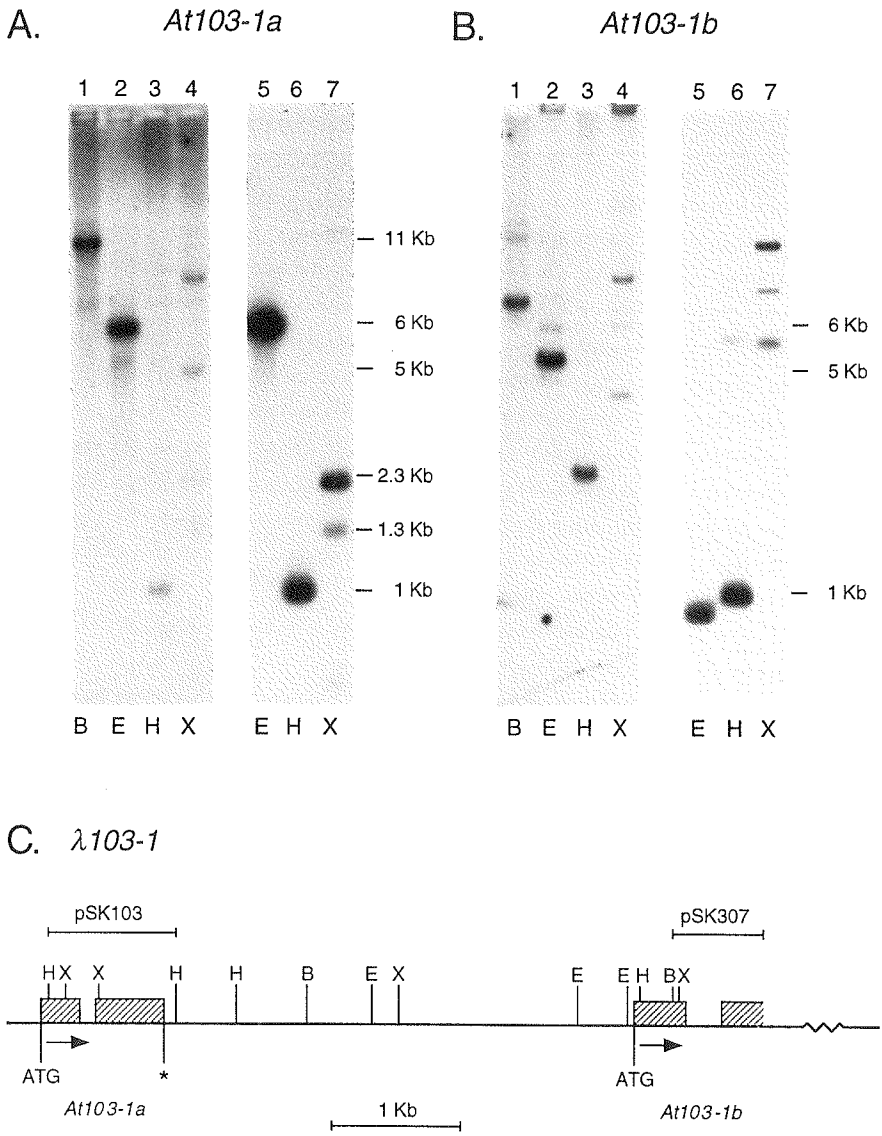

Figure 1. Genomic organization and structure of the *At103-1* genes from arabidopsis. A) Southern blot analysis of genomic DNA of *Arabidopsis thaliana* and λ103-1 digested with *Bam*HI (lane 1), *Eco*RI (lane 2+5), *Hind*III (lane 3+6) or *Xho*I (lane 4+7) hybridized to the 1 kb *Hind*III fragment of *At103-1a* (pSK103) or B) the *Cla*I/*Hind*III fragment of *At103-1b (pSK307)*. C) Restriction map of λ103-1. The coding region of the genes with the start (ATG) and stop (*) codons are indicated as well as the exons and introns of the genes. The position of the probes that were used in the Southern analysis are indicated as well. H = *Hind*III, E = *Eco*RI and X = *Xho*I.

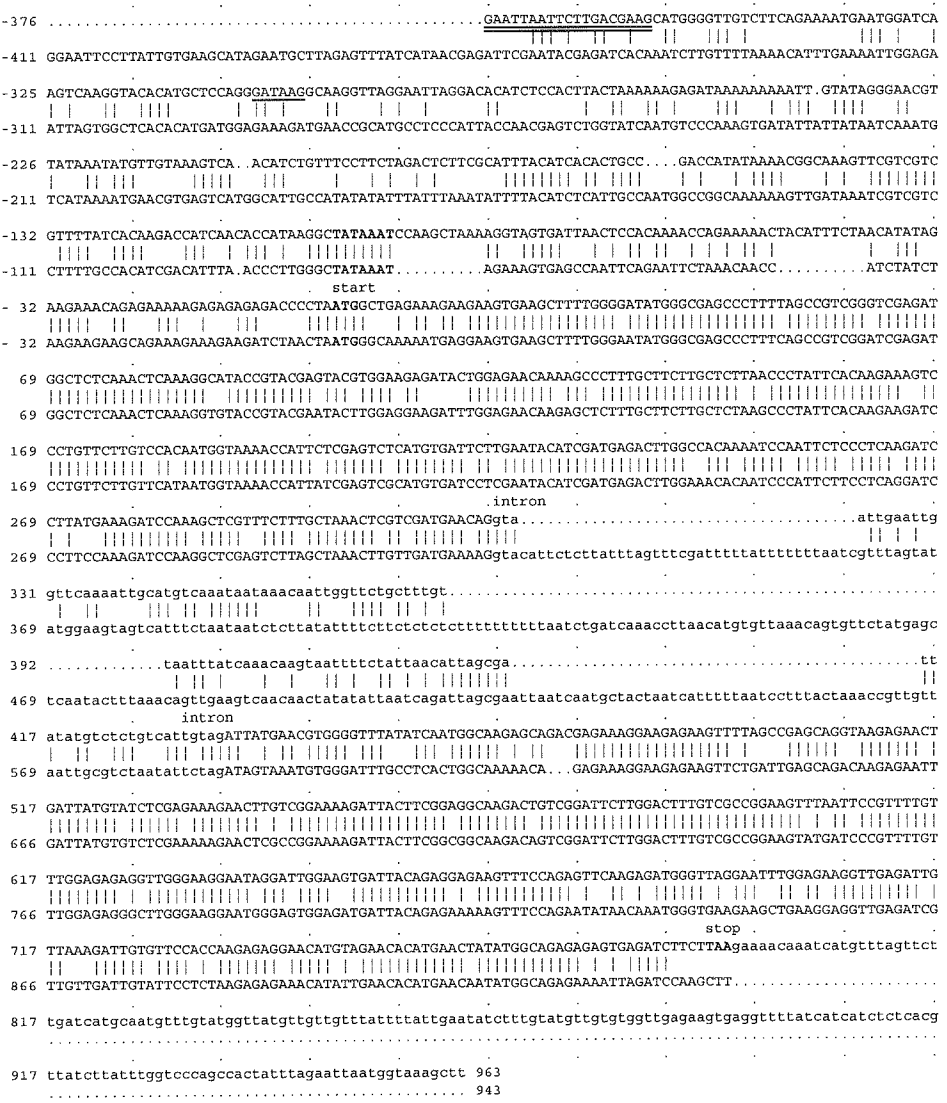

Figure 2. Alignment of the genomic sequences of the *At103-1a* and *At103-1b* genes. The presumptive TATA boxes, the translational start and the translational stop are represented in bold. Distances are given in base pairs with respect to the translational start codon (+1). The introns are indicated in small characters. The *as-1* like element (Lam *et al.*, 1989, Ellis *et al.*, 1993) is double underlined, while the *I* box (Guiliano *et al.*, 1988) is single underlined.

The 0.4 kb *Eco*RI fragment and part of the 2.9 kb *Bam*HI fragment of λ103-1 (Figure 1C) were sequenced to obtain detailed information on the promoter region of the *At103-1b* gene. The sequence of the promoter region started 411 bp upstream of the ATG initiation codon. The coding region of the *At103-1b* was only partially present on the *Bam*HI fragment. A supplementary sequence was obtained via IPCR. The sequence obtained did not contain the complete coding region. However, based on comparison to the *At103-1a* gene we believe the *At103-1b* gene to be nearly complete. The coding region is interrupted by one intron of 270 bp starting at the same position as the intron of *At103-1a*. The intron of the *At103-1b* gene is larger than the intron of the *At103-1a* gene (Figure 2). The exons of the *At103-1b* gene harbour an open reading frame specifying a protein of at least 224 amino acids. This protein was 78% identical to the protein encoded by the *At103-1a* gene and 43% identical to the tobacco NT103-1 protein.

In the promoter of *At103-1a* the TATA-box (Breathnach & Chambon, 1981) was positioned 100 basepairs upstream of the initiation codon. In the promoter of the *At103-1b* gene the TATA-box was located 80 bp upstream of the ATG. The promoters did not show extensive homology to each other or to the promoter of the *Nt103-1* gene. However, typically a sequence related to an *as-1* like element (Ellis *et al.*, 1993, Lam *et al.*, 1989) , as present in the promoters of genes belonging to the auxin-inducible *Nt103* gene family, was present in the promoter of the *At103-1a* gene as well. Also a sequence with homology to a light-responsive element (*I* box) (Giuliano *et al.*, 1988) was present in the promoter of the *At103-1a* gene. In the promoter of the *At103-1b* gene no *as-1* or *I* like elements were present.

mRNA expression of the *At103-1* genes

The expression of the *At103-1* genes was studied in seedlings of arabidopsis using Northern analysis.

Total RNA was isolated from the green parts and from the roots of 14 days old seedlings. The mRNA hybridizing to pSK103, was constitutively present in the green parts of the plants. In the roots the mRNA was present at a much lower level. Induction by auxin and other compounds was tested in roots. Figure 3A shows that the mRNA hybridizing to pSK103 was induced by the auxins 2,4-D and NAA, but not by IAA, in roots. The failure of IAA to induce the mRNA could be due to the rapid degradation of IAA in liquid medium. Abscisic acid (ABA) was also able to induce the mRNA hybridizing to pSK103 in roots, but the

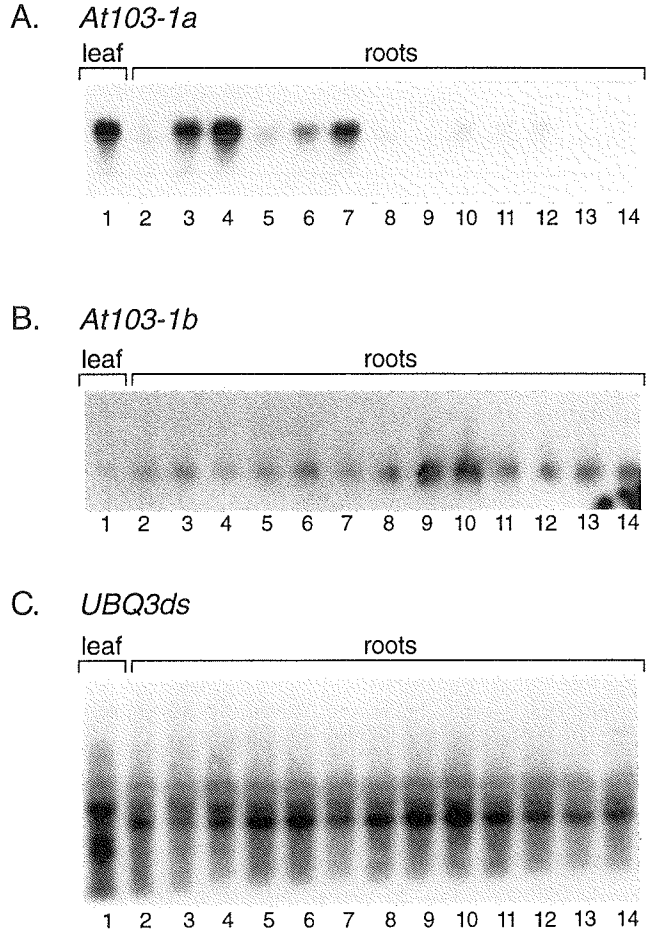

Figure 3. The mRNA expression in green parts (lane 1) and roots (lane 2) of *Arabidopsis thaliana*. Roots treated with 1 μM 2,4-D (lane 3), 10 μM NAA (lane 4), 1 μM IAA (lane 5), 100 μM kinetin (lane 6), 100 μM ABA (lane 7), 100 μM GA_3 (lane 8), 100 μM benzoic acid (lane 9), 10 μM 3,5-D (lane 10), 100 μM TIBA (lane 11), 1 mM GSH (lane 12), 10 μM $CuSO_4$ (lane 13) and 100 μM SA (lane 14).

A) hybridized to pSK103 (*At103-1a*)

B) hybridized to pSK307 *(At103-1b)*

C) hybridized to the control probe pUBQ3ds.

concentration of hormone needed was 100 times higher than the 2,4-D concentration required for induction. Presence of the cytokinin kinetin at 100 μM also led to a clear increase in the steady state level of mRNA. Other compounds

like the inactive auxin 3,5-dichlorophenoxyacetic acid (3,5-D), the auxin transport inhibitor 2,3,5 triiodobenzoic acid (TIBA), glutathione (GSH), gibberellic acid (GA_3), Cu^{2+}, benzoic acid (BA) and salicylic acid (SA) did not induce the mRNA although they were used at concentrations considerable higher than that of the 2,4-D concentration used.

The mRNA hybridizing to pSK307 was constitutively present in green parts and in roots. Presence of any of the compounds mentioned above did not lead to a significant increase in the steady state level of this mRNA as correlated to the level of the mRNA of the constitutive *ubq3ds* gene.

Discussion

After screening a genomic library of *Arabidopsis thaliana* with a cDNA clone of the tobacco auxin-inducible *Nt103-1* gene, two types of phages were isolated. A representative of the group containing a genomic DNA fragment hybridizing to an auxin-induced mRNA, turned out to contain two genes related to the tobacco *Nt103-1* gene. The genes are arranged in a tandem repeat in the arabidopsis genome. Clusters of auxin-regulated genes have been reported earlier. In soybean five different SAUR genes were found on one phage (McClure *et al.*, 1989). Also a cluster of two ethylene-responsive glutathione *S*-transferase genes has been reported in carnation (Itzhaki & Woodson, 1993).

Like the tobacco *Nt103* genes, the arabidopsis genes share high homology to the soybean *Gmhsp26-A* gene (Czarnecka *et al.*, 1988) and the potato *prp1* gene (Taylor *et al.*, 1990). The proteins belong to the class of type III GST enzymes (Droog *et al.*, 1995). The structure of the *At103-1* genes is very similar to the structure of type III *gst* genes. Like the *Nt103* genes, as well as the *prp1* gene and the *Gmhsp26-A* gene, the *At103-1* genes contain two exons interrupted by one intron. The structure of *gst* genes from other classes that have been isolated thus far is completely different. The *gst* genes of wheat (Dudler *et al.*, 1991), *Silene cucubalus* (Prändl & Kutchan, 1992) and maize (Wiegand *et al.*, 1986) contain two introns. The carnation genes *gst1* and *gst2* contain ten exons (Itzhaki & Woodson, 1993). These results suggest that the one intron containing class of *gst* genes may define a specific function in the cell which is different from that determined by *gst* genes belonging to different classes.

The relation between cis-acting elements and gene expression.

Promoter analysis of the 5' regions flanking different auxin-inducible genes has led to the identification of a number of sequences that are essential for auxin-inducibility. We looked for presence of such Auxin-Responsive Elements (AuxREs) in the promoter of the auxin-inducible *At103-1a* gene.

In this way we found that the promoter region of the *At103-1a* gene contains a possible *as-1* like element (Ellis *et al.*, 1993, Lam *et al.*, 1989) at position 376 to 358 upstream of the ATG initiation codon (GAatTAAttctTGACGaAg). No other sequences corresponding to the AuxREs from other types of auxin-inducible genes were detected (Ballas *et al.*, 1993, Korber *et al.*, 1991, Li *et al.*, 1994, Nagao *et al.*, 1993). It should be realized however that the promoter sequence which we obtained is rather short.

By promoter deletion analysis a region necessary for the response to auxin was defined in the promoters of the tobacco *Nt103-1* and *Nt103-35* genes (Droog *et al.*, 1995). This region contained an *as-1* like element. In the promoters of related auxin-inducible genes (*Nt107* and *Nt114*) also *as-1* like elements were present. Recently we found that these *as-1* like elements were sufficient to mediate the auxin-responsive transcriptional activation (Droog *et al.*, 1995, Droog, 1995). The *as-1* element or related elements like *ocs* and *nos* were first detected in genes present in the plant virus CaMV and the T-DNA genes introduced in plants via *Agrobacterium tumefaciens* (Bouchez *et al.*, 1989, An *et al.*, 1990, Ellis *et al.*, 1993, Kim *et al.*, 1993, Liu *et al.*, 1994). Liu & Lam (1994) reported that the *as-1* element mediates induction by auxin but not by abscisic acid or cytokinin. The element was also reported to mediate induction by salicylic acid and methyl jasmonate (Kim *et al.*, 1993, Qin *et al.*, 1994, Zhang & Singh, 1994). The tobacco *Nt103* genes could be induced by auxin, salicylic acid, heavy metals and glutathione. Also the inactive auxin analog 3,5-D was able to induce the genes (van der Zaal *et al.*, 1987, 1991, Boot *et al.*, 1993, Droog, 1995). Recently Ulmasov *et al.* (1994) demonstrated that the *ocs* element was induced by active and inactive auxin and salicylic acid analogs. They argued that the *ocs* element does not selectively mediate induction by plant hormones but also by non-hormonal stress-inducing or electrophilic agents.

We found that the mRNA corresponding to the arabidopsis *At103-1a* gene could be induced predominantly by auxin and relatively high concentrations of abscisic acid and cytokinin but not by Cu^{2+}, salicylic acid or benzoic acid. The differences in induction observed between the various *as-1* like elements and promoters with these elements can not be fully explained yet.

At103-1a was constitutively expressed in the green parts. This corresponded

to the presence of an *I* box in the promoter of *At103-1a* (Giuliano *et al.*, 1988). This is an element present in different light-responsive promoters and functions in the control of *rbcS* expression in light-grown plants (Donald & Cashmore, 1990, Schäffner & Sheen, 1991).
The functional importance of the *I* box as well as of the *as-1* like element remains to be determined.

Because of the homology of the *At103-1* genes to glutathione *S*-transferase genes the promoters were screened for elements involved in stress-induced expression or induction by other hormones. The box-1 element involved in wound-inducible expression (Kawaoka *et al.*, 1994) and ABA-regulated gene expression (Guiltinan *et al.*, 1990) was not present in the *At103-1* promoters. This was also true for a related ABA-responsive element and for a gibberellin-responsive element (Skriver *et al.*, 1991). Also the salicylic acid-inducible element conserved in stress-inducible genes (Goldsbrough *et al.*, 1993) was not present in the promoter regions we sequenced.

By isolating the *At103-1* genes we have shown that the gene family of auxin-inducible *gst* genes is also present in arabidopsis. The function of the genes, their regulation and their relation to auxin is still unknown. The use of arabidopsis genetics will hopefully help to elucidate these questions in the near future.

Materials and methods

All DNA manipulations were performed essentially according to Sambrook *et al.* (1989).

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (*gl1*) were used in all experiments. Plants were grown at 21°C with a 16 h light / 8 h dark cycle. The light intensity in the tissue culture room was 3000 lux (Philips TLD50W/83HF).

Bacterial strains

Escherichia coli strain DH5α (Clontech) was used for bacterial cloning. Strains were grown at 37°C in LC medium (Hooykaas *et al.*, 1977) supplemented with 100 mg/l carbenicillin or 25 mg/l kanamycin.

Screening of a genomic library

The phage clones were isolated from an *Arabidopsis thaliana* cv Landsberg *erecta*

genomic library. The library was screened by plaque hybridization using the ^{32}P-labelled cDNA clone pCNT103 as a probe (van der Zaal *et al.*, 1991). The filters were prehybridized and hybridized according to Church & Gilbert (1984). Subsequently they were washed in 2x SSC (1x SSC = 150 mM NaCl, 15 mM Sodium citrate pH 7.0), 0.1% SDS at room temperature for 30 min and 15 min followed by 2 washes of respectively 30 and 15 min in 0.2x SSC, 0.1% SDS. The filters were exposed to Fuji-RX films at -80°C.

DNA isolation and Southern analysis

Total DNA was isolated from leaves according to the method of Rogers & Bendich (1988). Approximately 5 to 10 μg of DNA was digested with restriction enzymes and subjected to o/n gel electrophoresis through a 0.8% agarose gel in 1x TAE (1x TAE = 0.04 M tris-acetate, 0.01 M EDTA). DNA was alkali blotted to Hybond N$^+$ (Amersham) following the manufacturer's instructions. α-^{32}P-dCTP labelled probes were generated using the random priming method (Feinberg & Vogelstein, 1983). Blots were washed at 65°C in 0.1x SSPE, 0.1% SDS and analysed using a phosphorimager (Molecular Dynamics). Blots were subsequently exposed to Fuji-RX films at -80°C using KMC x-ray intensifying screens.

Sequence analysis

Fragments subcloned in pBlueScript SK$^+$ (Stratagene) were sequenced with the dideoxy chain termination method (Sanger *et al.*, 1977), using sequenase version 2.0 (United States Biochemicals). The sequence was determined on both strands. Sequence data were analysed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984).

IPCR

Parts of the *At103* genes were isolated using inverse PCR as described by Does *et al.* (1991). Two primers were synthesized of sequences identical in both *At103-1a* and *At103-1b*. These primers, AtGST-1: 5'TTGAGTTTGAGAGCCATCTCG 3' and AtGST-2: 5'GAATACATCGATGAGACTTGG 3', were 5' phosphorylated (Pharmacia).

Approximately 1 μg chromosomal DNA of *Arabidopsis thaliana* cv Landsberg *erecta* was digested with a restriction enzyme unique for one of the genes. For the isolation of the promoter region of *At103-1a* the DNA was digested with 12 units *Xmn*I. For the isolation of the coding region of *At103-1b*, the DNA was digested with 10 units *Hind*III. The digestion was incubated for 4 h in the appropriate NEB buffer (Boehringer) and 0.2 μg/μl RNase in a 50 μl reaction. The restriction enzyme was inactivated by phenol/chloroform extraction and the DNA was ethanol-precipitated. The DNA was religated o/n at 14°C in 250 μl using 3 units T4-DNA ligase (Pharmacia). The ligase was inactivated (20 min 65°C) and the reaction mixture was precipitated with ethanol. One third of the religated DNA was digested for 2 h with an unique restriction enzyme for

one of the genes to linearize the fragment. For isolation of the promoter of the *At103-1a* gene, 15 units *Xho*I were used to digest the DNA. For the isolation of the coding region of the *At103-1b* gene, 10 units *Sac*I were used to digest the DNA. After inactivation of the enzyme by phenol/chloroform extraction, the DNA was ethanol precipitated and dissolved in 10 μl TE (10 mM Tris, 1mM EDTA, pH 8.0). PCR was performed on 2 μl of the solution in a thermocycler 480 (Perkin Elmer Cetus) using 25 pmol of each primer, 100 μM of each dNTP, 0.1 units SuperTaq (HT biotechnology) and 5 μl 10x SuperTaq reaction buffer (HT Biotechnology) in a 50 μl reaction mixture. The reactions were overlaid with 50 μl mineral oil. The amplification was started with one cycle of 2 min. at 95°C, 1 min. at 58°C and 2 min. at 72°C. This was followed by 35 cycles of 1 min. at 95°C, 1 min. at 58°C and 2 min. at 72°C and completed with one cycle of 1 min. at 95°C, 1 min. at 58°C and 10 min. at 72°C. Samples of 20 μl were analysed on a 1.0% agarose gel for each PCR. Fragments were isolated from gel using Prep-A-Gene (Biorad) according to the manufacturers instructions. The fragments were ligated into a pBlueScript SK$^+$ vector (Stratagene) digested with *Sma*I. For the determination of the sequence five independent clones were sequenced for each fragment.

Plants for RNA isolation

RNA was isolated from roots of 14 days old sterile plants incubated on a rotary shaker (100 rpm) in 250 ml flasks containing 50 ml Gamborg B5 medium (Gamborg *et al.*, 1968). Induction with different compounds was performed by addition of the compound to the medium followed by a 15 h incubation period. Leaves and roots were separated and frozen in liquid nitrogen. The material was kept at -80°C until RNA was isolated.

RNA isolation and northern blot analysis

The isolation of total RNA from different tissues and electroporation were performed according to van Slogteren *et al.* (1983). An amount of 25 μg glyoxylated RNA was electrophoresed in a 1.5% agarose gel and transferred to Genescreen (Dupont) by capillary blotting with 50 mM sodiumphosphate, 5 mM EDTA pH 6.5. Blots were baked at 80°C for 2 h and subsequently prehybridized at 42°C in 50% deionised formamide, 5x SSPE (1x SSPE = 180 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate pH 6.5), 5% SDS and hybridized o/n at 42°C in the same solution after addition of the probe. DNA was labelled with α-^{32}P-dCTP by the random priming method (Feinberg & Vogelstein, 1983). Blots were washed at 65°C in 0.5x SSPE, 0.5% SDS and analysed using a phosphorimager (Molecular Dynamics). Blots were subsequently exposed at -80°C to Fuji-RX films using KMC x-ray intensifying screens.

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Note

The nucleotide sequence of the *At103-1a* gene will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X89216.

References

Ainley WM, Walker JC, Nagao RT, Key JL (1988) Sequence and characterization of two auxin-regulated genes from soybean. J Biol Chem 263: 10658-10666

An G, Costa MA, Ha S-B (1990) Nopaline synthase promoter is wound inducible and auxin inducible. Plant Cell 2: 225-233

Ballas N, Wong L-M, Theologis A (1993) Identification of the auxin-responsive element *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA45*, of pea (*Pisum sativum*). J Mol Biol 233: 580-596

Bouchez D, Tokuhisa JG, Llewellyn DJ, Dennis ES, Ellis JG (1989) The *ocs*-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J 8: 4197-4204

Boot KJM, van der Zaal BJ, Velterop J, Quint A, Mennes AM, Hooykaas PJJ, Libbenga KR (1993) Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell-suspension cultures. Plant Physiol 102: 513-520

Breathnach R, Chambon P (1981) Organisation and expression of eucayotic split genes coding for proteins. Ann Rev Biochem 50: 349-383

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci *USA* 81: 1991-1995

Conner TW, Goekjian VH, LaFayette PR, Key JL (1990) Structure and expression of two auxin-inducible genes from arabidopsis. Plant Mol Biol 15: 623-632

Czarnecka E, Nagao RT, Key JL, Gurley WB (1988) Characterization of *Gmhsp26-A*, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol Cell Biol 8: 1113-1122

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs of the VAX. Nucl Acids Res 12: 387-395

Does MP, Dekker BMM, de Groot JA, Offringa R (1991) A quick method to estimate the T-DNA copy number in transgenic plants at an early stage after transformation, using inverse PCR. Plant Mol Biol 17: 151-153

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. The Plant Cell 4: 451-461

Donald RGK, Cashmore AR (1990) Mutation of either G box or I box sequences profoundly affects expression from the Arabidopsis rbcS-1A promoter. The EMBO J 9: 1717-1726

Droog FNJ, Hooykaas PJJ, Libbenga KR and van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferases and one member indeed shows in vitro GST activity. Plant Mol Biol 21: 965-972

Droog FNJ, Spek A, van der Kooy A, de Ruyter A, Hoge H, Libbenga KR, Hooykaas PJJ, van der Zaal EJ (1995) Promoter analysis of the auxin-regulated tobacco glutathione *S*-transferase genes *Nt103-1* and *Nt103-35*. Plant Mol Biol *in press*

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD Thesis, Leiden University, 165 pp

Dudler R, Hertig C, Rebmann G, Bull J, Mauch F (1991) A pathogen-induced wheat gene encodes a protein homologous to glutathione *S*-transferases. Mol Plant-Microbe Interact 4: 14-18

Ellis JG, Tokuhisa JG, Llewellyn DJ, Bouchez D, Singh K, Dennis ES, Peacock WJ (1993) Does the ocs-element occur as a functional component of the promoters of plant genes. The Plant Journal 4: 433-443

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13

Franco AR, Gee MA, Guilfoyle TJ (1990) Induction and superinduction of auxin responsive mRNAs with auxin and protein synthesis inhibitors. J Biol Chem 265: 15845-15849

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA and Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci *USA* 85: 7089-7093

Goldsbrough AP, Albrecht H, Stratford R (1993) Salicylic acid-inducible binding of a tobacco nuclear protein to a 10 bp sequence which is highly conserved amongst stress-inducible genes. The Plant Journal 3: 563-571

Guiltinan MJ, Marcotte Jr WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250: 267-271

Hagen G, Kleinschmidt A, Guilfoyle TJ (1984) Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta 162: 147-153

Hagen G, Guilfoyle TJ (1985) Rapid induction of selective transcription by auxins. Mol Cell Biol 5: 1197-1203

Hagen G, Uhrhammer N, Guilfoyle TJ (1988) Regulation of expression of an auxin-induced soybean sequence by cadmium. J Biol Chem 263: 6442-6446

Hagen G, Martin G, Li Y, Guilfoyle TJ (1991) Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. Plant Mol Biol 17: 567-579

Hahn K, Strittmatter G (1994) Pathogen-defence gene *prp1-1* from potato encodes an auxin- responsive glutathione *S*-transferase. Eur J Biochem 226: 619-626

Hooykaas PJJ, Klapwijk PM, Nuti MP, Schilperoort RA, Rörsch A (1977) Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium explanta*. Gen Microbiol 98: 477-484

Itzhaki H, Woodson WR (1993) Characterization of an ethylene-responsive glutathione S-transferase gene cluster in carnation. Plant Mol Biol 22: 43-58

Kawaoka A, Kawamoto T, Sekine M, Yoshida K, Takano M, Shinmyo A (1994) A *cis*-acting element and a *trans*-acting factor involved in the wound-induced expression of a horseradish peroxidase gene. The Plant Journal 6: 87-97

Kim S-R, Kim Y, An G (1993) Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (*nos*) promoter. Plant Physiol 103: 97-103

Kim Y, Buckley K, Costa MA, An G (1994) A 20 nucleotide upstream element is essential for the nopaline synthase (*nos*) promoter activity. Plant Mol Biol 24: 105-117

Korber H, Strizhov N, Staiger D, Feldwish J, Olsson O, Sanberg G, Palme K, Schell J, Koncz C (1991) T-DNA gene 5 of *Agrobacterium* modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants. EMBO J. 10: 3983-3991

Lam E, Benfey PN, Gilmartin PM, Fang R-X, Chua N-H (1989) Site-specific mutations alter in vitro factor binding and change promoter expression pattern in transgenic plants. Proc Natl Acad Sci *USA* 86: 7890-7894

Li Y, Liu Z-B, Shi X, Hagen G, Guilfoyle TJ: An auxin-inducible element in soybean SAUR promoters. Plant Physiol 106: 37--43 (1994).

Liu X, Lam E (1994) Two binding sites for plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. J Biol Chem 269: 668-675

Liu Z-B, Ulmasov T, Shi X, Hagen G, Guifoyle TJ (1994) Soybean *GH3* promoter contains multiple auxin-inducible elements. The Plant Cell 6: 645-657

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol Biol 9: 611-623

McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ (1989) Transcription, organisation and sequence of an auxin- regulated gene cluster in soybean. The Plant Cell 1: 229-239

Nagao RT, Goekjian VH, Hong JC, Key JL (1993) Identification of protein-binding DNA

sequences in an auxin-regulated gene of soybean. Plant Mol Biol 21: 1147-1162

Oeller PW, Keller JA, Parks JE, Silbert JE, Theologis A (1993) Structural characterization of the early indoleacetic acid-inducible genes,*PS-IAA45* and *PS-IAA6*, of pea (*Pisum sativum* L.). J Mol Biol 233: 789-798

Prändl R & Kutchan TM (1992) Nucleotide sequence of the gene for glutathione *S*-transferase from cell suspension cultures of *Silene cucubalus*. Plant Physiol 99: 1729-1731

Qin X-F, Holuigue L, Horvath DM, Chua N-H (1994) Immediate early transcription activation by salicylic acid via the Cauliflower Mosaic virus *as-1* element. The Plant Cell 6: 863-874

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant Mol Biol manual A6: 1-10

Sambrook L, Fritsch EF, Maniatis T (1989) Molecular Cloning: A laboratory Manual, second edition. Cold Spring Harbour Laboratory Press, Cold Spring harbor, New York

Sanger F, Nickler S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci *USA* 74: 5463-5467

Schäffner AR, Sheen J (1991) maize rbcS promoter activity depends on sequence elements not found in dicot rbcS promoters. The Plant Cell 3:997-1012

Skriver K, Olsen FL, Rogers JC, Mundy J (1991) Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. Proc Natl Acad Sci *USA* 88: 7266-7270

Slogteren van GMS, Hoge JHC, Hooykaas PJJ, Schilperoort RA (1983) Clonal analysis of heterogenous crown gall tumour tissues induced by wild-type and shooter mutant strains of *Agrobacterium tumefaciens* - expression of T-DNA genes. Plant Mol Biol 2: 321-333

Takahashi Y, Kusaba M, Hiraoka Y, Nagata T (1991) Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. The Plant Journal 1: 327-332

Takahashi Y, Nagata T (1992) *parB*: an auxin-regulated gene encoding glutathione *S*-transferase. Proc Natl Acad Sci *USA* 89: 56-59

Taylor JL, Fritzemeier K-H, Häuser I, Kombrink E, Rohwer F, Schröder M, Strittmatter G, Hahlbrock K (1990) Structural analysis and activiation by fungal infection of a gene encoding a pathogenesis-related protein in potato. Mol Plant-Microbe Interactions 3: 72-77

Theologis A, Huynh TV, Avis RW (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J Mol Biol 183: 53-68

Ulmasov T, Hagen G, Guilfoyle T (1994) The *ocs* element in the soybean *GH2/4* promoter is activated by both active and inactive auxin and salicylic acid analogues. Plant Mol Biol 26: 1055-1064

Walker JC, Key JL (1982) Isolation of cloned cDNAs to auxin- responsive poly(A) RNAs of elongating soybean hypocotyl. Proc Natl Acad Sci *USA* 79: 7185-7189

Wiegand RI, Shah DM, Mozer TJ, Harding EI, Diaz-Collier J, Saunders C, Jaworski EG,

Tiemeier DC (1986) Messenger RNA encoding a glutathione S-transferase responsible for herbicide tolerance in maize is induced in response to safener treatment. Plant Mol Biol 7: 235-243

Yamamoto KT, Mori H, Imaseki H (1992) cDNA cloning of indole-3-aceticacid-regulated genes: Aux22 and SAUR from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol 33: 93-97

Zaal van der EJ, Memelink J, Mennes AM, Ouint A, Libbenga KR (1987) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol 10: 145-157

Zaal van der EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998

Zhang B, Singh KB (1994) ocs element promoter sequences are activated by auxin and salicylic acid in arabidopsis. Proc Natl Acad Sci *USA* 91: 2507-2511

CHAPTER 3

An arabidopsis cDNA clone encoding an auxin-inducible glutathione *S*-transferase

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Summary

A cDNA clone corresponding to the auxin-inducible *At103-1a* gene of *Arabidopsis thaliana* was isolated. Overexpression of the cDNA clone in *E. coli* revealed that the AT103-1A protein had glutathione *S*-transferase activity. The protein encoded by the cDNA clone was highly homologous to the proteins encoded by the auxin-inducible glutathione *S*-transferase genes from other plant species, but distinct from those encoded by the *gst* genes that have been isolated from arabidopsis thus far. The *At103-1a* gene thus encodes a new class of glutathione *S*-transferases in arabidopsis. Transgenic plants overexpressing the gene as well as a plant with an antisense construct developed normally and had no altered response towards auxin.

Introduction

One way to elucidate the mode of action of auxin is to clone auxin-regulated genes and identify their function. To this end many auxin-responsive genes have been isolated but the functions of the proteins they encode are still unknown. Recently the proteins encoded by certain auxin-inducible genes were found to have glutathione *S*-transferase (GST) activity (Takahashi & Nagata, 1992, Droog *et al.*, 1993).

GSTs have been identified in many eukaryotes, including plants. They catalyse the conjugation of glutathione to a large variety of electrophilic compounds. These may be xenobiotic compounds as well as endogenous

organic hydroperoxides produced during normal respiration and especially during oxidative stress (for review see Daniel, 1993). In this manner GSTs are thought to protect tissues by functioning as an intracellular detoxification system. GSTs also bind a variety of hydrophobic compounds such as heme, hormones and drugs with high affinity. This suggests that they may serve as intracellular carrier proteins for the transport of various ligands (Listowsky, 1993).

Relatively little is known about the function, regulation and subcellular localization of plant GSTs. They have been studied most extensively in maize (Moore *et al.*, 1986, Mozer *et al.*, 1987), where they are important in the detoxification of herbicides. Recently the maize *Bz2* gene was found to encode a GST that conjugates anthocyanins with glutathione. These conjugates are thought to be recognized for transport into the vacuole by a glutathione pump (Marrs *et al.*, 1995). Genes encoding for GSTs were cloned from other plant species as well (Maude *et al.*, 1991, Prändl & Kutchan, 1992, Kutchan & Hochberger, 1992, Takahashi & Nagata, 1992). In carnation and arabidopsis they were isolated as dehydration or ethylene inducible genes (Meyer *et al.*, 1991, Kiyosue *et al.*, 1993, Zhou & Goldsbrough, 1993). Also GSTs were isolated from *Hyoscyamus muticus* (Bilang *et al.*, 1993) after photoaffinity labelling of proteins by 5-azido-[7-^{3}H]indole-3-acetic acid, a biologically active analog of the auxin indole-3-acetic acid (IAA), and from other plant species via auxin anti-idiotypic antibodies (Jones & Prasad, 1994). Auxins not only bind to GSTs, but also inhibit the *in vitro* activity of certain GSTs (Hahn & Strittmatter, 1994, Droog *et al.*, 1995).

In search for auxin-inducible genes, our laboratory isolated genes from tobacco after differential screening of mRNA populations from cell-suspension cultures treated with or without auxin (van der Zaal *et al.*, 1987). The proteins encoded by some of the genes turned out to have GST activity *in vitro* (Droog *et al.*, 1993, 1995).

Since the adoption of *Arabidopsis thaliana* as a model plant species, this plant species is being used by a number of groups to elucidate the auxin signal transduction pathway. To this end we isolated genes homologous to the tobacco auxin-inducible GST gene, *Nt103-1*, from arabidopsis. One of the isolated genes, *At103-1a*, turned out to be induced by auxin in roots (Chapter 2). Now we describe the isolation and sequencing of a cDNA corresponding to this gene. By overexpressing the protein encoded by the cDNA in *E. coli* we will show that also the protein encoded by the arabidopsis gene had GST activity. The predicted amino acid sequence of the protein is compared to the amino acid sequences of proteins encoded by auxin-inducible *gst* genes of other plant species and to other arabidopsis GSTs. Transgenic plants that overexpress the *At103-1a* gene or have an antisense construct will be described.

Results and discussion

Isolation and characterization of the cDNA clones

To identify cDNA clones corresponding to the arabidopsis *At103-1a* gene, a cDNA library was screened using the *Hind*III fragment of pSK103 (Chapter 2) as a probe. After screening approximately 200 000 plaque forming units (pfu), 60 pfu hybridized strongly to the probe. After purification, cDNA clones of 10 pfu were obtained as recombinant plasmids. The three cDNA clones that were sequenced corresponded to the *At103-1a* gene.

```
   1 GGAATTCGGCACAGAACATATAGAAGAAACAGAGAAAAAGAGAGAGAGACCCCTAATGGC
                                                           M  A
  61 TGAGAAAGAAGAAGTGAAGCTTTTGGGGATATGGGCGAGCCCTTTTAGCCGTCGGGTCGA
       E  K  E  E  V  K  L  L  G  I  W  A  S  P  F  S  R  R  V  E
 121 GATGGCTCTCAAACTCAAAGGCATACCGTACGAGTACGTGGAAGAGATACTGGAGAACAA
       M  A  L  K  L  K  G  I  P  Y  E  Y  V  E  E  I  L  E  N  K
 181 AAGCCCTTTGCTTCTTGCTCTTAACCCTATTCACAAGAAAGTCCCTGTTCTTGTCCACAA
       S  P  L  L  L  A  L  N  P  I  H  K  K  V  P  V  L  V  H  N
 241 TGGTAAAACCATTCTCGAGTCTCATGTGATTCTTGAATACATCGATGAGACTTGGCCACA
       G  K  T  I  L  E  S  H  V  I  L  E  Y  I  D  E  T  W  P  Q
 301 AAATCCAATTCTCCCTCAAGATCCTTATGAAAGATCCAAAGCTCGTTTCTTTGCTAAACT
       N  P  I  L  P  Q  D  P  Y  E  R  S  K  A  R  F  F  A  K  L
 361 CGTCGATGAACAGATTATGAACGTGGGGTTTATATCAATGGCAAGAGCAGACGAGAAAGG
       V  D  E  Q  I  M  N  V  G  F  I  S  M  A  R  A  D  E  K  G
 421 AAGAGAAGTTTTAGCCGAGCAGGAAAGAGAACTGATTATGTATCTTGAGAAAGAACTTGT
       R  E  V  L  A  E  Q  E  R  E  L  I  M  Y  L  E  K  E  L  V
 481 CGGAAAAGATTACTTCGGAGGCAAAACTGTCGGATTCTTGGACTTTGTCGCCGGAAGTTT
       G  K  D  Y  F  G  G  K  T  V  G  F  L  D  F  V  A  G  S  L
 541 AATTCCGTTTTGTTTGGAGAGAGGTTGGGAAGGAATAGGATTGGAAGTGATTACAGAGGA
       I  P  F  C  L  E  R  G  W  E  G  I  G  L  E  V  I  T  E  E
 601 GAAGTTTCCAGAGTTCAAGAGATGGGTTAGGAATTTGGAGAAGGTTGAGATTGTTAAAGA
       K  F  P  E  F  K  R  W  V  R  N  L  E  K  V  E  I  V  K  D
 661 TTGTGTTCCACCAAGAGAGGAACATGTAGAACACATGAACTATATGGCAGAGAGAGTGAG
       C  V  P  P  R  E  E  H  V  E  H  M  N  Y  M  A  E  R  V  R
 721 ATCTTCTTAAGAAAACAAATCATGTTTAGTTCTTGATCATGCAATGTTTGTATGGTTATG
       S  S  *
 781 TTGTTGTTTATTTTATTGAATATATGGTTATGTTGTTGTTTATTTAAAAAAAAAAAAAAA
 841 AAAAACTCGTG
```

Figure 1. Nucleotide sequence and deduced amino acid sequence of pCAT103-1a.

Three bp in the sequence of the cDNA clones differed from the sequence of the genomic clone. These differences were probably due to the fact that the genomic library was constructed from material of the Landsberg *erecta* ecotype,

whereas the cDNA library was made from the Columbia ecotype. By Southern blot analysis we were able to confirm one of the differences, since one base pair difference converted the sequence of a *Xho*I site in L. *erecta* into a loss of this restriction site in the Columbia cDNA (data not shown). The nucleotide sequences of the cDNAs revealed an open reading frame encoding a 224 amino acid protein with a predicted molecular weight of 26 kDa (Figure 1). All cDNA clones contained a poly (A)tail. The isolation of cDNA clones corresponding to the *At103-1a* gene and not of those corresponding to the *At103-1b* gene was expected since the hybridization of *At103-1b* to the probe was limited under the washing conditions used (Chapter 2).

Determination of the GST enzyme activity

Based on the homology to the tobacco NT103-1 protein, it was interesting to test if the protein encoded by the pCAT103-1a cDNA also showed *in vitro* GST activity. Therefore, the open reading frame (ORF) of pCAT103-1a was cloned in frame with the *lacZ* ORF of the pSK$^+$ expression vector. This resulted in the expression of the AT103-1A protein as a fusion protein in *E. coli*. As a negative control we used the empty pSK$^+$ vector and a cDNA clone pCAT32 in which the ORF was out of frame with the *lacZ* ORF. As a positive control the tobacco cDNA pCNT103 (van der Zaal *et al.*, 1987) was used. This construct was shown earlier to provide the cell with *in vitro* GST activity (Droog *et al.*, 1993).

Table 1. *In vitro* GST activity of different constructs on CDNB (A_{340}/mg per min).

construct	GST activity
pCAT103-1a	1,9 ± 0.1
pCAT32	0,6 ± 0.1
pSK$^+$	0,1 ± 0.0
pCNT103	5,6 ± 1.3

Both proteins encoded by the arabidopsis pCAT103-1a clone and the tobacco pCNT103 clone showed significant GST activity (Table 1). Remarkably, the clone pCAT32 provided *E.coli* with some GST activity although the coding

sequence was out of frame. This could possibly be due to frameshift suppression during protein translation in *E. coli*.

Homology to other GSTs

Since the protein was found to have GST activity, we studied the homology to the GSTs thus far isolated from arabidopsis. The homology to the proteins encoded by the auxin-inducible *Gmhsp26-A* gene of soybean (Czarnecka *et al.*, 1988) and the pathogen-inducible *prp1* gene of potato (Taylor *et al.*, 1990), recently found to encode glutathione *S*-transferases, was also studied. The protein encoded by pCAT103-1a showed identity to the proteins encoded by other arabidopsis GST genes that ranged from 23% to 27%. This homology was significantly lower than the homology to the proteins encoded by the auxin-inducible glutathione *S*-transferase genes of tobacco (van der Zaal *et al.*, 1991, Droog *et al.*, 1995) and soybean (Czarnecka *et al.*, 1988) and the related potato gene (Taylor *et al.*, 1990) that ranged from 46% to 48%. This result indicates that the AT103-1A protein belongs to a different class of GSTs than the proteins encoded by the *gst* genes isolated from arabidopsis so far. The AT103-1A protein like the tobacco NT103 and NT107 proteins, belongs to the type III class of GSTs (Droog *et al.*, 1993).

As shown in figure 2 the amino acid homology was predominantly found towards the N-terminal part of the proteins. The homology in the C terminal part was limited. This was also reported by Droog *et al.* (1993). The Arg21 residue, important in binding glutathione and necessary for conjugating activity of a class of human GSTs (Stenberg *et al.*, 1991), was conserved in all investigated GSTs.

Transgenic plants with altered *At103-1a* expression

It is not known yet what precise role auxin-inducible GST proteins play in plants. In order to find out more about their importance transgenic plants were constructed that were either overexpressing or repressing the *At103-1a* gene. Sense and antisense constructs were thus introduced into *Arabidopsis thaliana* via root transformation. Transgenic arabidopsis plants homozygous for the construct were analysed.

```
                1
AT103-1A        MA-EKEEVKLLGIWASPFSRRVEMALKLKGIPYEYVEEIL---ENKSPLLLLALNPIHKKV
NT103-1         MA-E---VKLLGFWYSPFTHRVEWALKLKGVKYEYIEEDR---DNKSSLLLQSNPVHKKV
PRP1            MA-E---VKLLGLRYSPFSHRVEWALKIKGVKYEFIEEDL---QNKSPLLLQSNPIHKKI
HSP26-A         MAATQEDVKLLGIVGSPFVCRVQIALKLKGVEYKFLEENL---GNKSDLLLKYNPVHKKV
ERD13           ------MVLTIYA-PLFASSKRAVVTLVEKGVSFETVNVDLMKGEQRQPEYLAIQPF-GKI
GST2            MA----GIKVFGHPASIATRRVLIALHEKNLDFELVHVELKDGEHKKEPFLSRNPF-GQV
ERD11           MA----GIKVFGHPAFTATRRVLIALHEKNVDFEFVHVELKDGEHKKEPFILRNPF-GKV
PM239x14        ----mvtvKIygMAYSTCTKRVYTTAKEIGVDVKIVPVDLMKGEHKEPAYLDNYHPFGVI

                61
AT103-1A        PVLVHNGK-TILESHVILEYIDETWPQ--NPILPQDPY---------------ERSKARF
NT103-1         PVLIHNGK-PIVESMVILEYIDETFEG--PSILPKDPY---------------DRALARF
PRP1            PVLIHNGK-CICESMVILEYIDEAFEG--PSILPKDPY---------------DRALARF
HSP26-A         PVFVHNEQ-PIAESLVIVEYIDETWKN--NPILPSDPY---------------QRALARF
ERD13           PVLVDGD-YKIFESRAIMRYIAEKYRSQGPDLL---GKTIEERGQVEQWLDVEATSYHPP
GST2            PAFEDGD-LKLFESRAITQYIAHRYENQGTNLLQTDSKNISQYAIMAIGMQVEDHQFDPV
ERD11           PAFEDGD-FKIFESRAITQYIAHEFSDKGNNLLSTG----KDMAIIAMGIEIESHEFDPV
PM239x14        PVLEDEDGTKIYESRAISRYLVAKY.GKGSSLLPSPSDP-KAYGLFEQAASVEYSSFDPP

                121
AT103-1A        FAKLVDEQIMNVGFISMARADEKGKEVLAEQERELIMYLEKELVGKDYFGGKTVGFLDFV
NT103-1         WSKFLGDK-VAAVVNTFFRKGE-EQEKGKEEVYEMLKVLDNELKDKKFFVGDKFGFADIA
PRP1            WAKYVEDK-GAAVWKSFFSKGE-EQEKAKEEAYEMLKILDNEFKDKKCFVGDKFGFADIV
HSP26-A         WSKFIDDKIVGAVSKSVFTVDEKERKNVEETYEALQFLENELKDKKFFGGEEFGLVDIA
ERD13           LLALTLNIVFAPLMG--FPADEKVIKESEEKLAEVLDVYEAQLSKNEYLAGDFVSLADLA
GST2            ASKLAFEQIFKSIYG--LTTDEAVVAEEEAKLAKVLDVYEARLKEFKYLAGETFTLTDLH
ERD11           GSKLVWEQVLKPLYG--MTTDKTVVEEEEAKLAKVLDVYEHRLGESKYLASDHFTLVDLH
PM239x14        ASSLAYERVFAGMRG--LKTNEELAKKYVDTLNAKMDGYERILSKQKYLAGNDFTLADLF

                181
AT103-1A        AGSLIPFCIERGWEGIGLEVITEEKFPEFKRWVRNLEKVEIVKDCVPPREEHVEHMNYMA
NT103-1         AN-LVGFWIGVFEEGYGVVLVTSEKFPNFSRWRDEYINCSQVKESLPSRDELLAFFRARF
PRP1            AN-GAALYLGILEEVSGIVLATSEKFPNFCAWRDEY--CTQNEEYFPSRDELLIRYRAYI
HSP26-A         AV-FIAFWIPIFQEIAGLQLFTSEKFPILYKWSQEFLNHPFVHEVLPPRDPLFAYFKARY
ERD13           HLPFTEYLVGPIGKAHLI-----KDRKHVSAWWDKISSRAAWKEVSAKYSLPV*------
GST2            HIPAIQYLLGTPTK-KLF-----TERPRVNEWVAEITKRPASEKVQ*-------------
ERD11           TIPVIQYLLGTPTK-KLF-----DERPHVSAWVADITSRPSAQKVL*-------------
PM239x14        HLPYGA--MVAQLEPTVL-----DSKPHVKAWWAA.SLRVIPGRLLRNSSKEFM*-----

                241
AT103-1A        ERVRSS*-----------
NT103-1         QAVVASISAPK*-------
PRP1            QPVDASK*-----------
HSP26-A         ESLSASK*-----------
ERD13           ------------------
GST2            ------------------
ERD11           ------------------
PM239x14        ------------------
```

Figure 2. Alignment of different proteins homologous to the protein encoded by the cDNA of pCAT103-1a. Protein sequences derived from cDNAs or genes of other plant species homologous to the AT103-1A protein are given. Gaps necessary for optimal alignment are indicated by dashes. Amino acids identical to the amino acids of the AT103-1A protein are shaded. The proteins are encoded by the *Nt103-1* gene of tobacco (van der Zaal *et al.*, 1991), the *prp1* gene of potato (Taylor *et al.*, 1990) and the *Gmhsp26-A* gene of soybean (Czarnecka *et al.*, 1988). Also proteins encoded by different GSTs of arabidopsis are compared to the AT103-1A protein. These proteins are derived from the cDNAs or genes *gst2* (Zhou & Goldsbrough, 1993), PM239x14 (Bartling *et al.*, 1993), ERD11 and ERD13 (Kiyosue *et al.*, 1993).

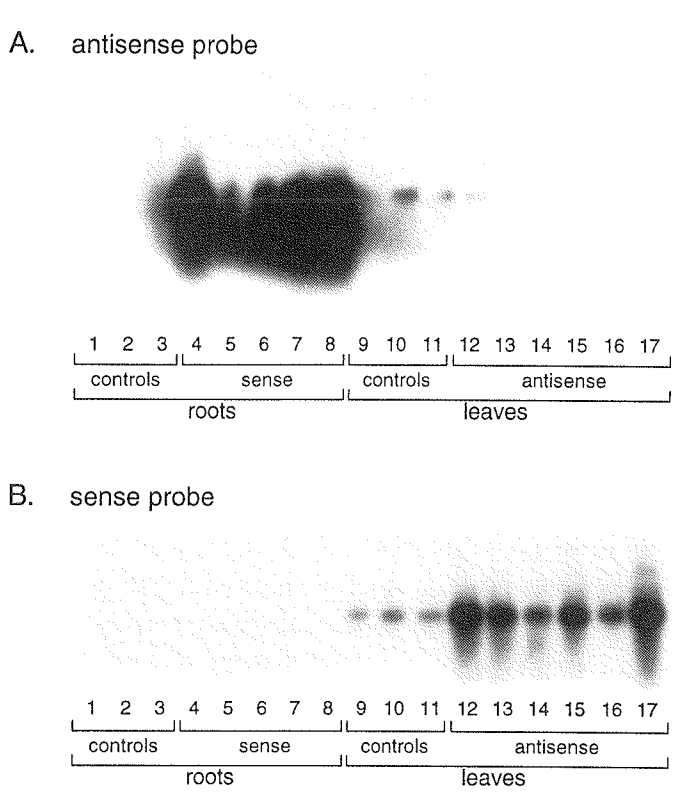

Figure 3. Total RNA hybridized with strand specific probes. RNA was isolated from roots of untransformed control plants (Lane 1); independent transgenic plant lines harbouring the pBDH5a vector (lanes 2 and 3) and five independent transgenic plants harbouring the sense overproducing construct 11.2 (lane 4); 11.4 (lane 5); 11.8 (lane 6); 11.9 (lane 7) and 11.10 (lane 8). RNA was isolated from leaves of untransformed control plants (Lane 9); independent transgenic plant lines harbouring the pBDH5a vector (lanes 10 and 11) and six independent transgenic plants harbouring the antisense overproducing construct 25.1 (lane 12); 25.5 (lane 13); 25.6 (lane 14); 25.20 (lane 15); 25.28 (lane 16) and 25.30 (lane 17).
A) antisense probe
B) sense probe

Using strand specific probes, sense and antisense RNA levels were determined in the transgenic plants. In figure 3 it is shown that all transgenic plants harbouring the sense construct overexpressed the *At103-1a* cDNA (11.2-11.4-11.8- 11.9- 11.10). Antisense RNA was expressed in leaves of all transgenic plants harbouring the antisense construct (25.1- 25.5- 25.6- 25.20- 25.28-25.30). In all transgenic plants expressing the antisense RNA, the level of sense

RNA was decreased. In the transformants 25.5, 25.6 and 25.20 this effect was most drastic: sense RNA could no longer be detected in the leaves.

To our surprise also in non-transgenic controls and controls transformed with the empty pBDH5a vector antisense RNA was detected. PCR analysis showed that the presence of this RNA was not due to mixing of the transgenic plants or the RNA samples (data not shown). Natural antisense RNA has been reported before in barley (Rogers, 1988) and in maize for the *Bz2* locus (Schmitz and Theres, 1992). The protein encoded by the *Bz2* locus of maize has homology to the AT103-1A protein and was recently found to encode a GST that conjugates anthocyanins to glutathione (Marrs *et al.*, 1995). The occurrence of antisense RNA for both the *At103-1a* and *Bz2 gst* genes might be a coincidence. It could however also point at a biological function for antisense RNA in the regulation of this specific gene family.

Transgenic plants either overexpressing or repressing the *At103-1a* gene were viable and fertile. They had no obvious phenotype. The protein encoded by *At103-1a*, therefore, does not seem to be essential for normal development of the plant, although we have to keep in mind that the lack of phenotype may be due to redundancy of gene function.

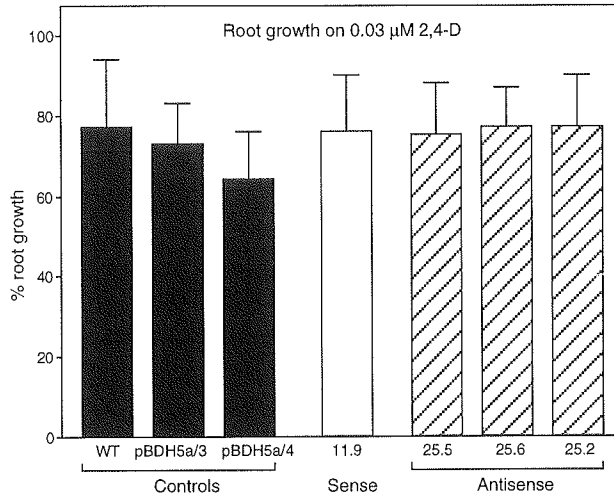

Figure 4. Root growth on ½ MS medium containing 0.03 µM 2,4-D. The filling of the bars indicates if the plants are non-transformed (WT) or transformed with the empty vector (pBDH5a/3 - pBDH5a/4), or that they are transformed with the sense T-DNA construct (11.9) or with the antisense T-DNA construct (25.5- 25.6- 25.2). The growth of the roots on ½ MS medium was identical in all plant lines tested.

Since GSTs are thought to be involved in detoxification and transport, we assayed the transgenic plants for their sensitivity to auxin. We found that roots of transgenic plants, producing antisense RNA or overexpressing the *At103-1a* gene, did not have an altered growth on medium containing 2,4-D (Figure 4). This means that the protein encoded by the pCAT103-1a cDNA is probably not essential in detoxification of auxin or substances formed after exposure of tissues to auxin. Also a function for the protein in transport seems unlikely. However, we have to be careful drawing these conclusions, since also in arabidopsis genes are present that are very homologous to the *At103-1a* gene. These could possibly take over the function of the repressed protein. Based on the homology to genes in other plant species, it will be very interesting to see whether the transgenic plants have an altered response to other stress factors and thus shed light on the function of the gene.

Materials and methods

All DNA manipulations were performed essentially according to Sambrook *et al.* (1989).

Bacterial strains

Escherichia coli strains DH5α (Clontech) and XL1-Blue (Stratagene) were used for bacterial cloning. Strains were grown at 37°C in LC medium (Hooykaas *et al.*, 1977) supplemented with 100 mg/l carbenicillin or 25 mg/l kanamycin. *Agrobacterium tumefaciens* strain MOG101 (Hood *et al.*, 1993), harbouring a non-oncogenic octopine Ti-plasmid in a C58 chromosomal background, was grown at 29°C in LC medium supplemented with 100 mg/l kanamycin.

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (*gll*) were used in all experiments. Plants were grown at 21°C with a 16 h light / 8 h dark cycle. The light intensity in the tissue culture room was 3000 lux (Philips TLD50W/83HF).

Screening of the cDNA library

The mRNA from leaves of greenhouse grown plants of *Arabidopsis thaliana* ecotype Columbia was used as plant material to generate an amplified λZap library, using the Uni Zap™ XR cDNA cloning kit (Stratagene). The 1 kb *Hind*III fragment of pSK103 was used as a probe to screen the library (Chapter 2). The fragment was labelled with α^{32}P-dCTP by using the random primed labelling method (Feinberg & Vogelstein, 1983). Plaques that hybridized to the probe were selected and rescreened. After further purification, the

cDNAs were obtained as recombinant plasmids by *in vitro* excision. The DNA sequences of the clones were determined using double stranded DNA templates and the Sequenase Kit (version 2,0, USB). Sequence data were analysed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984).

Enzymatic assay of GST activity

The enzymatic GST activity was determined according to standard procedures (Mannervik & Guthenberg, 1981). An overnight culture of DH5α cells was diluted hundred fold and grown in the presence of 1 mM IPTG for four hours. 1.5 ml of culture was collected by centrifugation. The cells were resuspended in 0.5 ml of 100 mM Tris-HCl (pH7.5), 1 mM DTT and lysed by sonication. The cell debris was removed by centrifugation and 200 μl supernatant was used in an enzyme activity assay. The supernatant was tested in the presence of 1 mM glutathione in 100 mM sodium phosphate buffer (pH6.5). The reaction was started by the addition of CDNB to a final concentration of 1 mM. The reaction was run at room temperature and the change in A_{340} was measured spectrophotometrically. Background levels of spontaneous CDNB decay were subtracted. Values were corrected for the protein content, measured according to Bradford (1976).

Construction of the transformation vectors

The coding region of the cDNA clone pCAT103-1a was subcloned in the binary vector pBDH5a (Goddijn, 1992) using pMTL24p (Chambers *et al.*, 1988) as an intermediate cloning vector. The *Pst*I/*Bcl*I fragment of pCAT103-1a was subcloned into *Pst*I/*Bgl*II digested pMTL24p. The resulting plasmid was digested with *Sal*I and the fragment was cloned in the *Sal*I site between the CaMV 35S promoter and terminator of pBDH5a. Both orientations were selected in order to obtain sense and antisense expression of the gene. Clone 11 represented the sense orientation, while clone 25 represented the antisense orientation. Both constructs were electroporated into *Agrobacterium tumefaciens* strain MOG101 using the method described by Mattanovich *et al.* (1989). As a control a pBDH5a plasmid without a cloned fragment was introduced into *A. tumefaciens* as well.

Plant transformation

Arabidopsis thaliana was transformed with *Agrobacterium tumefaciens* strain MOG101 containing the sense or antisense construct or the control vector pBDH5a using the root transformation protocol (Valvekens *et al.*, 1988) with some modifications.

An amount of 3 mg seeds were surface sterilized for 30 seconds in 70% EtOH, followed by a 20 min. incubation in 1% sodiumhypochloride and 0.15% Tween 20. The seeds were washed 5 times with sterile distilled water and transferred to a 250 ml Erlenmeyer flask containing 50 ml Gamborg B5 medium (Gamborg *et al.*, 1968). The

seeds were vernalized at 4°C for 4 days in the dark to promote synchronized germination. After 14 days incubation on a rotary shaker (100 rpm) in the light, the roots were harvested and used in a cocultivation procedure. Intact roots were incubated for 3 days on Callus Inducing Medium (CIM) being Gamborg B5 medium containing 20 g/l glucose, 0.5 g/l 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.7 and 8 g/l Daichin agar. After autoclaving at 110°C for 20 min. 0.5 mg/l 2,4-D and 0.05 mg/l kinetin were added. After cutting the roots into pieces of about 5 mm they were transferred to liquid CIM. 1 ml of an overnight *Agrobacterium tumefaciens* culture was added and mixed with the root explants. After a 2 min. incubation period, the root explants were blotted on sterile filter paper to remove most of the liquid medium and cocultivated for 2 days on solid CIM. After cocultivation the root explants were rinsed in liquid CIM and blotted dry. After repeating these last steps, the root explants were transferred to Shoot Induction Medium (SIM) being Gamborgs B5 medium containing 20 g/l glucose, 0.5 g/l MES pH 5.7 and 8 g/l Daichin agar. After autoclaving at 110°C for 20 min., 0.15 mg/l indole-3-acetic acid and 5 mg/l N^6-(2-isopentenyl)adenine were added. To kill off the bacteria 750 mg/l vancomycin and 50 mg/l augmentin were added to the medium. To select for transgenic shoots 50 mg/l kanamycin or 20 mg/l hygromycin was added. The root explants were transferred to fresh medium every week. The concentration of vancomycin was lowered every week with 250 mg/l till the concentration was 100 mg/l. Green calli were visible on the regeneration controls after 2 weeks followed directly by shoot formation. On media containing antibiotics the calli were visible after 3-4 weeks. The first shoots on these calli were formed 2-3 weeks after callus formation. The transgenic shoots were transferred to Root Induction Medium (RIM) being MS medium (Murashige and Skoog, 1962) containing 20 g/l sucrose, 0.5 g/l MES pH 5.7 and 8 g/l Daichin agar. After autoclaving at 110°C for 20 min., 1 mg/l indolebutyric acid (IBA), 100 mg/l vancomycin and 50 mg/l augmentin were added. After one week the transgenic shoots were transferred to the same medium without IBA and grown for seed set. T_1 seeds were germinated on Basal Medium (BM) being ½ MS medium containing 20 g/l sucrose, 0.5 g/l MES pH 5.7 and 8 g/l Difco Bacto agar. After autoclaving at 121°C for 20 min., 50 mg/l kanamycin was added.

RNA isolation and northern blot analysis

Plant material and procedures for RNA isolation and northern blot analyses were performed as described in chapter 2.

Synthesis of strand specific probes

The *Bgl*II/*Eco*RI fragment of pCAT103-1a containing the complete coding sequence was inserted in pSK103 digested with *BamH*I/*Eco*RI. Digestion of this plasmid with *Eco*RI resulted in a template for T7/T3 polymerase. The antisense RNA probe was synthesized from the cDNA fragment using T3 polymerase. The sense probe was synthesized from the genomic fragment using T7 polymerase. The reactions were performed according to

the manufacturers instructions. 120 μCi of α^{32}P-dCTP (800 Ci/mmol) was used for labelling.

Auxin root inhibition assay

Sterilized seeds were germinated on ½ MS plates placed in vertical position. After 5 days the seedlings were transferred to fresh ½ MS medium or to medium containing 0.03 μM 2,4-D. The end of the root tip was marked on the back of the Petri dish. After 6 days the root growth was measured and the root inhibition on medium containing 2,4-D was calculated as a percentage of the growth on ½ MS medium. Per treatment 30 roots were measured.

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References

Bartling D, Radzio R, Steiner U, Weiler EW (1993) A glutathione *S*-transferase with glutathione-peroxidase activity from *Arabidopsis thaliana*. Molecular cloning and functional characterization. Eur J Biochem 216: 579-586

Bilang J, Macdonald H, King PJ, Sturm A (1993) A soluble auxin-binding protein from *Hyoscyamus muticus* is a glutathione *S*-transferase. Plant Physiol 102: 29-34

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein dye binding. Anal Biochem 72: 248-254

Chambers SP, Prior SE, Barstow DA, Minton NP (1988) The pMTL *nic*- cloning vectors I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene 68: 139-149

Czarnecka E, Nagao RT, Key JL, Gurley WB (1988) Characterization of *Gmhsp26-A*, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol Cell Biol 8: 1113-1122

Daniel V (1993) Glutathion *S*-transferases: Structure and regulation of expression. Critical Reviews in Biochemistry and Molecular Biology 28: 173-207

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs of the VAX. Nucl Acids Res 12: 387-395

Droog FNJ, Hooykaas PJJ, Libbenga KR, van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferases and one member indeed shows *in vitro* GST activity. Plant

Mol Biol 21: 965-972

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD Thesis, Leiden University, 165 pp

Droog FNJ, Hooykaas PJJ, van der Zaal (1995) 2,4-Dichlorophenoxyacetic acid and related chlorinated compounds inhibit two auxin-regulated type-III tobacco glutathione *S*-transferases. Plant Physiol 107: 1139-1146

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158

Goddijn O (1992) Regulation of terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*: The tryptophan decarboxylase gene. phD Thesis, Leiden University, 99 pp

Hagen G, Kleinschmidt A, Guilfoyle TJ (1984) Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta 162: 147-153

Hahn K, Strittmatter G (1994) Pathogen-defence gene *prp1-1* from potato encodes an auxin-responsive glutathione *S*-transferase. Eur J Biochem 226: 619-626

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2: 208-218

Hooykaas PJJ, Klapwijk PM, Nuti MP, Schilperoort RA, Rörsch A (1977) Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium explanta*. Gen Microbiol 98: 477-484

Jones AM Prasad P (1994) The possible function of a nuclear auxin-binding protein. 4th international congress of plant molecular biology. Poster 900

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of two cDNAs (ERD11 and ERD13) for dehydration-inducible genes that encode putative glutathione *S*-transferases in *Arabidopsis thaliana* L. FEBS 335: 189-192

Kutchan TM, Hochberger A (1992) Nucleotide sequence of a cDNA encoding a constitutively expressed glutathione *S*-transferase from cell suspension cultures of *Silene cucubalus*. Plant physiol 99: 789-790

Listowsky I (1993) Glutathione *S*-transferases: intracellular binding, detoxification and adaptive responses. In: Hepatic and bile secretion transport: physiology and pathophysiology. Tavoloni N, Berk PD (ed) Raven Press, New York, p 397

Mannervik B, Guthenberg C (1981) Glutathione transferase (human placenta). Meth Enzymol 77: 231-235

Marrs KA, Alfenito MR Lloyd AM, Walbot V (1995) A glutathione *S*-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. Nature 375: 397-400

Mattanovich D, Rüker F, Câmara da, Laimer M, Regner F, Steinkellner H, Himmler G, Katinger H (1989) Efficient transformation of *Agrobacterium* spp. by electroporation. Nucl Acids Res 17: 6747

Maude F, Hertig C, Rebmann G, Bull J, Dudler R (1991) Plant mol biol 16: 1089-1091

Meyer RC, Goldsbrough PB, Woodson WR (1991) An ethylene-responsive flower senescence-related gene from carnation encodes a protein homologous to glutathione *S*-transferase. Plant Mol Biol 17: 277-281

Moore RE, Davies MS, O'Connell KM, Harding EI, Wiegand RC, Tiemeier DC (1986)

Cloning and expression of a cDNA encoding a maize glutathione *S*-transferase in E. coli. Nucl Acid Res 14: 7227-7235

Mozer TJ, Tiemeier DC, Jaworski EG (1987) Purification and characterization of corn glutathione *S*-transferase. Biochem 22: 1068-1072

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Picket CB, Lu AYH (1989) Glutathione *S*-transferases: gene structure, regulation, and biological function. Ann Rev Biochem 58: 743-764

Prändl R, Kutchan TM (1992) Nucleotide sequence of the gene for a glutathione *S*-transferase from cell-suspension cultures of *Silene cucubalus*. Plant Physiol 99: 1729-1731

Rogers JC (1988) RNA complementary to α-amylase mRNA in barley. Plant Mol Biol 11: 125-138

Sambrook L, Fritsch EF, Maniatis T (1989) Molecular Cloning: A laboratory Manual. Cold Spring Harbour Laboratory Press, New York **Sies H, Ketter B** (1988) Glutathione conjugation: Mechanisms and biological Significance. Academic Press, New York

Schmitz G, Theres K (1992) Structural and functional analysis of the *Bz2* locus of *Zea mays*: characterization of overlapping transcripts. Mol Gen Genet 233: 269-277

Stenberg G, Board PG, Carlberg I, Mannervik B (1991) Effects of directed mutagenesis on conserved arginine residues in a human class alpha glutathione transferase. Biochem J 274: 549-555

Takahashi Y, Kusaba M, Hiraoka Y, Nagata T (1991) Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. The Plant J 1: 327-332

Takahashi Y, Nagata T (1992) *parB*: An auxin-regulated gene encoding glutathione *S*-transferase. Proc Natl Acad Sci *USA* 89: 56-59

Taylor JL, Fritzemeier K-H, Häuser I, Kombrink E, Rohwer F, Schröder M, Strittmatter G, Hahlbrock K (1990) Structural analysis and activiation by fungal infection ofa gene encoding a pathogenesis-related protein in potato. Mol Plant-Microbe Interactions 3: 72-77

Valvekens D, van Montagu M, van Lijsebettens M (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci *USA* 85: 5536-5540

van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998

van der Zaal EJ, Memelink J, Mennes AM, Ouint A, Libbenga KR (1987) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol 10: 145-157

Zettl R, Schell J, Palme K (1994) Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azido- [7-^{3}H]indole-3-acetic acid; Identification of a glutathione *S*-transferase. Proc Natl Acad Sci *USA* 91: 689-693

Zhou J, Goldsbrough PB (1993) An arabidopsis gene with homology to glutathione *S*-transferase is regulated by ethylene. Plant Mol Biol 22: 517-523

CHAPTER 4

Expression of an auxin-inducible promoter of tobacco in *Arabidopsis thaliana*

Dianne A.M. van der Kop, Frans N.J. Droog, Bert J. van der Zaal and Paul J.J. Hooykaas

Summary

The expression of the auxin-inducible *Nt103-1* gene of tobacco was studied in *Arabidopsis thaliana*. For this purpose we introduced a gene fusion between the promoter of the gene and the β-glucuronidase reporter gene (GUS) into *Arabidopsis thaliana*. The expression and location of GUS activity were studied histochemically in time and after incubation of seedlings on medium containing auxin or other compounds. The auxin 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid and 1-naphthylacetic acid were able to induce GUS activity in the root tips of transgenic seedlings. The auxin transport inhibitor 2,3,5-triiodobenzoic acid was able to induce GUS activity not only in the root tip, but also in other parts of the root. Induction by the inactive auxin analog 3,5-dichlorophenoxyacetic acid was much weaker. Compounds like glutathione and the heavy metal $CuSO_4$ were weak inducers. GUS activity observed after induction by glutathione was located in the transition zone. Salicylic acid and compounds increasing the concentration of hydrogen peroxide in the cell were also very well able to induce GUS activity in the roots. The possible involvement of hydrogen peroxide as a second messenger in the pathway leading to the induction of the *Nt103-1* promoter is discussed.

Introduction

The plant hormone auxin has been studied extensively over many years. It is involved in various processes like cell division, elongation, differentiation and

initiation of buds and lateral roots (Thimann, 1969). In the past few years molecular biological tools have opened new ways to investigate the mode of action of auxin and auxin signal transduction. Thus auxin-responsive genes have been cloned and characterized (Walker & Key, 1982, Hagen *et al.*, 1984, Theologis *et al.*, 1985, McClure & Guilfoyle, 1987, van der Zaal *et al.*, 1987, Alliotte *et al.*, 1989, Takahashi *et al.*, 1989, Conner *et al.*, 1990, Reddy & Poovaiah, 1990, Reddy *et al.*, 1990, Takahashi & Nagata, 1992a,b). While the function of most of the isolated genes is still unknown, one group of genes can be distinguished because they encode proteins that have significant homology to animal and plant glutathione *S*-transferases (GSTs) (Takahashi & Nagata, 1992b, Droog *et al.*, 1993).

The *Nt103* genes from tobacco form a family of auxin-responsive genes encoding proteins with *in vitro* GST activity (Droog *et al.*, 1993). The cDNAs corresponding to the *Nt103* genes were isolated after differential screening of a cDNA library constructed from RNA isolated from auxin-starved tobacco (*Nicotiana tabacum*) cell-suspension cells which were treated for four hours with 2,4-dichlorophenoxyacetic acid (2,4-D) (van der Zaal *et al.*, 1987). The mRNA produced via the *Nt103* genes was induced within 30 minutes after the addition of 2,4-D to auxin-starved cell-suspension cultures. Also other auxins were found to be able to induce the mRNA. Interestingly salicylic acid (SA) which is thought to be the endogenous signal required for induction of the systemic acquired resistance (SAR) response of plants was found to be able to induce the mRNA (Boot, 1994).

When the promoter of one of the genes of the Nt103 gene family, the *Nt103-1* gene, was fused to the *β*-glucuronidase reporter gene (*gusA*) and introduced into tobacco, GUS expression could be detected in the root tips of transgenic plants. The expression was enhanced by the addition of 2,4-D to the medium (van der Zaal *et al.*, 1987).

We were interested to use the *Nt103-1* promoter in *Arabidopsis thaliana* for a genetic analysis of auxin-induced gene expression. For this reason we had to test first whether the *Nt103-1* promoter had the same expression pattern and induction characteristics in this plant species. Thus *Arabidopsis thaliana* was transformed with constructs containing the *Nt103-1* promoter translationally fused to the *gusA* coding region. The expression pattern of the hybrid gene was studied by histochemical analyses of seedlings. The expression of the promoter after induction with the synthetic auxins 2,4-D and 1-naphthylacetic acid (1-NAA) and the naturally occurring auxin indole-3-acetic acid (IAA) was compared to the expression in tobacco. The specificity of the promoter to auxin was tested

by incubation with structural analogs of auxin. Because of the possible role of GSTs in plants, also compounds involved in stress or pathogen induction like heavy metals, glutathione and salicylic acid were tested. It was also tested if hydrogen peroxide acted as a second messenger in the pathway leading to the induction of the promoter.

Results

Introduction of an auxin-responsive tobacco gene in *Arabidopsis thaliana*

The expression of the Nt103 gene family was studied in tobacco cell-suspension cultures earlier in our laboratory (van der Zaal *et al.*, 1987, Boot *et al.*, 1993, Boot, 1994). Transcripts were found to accumulate in cell-suspension cultures after induction by auxin and certain other compounds (see discussion). Transgenic tobacco plants containing the promoter of one of the *Nt103* genes, *Nt103-1*, fused to the coding region of the *gusA* gene (pBGUS1) were obtained previously (van der Zaal *et al.*, 1991). After introduction of the pBGUS1 construct into *Arabidopsis thaliana*, T_1 seeds were harvested. Self fertilization of the T_1 plants gave rise to T_2 seeds that were tested in induction assays (lines 10, 13 and 21). In one experiment we used homozygous transgenic T_3 lines harbouring the pAIR1 construct (lines AIR1-2, AIR1-8 and AIR1-11) which contained the same *Nt103-1/gusA* fusion gene as pBGUS1. The GUS activity reported below was not due to endogenous GUS activity in arabidopsis because transgenic seedlings containing a control construct without the *gusA* gene , pBDH5a (Chapter 3) expressed no GUS activity after induction by the compounds tested (data not shown).

Expression of the *Nt103-1/gusA* fusion in *Arabidopsis thaliana* and induction by auxin

Transgenic tobacco plants harbouring the pBGUS1 construct expressed GUS activity in the root tips of rapidly growing root systems (van der Zaal *et al.*, 1991). The GUS expression could be increased after incubation of the plants on medium containing 2,4-D. In arabidopsis weak GUS activity was detected in the root tips in only a small percentage of transgenic seedlings after germination on hormone free medium (Figure 2A). However after induction by incubation of the seedlings on medium containing 2,4-D, GUS activity was strongly enhanced. After induction GUS activity was present in the primary roots one to three days

after germination. After the formation of lateral roots, 7 days after germination, GUS activity could be induced in the root tips of lateral roots and remained inducible for at least two weeks in young lateral roots (Figure 2J). The pattern of GUS expression depended on the age of the seedlings. In very young seedlings, induced one day after germination, weak GUS activity was detectable in almost the complete root with the strongest GUS activity in the zone of transition between the hypocotyl and the root, the transition zone, and in the root tip (Figure 2B). In seedlings induced two or three days after germination, GUS activity could only be detected in the root tip (Figure 2C). In the root tip GUS activity was mainly present in the epidermis (results not shown).

Because Li *et al.* (1991) found that light could inhibit the expression of some auxin-regulated genes we germinated seedlings in the dark as well. However, dark treatment did not lead to higher levels of GUS expression (data not shown).

In tobacco induction by different auxins was tested in cell-suspension cultures. 2,4-D was able to induce the mRNA produced via the *Nt103* genes efficiently. Also NAA and IAA were able to do so (van der Zaal *et al.*, 1987). To compare the activity of different auxins in arabidopsis, transgenic seedlings were incubated on media containing varying concentrations of 2,4-D, NAA or IAA.

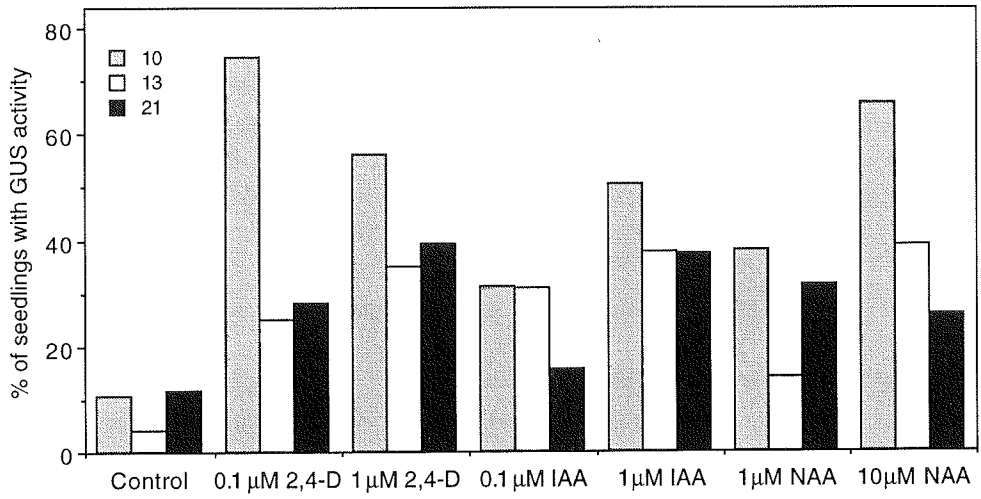

Figure 1. Induction of *Nt103-1/gusA* expression by different auxins. Seedlings were incubated on medium containing 0.1 μM or 1 μM 2,4-D, 0.1 μM or 1 μM IAA or 1 μM or 10 μM NAA. After histochemical staining for GUS activity, the percentage of seedlings with GUS activity was determined for three independent transgenic lines (10, 13 and 21). Per treatment 150-200 seedlings were tested. Similar results were obtained in three independent experiments.

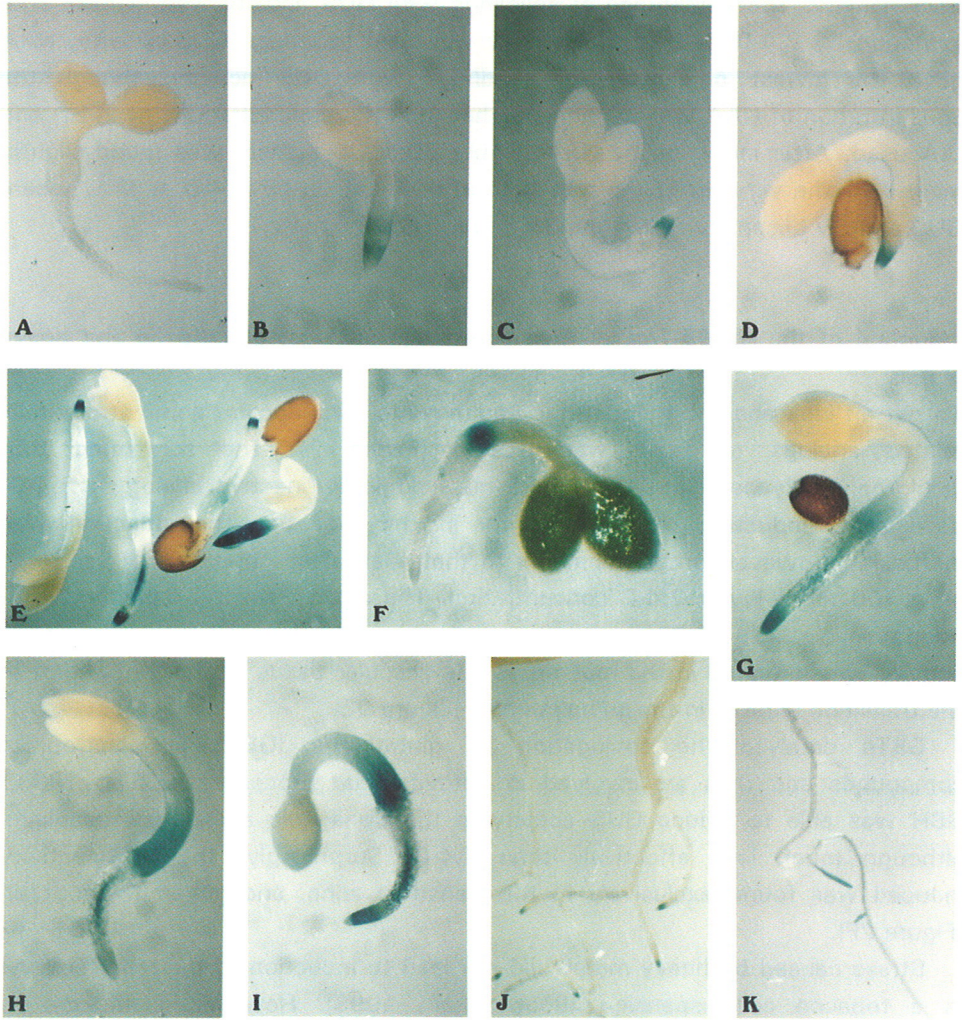

Figure 2. Histochemical analysis of GUS activity of transgenic seedlings of *Arabidopsis thaliana* containing the *Nt103-1/gusA* fusion gene construct. A) seedling in the absence of inducers. B) seedling induced by 2,4-D (1 μM) one day after germination. C) seedling induced by 2,4-D (1 μM) two days after germination. D) idem C but induced by NAA (10 μM). E) idem C but induced by TIBA (100 μM). F) idem C but induced by GSH (100 μM). G) idem C but induced by SA (100 μM). H) idem C but induced by 3-amino-1,2,4-triazole (1 mM). I) idem C but induced by Methyl Viologen (1 mM). J) seedling induced by 2,4-D (1 μM) seven days after germination. K) idem J but induced by SA (100 μM).

Figure 1 shows that 0.1 μM 2,4-D and 0.1 μM IAA already gave induction of GUS activity. The percentage of seedlings with GUS activity was highest after induction by 2,4-D. IAA was also very well able to induce GUS activity. NAA gave the lowest percentage of seedlings with GUS activity, though the concentration of NAA was 10 times higher than the concentrations of 2,4-D and IAA used. After induction by NAA (Figure 2D) GUS activity was found slightly more near the very tip of the root than after induction by 2,4-D or IAA, where GUS activity became visible 1 mm from the tip of the root.

Induction of the *Nt103-1/gusA* fusion by auxin analogs, glutathione and heavy metals

To get an idea about the structural requirements for inducers, some structurally related analogs of auxin were tested (Figure 3). The compound 3,5-dichlorophenoxyacetic acid (3,5-D), a physiologically inactive analog of 2,4-D, gave some induction of GUS activity in the root tip. The induction was inefficient, however, and comparable to that seen after incubation of seedlings on a 100 times lower 2,4-D concentration. The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) was very well able to induce GUS activity. GUS activity was detected in the root tip, in the vascular tissue of the root and/or in the transition zone or in the complete root (Figure 2E).

GSTs catalyze the conjugation of glutathione (GSH) to electrophilic compounds and thus are involved in detoxification processes (Daniel, 1993). GSH was able to induce GUS activity in the transgenic arabidopsis seedlings although much less effectively than 2,4-D. Surprisingly, the GUS activity induced was found exclusively in the transition zone, and not in the root tip (Figure 2F).

Stress caused by heavy metals did not lead to induction of the reporter gene in a tobacco cell-suspension (Boot *et al.*, 1993). However, incubation of arabidopsis seedlings with $CuSO_4$ did lead to induction of the reporter gene.

Induction of the *Nt103-1/gusA* gene fusion via hydrogen peroxide

Boot *et al.* (1993) showed that the mRNA corresponding to the Nt103 gene family could be induced by SA in tobacco cell-suspension cultures. Recently it was found that the signal transduction pathway leading from SA to the expression of PR genes was mediated via activated oxygen species (Chen *et al.*, 1993). SA was found to inhibit a catalase whose activity normally dismutates

hydroxygen peroxide, H_2O_2, into H_2O and O_2. By doing so the concentration of H_2O_2 is elevated which leads to induction of the PR genes. We tested if SA was also able to induce the *Nt103-1/gusA* gene fusion in arabidopsis. In experiments corresponding to those described by Chen *et al.* (1993), we tested if H_2O_2 was involved in the signal transduction pathway leading to the induction of the gene.

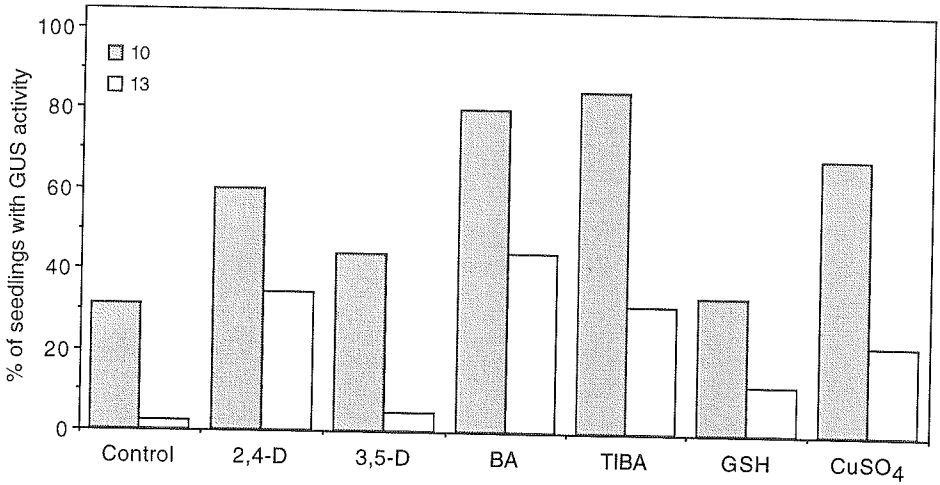

Figure 3. Induction of *Nt103-1/gusA* by auxin analogs, glutathione and $CuSO_4$. Seedlings were incubated on medium containing 0.1 μM 2,4-D, 10 μM 3,5-D, 100 μM BA or 100 μM TIBA. Also 1 mM GSH and 10 μM $CuSO_4$ were tested. After histochemical staining for GUS activity, the percentage of seedlings with GUS activity was determined for two independent transgenic lines (10 and 13). Per treatment 100 seedlings were tested. Similar results were obtained in 2 independent experiments.

In figure 4 it is shown that SA was able to induce GUS activity in arabidopsis seedlings. The inactive analog of SA, 3-hydroxybenzoic acid (3-HBA), was not able to induce. Chen *et al.* (1993) found that 3-HBA was not able to inhibit catalase and thereby elevate the concentration of H_2O_2. The compound 3-amino-1,2,4-triazole (3-AT) which is a specific inhibitor of catalase activity was able to induce GUS activity in our system. Methyl Viologen (MV) which is known to promote the generation of H_2O_2 turned out to be an even better inducer of GUS activity than 2,4-D. It has to be noted however, that the concentrations of the compounds tested were 100 to 1000 times higher than the tested concentration of 2,4-D. Incubation of seedlings on medium containing H_2O_2 itself did not lead to induction of GUS activity. This was probably due to the rapid conversion of

H_2O_2 into H_2O and O_2 in the medium. After induction by SA the GUS activity was located in the transition zone, in the vascular tissue of the root and in the root tip (Figure 2G). This was also the case after induction by 3-AT (Figure 2H). After induction by MV GUS activity was more intense and located in the transition zone, the vascular tissue of the root and the root tip or in the complete root (Figure 2I).

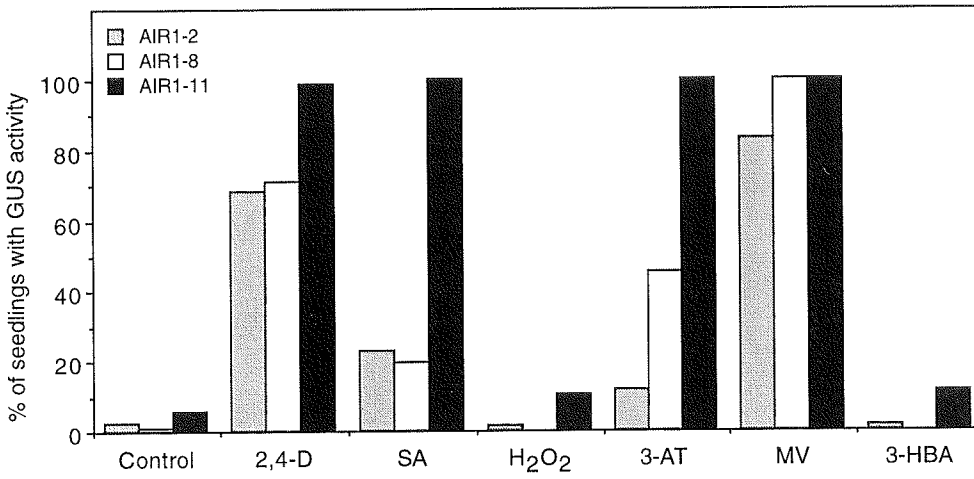

Figure 4. Induction of *Nt103-1/gusA* expression by SA and H_2O_2. Seedlings were incubated on medium containing 1 μM 2,4-D, 100 μM SA, 100 μM 3-HBA, 1 mM H_2O_2, 1 mM 3-AT or 1 mM MV. After histochemical staining for GUS activity, the percentage of seedlings with GUS activity was determined for three independent transgenic lines containing the pAIR1 construct (AIR1-2, AIR1-8 and AIR1-11). Per treatment 150-200 seedlings were tested.

After induction of older plantlets by SA, GUS activity was not restricted to the young root tips like after induction by 2,4-D. GUS activity was also present in the vascular tissue of older roots and in various tissues of lateral roots (Figure 2K). Plant lines AIR1-2, AIR1-8 and AIR1-11 showed higher levels of GUS activity than the plant lines transformed with the pBGUS1 construct. This was probably due to the presence of the 35S CaMV promoter near the *Nt103-1* promoter in the construct pAIR1 which was used to transform these plant lines.

Discussion.

Induction by auxin and auxin analogs

The expression of GUS activity by the *Nt103-1/gusA* fusion gene in *Arabidopsis thaliana* was, as in tobacco, mainly localized in the root tip (van der Zaal *et al.*, 1991). After induction by auxin GUS activity was enhanced in the root tip and, depending on the developmental stage of the seedlings, could also be detected in the transition zone and in the vascular tissue of the root. The age of the roots was important for their capacity to be induced by auxin. In arabidopsis this was more critical than in tobacco. Like in tobacco cell-suspension cultures (van der Zaal *et al.*, 1987), different auxins were able to induce GUS activity in arabidopsis, whereby 2,4-D seemed to be the most effective inducer.

Differences were seen between the tobacco and arabidopsis systems when auxin analogs were used. In tobacco cell-suspensions the inactive auxin analog, 3,5-D was as effective as 2,4-D in inducing GUS expression (Boot, 1994), but in arabidopsis it was only a weak inducer. The opposite was true for the auxin transport inhibitor TIBA. This was a weak inducer in tobacco (Boot, 1994), but a strong inducer in arabidopsis. These compounds have no auxin activity, but have been reported to be able to bind to the auxin-binding protein (Edgerton *et al.*, 1994). Their structural resemblance to auxin could be the reason for their ability to induce GUS activity. Differences in induction between tobacco and arabidopsis can possibly be explained by the different experimental systems used. They might be caused by differences between the arabidopsis and tobacco auxin-binding proteins involved.

Induction by stress-inducing compounds

Glutathione as well as heavy metals, especially Cu^{2+} ions, were able to induce GUS activity in arabidopsis. This was also the case in transgenic tobacco plants (Droog, 1995). Recently it was found that the Nt103 gene family encodes glutathione *S*-transferases (Droog *et al.*, 1993). In animals, GSTs are believed to play an important role in the protection of cellular macromolecules from attack by reactive electrophiles. They are thought to be involved in detoxification and via their associated GSH-dependent peroxidase activity may play an important role in protecting tissues from endogenous organic hydroperoxides produced during oxidative stress (Daniel, 1993). Although it is not known whether the protein encoded by the *Nt103-1* gene is involved in detoxification indeed, this would be in line with the ability of stress-inducing compounds to induce the

promoter. The induction by auxin and auxin analogs may also be related to the detoxifying function of the gene. Alternatively, GSTs have been found to bind a variety of hydrophobic compounds such as hormones and to serve as intracellular carrier proteins for the transport of such ligands (Daniel, 1993, and references therein). The function of the *Nt103-1* protein may also be in binding and transporting auxin. Recently two papers were published in which auxin-binding proteins were found to be GSTs (Bilang *et al.*, 1993, Zettl *et al.*, 1994).

Induction via hydrogen peroxide

The *Nt103-1* promoter has an *ocs/as-1* element in common with the nopaline synthase (*nos*) promoter, and the 35S cauliflower mosaic virus promoter (Liu & Lam, 1994, Zhang & Singh, 1994). The *ocs* element was found to mediate induction by auxin and SA (Zhang & Singh, 1994). SA was previously found by us to be able to induce the mRNA corresponding to one of the genes of the Nt103 gene family in cell-suspension cultures (Boot *et al.*, 1993). Recently, it was found that addition of SA can lead to elevated levels of H_2O_2, which in turn are involved in induction of the PR genes (Chen *et al.*, 1993). From a comparison of our results with the results obtained by Chen *et al.* (1993) a clear resemblance can be seen and H_2O_2 thus seems to be one of the signals that can lead to the induction of the *Nt103-1* promoter. Gunsé and Elstner (1992) found that H_2O_2 was able to cause an "activated state" of IAA. This might lead to induction of the promoter. Alternatively, the oxidative stress caused by H_2O_2 might lead directly to a change in activity of certain transcription factors as was described for AP1, NF-κb and Myc in mammalian cells (reviewed by Daniel 1993).

The location of GUS activity

The location of GUS activity after induction by compounds other than auxin was not restricted to the root tip. After induction by other compounds the GUS activity did also seem to be less dependent on the developmental stage of the roots. Boot (1994) found evidence for the existence of different transduction/ perception pathways for SA and 2,4-D leading to the expression of the *Nt103* genes. Induction of the *Nt103* promoter by 2,4-D could be inhibited with D16 antibodies which recognize the auxin-binding site of the auxin-binding protein (Venis *et al.*, 1992), but induction by SA was not inhibited. A different distribution of ligand binding proteins for auxin, SA and other inducers of the

Nt103 genes may explain the differences observed in the location of expression after addition of the different inducers.

From these experiments we conclude that the promoter of the tobacco gene is auxin -inducible in *Arabidopsis thaliana*. We can thus use this promoter for a genetic analysis of auxin-induced gene expression.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (*gl1*) were used in all experiments. Plants were grown at 21°C in a 16 h light / 8 h dark cycle. The light intensity in the tissue culture room was 3000 lux (Philips TLD50W/83HF).

Construction of plasmids

Construction of the *Nt103-1/gusA* gene fusion, pBGUS1, and introduction into *Agrobacterium tumefaciens* strain LBA4404 were described earlier (van der Zaal *et al.*, 1991). The pAIR1 (**A**uxin-**I**nducible **R**eporter **1**) construct contains the same *Nt103-1/gusA* fusion gene as pBGUS1 (Chapter 5). Unless otherwise stated, independent transgenic plant-lines containing the pBGUS1 construct were used.

Transformation of arabidopsis

Arabidopsis thaliana was transformed with *Agrobacterium tumefaciens* strain LBA4404 using the root transformation protocol (Valvekens *et al.*, 1988) as described in chapter 2. Transgenic shoots were selected on medium containing 50 mg/l kanamycin.

GUS histochemical assay

Histochemical analysis of seedlings for GUS activity was performed as described by Jefferson *et al.* (1987). Seedlings were incubated in a solution containing 0.3 g/l 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc); 0.3 mM K ferricyanide; 10 mM Na_2EDTA; 0.1% Sodium Lauryl Sarcosine and 0.1% Triton-X100 in 0.1 *M* $NaPO_4$ pH 7.0 for 16 h at 37°C.

Induction assay

T_2 or T_3 transgenic seeds were surface sterilized, resuspended in 0.1% agarose and transferred to Basal Medium (BM = ½ MS + 0.5 mg/l MES). After 48 h the germinating seeds were transferred to BM supplemented with hormones or other compounds. After an induction period of 24 h the seedlings were histochemically stained for GUS activity. In a segregating population the percentage of GUS positive seedlings was determined and corrected for the percentage of transgenic seedlings as determined by germination

of seeds on medium containing kanamycin.

Acknowledgements

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References

Alliotte T, Tiré C, Engler G, Peleman J, Caplan A, van Montagu M, Inzé D (1989) An auxin-regulated gene of *Arabidopsis thaliana* encodes a DNA-binding protein. Plant Physiol 89: 743-752

Bilang J, Macdonald H, King PJ, Sturm A (1993) A soluble auxin-binding protein from *Hyoscyamus muticus* is a glutathione *S*-transferase. Plant Physiol 102: 29-34

Boot CJM, van der Zaal EJ, Velterop J, Quint A, Mennes AM, Hooykaas PJJ, Libbenga KR (1993) Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell-suspension cultures. Plant Physiol 102: 513-520

Boot CJM (1994) Regulation of auxin-induced genes in cell-suspension cultures from *Nicotiana tabacum*. PhD Thesis, Leiden University, 161 pp

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262: 1883-1886

Conner TW, Goekjian VH, LaFayette PR, Key JL (1990) Structure and expression of two auxin-inducible genes from arabidopsis. Plant Mol Biol 15: 623-632

Daniel V (1993) Glutathione *S*-transferases: Structure and regulation of expression. Critical Reviews in Biochemistry and Molecular Biology: 173-207

Droog FNJ, Hooykaas PJJ, Libbenga KR, van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferases and one member indeed shows *in vitro* GST activity. Plant Mol Biol 21: 965-972

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD Thesis, Leiden University, 165 pp

Edgerton MD, Tropsha A, Jones AM (1994) Modelling the auxin-binding site of auxin-binding protein 1 of maize. Phytochemistry 35: 1111-1123

Gunsé B, Elstner EF (1992) Formation of activated states of indoleacetic acid and cytokinins: An experimental approach to a hypothesis concerning signal transduction. J Plant Physiol 140: 536-540

Hagen G, Kleinschmidt A, Guilfoyle TJ (1984) Auxin-regulated gene expression in intact

soybean hypocotyl and excised hypocotyl sections. Planta 162: 147-153

Jefferson RA, Kavanagh TA, Bevan M (1987) GUS fusions β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907

Li Y, Hagen G, Guilfoyle TJ (1991) An auxin-responsive promoter is differentially induced by gradients during tropisms. The Plant Cell 3: 1167-1175

Liu X, Lam E (1994) Two binding sites for plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. The J of Biol Chem 269: 668-675

McClure BA, Guilfoyle TJ (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol Biol 9: 611-623

Reddy ASN, Poovaiah BW (1990) Molecular cloning and sequencing of a cDNA for an auxin-repressed mRNA: correlation between fruit growth and repression of the auxin-regulated gene. Plant Mol Biol 14: 1987-136

Reddy ASN, Jena PK, Mukherjee SK, Poovaiah BW (1990) Molecular cloning of cDNAs for auxin-induced mRNAs and developmental expression of the auxin-inducible genes. Plant Mol Biol 14: 643-653

Takahashi Y, Kuroda H, Tanaka T, Machida Y, Takebe I, Nagata T (1989) Isolation of an auxin-regulated gene cDNA expressed during the transition from G0 to S phase in tobacco mesophyll protoplasts. Proc Natl Acad Sci *USA* 86: 9279-9283

Takahashi Y, Nagata T (1992a) Differential expression of an auxin-regulated gene, *parC*, and a novel related gene *C-7*, from tobacco mesophyll protoplasts in response to external stimuli and in plant tissues. Plant Cell Physiol 33: 779-787

Takahashi Y, Nagata T (1992b) *parB*: An auxin-regulated gene encoding glutathione *S*-transferase. Proc Natl Acad Sci *USA* 89: 56-59

Theologis A, Huynh TV, Avis RW (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J Mol Biol 183: 53-68

Thimann KV (1969) The Physiology of Plant growth and developement. New York: McGraw-Hill

Valvekens D, van Montagu M, van Lijsebettens M (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci *USA* 85: 5536-5540

Venis MA, Napier RM, Barbier-Brygoo H, Maurel C, Perrot-Rechenmann C, Guern J (1992) Antibodies to a peptide from the maize auxin-binding protein have auxin agonist activity. Proc Natl Acad Sci *USA* 89: 7208-7212

Walker JC, Key JL (1982) Isolation of cloned cDNAs to auxin-responsive poly(A) RNAs of elongating soybean hypocotyl. Proc Natl Acad Sci *USA* 79: 7185-7189

van der Zaal EJ, Memelink J, Mennes AM, Ouint A, Libbenga KR (1987) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol 10: 145-157

van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998

Zettl R, Schell J, Palme K (1994) Photoaffinity labeling of *Arabidopsis thaliana* plasma

membrane vesicles by 5-azido-[7-^{3}H]indole-3-acetic acid: Identification of a glutathione *S*-transferase. Proc Natl Acad Sci *USA* 91: 689-693

Zhang B, Singh KB (1994) ocs element promoter sequences are activated by auxin and salicylic acid in arabidopsis. Proc Natl Acad Sci *USA* 91: 2507-2511

CHAPTER 5

The selection of mutants up-regulated for auxin-inducible glutathione *S*-transferase genes

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Summary

In order to identify genes involved in a signal transduction pathway leading to the expression of auxin-regulated genes, we isolated mutants of *Arabidopsis thaliana* with higher expression levels of the auxin-inducible *Nt103-1* gene of tobacco. To obtain these mutants we generated transgenic arabidopsis plants containing a T-DNA construct (pAIR) in which the promoter of the *Nt103-1* gene was fused to the neomycin phosphotransferase (*nptII*) gene as well as to the *β*-glucuronidase (*gusA*) reporter gene. The *hptII* gene driven by the CaMV 35S promoter was incorporated in the construct to be able to select for transgenic plants. Homozygous transgenic seedlings were resistant to hygromycin, sensitive to kanamycin and showed low or no basal GUS activity. After treatment of the seedlings with auxin the kanamycin resistance and GUS activity were enhanced. Seeds of 3 selected transgenic lines were treated with the mutagen ethyl methane sulfonate and the resulting M_1 plants were selfed. M_2 seeds were germinated on medium containing kanamycin to select for mutants. The selected kanamycin resistant seedlings were selfed and the M_3 progenies were histochemically assayed for GUS activity. This allowed us to distinguish between mutants that were resistant to kanamycin due to a *trans*-acting mutation causing an up-regulation of the *Nt103-1* promoter and kanamycin resistant escapes due to a cis-acting (promoter) mutation or rearrangements in the construct. Kanamycin resistant mutants with a high basal level of GUS activity will be characterized in the near future to find out whether they have mutations in one of the components of an auxin signal transduction pathway leading to the expression of the *Nt103-1* gene.

Introduction

Auxin, the first plant hormone to be discovered, is known to play an essential role in many processes in plant development. Physiological studies have illustrated that auxins influence plant growth and development and that they are important in processes like cell elongation, apical dominance, root initiation and tropic behaviour. Despite its importance, little is known about the molecular mechanism of auxin action but much effort is made to make up for this gap in knowledge.

Because auxins are thought to bind to receptors, which as a result of this binding transduce signals that regulate gene expression, several groups have tried to isolate these receptors. Different proteins that are able to bind auxins have been isolated and are presently being studied (reviewed by Jones & Prasad, 1992).

Another approach has been the isolation of auxin regulated genes. A number of genes that are induced by auxin have been isolated (Walker & Key, 1982, Hagen *et al.*, 1984, Theologis *et al.*, 1985, McClure & Guilfoyle, 1987, van der Zaal *et al.*, 1987, Conner *et al.*, 1990, Reddy *et al.*, 1990, Takahashi *et al.*, 1989). While promoter analyses of these genes has given ideas about the *cis*-acting elements involved in the regulation by auxins, the function of most of these genes is still unknown (Ballas *et al.*, 1993, Guilfoyle *et al.*, 1993, Nagao *et al.*, 1993)

A third approach has been the isolation of mutants impaired in auxin action. Because of the importance of auxins for plant development, it is likely that mutations which eliminate auxin biosynthetic activity or functions required for auxin action will be lethal. For this reason most mutants isolated sofar are mutants resistant to exogenously applied auxins. These mutants could be resistant to auxins because they are receptor mutants or have alterations more downstream in the transduction pathways. However, they could also be affected in the uptake or metabolism of auxin. Mutants resistant to auxins have been isolated and described for different plant species (reviewed by Reid, 1993). Since the introduction of *Arabidopsis thaliana* as a model system for plants, the research on mutants involved in auxin action has been intensified. Arabidopsis, being a small plant with a short generation time and having a small genome with little repetitive DNA, is an ideal plant to use in mutagenesis experiments (Meyerowitz, 1989).

Most auxin resistant arabidopsis mutants were isolated because of their resistance to the synthetic auxin 2,4-dichlorophenoxy-acetic acid (2,4-D) (Maher

& Martindale, 1980, Mirza & Maher, 1987, Estelle & Somerville, 1987). In more recent experiments the naturally occurring auxin indole-3-acetic acid (IAA) was used as a selective agent to isolate mutants (Wilson *et al.*, 1990). All isolated auxin resistant mutants are impaired in gravitropism of the roots. The mutants *dwf, axr1* and *axr2* are also impaired in shoot growth and have a dwarf phenotype. Auxins are known to affect cell elongation and gravitropism and it is very well possible that a defect in auxin action in these mutants is responsible for some aspects of the mutant phenotype. The gene mutated in the *axr1* mutant has been cloned and was found to encode a protein related to the ubiquitin-activating enzyme E1, suggesting a role for the ubiquitin pathway in plant hormone action (Leyser *et al.*, 1993).

A link between the expression of auxin-regulated genes and auxin resistant mutants was established by studying the expression of auxin-regulated genes in the mutants. The expression of the auxin-inducible *SAUR-AC1* gene was altered in the different mutants. In the *axr2* mutant the accumulation of the RNA corresponding to the *SAUR-AC1* gene was almost completely blocked and a reduced expression was also found in the *aux1* mutant compared to wild-type (Gil *et al.*, 1994).

So far "auxin" mutants were isolated after screening seedlings for auxin resistance to externally applied auxins. Since they may be affected in uptake rather than auxin-signal transduction, we chose to use the selection of mutants in a completely different manner using an auxin-inducible gene as a genetic tool. For this reason we made transgenic plants containing a construct with the promoter of the auxin-inducible tobacco glutathione *S*-transferase gene, *Nt103-1* (van der Zaal *et al.*, 1991), fused to two reporter genes. The seeds of the transgenic plants were treated with the mutagen ethyl methane sulfonate (EMS) in order to obtain mutants with an **up**-regulation of this **g**lutathione *S*-transferase gene (*gup* mutants). Construction and characterization of the transgenic plants and selection of mutants after EMS mutagenesis are described in this chapter.

Results and discussion

Construction of the transgenic lines

An **A**uxin **I**nducible **R**eporter plasmid (pAIR1) containing two reporter genes controlled by the promoter of the *Nt103-1* gene was constructed (Figure 1). The coding region of the *β*-glucuronidase (*gusA*) reporter gene was placed under control of the full-length promoter of the *Nt103-1* gene. This fusion gene was used earlier to study the expression of the promoter in tobacco plants (van der

Zaal *et al.*, 1991) and cell-suspensions (Boot *et al.*, 1993) and it was also tested in arabidopsis (Chapter 4).

In a promoter deletion analysis it was found that the -650 bp fragment of the promoter was sufficient to give the same level of expression as the full-length promoter when fused to the *gusA* reporter gene (Droog *et al.*, 1995). For this reason this promoter fragment was used to control the expression of the neomycin phosphotransferase (*nptII*) gene in a transcriptional fusion. The *Nt103-1* promoter was fused to two reporter genes to be able to distinguish between mutants having a *cis*-acting mutation in the *Nt103-1* promoter fused to the kanamycin resistance gene from mutants having a *trans*-acting mutation affecting the expression of both *Nt103-1* promoters (thus leading to both kanamycin resistance and higher GUS activity). Roots of *Arabidopsis thaliana* were transformed using *Agrobacterium tumefaciens*. Transgenic plants were selected on medium containing the antibiotic hygromycin. The transgenic lines obtained did not differ from the wild-type in appearance. The lines segregating in a 3:1 manner for hygromycin resistance were selected for further study. Southern analysis showed that the transgenic lines AIR1-2 and AIR1-8 contained one copy of the pAIR1 construct. The line AIR1-11 contained two T-DNA copies (data not shown).

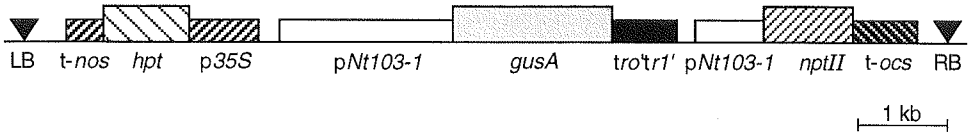

Figure 1. The pAIR1 construct. LB, RB: left and right T-DNA border; *hpt, nptII, gusA*: encoding hygromycin phosphotransferase, neomycin phosphotransferase and β-glucuronidase, respectively; p35S: 35S CaMV promoter; p*Nt103-1*: promoter region of the *Nt103-1* gene of tobacco; t-*nos* and t-*ocs*: terminator of the nopaline synthase and octopine synthase genes, respectively, *tr0'-tr1'*: dual terminator from the T, region of the Ti plasmid.

Expression of the fusion genes

Before using the transgenic lines in a mutagenesis experiment, we checked if the *Nt103-1/gusA* fusion gene could be induced by auxins. We also checked if the level of kanamycin resistance was low enough to be able to select for increased kanamycin resistance after mutagenesis.

The GUS expression pattern of the transgenic lines is described in detail in chapter 4. After germination of seedlings on hormone free medium, weak or no GUS activity was detected in the root tips of the seedlings. When the seedlings

were induced by incubation on medium containing 0.1 μM 2,4-D, GUS activity was detected in the root tip, in the vascular tissue of the root and in the transition zone. Three lines, named AIR1-2, AIR1-8 and AIR1-11, were selected because of their efficient inducibility by 2,4-D. GUS expression was also induced by other auxins in these lines (Figure 2). The percentage of seedlings which showed induction of GUS activity in the transgenic lines, harbouring the pAIR1 construct, was higher than in lines harbouring the pBGUS1 construct described in chapter 4. Also the intensity of the GUS activity was higher. This was probably due to the presence of the 35S CaMV promoter adjacent to the *Nt103-1* promoter controlling the *gusA* coding region.

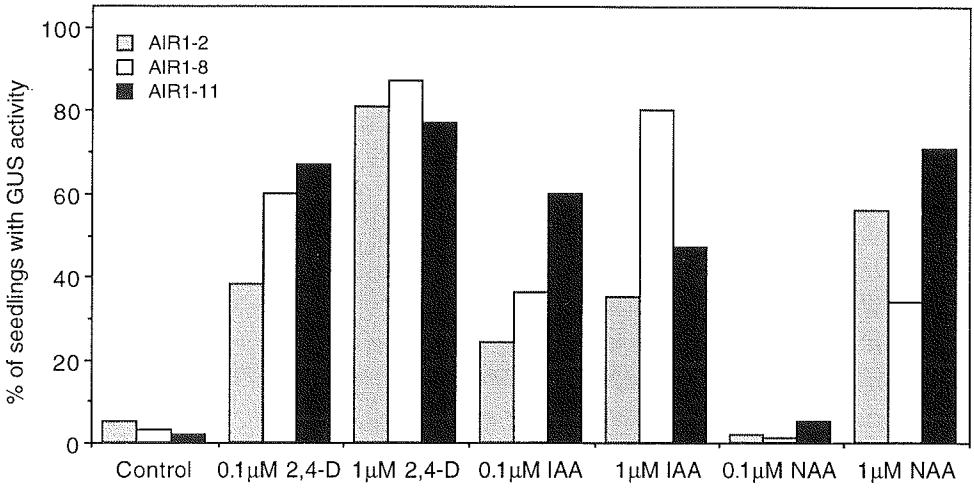

Figure 2. Auxin inducibility of three independent transgenic lines. Two days old seedlings were transferred to medium containing 0.1 μM or 1 μM 2,4-D, 0.1 μM or 1 μM IAA or 0.1 μM or 1 μM NAA and incubated overnight. After histochemical staining for GUS activity, the percentage of seedlings with GUS activity was determined for the independent transgenic lines AIR1-2, AIR1-8 and AIR1-11. Per treatment 50-100 seedlings were tested. Similar results were obtained in two independent experiments.

After the auxin inducibility was determined, the level of kanamycin resistance was studied. The shoots of both wild-type and transgenic lines bleached and did not develop on medium containing kanamycin. However, whereas the wild-type formed short roots on medium containing kanamycin, the transgenic lines formed a much larger root (Table 1). There was no difference in root length between the wild-type and transgenic lines on medium without kanamycin (data

not shown). These results indicate that although GUS expression was usually not detected in non-induced roots, there was low expression of the *Nt103-1* promoter in the roots. The expression of the promoter was too weak to give kanamycin resistance to the complete plant.

Table 1. Root growth on medium containing kanamycin.

plant line	length of root[a] in mm ± SE
AIR1-2	114 ± 24
AIR1-8	100 ± 19
AIR1-11	85 ± 15
wild-type	25 ± 5

[a] The root length of the control and three transgenic lines was measured 14 days after germination. The medium contained 25 mg/l kanamycin. Values are mean values for 40 plants per treatment ± SE (Standard Error).

Knowing that the GUS expression of the transgenic lines could be induced by different auxins and that the basal level of expression of the *Nt103-1/nptII* fusion gene was too weak to give kanamycin resistant seedlings, we decided to use these transgenic lines in a mutagenesis experiment.

EMS mutagenesis and selection of the mutants

Figure 3 represents a scheme of the mutation and selection procedure.

We screened 180 000 M_2 seedlings for growth on medium containing kanamycin. As a control we germinated 24 000 non-mutagenized seeds of the transgenic parent lines on medium containing kanamycin. Ten days after germination seedlings were scored for green cotyledons and were transferred to medium without kanamycin and allowed to set seed. In this way 153 M_2 seedlings were transferred to medium without kanamycin. From the control plates, 4 seedlings with green cotyledons were transferred. Out of the 153 M_2 seedlings transferred, 103 recovered, were fertile and set seed. Of these lines 48 lines lacked increased GUS activity and may represent lines with a *cis*-acting mutation or escapes.

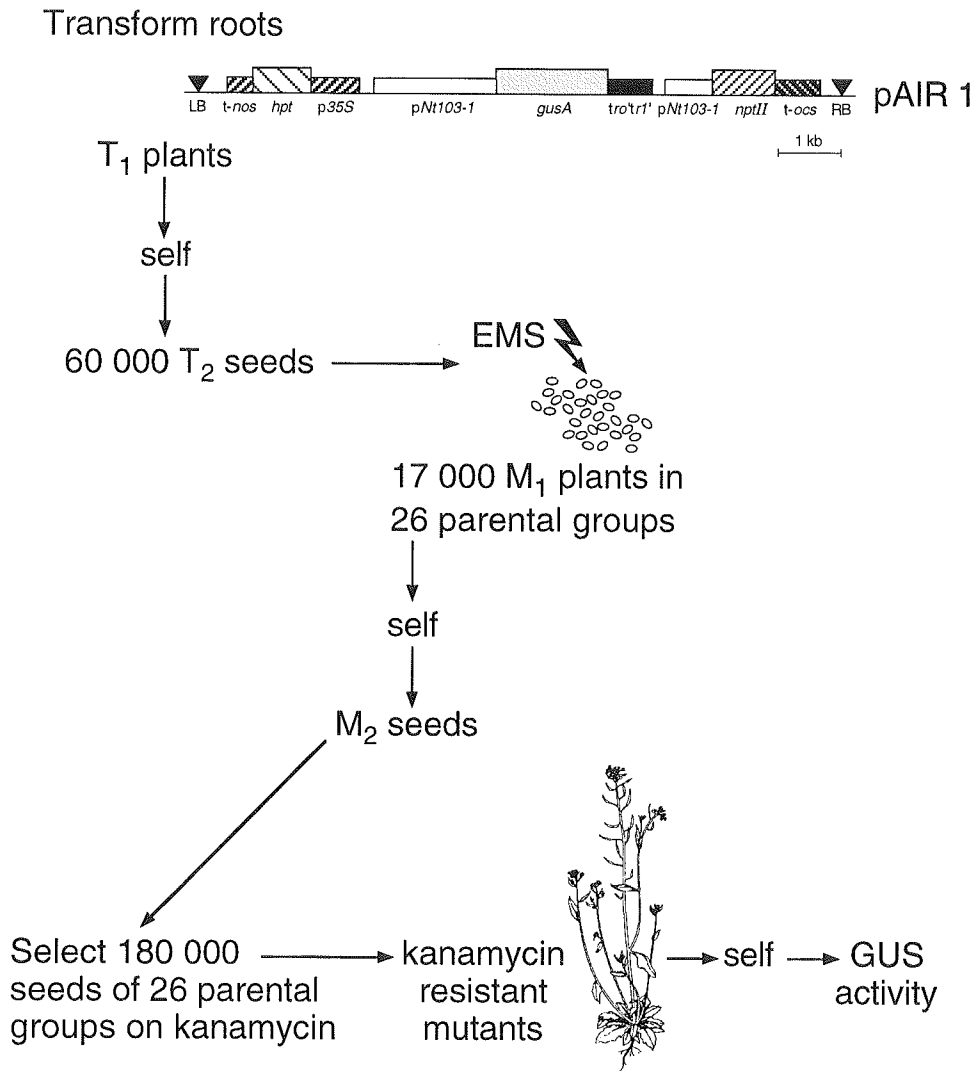

Figure 3. Selection procedure of the *gup* mutants.

Alternatively, the level of expression of both *Nt103-1* gene fusions might have been increased but to a too low level to be detectable by the GUS assay. The seedlings that were transferred from the non-mutagenized control group did not show enhanced levels of GUS activity.

Southern analysis of four of the kanamycin resistant lines that lacked GUS activity revealed that the coding region of the *gusA* gene was no longer present. This was probably caused by recombination between the *Nt103-1* promoters

present on the T-DNA construct of these transgenic lines (results not shown). The kanamycin resistance could in this case be due to the influence of the strong 35S CaMV promoter on the *Nt103-1/nptII* fusion gene that became positioned adjacent to this promoter. This influence was reported earlier when comparing the expression of the *Nt103-1/gusA* fusion in the pAIR1 construct to that in the pGUS1 construct (Chapter 4).

After histochemical staining 55 mutants with a more pronounced GUS activity than the transgenic parents were isolated. In table 2 the distribution of the kanamycin resistant M_2 seedlings and the M_3 seedlings with higher GUS activity over the parental groups is shown. The parental groups could be divided in three major classes. In the first class (parental groups 5-9-12-14-15-24-25 and 26), the number of kanamycin resistant seedlings was very low or zero. M_3 seedlings in this class did not have higher levels of GUS activity and the few

Table 2. Selection of mutants after mutagenesis.

parental group	kanamycin [a] resistant	fertility [b]		enhanced GUS activity [c]	
		1	2	1	2
Class I					
5	0				
9	1	0	0%		
12	0				
14	1	0	43%	3	100%
15	1	1	100%	0	0%
24	1	1	100%	0	0%
25	0				
26	0				
Class II					
6	3	3	100%	3	100%
10	3	1	33%	1	100%
18	3	1	33%	1	100%
21	3	2	67%	2	100%
23	3	2	67%	1	50%

Table 2 continued.

parental group	kanamycin [a] resistant	fertility [b]		enhanced GUS activity [c]	
		1	2	1	2
Class III					
1	7	3	43%	3	100%
2	10	8	80%	2	25%
4	8	8	100%	4	50%
7	10	6	60%	4	67%
8	15	7	47%	6	85%
13	20	16	80%	10	63%
16	17	7	41%	3	43%
17	8	7	88%	3	43%
19	6	3	50%	1	33%
20	8	8	100%	3	38%
22	9	8	89%	7	88%
Class IV					
3	3	0	0%		
11	13	11	85%	0	0%
total	153	103	67%	55	53%
control	4	4	100%	0	0%

Analysis on the number of kanamycin resistant M_2 seedlings per parental group (out of approximately 7000 seedlings per parental group and 24000 control seedlings) after selection of the seedlings on medium containing kanamycin and subsequent analysis of the M_3 progeny of the fertile kanamycin resistant M_2 plants.

[a]) number of kanamycin resistant seedlings

[b]) 1) number of fertile kanamycin resistant M_2 lines

2) number of fertile kanamycin resistant M_2 lines calculated as a percentage of the total number of kanamycin resistant seedlings

[c]) analysis of kanamycin resistant lines by histochemical staining of M_3 seedlings for GUS activity

1) M_3 populations with enhanced GUS activity

2) M_3 populations with enhanced GUS activity calculated as a percentage of the number of fertile lines

kanamycin resistant lines were, like the kanamycin resistant seedlings from the control group, likely to be escapes. In the second class the number of kanamycin resistant seedlings was also low, but a considerable percentage of M_3 seedlings of fertile kanamycin resistant plants had more pronounced GUS activity than the control (parental groups 6-10-18-21-23). The third and largest class of parental groups gave relatively high numbers of kanamycin resistant seedlings, of which the fertile plants sometimes gave M_3 seedlings with constitutive and more pronounced GUS activity (parental groups 1-2-4-7-8-13-16-17-19-20-22). Two parental groups did not fit into this classification. In parental group 3 the three kanamycin resistant seedlings did not set seed. This could be due to a mutation essential for fertility. In parental group 11, none of the selected kanamycin resistant seedlings showed constitutive GUS activity. The kanamycin resistant mutants in this group completely lacked a root on medium containing kanamycin, whereas the transgenic parents did form a root (Table 1). After transfer of the seedlings to medium without kanamycin the plants developed a root. The kanamycin resistance could be caused by a *cis*-acting mutation in the *Nt103-1* promoter controlling the *nptII* gene in these lines. It is, however more likely that the initial lack of roots was responsible for kanamycin resistance in these mutants because uptake of kanamycin was diminished.

The number of interesting mutants found was rather high compared to the frequency with which auxin resistant mutants were found (Maher & Martindale, 1980, Mirza & Maher, 1987, Estelle & Somerville, 1987, Wilson *et al.*, 1990). However, the number of mutants we found was lower than the number of mutants isolated after a procedure comparable to ours aimed to obtain mutants affected in light signal transduction (Susek *et al.*, 1993). The number of loci mutated in our experiment may differ significantly from the number of loci that may provide auxin resistance or affect light signal transduction. It is however difficult to compare mutation frequencies because the size of the M_1 population and the number of parental groups in the M_2 population varies in the different experiments. Besides it is known that the frequency of mutations varies per locus (Koornneef *et al.*, 1981).

In our selection procedure we selected for fertile mutants. Some of the kanamycin resistant mutants formed multiple rosettes but did not form an inflorescence. Unfortunately, these could not be rescued. It is probable that mutants with severe alterations in auxin signal transduction were been lost in early stages of the experiment.

Nevertheless some of the isolated mutants had phenotypic alterations such as aberrant roots or multiple rosettes. A high number of mutants had a reduced apical dominance in the inflorescences and a reduced fertility. These phenotypes could be due to changes in auxin signal transduction or perception. The phenotypes could however also be caused by germination on medium containing kanamycin and subsequent growth of the plants in tissue culture or to second site mutations. Therefore, it will be very interesting to study the phenotype of the M_3 mutants after germination in soil and after back crosses to the transgenic parent plants.

In future experiments (Chapter 6) we will investigate the resistance of the isolated mutants to kanamycin and the expression of GUS activity in more detail. Further characteristics of the mutants will also be studied in detail.

Material and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (*gl1*) was used in all experiments. In tissue culture, plants were grown at 21°C to 24°C and a 16 h photoperiod. The light intensity in the tissue culture room was 3000 lux (Philips TLD50W/83HF). Unless stated otherwise plants were grown on basal medium (Chapter 4). After autoclaving, the medium was - when necessary - supplemented with antibiotics or hormones.
In the greenhouse, plants were grown at 23°C in a 16 h light cycle.

Bacterial strains

Escherichia coli strains DH5α (Clontech) and XL1 Blue (Stratagene) were used for bacterial cloning. Strains were grown at 37°C in LC medium (Hooykaas *et al.*, 1977) supplemented with 100 mg/l carbenicillin or 25 mg/l kanamycin. *Agrobacterium tumefaciens* strain MOG101 (Hood *et al.*, 1993), harbouring a non-oncogenic octopine Ti-helper plasmid in a C58 chromosomal background, was grown at 29°C in LC medium supplemented with 100 mg/l kanamycin.

Construction of the pAIR1 plasmid

All DNA manipulations were performed essentially according to Sambrook *et al.*. (1989). The -650 promoter fragment of *Nt103-1* (van der Zaal *et al.*, 1991) was transcriptionally fused to the *nptII* coding region with a 3'*ocs* terminator. This was achieved by cloning the *Sau*3A fragment of the *Nt103-1* gene (-650 to + 106 relative to the ATG (+1)) in the *Bgl*II-site of pSDM56. The fusion gene was subcloned in the *Bam*HI-site of pIC20H (Marsh *et al.*, 1984). The *Nt103-1/gusA* fusion gene was cloned into this same plasmid

by insertion of the 4400 bp *EcoR*I/*Kpn*I fragment of pGNTGUS1 (van der Zaal *et al.*, 1991). This *EcoR*I/*Kpn*I fragment contained the full-length promoter of the *Nt103-1* gene translationally fused to the *gusA* coding region and the polyadenylation signal from the *TRO'-TR1'* dual terminator (van der Zaal *et al.*, 1991). The generated 6850 bp *Hind*III fragment, containing both reporter genes under the control of the *Nt103-1* promoter, was introduced into the binary vector pMOG22 (Goddijn *et al.*, 1993). This vector contained a hygromycin resistance gene (*hpt*) (van den Elzen *et al.*, 1985) controlled by a CaMV 35S promoter and a *nos* terminator and a multiple cloning site between the borders of the T-DNA. The resulting plasmid, pAIR1, was electroporated into *Agrobacterium tumefaciens* strain MOG101 using the method described by Mattanovich *et al.* (1989).

Transformation of Arabidopsis thaliana

The *Agrobacterium tumefaciens* strain MOG101 harbouring the binary plasmid pAIR1 was used in a cocultivation experiment with root explants of *Arabidopsis thaliana*. Transformation was performed via the root transformation protocol described by Valvekens *et al.* (1988) as described in Chapter 3. The transgenic shoots were selected by the addition of 20 mg/l hygromycin to the shoot inducing medium (SIM). Transgenic lines were generated and their GUS expression was studied. The kanamycin resistance of the seedlings was determined by germination of transgenic seeds on medium containing 25 mg/l kanamycin.

Auxin induction assay

T_2 transgenic seeds were surface sterilized, resuspended in 0.1% agarose and transferred to basal medium. The seeds were vernalized for 4 days at 4°C in the dark to promote synchronized germination. After 48 h at 21°C the seedlings were transferred to basal medium supplemented with different auxins. After an induction period of 15-24 h the seeds were histochemically stained for GUS activity. The percentage of GUS positive seedlings was determined.

Histochemical staining for GUS activity

Seedlings were stained for GUS activity essentially as described by Jefferson *et al.* (1987). The seedlings were incubated in a solution containing 0.3 g/liter 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc); 0.3 mM K ferricyanide; 10 mM Na_2EDTA; 0.1% Sodium Lauryl Sarcosine and 0.1% Triton-X100 in 0.1 M $NaPO_4$ (pH 7.0) for 16 h at 37°C.

Mutagenesis and screening

After selecting three transgenic lines, homozygous T_2 seeds were pooled to obtain enough seeds for the mutagenesis experiment (0.74 g of AIR1-2, 0.33 g of AIR1-8 and 0.13 g of AIR1-11). 110 000 homozygous T_2 seeds (2.2 g) were treated with the

chemical mutagen EMS by Lehle Seeds (Tucson, USA) according to the following protocol. Seeds were surface sterilized by soaking in water for 30 min, followed by 5 min in 95% EtOH and subsequently 5 min in 10% Chlorox bleach. Then the seeds were rinsed 5 times with water. EMS (Sigma) was applied in a 0.2% (v/v) solution for 12 hours. After this treatment the seeds (the M_1 generation) were rinsed with 15 washes of water and planted in 26 flats in the greenhouse using a 0.15% agar transfer solution. The treated seeds were grown to maturity and allowed to set seed. M_2 seeds were harvested in 26 parental groups. The number of M_1 plants per parental group was determined by performing stand counts on three separate locations on each of the 26 flats. In this way it was determined that each parental group represented approximately 641 parent plants. Putative mutants were selected by germination of sterilized M_2 seeds on solidified basal medium supplemented with 25 mg/l kanamycin. Approximately 1000 seeds were sown per 15 cm Petri dish using 3 ml 0.1% agarose as a transfer solution. Ten days after germination kanamycin resistant seedlings were transferred to basal medium without antibiotics and allowed to set seed. The M_3 seeds were germinated on basal medium and histochemically stained for GUS activity 3 and 10 days after germination.

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References

Ballas, N., Wong, L.-M. and Theologis, A. (1993) Identification of the auxin-responsive element, *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). J Mol Biol 233: 580-596.

Boot, K.J.M., van der Zaal, E.J., Velterop J., Quint, A., Mennes, A.M., Hooykaas, P.J.J. and Libbenga, K.R. (1993) Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell-suspension cultures. Plant Physiol 102: 513-520.

Conner, T.W., Goekjian, V.H., LaFayette, P.R. and Key, J.L. (1990) Structure and expression of two auxin-inducible genes from *Arabidopsis*. Plant Mol Biol 15: 623-632.

Droog FNJ, Spek A, van der Kooy A, de Ruyter A, Hoge H, Libbenga KR, Hooykaas PJJ, van der Zaal EJ (1995) Promoter analysis of the auxin-regulated tobacco glutathione *S*-transferase genes *Nt103-1* and *Nt103-35*. Plant Mol Biol *in press*

van den Elzen, P.J.M., Townsend, J., Lee, K.Y. and Bedbrook, J.R. (1985) A chimaeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol Biol 5: 299-302.

Estelle, M.A. and Somerville, C. (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genet 206: 200-206.

Gil, P., Liu, L., Orbovic, V., Verkamp, E., Poff, K.L. and Green, P.J. (1994) Characterization of the auxin-inducible *SAUR-AC1* gene for use as a molecular genetic tool in *Arabidopsis*. Plant Physiol 104: 777-784.

Goddijn, O.J.M., Lindsey, K., van der Lee, F.M., Klap, J.C. and Sijmons, P.C. (1993) Differential gene expression in nematode-induced feeding structures of transgenic plants harbouring promoter-gusA fusion constructs. The Plant Journal 4: 863-873.

Guilfoyle, T.J., Hagen, G., Li, Y., Ulmasov, T., Liu, Z., Strabala, T. and Gee M. (1993) Auxin-regulated transcription. Aust J Plant Physiol 20: 489-502.

Hagen, G., Kleinschmidt, A. and Guilfoyle, T.J. (1984) Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta 162: 147-153.

Hood, E.E., Gelvin, S.B., Melchers, L.S. and Hoekema, A. (1993) New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res 2: 208-218.

Hooykaas, P.J.J., Klapwijk, P.M., Nuti, M.P., Schilperoort, R.A. and Rörsch, A. (1977) Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium ex planta*. Gen Microbiol 98: 477-484.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907.

Jones, A.M. and Prasad, P.V. (1992) Auxin-binding proteins and their possible reles in auxin-mediated plant cell growth. BioEssays 14: 43-47.

Koornneef, M., Dellaert L.W.M. and van der Veen, J.H. (1981) EMS- and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana* (L.) Heynh. : 109-123.

Leyser, H.M.O., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J. and Estelle, M. (1993) *Arabidopsis* auxin-resistance gene *axr1* encodes a protein related to ubiquitin-activating enzyme E1. Nature 364: 161-164.

Liu, X. and Lam, E. (1994) Two binding sites for plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. J of Biol Chem 269: 668-675.

Maher, E.P. and Martindale, S.J.B. (1980) Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. Biochem Genetics 18: 1041-1053.

Marsh, J.L., Erfle, M. and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32: 481-485.

Mattanovich, D., Rüker, F., da Câmara Machado, A., Laimer, M., Regner, F., Steinkellner, H., Himmler, G. and Katinger, H. (1989) Efficient transformation of *Agrobacterium* spp. by electroporation. Nucl Acids Res 17: 6747.

McClure, B.A. and Guilfoyle, T. (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol Biol 9: 611-623.

Meyerowitz, E.M. (1989) *Arabidopsis*, a useful weed. Cell 56: 263-269.

Mirza, J.I. and Maher, E.P. (1987) Physiological characteristics of two auxin-resistant mutants of *Arabidopsis thaliana, aux-2* and *Dwf*. Plant Growth Regul 5: 41-49.

Nagao, R.T., Goekjian, V.H., Hong, J.C. and Key, J.L. (1993) Identification of protein-binding DNA sequences in an auxin-regulated gene of soybean. Plant Mol Biol 21: 1147-1162.

Reddy, A.S.N., Jena, P.K., Mukherjee, S.K. and Poovaiah, B.W. (1990) Molecular cloning of cDNAs for auxin-induced mRNAs and developmental expression of the auxin-inducible genes. Plant Mol Biol 14: 643-653.

Reid JB (1993) Plant hormone mutants. Journal of Plant Growth Regul 12: 207-226

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual. Second edition. Cold Spring Harbour Laboratory Press, Cold Spring Harbor NY.

Susek, R.E., Ausubel, F.M. and Chory, J. (1993) Signal transduction mutants of *Arabidopsis* uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell 74: 787-799.

Takahashi, Y., Kuroda, H., Tanaka, T., Machida, Y., Takebe, I. and Nagata, T. (1989) Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts. Proc Natl Acad Sci *USA* 86: 9279-9283.

Theologis, A., Huynh, T.V. and Avis, R.W. (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J Mol Biol 183: 53-68.

Valvekens, D., van Montagu, M. and van Lijsebettens, M. (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci *USA* 85: 5536-5540.

Walker, J.C. and Key, J.L. (1982) Isolation of cloned cDNAs to auxin- responsive poly(A) $^{+}$RNAs of elongating soybean hypocotyl. Proc Natl Acad Sci *USA* 79: 7185-7189.

Wilson, A.K., Pickett, F.B., Turner, J.C. and Estelle, M. (1990) A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Mol Gen Genet 222: 377-383.

van der Zaal, E.J., Memelink, J., Mennes, A.M., Ouint, A. and Libbenga K.R. (1987) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol 10: 145-157.

van der Zaal, E.J., Droog, F.N.J., Boot, C.J.M., Hensgens, L.A.M. Hoge, J.H.C. Schilperoort, R.A. and Libbenga K.R. (1991) Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998.

Zhang, B. and Singh, K.B. (1994) Ocs element promoter sequences are activated by auxin and salicylic acid in *Arabidopsis*. Proc Natl Acad Sci *USA* 91: 2507-2511.

CHAPTER 6

Characterization of mutants up-regulated for an auxin-inducible promoter

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Summary

In the previous chapter 55 mutants were identified after EMS mutagenesis that showed up-regulation of the auxin-inducible glutathione *S*-transferase *Nt103-1* promoter (*gup* mutants). In this chapter, 10 mutants were selected for further analysis. A construct containing the *Nt103-1* promoter fused to the *β*-glucuronidase *(gusA)* as well as the kanamycin resistance *(nptII)* reporter gene, had been introduced in the parental lines for selection of the mutants. The *gup* phenotype exhibited kanamycin resistance and enhanced levels of GUS activity compared to the parental line. Northern analysis showed that the expression of an endogenous arabidopsis gene, homologous to the *Nt103*-1 gene, was also increased in some of the mutants. Another auxin-inducible gene, *SAUR-AC1*, was not up-regulated. Also the expression of the pathogenesis-related protein gene *PR1* was not altered in the mutants, indicating that a specific group of genes is affected by the mutation. In six of the ten mutants analyzed the phenotype was caused by a single dominant mutation while in two mutants it was caused by a monogenic recessive mutation. In two other mutants the genetic basis of the mutation is not clear yet. The dominant mutation in the *GUP1* gene was mapped to the top of chromosome 1, while the recessive mutation in the *GUP2* gene was mapped to chromosome 2. Most of the mutants showed morphological alterations related to an altered response to auxins. Most prominent were the epinastic leaves and cotyledons found in seven of the *gup* mutants. In the *gup1-1* and *gup2-1* mutants this phenotype was shown to be linked to the mutation. In five mutants the roots had a reduced ability to penetrate the agar. Also this phenotype was shown to be linked to the mutation in one mutant. The response of the mutants to external auxin was not drastically altered. Only the roots of the *gup0-1* mutant were slightly more sensitive to 2,4-

dichlorophenoxyacetic acid as compared to the parent line. Three mutants were slightly more sensitive to abscisic acid than the parent lines.

Introduction

Recently the isolation and characterization of mutants has become increasingly important in the research on the mode of action of plant hormones. Especially since the adoption of the small crucifer *Arabidopsis thaliana* as a model plant, the search for mutants and the isolation of genes, has been intensified. Mutants have been isolated that yield information on different steps in the signal transduction pathway of these substances in plants.

The *Vp1* gene, identified after transposon tagging (McCarty *et al.*, 1991), and the *ABI3* gene, isolated by positional cloning (Giraudat *et al.*, 1992), were found to encode transcriptional activators involved in the response to abscisic acid. Also genes involved in the ethylene signal transduction pathway have been cloned that showed interesting features. The *Etr1* mutant of arabidopsis was isolated after screening for ethylene resistance. The *ETR1* gene shares homology to a class of prokaryotic proteins that are part of a two-components signal transduction system. ETR1 is proposed to be the, or one of the components of the ethylene receptor (Chang *et al.*, 1993). Another interesting gene, cloned after screening for mutants that constitutively exhibited the phenotypes of plants treated with ethylene, was the *CTR1* gene (Kieber *et al.*, 1993). This gene encodes a protein similar to members of the Raf family of serine-threonine protein kinases.

Although interesting mutants are available for the phytohormones abscisic acid, ethylene and gibberellin, the number of signal transduction mutants for the phytohormones auxin and cytokinin is rather low. This is mainly because the isolation of mutants is restricted to the possibility to select for a phenotype. For abscisic acid and gibberellin one can select for phenotypic changes in seed dormancy and water relations (Koornneef *et al.*, 1984) or for dwarf phenotypes (Stoddart, 1987). The "triple response" has proven to be a good phenotype to select for ethylene mutants (Kieber *et al.*, 1993). The phenotype caused by alterations in the action of the hormones auxin and cytokinin is more difficult to describe. These hormones are involved in many developmental processes and alterations in hormone levels result in many pleiotropic effects. The introduction of the auxin and cytokinin biosynthetic genes of *Agrobacterium tumefaciens* into plants has given the possibility to study the phenotypes that are caused by

increased levels of these hormones (Klee *et al.*, 1987, Medford *et al.*, 1989). The results can be used in the search for mutants affected in auxin or cytokinin biosynthesis.

For the phytohormone auxin several interesting auxin resistant mutants have been isolated (Maher & Martindale, 1980, Estelle & Sommerville, 1987, Mirza & Maher, 1987, Wilson *et al.*, 1990). The auxin resistance in these mutants is accompanied by many pleiotropic morphological effects. Many of the mutants are also resistant to other hormones, making it difficult to determine which phenotypic changes are related to auxin. For the *axr1* mutant the gene has been cloned. This *AXR1* gene encodes a protein which shares identity with the E1 enzyme of the ubiquitin activation pathway (Leyser *et al.*, 1993).

We have used a procedure by which mutants can be selected with altered transcription levels for genes that are regulated by auxins (Chapter 5). Here we describe the characterization of mutants up-regulated for the expression of an auxin-inducible glutathione *S*-transferase (*gst*) gene, *Nt103-1*. We designated these mutants *gup* mutants, for **g**st **up**-regulated mutants. The expression of the *Nt103-1* promoter has been studied in tobacco (van der Zaal *et al.*, 1991, Boot *et al.*, 1993, Droog, 1995) and in *Arabidopsis thaliana* (Chapter 4). By using two reporter genes controlled by the *Nt103-1* promoter, we could discriminate between *trans*-acting and *cis*-acting mutations.

In the previous chapter, the construction of the transgenic lines containing the promoter/reporter fusion genes, the mutagenesis and the isolation of the mutants was described. In this chapter we describe the phenotype of some of these mutants, the mRNA expression of other auxin-inducible and pathogen-inducible genes and the response of the mutants to different externally applied hormones. Also the position of two of the mutations on the genomic map was determined.

Results

In chapter 5 we selected 55 *gup* mutants. Ten mutants from nine independent M_2 populations were selected for further analysis.

The seed stock used for mutagenesis was a pool of seeds from three transgenic parent lines (AIR1-2, AIR1-8 and AIR1-11). Based on the specific hybridization pattern after digestion of the DNA with *EcoR*I and subsequent Southern analysis using a probe hybridizing to the β-glucuronidase (*gusA*) gene coding region, we were able to determine the transgenic parent line from which

each mutant originated (results not shown). The mutants *gup1-1*, *gup2-1* and *gup 0-7* originated from the diploid transgenic parent AIR1-8. The mutant *gup0-1* originated from the diploid transgenic parent AIR1-11. The mutants *gup0-2*, *gup0-3*, *gup0-4*, *gup0-5*, *gup0-6* and *gup0-8* originated from the tetraploid transgenic parent AIR1-2.

Genetic analysis

We have begun a genetic characterization of mutants affected in regulation of the *Nt103-1* gene. Since each mutant except *gup0-3* and *gup0-4* originated from independent M_2 populations, at least nine mutants represented independent mutation events. The mutants were crossed to their transgenic parents and the progenies were analysed. The backcrossed F_1 progenies of *gup0-1, gup0-3, gup0-5* and *gup1-1* were kanamycin resistant. The backcrossed F_1 progenies of *gup0-7* and *gup2-1* were sensitive to kanamycin. In the progenies of the tetraploid mutants *gup0-2, gup0-6* and *gup0-8* kanamycin resistant and sensitive seedlings segregated. This indicated that the parent mutants used to generate the F_1 population were not homozygous for the mutation. Backcrosses have not yet been performed with the *gup0-4* mutant. Since the M_3 population of this mutant segregated in a 3:1 manner ($\chi^2 = 3.1$), it was likely that the *gup* phenotype was caused by a dominant mutation in this mutant (data not shown).

The F_2 progenies segregated in a 3:1 (resistant : sensitive) manner for most of the mutants (*gup0-1, gup0-3, gup0-5, gup0-6* and *gup1-1)* indicating that the kanamycin resistance was caused by a dominant mutation of a single gene (Table 1). The F_2 progeny of the *gup0-7* and *gup2-1* mutants segregated in a 1:3 (resistant : sensitive) manner indicating that this mutation was a monogenetic recessive mutation. The progeny of *gup0-2* and *gup0-8* segregated in manner that can not be explained by monogenic mutations. The intermediate phenotype of the F_2 seedlings of *gup0-2* was probably caused by gene dosage effects of the tetraploid. A digenic inheritance could lead to the segregation ratios found for *gup0-8*. Further analysis has to be performed to get a decisive answer on the genetic basis of these mutants.

Table 1. Backcrosses of the mutants.

cross	F_2 population tested on kanamycin			expected	χ^2
	total	resistant	sensitive		
AIR1-11 x *gup0-1*	168	113	51	dominant	3,5
AIR1- 2 x *gup0-2*	191	24 (150 ± [a])	17	dominant	25,6
AIR1- 8 x *gup1-1*	264	200	64	dominant	0.04
AIR1- 2 x *gup0-3*	76	53	23	dominant	1.4
AIR1- 2 x *gup0-5*	240	186	54	dominant	0.8
AIR1- 8 x *gup2-1*	242	70	162	recessive	3.4
AIR1- 2 x *gup0-6*	202	153	49	dominant	0.03
AIR1- 8 x *gup0-7*	207	49	158	recessive	0.13
AIR1- 2 x *gup0-8*	208	100	108	dominant	81.8

In each cross the transgenic parent (AIR1-2, AIR1-8 or AIR1-11) was used as a female parent and the *gup* mutant was used as a male parent. The F_2 plants were analysed for kanamycin resistance. For the dominant mutations we expected a 3:1 segregation (resistant : sensitive). For the recessive mutants we expected a 1:3 segregation. χ^2 values indicate the observed deviation from the expected segregation ratios. A value of <3.8 is acceptable ($p > 0.05$). [a] intermediate kanamycin resistance.

The map position of the gup1-1 and gup2-1 mutations.

The global map position of a dominant and a recessive mutant gene causing enhanced expression in the diploid mutants *gup1-1* and *gup2-1* was determined. To this end the mutants were crossed to the ecotype Landsberg *erecta* (LE). The F_1 and F_2 populations were germinated on medium containing hygromycin, selecting for the pAIR construct. DNA isolated from F_3 populations segregating for kanamycin resistance was used to map the mutations. In order to map the location of the T-DNA insert of the pAIR1 construct, DNA isolated from F_3 populations sensitive to kanamycin was also analysed.

By using the ARMS marker set (Fabri & Schäffner, 1994) and additional CAPS markers (Konieczny & Ausubel, 1993), we were able to map the *gup1-1* mutation to the top of chromosome 1. The mutation turned out to be closely linked to the CAPS marker NCC1. As a control for linkage to this marker we analysed 20 F_2 plants of the LE x *gup1-1* cross that had not been selected for

the mutation for segregation of the marker. Also DNA of 20 F_3 populations of the LE x *gup2-1* cross was analysed.

Table 2. The map position of the gup1-1 and gup2-1 mutations.

		LE x *gup1-1* [a]			LE x *gup2-1* [b]			LE x *gup1-1* [c] + LE x *gup2-1*		
marker	position[d]	NR	R	χ^2	NR	R	χ^2	NR	R	χ^2
m241A	1: 17	24	12		21	19		46	36	
NCC1	**1: 19**	**42**	**2**	**34.6**	29	11	8.1			
m254A	1: 68	23	13		19	21		46	38	
m315A	1: 120	19	17		19	21		44	38	
m246B	2: 11	19	15		25	13		47	31	
m497A	**2: 13**	18	16	0.03	**26**	**12**	**4.4**			
m283C	2: 59	21	15		21	21		43	39	
m560B2	3: 36	22	14		22	18		44	38	
m448A	4: 38	19	17		20	20		43	39	
m326B	4: 76	17	17		23	17		43	37	
d104C	4: 109	20	16		17	23		39	43	
m217C	5: 11	22	14		21	19		45	37	
LYF3	**5: 115**							**58**	**32**	**6.9**
m558A	**5: 125**	24	12		30	10		**57**	**25**	**11.7**

DNA of the F_3 populations of the crosses [a] LE x *gup1-1* and [b] LE x *gup2-1* that segregated for kanamycin resistance was analysed using RFLP and CAPS markers. [c] The data for the F_3 populations of the crosses LE x *gup1-1* and LE x *gup2-1* were combined with the data obtained from kanamycin sensitive F_3 populations of these crosses to determine the map position of the pAIR1 construct. [d] The chromosome and the position of the marker on the chromosome according to Lister and Dean (Arabidopsis news group, 1995) are indicated. The number of non-recombinant (NR) and recombinant (R) alleles are represented for each cross. Linkage is determined using the χ^2 test for 1: 1 segregation: if $\chi^2 < 3.8$ linkage is not proven ($p > 0.05$).

The NCC1 marker turned out to segregate 28 NR : 12R and 29NR : 11R for the LE x *gup1-1* and LE x *gup2-1* populations respectively. Although this was not a 1 : 1 segregation (χ^2 = 6.4 and 8.1) the linkage of the NCC1 marker to the *gup1-1* mutation was obvious because the 42NR : 2R segregation ratio of the F_3 populations exhibiting the mutation was clearly different (χ^2 = 13.2 and 11.2) from the segregation ratios of the populations that had not been selected for the mutation.

The *gup2-1* mutation was linked to the RFLP marker m497A, and thus mapped to the top of chromosome 2. The linkage of the marker m497A was specific for the F_3 populations of the LE x *gup2-1* cross. The F_3 populations of the LE x *gup1-1* cross did not show linkage to this marker (Table 2).

The T-DNA insert of the transgenic parent (AIR1-8) of both mutants was linked to the RFLP marker m558A and to the CAPS marker LYF3. Both markers are located at the bottom of chromosome 5 (Table 2).

Only a limited number of F_2 and F_3 populations were tested in this mapping analysis. More markers and larger F_3 populations have to be tested to determine the position of the mutations before a chromosome walk can be started.

The phenotype of the mutants

The level of kanamycin resistance

During the mutagenesis experiment we selected for mutants with a high level of kanamycin resistance by screening 10 days old seedlings. Whereas the cotyledons of non-resistant seedlings bleached, the cotyledons of mutant seedlings remained green. The putative mutants were transferred to medium without kanamycin. To study the kanamycin resistance in more detail the M_3 seeds of the mutants were germinated on medium containing kanamycin. In contrast to the transgenic parent lines, the mutant seedlings developed into plants that were able to flower on medium containing kanamycin (Figure 3A). Also the kanamycin resistance of the roots was increased. In figure 1 it is shown that the root growth on medium containing kanamycin was increased in all mutants compared to their transgenic parents. We can therefore conclude that the expression level of the *Nt103-1/nptII* fusion gene was up-regulated in the *gup* mutants.

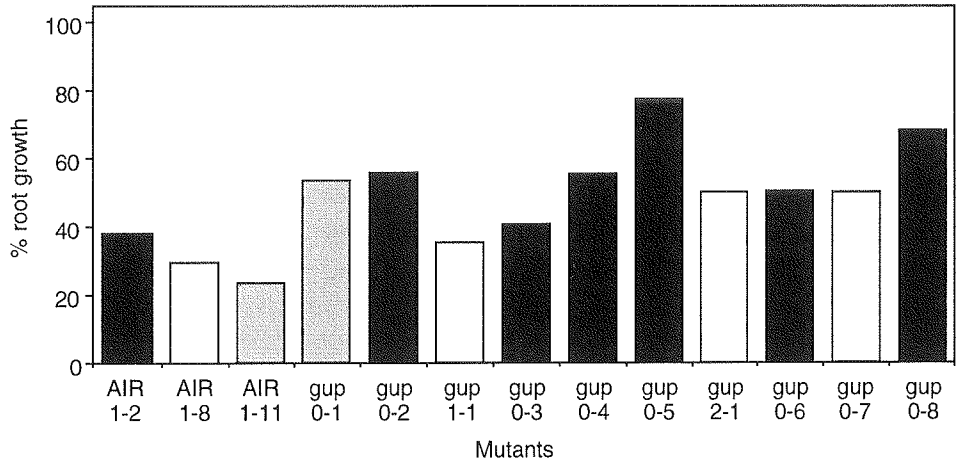


Figure 1. The kanamycin resistance of mutant seedlings. The kanamycin resistance is expressed as the percentage of the root growth on medium with kanamycin as compared to growth without kanamycin. The filling of the bars corresponds to the transgenic wild-type parent line from which the mutant originates. The root length on basal medium without kanamycin was identical in the mutants and transgenic parents.

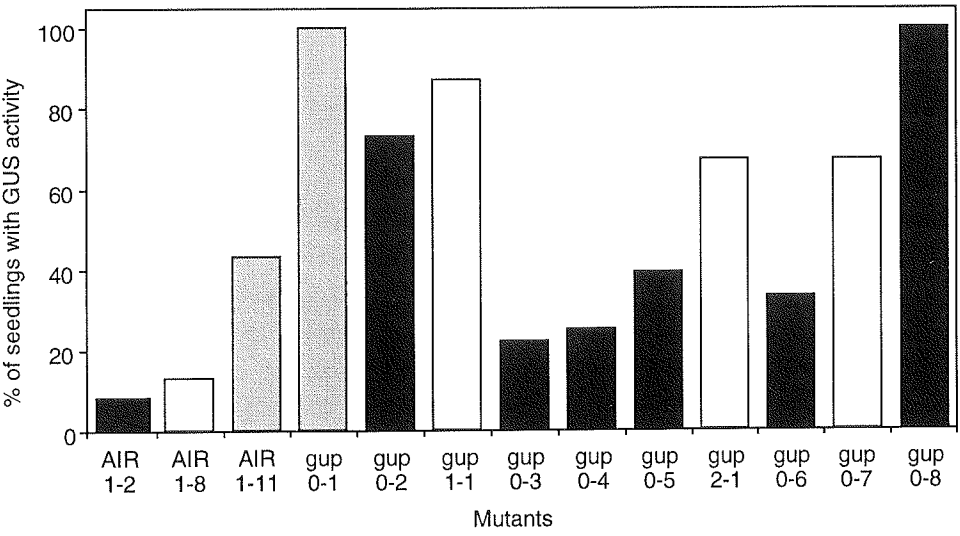


Figure 2. The % of seedlings with GUS activity on basal medium. Per mutant 4 to 60 seedlings were tested by histochemical staining. The filling of the bars corresponds to the transgenic wild-type parent line from which the mutants originate.

The level of GUS activity

The expression of the *Nt103-1/gusA* fusion gene in the transgenic parent seedlings was very low when the seedlings were grown on basal medium. After induction by auxin, GUS activity could be detected in the root tip, the vascular tissue of the root and the transition zone (Chapter 4 and 5).

In the *gup* mutants the level of GUS activity was considerably higher, when the seedlings were grown on basal medium (Figure 2). GUS activity was detected in the root tip, the transition zone and sometimes in the cotyledons of the seedlings. In older plants the GUS activity was detected mainly in the root tips and/or in the vascular tissue of the lateral roots (Figure 3B). Sometimes GUS activity was also detected in the cotyledons and leaves. GUS expression could still be further induced by auxin as well as by salicylic acid. The concentration needed for increased expression of GUS activity in the mutants was identical to the concentration needed to induce the transgenic parents (data not shown).

Morphological alterations

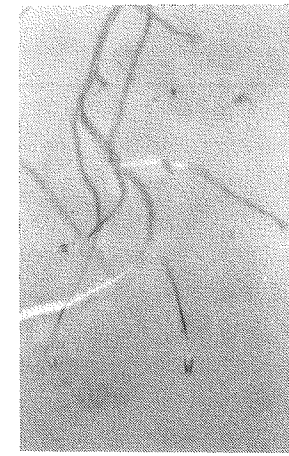
From work on other auxin-related mutants and the overexpression of T-DNA genes in transgenic plants, some phenotypes related to an altered response to auxin are known. We were interested to see whether the *gup* mutations, next to the up-regulation of the *Nt103-1* reporter gene fusions, also caused morphological alterations. In seven out of ten mutants the cotyledons and leaves curled down and were smaller compared to the wild-type (*gup1-1, gup2-1, gup0-1, gup0-2, gup0-4, gup0-7, gup0-8*). The epinastic growth was also found in the kanamycin resistant F_2 progeny of backcrosses of the mutants *gup1-1* and *gup2-1* with their transgenic parent plants or with the Landsberg *erecta* ecotype. Forty-six backcrossed F_3 populations of *gup1-1* and 34 backcrossed F_3 populations of *gup2-1* were studied. If the mutation causing the epinastic leaves was not linked to the *gup* mutations 25% of the F_3 populations should not have this phenotype. Since all F_3 populations showed epinasty, we concluded that this phenotype was linked to the *gup* mutation ($\chi^2 = 14$).

Of the ten investigated mutants the roots of five mutants had a reduced ability to penetrate the agar (*gup0-2, gup0-3, gup0-4, gup0-5* and *gup0-6*). The gravitropic response however, was not altered in these mutants (data not shown). The roots of seedlings of 20 F_3 populations of a backcross between *gup0-6* and its parental line that showed kanamycin resistance also showed a reduced ability to penetrate the agar. This indicated that this phenotype was also linked to the *gup* mutation in this mutant ($\chi^2 = 5.4$).

A)

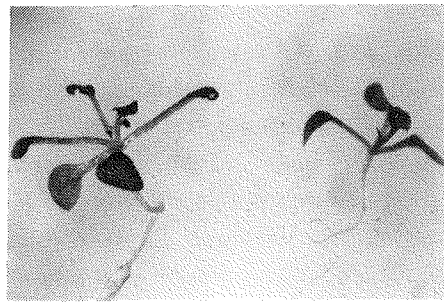
gup0-1 AIR1-11

B)



gup1-1

C)



gup1-1 AIR1-8

Figure 3. Expression of the *Nt103/reporter* gene fusion and morphological alterations.
A) Kanamycin resistance in the mutant *gup0-1* and in the its parent line AIR1-11. B) GUS activity in the roots of the *gup1-1* mutant C) epinasty in cotyledons and leaves of the mutant *gup1-1* and its transgenic parent AIR1-8

The roots of the *gup1-1* and *gup2-1* mutants were highly branched. Some of the seedlings of the latter mutant had a short callused root. Seedlings of *gup0-6* sometimes showed reduced apical dominance. The seedlings formed a callus with many rosettes and did not produce an inflorescence. After studying the F_3 populations of these mutants we found that these phenotypes were not linked to the *gup* mutations. In none of the mutants root hair formation was disrupted.

The expression of the *At103-1a* gene is enhanced in some mutants

Since the tobacco auxin-inducible genes remained auxin-inducible in arabidopsis we expected that also certain arabidopsis genes would be inducible by auxin via the same signal transduction pathway. Homologs of the *Nt103* gene had been isolated previously from arabidopsis (Chapter 2), and we studied whether expression of one of these homologs *At103-1a* , which we found to be auxin-inducible (Chapter 2), was affected in the mutants. Therefore, the steady state mRNA level of the *At103-1a* gene in the roots of the mutants was determined.

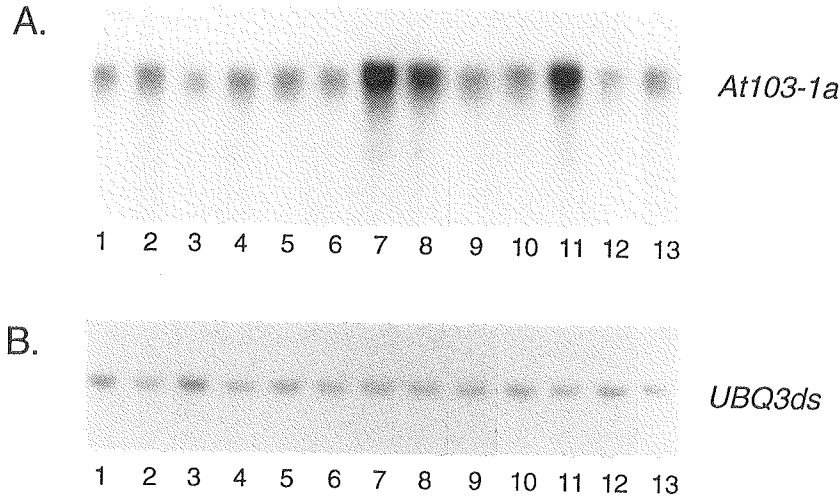


Figure 4. Level of mRNA in roots of non-induced seedlings of transgenic wild-types AIR1-2 (lane 1), AIR1-8 (lane 2) and AIR1-11 (lane 3) and *gup* mutants: *gup0-1* (lane 4), *gup0-2* (lane 5), *gup0-3* (lane 6), *gup1-1* (lane 7), *gup0-4* (lane 8), *gup0-5* (lane 9), *gup2-1* (lane 10), *gup0-6* (lane 11), *gup0-7* (lane 12), *gup0-8* (lane 13).
A) mRNA hybridizing to pSK103 (*At103-1a*)
B) mRNA hybridizing to pUBQ3ds (constitutive probe)

Figure 4 shows that the mRNA level corresponding to the *At103-1a* gene was increased in the roots of *gup1-1*, *gup0-4* and *gup0-6* compared to the mRNA level of the transgenic parents. As a control for the amount of RNA on the blot the expression of a constitutively expressed ubiquitine gene was measured as well. To test if the up-regulation was general for auxin-inducible genes we determined the steady state mRNA level of the auxin-inducible *SAUR-AC1* gene in elongating etiolated seedlings of the mutants as well. The SAUR mRNA level in the mutants however was not different from the mRNA level in the parents

(results not shown).

Because the *Nt103-1* gene could be induced not only by auxins but also by salicylic acid, we tested if the steady state mRNA level of the *PR1* gene was increased in the mutants. The mRNA level was however not altered in the mutants (results not shown).

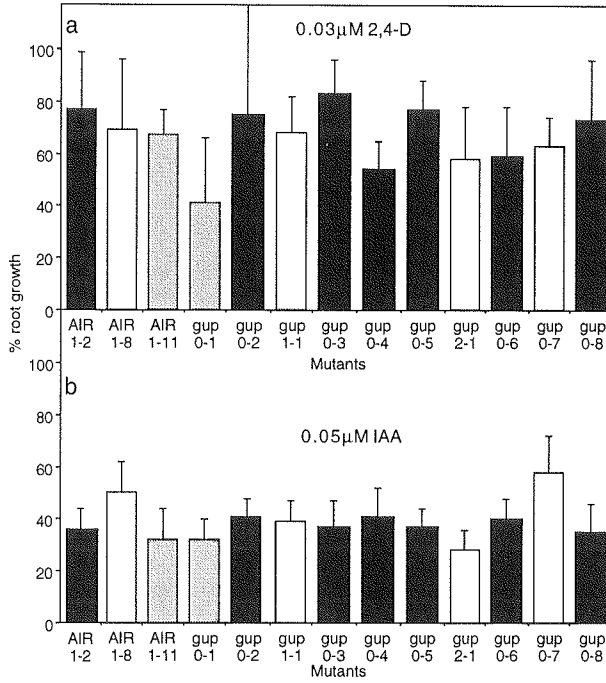


Figure 5. Root growth of the *gup* mutants on medium containing A) 0.03 µM 2,4-D or B) 0.05 µM IAA. The root growth is expressed as the percentage of root growth on medium with auxin as compared to growth on medium without auxin. There was no difference in root growth between the transgenic parents (AIR1-2, AIR1-8 and AIR1-11) and the mutants (*gup0-1, gup0-2, gup1-1, gup0-3, gup0-4, gup0-5, gup2-1, gup0-6, gup0-7* and *gup0-8*) on basal medium without auxins. The filling of the bars corresponds to the transgenic wild-type parent line from which the mutants originate.

Response to hormones

Response to auxin

The influence of the *gup* mutations on the response to different hormones was

determined by looking at root growth. The inhibition of root growth by auxin was tested on 0.03 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 μM indole-3-acetic acid (IAA). In none of the mutants the response to external 2,4-D or IAA was altered significantly (Figure 5). Only the roots of *gup0-1* seemed somewhat more sensitive to 2,4-D than the transgenic parent. Although the differences between the transgenic parent and *gup0-1* were small they were found in 4 independent experiments.

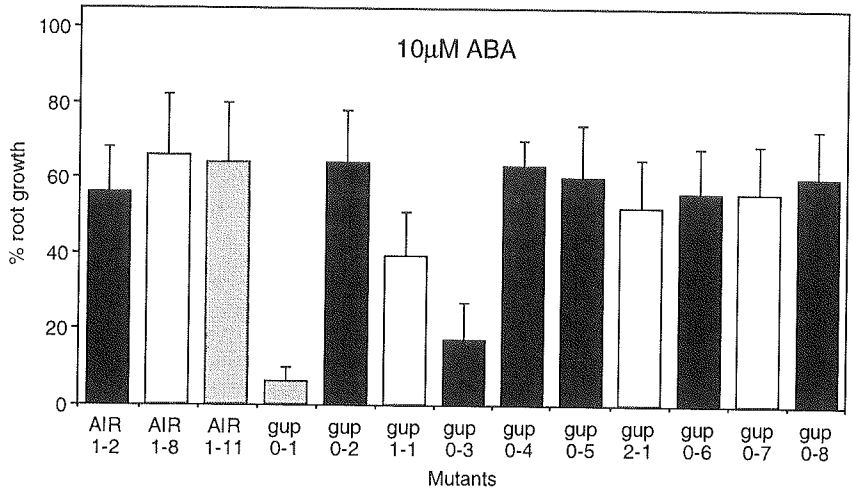


Figure 6. Root growth of the *gup* mutants on medium containing 10 μM ABA. The root growth is expressed as the percentage of root growth on medium with ABA as compared to growth on medium without ABA. The filling of the bars corresponds to the transgenic wild-type parent line from which the mutants originate. The root growth of the transgenic parents (AIR1-2, AIR1-8 and AIR1-11) was not different from the root growth of the mutants (*gup0-1, gup0-2, gup1-1, gup0-3, gup0-4, gup0-5, gup2-1, gup0-6, gup0-7* and *gup0-8*) on medium without ABA.

Response to other hormones

Because some of the auxin resistant mutants described in literature also show resistance to other hormones, we tested the response of the mutants to abscisic acid and the cytokinin benzylaminopurine (BAP). The *gup0-1* and *gup0-3* mutants showed a drastic inhibition of root growth by ABA compared to the parental lines (Figure 6). The inhibition of root growth of *gup1-1* was less drastic but was also found in repeated experiments. The response of the roots of the mutants to the cytokinin was not significantly different from the response of the transgenic parents to this hormone (results not shown).

Discussion

Out of 55 mutants up-regulated for the *Nt103-1* promoter/reporter gene fusions, 10 were studied in more detail. Next to enhanced levels of kanamycin resistance and GUS activity in the root, also the shoots of the mutants were kanamycin resistant and sometimes expressed GUS activity, the *gup* phenotype.
In some mutants the steady state mRNA level of an endogenous auxin-inducible *gst* gene was up-regulated as well. Also morphological alterations related to altered auxin responses were present in a number of mutants. The response to the phytohormones auxin and abscisic acid were found to be altered in some of the mutants. However, there was no obvious correlation between the increased expression level of the endogenous arabidopsis gene, the morphological alterations and the sensitivity towards hormones (Table 3).

Table 3. Characteristics of the *gup* mutants.

mutant	morphological alterations	hormone [a] response	At103-1a [b] up-regulated
gup1-1	epinasty	sensitive to ABA	yes
gup2-1	epinasty	no	no
gup0-1	epinasty	sensitive to 2,4-D and ABA	no
gup0-2	epinasty, root	no	no
gup0-3	root	sensitive to ABA	no
gup0-4	epinasty, root	no	yes
gup0-5	root	no	no
gup0-6	root	no	yes
gup0-7	epinasty	nd	no
gup0-8	epinasty	no	no

Morphological alterations are indicated by: no = when the mutants did not have an altered morphology; root = reduced ability to penetrate the agar; epinasty = as is indicated in the text. [a] The response to auxin was tested on a concentration of 0.03 μM 2,4-D. The response to abscisic acid was tested on 10 μM ABA. [b] The level of steady state mRNA of *At103-1* was measured in the roots as indicated in the text.

Morphological alterations of the *gup* mutants are related to auxin phenotypes

The morphological alterations caused by the *gup* mutations could be divided into two classes. Seven out of ten mutants showed epinastic leaves and cotyledons. In five out of ten mutants the roots had a reduced ability to penetrate the agar. Epinasty was reported earlier in correlation with auxin. Transgenic plants expressing both the *iaaH* and *iaaM* genes or the *iaaM* gene alone had epinastic leaves (Klee *et al.*, 1987, Sitbon *et al.*, 1992, Romano *et al.*, 1993). Also in auxin response mutants epinasty was reported. Auxin sensitive mutants of *Nicotiana plumbaginifolia* had mildly epinastic leaves (DeSouza & King, 1991). The morphological alterations of most of the auxin resistant mutants reported earlier were quite drastic compared to the morphological alteration of the *gup* mutants (Maher & Martindale, 1980, Estelle & Somerville, 1987, Mirza & Maher, 1987, Wilson *et al.*, 1990, Hobbie & Estelle, 1994). These mutants however were resistant to high concentrations of auxin, while the auxin sensitivity of the *gup* mutants was not or only slightly altered.

Root growth of the *gup0-1*, *gup0-3* and *gup1-1* mutants were more sensitive to abscisic acid. The *Nt103-1* gene is inducible by high concentrations of abscisic acid in tobacco (Droog, 1995). Also in arabidopsis roots the *Nt103-1* promoter could be induced by high concentrations of abscisic acid (unpublished results). It was thus possible that mutants with an altered sensitivity to abscisic acid could be isolated in our screen.

The nature of the *gup* mutations

As demonstrated by Northern analysis showing the steady state mRNA levels of the *At103-1a* gene, the *SAUR-AC1* gene and the *PR1* gene, only a specific set of genes was affected by the *gup* mutation. These results indicate that the *GUP* genes are important for the expression of specific genes.

By analysis of the promoters of different plant genes, multiple elements have been identified that are responsive to auxin (McClure *et al.*, 1989, Ballas *et al.*, 1993, Liu *et al.*, 1994). The variety of auxin-responsive elements suggests that different signal transduction pathways exist for various auxin-inducible genes.

In the promoter region of the tobacco *Nt103-1* gene, a 20 bp element has been identified as a functionally active auxin-responsive element (Droog *et al.*, 1995, Droog, 1995). This element, *as103-1*, has been capable of activating transcription and is responsive to exogenous auxin. Also in other promoters an *as* element has been found to be important for auxin inducibility (Kim *et al.*,

1994, Liu & Lam, 1994, Zhang & Singh, 1994). In three of the ten mutants tested the level of mRNA of the endogenous *At103-1* gene was increased next to the up-regulation of the *Nt103-1* promoter. In the promoter of the *At103-1* gene an *as-1* like element was present as well (Chapter 2).
Genes are thought to be regulated by proteins binding to the sequence elements in their promoter regions. In tobacco, *as-1* elements can bind the nuclear binding factor ASF-1 and the transcription factor TGA1a (Katagiri *et al.*, 1989). In arabidopsis genes coding for proteins homologous to TGA1a have been isolated. These binding proteins, aHBP1b (Kawata *et al.*, 1992), TGA1 (Schindler *et al.*, 1992), OBF4 and OBF5 (Zhang *et al.*, 1993) and TGA3 (Miao *et al.*, 1994), belong to a group of plant bZIP proteins binding to *as-1* like sequences. Also other proteins like OBF1 (Singh *et al.*, 1990) and CPRF2 (Weisshaar *et al.*, 1991, Armstrong *et al.*, 1992) are able to bind to *as-1* like elements.

The transcription factor VP1 is required for ABA-inducible gene expression (McCarty *et al.*, 1991). Overexpression of this transcription factor results in a 5-fold increase of the expression of an ABA-inducible promoter construct in maize protoplasts (Rock & Quatrano, 1995). In analogy the expression of the *Nt/At103-1* genes might be enhanced by an increased level of the transcription factor binding to the *as-1* element resulting in the *GUP* phenotype.

While in the transgenic parents the expression of both *Nt103-1* promoter/reporter gene fusions was restricted to the roots, in the mutants expression could be detected in cotyledons and leaves. Neuhaus *et al.* (1994) showed that the undetectable expression of an *as* linked transgene in cotyledon cells was most likely the result of its inability to compete for a limiting amount of its cognate transcription factor(s). By increasing the level of TGA1 in the cotyledon cells the expression of the *as-1* linked gene could be increased. The expression of the fusion genes in cotyledons and leaves could be explained if the *GUP* genes increased the level of expression of the transcription factor in the plants. The *gup* phenotype might also be caused by a conformational change of a transcription factor leading to enhanced binding to the *as-1* element. By studying the expression of genes encoding transcription factors binding to the *as-1* elements in the mutants, the nature of the mutations might be revealed.

Alternatively a change in conformation of the transcription factor could lead to an increased binding to promoter elements, leading to an increased level of transcription of the auxin-inducible genes. Mutations of this nature are gain of function mutations and usually dominant. Most of the *gup* mutations are dominant mutations. Only two out of ten mutants are recessive. The M_1 population existed for 60% of the tetraploid AIR1-2 transgenic wild-type. In a

segregating tetraploid M_2 population the chance to find a recessive mutant is four times lower than in a diploid M_2 population. Indeed all tetraploid mutants are dominant mutations. Of the remaining four diploid mutants two mutations are recessive. These recessive mutations could be caused by a mutation in a repressor protein, inhibiting the expression of the *Nt103-1* promoter directly or inhibiting the production or activation of the transcription factor binding to the auxin responsive element of the *Nt103-1* promoter.

Isolation of the *GUP* genes

One way to elucidate the nature of the *gup* mutants might be the isolation of the *GUP* genes by positional cloning. By analysis of RFLP and CAPS markers two *GUP* genes have been assigned to different chromosomes. Based on their phenotype and map position, none of the mutants was reported earlier.

After identification of closely linked markers, Yeast Artificial Chromosomes (YACs) can be identified containing the *GUP* genes. By screening a cDNA library with the YACs, genes present on the YACs can be isolated. Subsequent overexpression of one of these genes in arabidopsis might possibly reveal the mutant phenotype. The mutant phenotype should be easily selectable in the transgenic parent lines of arabidopsis. After stable transformation of the transgenic parent with a vector overexpressing the genes, we can select for kanamycin resistance. Instead of using stable transformation we can also use a transient system to isolate the *GUP* genes. By particle gun bombardment, the putative *GUP* genes could be introduced in the transgenic parent. By screening for GUS activity, up-regulation of the *Nt103-1* caused by a *GUP* gene should be visible. The last method is preferable when the YAC turns out to contain a lot of genes. Stable transformation should then confirm the results obtained with the particle gun bombardments.

Since positional cloning of genes still is rather time consuming, isolation of the mutants by T-DNA insertional mutagenesis could be an alternative. By transforming arabidopsis transgenic parents with an activator T-DNA construct (van der Graaff, unpublished) and subsequent selection for kanamycin resistance, we should be able to isolate *gup* mutants via insertional mutagenesis.

The use of promoter/reporter fusion genes to generate mutants has proven to be successful in the isolation of mutants altered in the signal transduction pathway. By isolating the *gup* mutants we have obtained new tools in the research on auxin signal transduction. Characterization of the mutants will also give new

information on regulation of the *gst* genes.

Material and Methods

Plant material and growth conditions

All mutants described in this chapter were derived from *Arabidopsis thaliana* ecotype Columbia (*gl1*). For growing sterile plants, seeds were sterilized for 30 seconds in 70% EtOH, followed by a 20 min. incubation in 1% sodiumhypochloride with 0.15% Tween 20. Sterilized seeds were incubated for 4 days at 4°C to synchronize the germination. Plants were grown at 21°C in a 16 h photoperiod. Unless stated otherwise plants were grown on basal medium (Chapter 4). When necessary, the medium was supplemented with hormones or the antibiotics hygromycin (10 mg/l) or kanamycin (25 mg/l), after autoclaving. Non-sterile plants were grown in soil at 23°C in a 16 h photoperiod.

Bacterial strains

Escherichia coli strain DH5α (Clonetech) was used for bacterial cloning. Strains were grown at 37°C in LC medium (Hooykaas *et al.*, 1977) supplemented with 100 mg/l carbenicillin or 25 mg/l kanamycin.

Kanamycin resistance assay

Petri dishes were placed in vertical position (60°)to facilitate measurement of the root length. Seeds were germinated on medium with or without 25 mg/l kanamycin. After 14 days the root length was measured and the kanamycin resistance was calculated as a percentage of the growth on medium without kanamycin.

GUS histochemical assay

Histochemical analysis of seedlings for GUS activity was performed as described by Jefferson *et al.* (1987). Tissues were incubated in a solution containing 0.3 g/liter 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc); 0.3 mM K ferricyanide; 10 mM Na_2EDTA; 0.1% Sodium Lauryl Sarcosine and 0.1% Triton-X100 in 0.1 M $NaPO_4$ pH 7.0 for 16 h at 37°C.

Determination of the ploidy level

The ploidy level of the transgenic plants was determined by counting the number of chloroplasts in the guard cells of the stomata. The epidermis of the bottom part of the leaf was stripped. The chloroplasts in the guard cells were visualized using a fluorescence microscope. Between 6 to 8 chloroplasts were present in the guard cells of the stomata of diploid plants. In tetraploid plants 10 to 14 chloroplasts were counted per stomata. Per plant 20 stomata were counted to determine the ploidy level of the

plant.

Hormone response assays

Sterilized seeds were distributed on the surface of basal medium. Petri dishes were placed in a vertical position (60°). After 5 days the seedlings were transferred to basal medium and to medium containing different hormones. The end of the root tip was marked on the back of the Petri dish. After 6 days the root growth was measured. The root growth on medium containing hormones was calculated as a percentage of the root growth on basal medium. Per treatment 20-30 seedlings were measured. The experiments were repeated at least 3 times.

RNA isolation and northern blot analysis

The isolation of total RNA from different tissues was performed according to van Slogteren *et al.* (1983). RNA was isolated from roots of 14 days old sterile seedlings incubated on a rotary shaker (100 rpm) in 250 ml flasks containing 50 ml Gamborg B5 medium (Gamborg *et al.*, 1968). Induction by different compounds was performed by addition of the compound to the medium followed by a 15 h incubation period. RNA was also isolated from etiolated seedlings grown as described by Gil *et al.* (1994). The aerial parts of the seedlings were frozen in liquid nitrogen immediately or incubated in 50 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 1 mM salicylic acid (SA) for 1 h at 21°C and then frozen in liquid nitrogen.

25 μg glyoxylated RNA was electrophoresed in 1.5% agarose gels according to van Slogteren *et al.* (1983) and transferred to Genescreen (Dupont) by capillary blotting with 50 mM sodiumphosphate, 5 mM EDTA pH 6.5. Blots were baked at 80°C for 2 h and subsequently prehybridized at 42°C in 50% deionised formamide, 5x SSPE (1x SSPE = 180 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate pH 6.5), 5% SDS and hybridized o/n at 42°C in the same solution with different probes.

Probes were labelled with α-^{32}P-dCTP by the random priming method (Feinberg & Vogelstein, 1983). Blots were washed at 65°C in 0.5x SSPE, 0.5% SDS at 60°C and analysed using a phosphorimager (Molecular Dynamics). Blots were subsequently exposed at -80°C to Fuji-RX films using KMC x-ray intensifying screens.

PCR amplification of the *PR-1* gene

Using the sequence of the *PR-1* gene of *Arabidopsis thaliana* (Uknes *et al.*, 1992), we designed primers to amplify the *PR-1* coding sequence using PCR. AtPR-1: 5'-TCCGTCGACAGCTCAAGATAGCC-3' and AtPR-2: 5'-TCCCTCGAGGATCATAGTTGC-3' (Isogen bioscience). 50 ng genomic DNA of *Arabidopsis thaliana* was used in a reaction with 25 pmol of each primer, 100 μM of each dNTP, 0.1 units SuperTaq (HT biotechnology) and 5 μl 10x SuperTaq reaction buffer in a 50 μl reaction mixture. The reactions were overlaid with 50 μl mineral oil. The amplification was started with one

cycle of 2 min. at 95°C, 1 min. at 58°C and 2 min. at 72°C. This was followed by 35 cycles of 1 min. at 95°C, 1 min. at 58°C and 2 min. at 72°C and completed with one cycle of 1 min. at 95°C, 1 min. at 58°C and 10 min. at 72°C. Samples of 20 μl were analysed on a 1.0% agarose gel. Fragments were isolated from gel using JETpure (Genomed) according to the manufacturers instructions. The fragments were ligated into a pBlueScript SK$^+$ vector (Stratagene) using the restriction sites incorporated in the primers. The sequence of the amplified fragment was checked using the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase version 2.0 (United States Biochemicals).

DNA isolation and RFLP mapping

The chromosomal location of the mutated genes was determined by measuring the recombination frequency between the mutant gene and RFLP markers of the ARMS marker set (Fabri & Schäffner, 1994) or CAPS markers (Konieczny & Ausubel, 1993). Homozygous mutant plants were crossed to a wild-type plant of the Landsberg *erecta* ecotype (La-0). The resulting hygromycin resistant F_1 plants were selfed to generate F_2 plants segregating for the pAIR construct, the mutation and the markers. Hygromycin resistant F_2 plants were selfed to establish F_3 lines. DNA was isolated from F_3 lines segregating for kanamycin resistance.

Total DNA was isolated from leaves according to the method of Rogers & Bendich (1988). Approximately 5 to 10 μg of DNA was digested with *EcoR*I and subjected to o/n gel electrophoresis through a 0.6% agarose gel in 1x TAE (1x TAE= 0.04 M tris-acetate, 0.01 M EDTA). DNA was alkali blotted to Hybond N$^+$ (Amersham) according to the manufacturer's instructions. α-^{32}P-dCTP labelled probes were generated using a down scaled random priming method (Feinberg & Vogelstein, 1983). Blots were washed at 65°C in 0.5x SSPE, 0.5% SDS and subsequently exposed at -80°C to Fuji-RX films using KMC x-ray intensifying screens.

When the PCR based CAPS markers were used for mapping, an amount of 50 ng of DNA was used and amplified as described for the *PR-1* gene with some modifications. The annealing temperature was 56°C and 50 cycles were run. 10 μl of the PCR mixture was digested and analysed on gel according to Konieczny & Ausubel (1993).

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Literature

Armstrong GA, Weisshaar B, Hahlbrock K (1992) Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with ACGT cores. Plant Cell 4: 525-537

Ballas N, Wong L-M, Theologis A (1993) Identification of the auxin-responsive element *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA45*, of pea (*Pisum sativum*). J Mol Biol 233: 580-596

Boot KJM, Zaal van der BJ, J Velterop, A Quint, AM Mennes, PJJ Hooykaas, KR Libbenga (1993) Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell-suspension cultures. Plant Physiol 102: 513-520

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) *Arabidopsis* ethylene-response gene ETR1: Similarity of product to two-component regulators. Science 262: 539-544

DeSouza L, King PJ (1991) Mutants of *Nicotiana plumbaginifolia* with increased sensitivity to auxin. Mol Gen Genet 231: 65-75

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD Thesis, Leiden University, 165 pp

Droog FNJ, Spek A, van der Kooy A, de Ruyter A, Hoge H, Libbenga KR, Hooykaas PJJ, van der Zaal EJ (1995) Promoter analysis of the auxin-regulated tobacco glutathione *S*-transferase genes *Nt103-1* and *Nt103-35*. Plant Mol Biol *in press*

Estelle MA, Somerville C (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genet 206: 200-206

Fabri CO, Schäffner AR (1994) An *Arabidopsis thaliana* RFLP mapping set to localize mutations to chromosomal regions. The Plant Journal 5: 149-156

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158

Gil P, Y Liu, V Orbovic, E Verkamp, KL Poff, PJ Green (1994) Characterization of the auxin-inducible SAUR-AC1 gene for use as a molecular genetic tool in Arabidopsis. Plant Physiol 104: 777-784

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the

Arabidopsis ABI3 gene by positional cloning. The Plant Cell 4: 1251-1261

Hobbie L, Estelle M (1994) Genetic approaches to auxin action. Plant, Cell and Environment 17: 525-540

Hooykaas PJJ, Klapwijk PM, Nuti MP, Schilperoort RA, Rörsch A (1977) Transfer of the *Agrobacterium tumefaciens* Ti-plasmid to avirulent agrobacteria and to *Rhizobium ex* planta. Gen Microbiol 98: 477-484

Jefferson RA, TA Kavanagh, M Bevan (1987) GUS fusions β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907

Katagiri F, Lam E, Chua N-H (1989) Two tobacco DNA-binding proteins with homology to nuclear factor CREB. Nature 340: 727-730

Kawata T, Imada T, Shiraishi H, Okada K, Shimura Y, Iwabuchi M (1992) A cDNA clone encoding HBP-1b homologue in *Arabidopsis thaliana*. Nucl Acids Res 5: 1141

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) *CTR1*, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the RAF family of protein kinases. Cell 72: 427-441

Kim Y, Buckley K, Costa MA, An G (1994) A 20 nucleotide upstream element is essential for the nopaline synthase (nos) promoter activity. Plant Mol Biol 24: 105-117

Klee HJ, Horsch RB, Hinchee MA, Hein MB, Hoffmann NL (1987) The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Genes and Development 1: 86-96

Konieczny A, Ausubel FM (1993) A procedure for mapping arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. The Plant Journal 4: 403-410

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol Plant 61: 377-383

Leyser HMO, Lincoln CA, Timpte C, Lammer D, Turner J, Estelle M (1993) arabidopsis auxin-resistance gene *axr1* encodes a protein related to ubiquitin-activating enzyme E1. Nature 364: 161-164

Liu X, E Lam (1994) Two binding sites for plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. J Biol Chem 269: 668-675

Liu Z-B, Ulmasov T, Shi X, Hagen G, Guifoyle TJ (1994) Soybean *GH3* promoter contains multiple auxin-inducible elements. The Plant Cell 6: 645-657

Maher EP, Martindale SJB (1980) Mutants of arabidopsis with altered responses to auxins and gravity. Biochem Genet 18: 1041-1053

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell 66: 895-905

McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ (1989) Transcription, organisation and sequence of an auxin-regulated gene cluster in soybean. The Plant Cell 1: 229-239

Medford JI, Horgan R, El-Sawi Z, Klee HJ (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric *isopentenyl transferase* gene. The Plant Cell 4: 403-413

Miao Z-H, Liu X, Lam E (1994) TGA3 is a distinct member of the TGA family of bZIP transcription factors in *Arabidopsis thaliana*. Plant Mol Biol 25: 1-11

Mirza JI, Maher EP (1987) Physiological characteristics of two auxin-resistant mutants of *Arabidopsis thaliana*, *aux-2* and *Dwf*. Plant Growth Regulation 5: 41-49

Neuhaus G, Neuhaus-Url G, Katagiri F, Seipel K and Chua N-H (1994) Tissue-specific expression of *as-1* in transgenic tobacco. The Plant Cell 6: 827-834

Rogers SO, AJ Bendich (1988) Extraction of DNA from plant tissues. Plant Mol Biol manual A6: 1-10

Rock CD, Quatrano RS (1995) The role of hormones during seed development. In: Davies PJ (ed) Plant Hormones. KLuwer Academic Press pp 671-697

Romano CP, Cooper ML, Klee HJ (1993) Uncoupling auxin and ethylene effects in transgenic tobacco and *Arabidopsis* plants. The Plant Cell 5: 181-189

Sanger F, Nickler S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci *USA* 74: 5463-5467

Schindler U, Beckman H, Cashmore AR (1992) TGA1 and G-box binding factors: two distict classes of *Arabidopsis* leucine zipper proteins compete for the G-box-like element TGACGTGG. The Plant Cell 4: 1309-1319

Singh K, Dennis ES, Ellis JG, Llewellyn DJ, Tokushisa JG, Wahleithner JA, Peacock WJ (1990) OCSBF-1, a maize Ocs enhancer binding factor: isolation and expression during development. The Plant Cell 2: 891-903

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. Plant Physiol 99: 1062-1069

Slogteren van GMS, Hoge JHC, Hooykaas PJJ, Schilperoort RA (1983) Clonal analysis of heterogenous crown gall tumour tissues induced by wild-type and shooter mutant strains of *Agrobacterium tumefaciens* - expression of T-DNA genes. Plant Mol Biol 2: 321-333

Stoddart JL (1987) Genetic and hormonal regulation of stature. In: Thomas H, Grierson D (ed) Developmental mutants in higher plants. Cambridge Univ. Press, Cambridge, pp 155-180

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in arabidopsis. The Plant Cell 4: 645-656

Weisshaar B, Armstrong GA, Block A, Silva OdCe, Hahlbrock K (1991) Light inducible and constituvily expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J 10: 1777-1786

Wilson AK, Pickett FB, Turner JC, Estelle M (1990) A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Mol Gen Genet 222: 377-383

Yamazaki K-I, Katagiri F, Imaseki H, Chua N-H (1990) TGA1a, a tobacco DNA-binding protein, increases the rate of initiation in a plant in vitro transcription system. Proc Natl Acad Sci *USA* 87: 7035-7039

Zaal van der EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998

Zhang B, Foley RC, Singh KB (1993) Isolation and characterization of two related arabidopsis ocs- element bZIP binding proteins. The Plant J 4: 711-716

Zhang B, KB Singh (1994) ocs element promoter sequences are activated by auxin and salicylic acid in arabidopsis. Proc Natl Acad Sci *USA* 91: 2507-2511

CHAPTER 7

Summary and discussion

Introduction

Over the years mutants have proven to be valuable in research on hormone perception and signal transduction. With the adoption of the small crucifer *Arabidopsis thaliana* as the model species for plants, such mutants have become even more important in this research area. A number of different types of hormone mutants have been isolated and for some the mutated genes have been cloned providing insight into the molecular basis of the affected process. Hormone mutants could sometimes be easily isolated because of a specific phenotype. For instance abscisic acid mutants exhibit phenotypic changes in seed dormancy and water relations (Koornneef *et al.*, 1984). Gibberellin mutants have an altered plant stature (Phinney *et al.*, 1986), and ethylene mutants can be isolated selecting for an altered "triple response" (Kieber *et al.*, 1993). The development of molecular genetic tools for plants made it possible to devise alternative strategies for mutant isolation, not relying on selection for a phenotype. For instance, screening for mutants having an altered expression of genes known to be regulated by a hormone, was facilitated by the use of reporter genes. We have used this type of strategy to isolate mutants affected in auxin signal transduction.

In the past few years a number of different auxin-inducible genes have been isolated (reviewed by Hagen, 1995). After differential screening of a cDNA library constructed from RNA isolated from auxin-starved tobacco cell-suspension cells which were treated for four hours with 2,4-dichlorophenoxyacetic acid (2,4-D), our laboratory isolated genes belonging to a multigene family of auxin-inducible genes. The mRNA corresponding to these genes was induced within 30 minutes after the addition of auxin to the auxin-starved cell-suspension culture (van der Zaal *et al.*, 1987). When the promoter of one of these genes, the *Nt103-1* gene, was fused to the *β*-glucuronidase reporter gene (*gusA*) and introduced into tobacco, GUS activity could be detected in the root tips of the transgenic plants. The GUS activity could be enhanced by the addition of auxin to the medium (van der Zaal *et al.*, 1991). Recently the proteins encoded by the genes belonging to the Nt103 gene family were found to have glutathione *S*-transferase (GST) activity (Droog *et al.*, 1993). However, the specific function of these genes in plants is still unknown.

In this thesis we have used the promoter of the auxin-inducible *Nt103-1* gene in order to isolate factors that are involved in the auxin signal transduction pathway leading to the expression of this gene. To this end the promoter was coupled to reporter genes in order to isolate mutants of arabidopsis that showed an enhanced expression of the fusion genes. Arabidopsis genes homologous to

the tobacco *Nt103-1* gene were also isolated. The expression of these genes was studied as well as an attempt was made to elucidate the function of the genes.

Summary

Chapter 2 reports on the isolation of a λclone from a genomic library of arabidopsis, which contains two genes that are homologous to the tobacco *Nt103-1* gene. The genes were called *At103-1a* and *At103-1b*. The expression profile of the *At103-1* genes was found to be different from the expression of the tobacco *Nt103* genes. Both the tobacco gene and the arabidopsis *At103-1a* gene were inducible by auxins in roots, but in addition and in contrast to the tobacco gene the *At103-1a* gene was expressed constitutively in leaves. The *At103-1b* gene was constitutively expressed in leaves as well as in roots.

In the promoter of the *At103-1a* gene an *as-1* like element was present. Such element was also present in different genes belonging to the Nt103 gene family. It was recently found to be necessary for the auxin induction of these genes (Droog *et al.*, 1995, Droog, 1995). As expected, in the promoter of the *At103-1b* gene, which is not inducible by auxin, no *as-1* like element was present. The expression of the *At103-1a* gene in leaves corresponded to the presence of an *I* box element in the promoter of this gene. Such *I* box mediates the expression of light-responsive genes.

The coding regions of the isolated genes, *At103-1a* and *At103-1b*, were very homologous to each other and to the *Nt103-1* gene, which encodes an auxin-inducible glutathione *S*-transferase protein. In **chapter 3** we showed that the protein encoded by the *At103-1a* gene also had glutathione *S*-transferase activity.

The similarity of the *AT103-1A* protein to other glutathione *S*-transferases that were cloned from arabidopsis was significantly lower than to the NT103 like proteins from tobacco. For this reason the *At103-1a* gene is thought to encode a novel class of arabidopsis glutathione *S*-transferases. We constructed transgenic plants with *At103-1a* cDNA constructs in the sense and antisense orientation, overexpressing or repressing the *At103-1a* gene, respectively. These transgenic plants did not have an obvious phenotype nor were they altered in their response to auxin. These results indicate that the *At103-1a* gene is not essential for normal development of the plant. However, we have to be careful drawing conclusions from these experiments since the genes homologous to the *At103-1a* gene might take over the function of the gene.

Our intention to use the tobacco *Nt103-1* promoter in a mutagenesis experiment in arabidopsis prompted us to study the expression of the promoter in this plant species. **Chapter 4** reports on the introduction of a T-DNA construct containing a fusion between the promoter of the *Nt103-1* gene and the *gusA* reporter gene into arabidopsis. The expression of the fusion gene was studied histochemically in the transgenic seedlings. Besides different auxins also the

auxin transport inhibitor 2,3,5-triiodobenzoic acid was able to induce GUS activity after incubation of the seedlings on medium containing this compound. The inactive auxin analog 3,5-dichlorophenoxyacetic acid, glutathione and $CuSO_4$ were weak inducers of the GUS activity. Salicylic acid and compounds that increased the concentration of hydrogen peroxide in the cell were also able to induce GUS activity, suggesting a role for hydrogen peroxide as a second messenger in the induction pathway.

Although the *Nt103-1* promoter was induced not only by auxins, but also by certain other compounds, the concentration of auxin needed for induction was much lower than the concentration of any of the other inducers needed. Therefore we decided to use this promoter in a mutant analysis for factors involved in auxin signal transduction.

In **chapter 5** we describe the construction of transgenic arabidopsis plants containing a T-DNA construct in which the promoter of the *Nt103-1* gene was fused to the *nptII* gene as well as to the *gusA* reporter gene. After mutagenesis the M_2 seedlings were selected for kanamycin resistance. In the M_3 generation the seedlings of the putative kanamycin resistant mutants were screened for GUS activity. The mutants that were isolated in this way exhibited kanamycin resistance and enhanced GUS activity, indicating that a mutation in the signal transduction pathway leading to the expression of the *Nt103-1* gene had occurred. The mutants were designated **g**lutathione *S*-transferase **up**-regulated (*gup*) mutants.

Some of the mutants were characterized in more detail in **chapter 6**. Next to a detailed analysis of the promoter/reporter gene fusions, the expression of several endogenous genes was studied. The steady state level of the mRNA of the endogenous arabidopsis *At103-1a* gene was enhanced in some of the mutants. The steady state mRNA levels of the auxin-inducible *SAUR-AC1* gene and of the salicylic acid-inducible pathogenesis related *PR1* gene, however, were not enhanced. This indicated that the expression of only a group of auxin-inducible genes was altered by the *gup* mutations. The most prominent morphological alteration in the mutants was the epinasty of leaves and cotyledons that was found in seven out of ten mutants analyzed. Also the roots of five out of ten mutants displayed a decreased ability to penetrate the agar. Most of the mutations giving rise to the *gup* phenotype were dominant, the mutant phenotype was caused by a recessive mutation in only two of the investigated mutants. One dominant and one recessive mutation were mapped to the top of chromosome 1 and to chromosome 2, respectively. The response to auxin was not drastically altered in the mutants. Only the roots of mutant *gup0-1* were slightly more sensitive to 2,4-D than those of the wild-type. The roots of three mutants were slightly more sensitive to abscisic acid than those of the transgenic parent lines. We found no correlation between the increased levels of expression of the endogenous arabidopsis gene, the morphological alterations and the sensitivity towards hormones.

The use of reporter genes to select for mutants

In this thesis we used the promoter of the tobacco auxin-inducible *Nt103-1* gene coupled to the selectable marker *nptII* as well as to the visible marker *gusA* to be able to select for mutants altered in an auxin signal transduction pathway. The *Nt103-1/nptII* fusion gene allowed us to screen large numbers of M_2 seedlings for enhanced expression of the *Nt103-1* promoter, while the *Nt103-1/gusA* fusion gene allowed discrimination between *cis*-acting mutations or rearrangements in the promoter giving rise to kanamycin resistance and *trans*-acting mutations giving rise to kanamycin resistance due to a mutation in the signal transduction pathway.

Recently other work was published using inducible reporter genes to select for mutants. Cao *et al.* (1994) used a construct constituting of the promoter of the gene for a pathogenesis related β-glucanase fused to the *gusA* gene to select for mutants with an altered expression of the fusion gene. In this way they isolated mutants that were non-responsive to inducers of systemic acquired resistance (SAR). In the same screen mutants with a constitutive expression of SAR were isolated (Bowling *et al.*, 1994). The above mentioned approach required the screening of all M_2 mutants for GUS activity individually. To confirm the *trans*-acting mutation the RNA level of an endogenous gene had to be checked in every putative mutant. Although successful this method was therefore rather laborious and time consuming. A method in which the altered expression of the promoter/reporter gene fusion could be selected for by screening for resistance or sensitivity is therefore more favourable. The presence of a second promoter/reporter gene fusion to discriminate between a *trans*- and a *cis*-acting mutation also greatly simplifies the selection procedure.

Other mutants that were isolated using reporter genes, were reported by Susek *et al.* (1993), who made fusion genes between the light dependent *cab140* promoter and two reporter genes: the *hpt* gene conferring resistance to the antibiotic hygromycin and to the *gusA* gene. They were able to isolate mutants altered in signalling between the chloroplast and nucleus and that uncoupled nuclear gene expression from plastid function.

Karlin-Neumann *et al.* (1991) used the negative selection marker *tms2* for mutant isolation. The *tms2* gene encodes an amidohydrolase that catalyses the conversion of biologically inactive amides into active auxins that are toxic at high concentrations. The coding region of this gene was fused to a phytochrome-regulated promoter and transformed to arabidopsis. Mutants with a crippled phytochrome-regulated transcriptional response can survive the selection and can thus be selected for. However, genetic analysis of the mutants isolated using this method showed that the lines survived the selection due to cosuppression of the introduced fusion gene and the native gene caused by epigenetic phenomena (Brusslan & Tobin, 1995). The use of a heterologous promoter to select mutants as described in this thesis probably has a better chance to avoid unwanted interference of the introduced promoter and the endogenous gene leading to cosuppression.

The nature of the *gup* mutation

In the promoters of auxin-regulated genes different **Aux**in **R**esponsive **E**lements (AuxRE) have been identified (reviewed by Hagen, 1995). The level of expression of a gene is determined by regulatory proteins, transcription factors, that bind to the AuxRE sequences. An AuxRE present in both the promoter of the *Nt103-1* gene and in the promoter of its arabidopsis homolog *At103-1a* could thus be important in the auxin signal transduction leading to the enhanced expression of both genes. The only known AuxRE that both promoters have in common is an *as-1* like element. This element turned out to be necessary and sufficient to confer auxin inducibility to the promoters of the genes of the NT103 gene family (Droog, 1995). Elements related to *as-1* were identified in the cauliflower mosaic virus and in some of the T-DNA genes introduced in plants via *Agrobacterium tumefaciens* as well (Bouchez *et al.*, 1989, An *et al.*, 1990, Ellis *et al.*, 1993, Kim *et al.*, 1993, Liu *et al.*, 1994). These related elements were reported to be important in auxin induction (Kim *et al.*, 1993, Qin *et al.*, 1994, Zhang & Singh, 1994).

The *as-1* element contains two copies of a TGACG motif. Proteins that can bind to the *as-1* like elements belong to the basic leucine zipper (bZIP) class of transcription factors. The bZIP domain is a well-defined DNA binding motif found in several eukaryotic transcriptional activators (Landschulz *et al.*, 1988, McKnight, 1991). The domain dimerizes by forming a coiled-coil structure in the leucine zipper region. Dimerization is essential for DNA binding. Regulation of gene expression by bZIP proteins is not yet understood (reviewed by Foster *et al.*, 1994). The formation of homo- or heterodimers might be a mechanism to control gene expression. Also post-translational modification such as phosphorylation might be important in regulation. Translocation of cytoplasmically retained transcription factors into the nucleus could also be a regulatory mechanism.

The TGA1 protein is one of the proteins that can bind to the *as-1* element. Different cDNAs encoding TGA like proteins have been isolated from tobacco (Fromm *et al.*, 1989, Katagiri *et al.*, 1989) and arabidopsis (Kawata *et al.*, 1992, Schindler *et al.*, 1992, Zhang *et al.*, 1993, Miao *et al.*, 1994). Neuhaus *et al.* (1994) showed that by increasing the level of TGA1 in cotyledon cells, the expression of an *as-1*-linked gene could be increased. An enhanced level of TGA like proteins might thus lead to an enhanced expression of the genes controlled by a promoter containing an *as-1* like sequence like *Nt103-1* and *At103-1a*. Recent preliminary results showed that in some of the investigated *gup* mutants, the mRNA level corresponding to the *tga1* gene was enhanced. The increased level of the TGA1 protein may therefore be the direct cause of the enhanced expression of genes linked to the *Nt103-1* and *At103-1a* promoters in the *gup* mutants. This may be due to a mutation in the *tga1* promoter. The nature of such a mutation is expected to be dominant. Indeed only mutants bearing a dominant mutation showed increased levels of *tga1* mRNA (unpublished results). However, besides mutations in the promoter of the *tga1* gene, also mutations in

factors controlling the expression of the *tga1* gene might have led to the enhanced expression of this gene. In arabidopsis at least five TGA like proteins are present (Miao *et al.*, 1994). It will be very interesting to see whether the expression of one of the other *tga* genes is enhanced in the mutants as well.

In some dominant *gup* mutants the *tga1* expression was not enhanced. The *gup* phenotype in these mutants might be due to mutations affecting the affinity of binding of the TGA1 protein to the *as-1* like element. These mutants might however also be mutated in a receptor or a component of the signal transduction pathway, giving rise to a constitutive signal indirectly or directly responsible for the *gup* phenotype.

Two of the *gup* mutants had recessive mutations. In these mutants a receptor or repressor, that normally negatively regulates the expression of the *Nt103-1* promoter-linked genes, might be mutated. The inhibitory effect of this receptor or repressor might thus be decreased in the mutants giving rise to the *gup* phenotype.

Future research

In this thesis we have shown that it was possible to isolate mutants altered in the expression of a specific group of auxin-inducible genes using a molecular genetic approach. Further research on the nature of the mutations will provide more insight in the signal transduction pathway leading to the expression of this class of genes. This could be achieved by isolation and characterization of the *gup* genes. Since genetic mapping and chromosome walking still is time-consuming, T-DNA tagging might be the alternative way to isolate the genes. Instead of using EMS mutagenesis, we could use T-DNA tagging to isolate similar *gup* mutants. An activator T-DNA construct containing an outward directed constitutive promoter adjacent to the right border (van der Graaff, unpublished results) could then be used to transform the transgenic parent lines. In this way it should be possible to obtain T-DNA tagged *gup* mutants due to sense or antisense overproduction or due to insertional inactivation.

Using the approach described in this thesis also other inducible promoters can be used to study different signal transduction pathways.

References

An G, Costa MA, Ha S-B (1990) Nopaline synthase promoter is wound inducible and auxin inducible. Plant Cell 2: 225-233

Bouchez D, Tokuhisa JG, Llewellyn DJ, Dennis ES, Ellis JG (1989) The *ocs*-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J 8: 4197-4204

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong X (1994) A mutation in arabidopsis that leads to constitutive expression of systemic acquired resistance.

The Plant Cell 8: 1845-1857

Brusslan JA & Tobin EM (1995) Isolation of new promoter-mediated co-suppressed lines in *Arabidopsis thaliana*. Plant Mol Biol 27: 809-813

Cao H, Bowling SA, Gordon AS, Dong X (1994) Characterization of an arabidopsis mutants that is nonresponsive to inducers of systemic aquired resistance. The Plant Cell 6: 1583-1592

Ellis JG, Tokuhisa JG, Llewellyn DJ, Bouchez D, Singh K, Dennis ES, Peacock WJ (1993) Does the ocs-element occur as a functional component of the promoters of plant genes. The Plant Journal 4: 433-443

Droog FNJ, Hooykaas PJJ, Libbenga KR and van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferases and one member indeed shows in vitro GST activity. Plant Mol Biol 21: 965-972

Droog FNJ, Spek A, van der Kooy A, de Ruyter A, Hoge H, Libbenga KR, Hooykaas PJJ, van der Zaal EJ (1995) Promoter analysis of the auxin-regulated tobacco glutathione *S*-transferase genes *Nt103-1* and *Nt103-35*. Plant Mol Biol *in press*

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD Thesis, Leiden University, 165 pp

Foster R, Izawa T, Chua N-H (1994) Plant bZIP proteins gather at ACGT elements. *FASEB J* 8: 192-200

Fromm H, Katagiri F, Chua N-H (1991) An octopine synthase enhancer element directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell 1:977-984

Hagen G (1995) The control of gene expression by auxin. In: Plant Hormones Davies PJ (ed) Kluwer academic publishers, The Netherlands: 228-245

Karlin-Neumann GA, Brusslan JA, Tobin EM (1991) Phytochrome control of the *tms2* gene in transgenic arabidopsis: A stratagy for selecting mutants in the signal transduction pathway. The Plant Cell 3: 573-582

Katagiri F, Lam E, Chua N-H (1989) Two tobacco DNA-binding proteins with homology to nuclear factor CREB. Nature 340: 727-730

Kawata T, Imada T, Shiraishi H, Okada K, Shimura Y, Ivabuchi M (1992) A cDNA clone encoding an HBP-1b homologue in *Arabidopsis thaliana*. Nucl Acid Res 20: 1141-1147

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid insensitive mutants of *Arabidopsis thaliana*. Physiol Plant 61: 377-383

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) *CTR1*, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the RAF family of protein kinases. Cell 72: 427-441

Kim S-R, Kim Y, An G (1993) Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (*nos*) promoter. Plant Physiol 103: 97-103

Liu Z-B, Ulmasov T, Shi X, Hagen G, Guifoyle TJ (1994) Soybean *GH3* promoter contains multiple auxin-inducible elements. The Plant Cell 6: 645-657

Landschulz WH, Johnson PF, McKnight SL (1988) The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 240: 1759-1764

McKnight SL (1991) Molecular zippers in gene regulation. Sci Am 264: 54-64

Miao Z-H, Liu X, Lam E (1994) TGA3 is a distinct member of the TGA family of bZIP transcription factors in *Arabidopsis thaliana*. Palnt Mol Biol 25: 1-11

Neuhaus G, Neuhaus-Url G, Katagiri F, Seipel K, Chua N-H (1994) Tissue-specific

expression of *as-1* in transgenic tobacco. The Plant Cell 6: 827-834

Phinney BO, Freeling M, Robertson DS, Spray CR, Silverthorne J (1986) Dwarf mutants of maize, the gibberellin pathway and its molecular future. In: Plant growth substances. Bopp M (ed) Springer-Verlag, Berlin, heidelberg. pp 55-64

Qin X-F, Holuigue L, Horvath DM, Chua N-H (1994) Immediate early transcription activation by salicylic acid via the Cauliflower Mosaic virus *as-1* element. The Plant Cell 6: 863-874

Schindler U, Beckmann H, Cashmore AR (1992) TGA1 and G-box binding factors: Two distinct classes of arabidopsis leucine zipper proteins compete for the G-box like element TGACGTGG. Plant cell 4: 1309-1319

Susek RE, Ausubel FM, Chory J (1993) Signal transduction mutants of arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell 74: 787-799

van der Zaal EJ, Memelink J, Mennes AM, Ouint A, Libbenga KR (1987) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol 10: 145-157

van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible and root tip-specific expression. Plant Mol Biol 16: 983-998

Zhang B, Foley RC, Singh KB (1993) Isolation and characterization of two related arabidopsis ocs- element bZIP binding proteins. Plant J 4:711-716

Zhang B, Singh KB (1994) ocs element promoter sequences are activated by auxin and salicylic acid in arabidopsis. Proc Natl Acad Sci *USA* 91: 2507-2511

SAMENVATTING

Het hormoon auxine speelt een belangrijke rol in de groei en ontwikkeling van planten. Het heeft invloed op processen als celstrekking, celdeling, vaatbundel-differentiatie, apicale dominantie en wortelvorming. Ondanks het belang van auxine voor de plant is er weinig bekend over de manier waarop het hormoon werkt. Algemeen wordt aangenomen dat auxine als signaalstof kan binden aan een receptor. Na binding wordt een signaal doorgegeven in de plantecel dat vervolgens kan leiden tot het aanzetten van genen. Naast isolatie en onderzoek van eiwitten die auxine kunnen binden, wordt er onderzoek gedaan aan genen die door auxine aangezet worden. Ook op andere manieren wordt geprobeerd om de signaal-transductie van auxine op te lossen. Door planten te maken die - door inbreng van een bacterieel gen - een verhoogd auxine-niveau hebben, kan het effect van auxine op planten beter bestudeerd worden. Een andere manier om dit te bereiken is door de isolatie en karakterisering van mutanten die - in vergelijking met normale planten - minder gevoelig zijn voor auxine.

In dit proefschrift zijn verschillende methoden om auxine signaal-transductie te bestuderen gecombineerd. Door mutanten te maken waarin het expressie-niveau van genen die door auxine aangezet worden is veranderd, kunnen mogelijk factoren die belangrijk zijn in de auxine signaal-transductie geïdentificeerd worden. Er werd gebruik gemaakt van de zandraket, *Arabidopsis thaliana*, welke door zijn kleine genoom, korte levenscyclus en een kleine gestalte zeer geschikt was voor onze experimenten. Daarnaast waren de uitgebreide kennis van de genetica van deze plant en de mogelijkheid om de plant onder steriele omstandigheden te groeien en te transformeren van belang bij de keuze voor arabidopsis.

Eén van de genen die door auxine aangezet wordt is het *Nt103-1* gen van tabak. De expressie van dit gen werd in dit laboratorium reeds uitvoerig bestudeerd in tabaksplanten en plantecel-suspensies. Ook in arabidopsis blijken genen aanwezig te zijn die verwant zijn aan het *Nt103-1* gen van tabak. In **hoofdstuk 2** wordt de isolatie van twee van deze genen beschreven. Deze genen bleken naast elkaar op het genoom te liggen. De expressie van één van deze genen, *At103-1a*, bleek verhoogd te worden onder invloed van auxine. De expressie van het andere gen, *At103-1b*, werd niet verhoogd onder invloed van dit hormoon. Door de isolatie van het auxine-induceerbare gen van arabidopsis, werd duidelijk dat het voorkomen van dit soort genen niet specifiek is voor tabak. Door de hoge mate van verwantschap tussen het tabaksgen en de arabidopsis-genen, leek het ook goed mogelijk dat de functies van deze genen overeenkomen in verschillende plantensoorten. Uit de experimenten beschreven in **hoofdstuk 3** bleek inderdaad dat het eiwit dat gecodeerd wordt door het

At103-1a gen van arabidopsis, net als het eiwit dat gecodeerd wordt door het *Nt103-1* gen van tabak, glutathion *S*-transferase (GST) activiteit heeft. Het AT103-1A eiwit werd vergeleken met GST eiwitten uit andere planten. Doordat het AT103-1A eiwit meer leek op GST eiwitten uit andere plantensoorten dan op GST eiwitten die eerder uit arabidopsis geïsoleerd werden, werd geconcludeerd dat dit eiwit tot een andere groep behoort dan de arabidopsis GSTs. Een andere of aanvullende functie van AT103-1A, ten opzichte van de arabidopsis GSTs, zou dan ook goed mogelijk zijn.

Uit experimenten met transgene tabaksplanten, welke een fusie-gen bevatten van de promoter van het *Nt103-1* gen met de coderende regio van het β-glucuronidase gen (*Nt103-1/gusA*), bleek dat deze promoter tot expressie kwam in de worteltopjes van de planten. De expressie van het fusie-gen kon verhoogd worden door de toevoeging van auxine. Door de isolatie van mutanten met een veranderde gen-expressie van het *Nt103-1* gen, zouden factoren geïdentificeerd kunnen worden die belangrijk zijn in de signaal-transductie van auxine. Voordat het *Nt103-1* gen van tabak gebruikt kon worden voor een dergelijke analyse in arabidopsis, moest gecontroleerd worden of de expressie van dit gen ook in arabidopsis verhoogd werd door auxine. In **hoofdstuk 4** wordt de expressie van het *Nt103-1/gusA* fusie-gen in arabidopsis bestudeerd. De expressie van de promoter in arabidopsis was beperkt tot de worteltop en kon verhoogd worden door de toevoeging van auxine. Doordat de expressie van het fusie-gen in arabidopsis hetzelfde was als in tabak, bleek het mogelijk om de *Nt103-1* promoter te gebruiken in een zoektocht naar mutanten van arabidopsis met een veranderde signaal-transductie van auxine.

Hoofdstuk 5 beschrijft de selectie van deze mutanten. Voor de selectie van mutanten werden transgene planten gemaakt die een T-DNA construct bevatten met daarop twee *Nt103-1* promoter/reporter fusie-genen: een *Nt103-1/nptII* fusie-gen, waarvan expressie leidt tot resistentie tegen het antibioticum kanamycine, en een *Nt103-1/gusA* fusie-gen. Het homozygote zaad van drie transgene lijnen werd gemutageniseerd. Door nakomelingen van dit gemutageniseerde zaad te kiemen op medium met kanamycine kon worden geselecteerd op een verhoogde expressie van het *Nt103-1/nptII* fusie-gen. Kanamycine resistentie kan veroorzaakt worden door mutaties in de signaal-transductie, welke leiden tot verhoogde expressie van het *Nt103-1/nptII* fusie-gen of door veranderingen in het fusie-gen zelf. Om onderscheid te kunnen maken tussen deze twee mogelijkheden werd gekeken naar de verhoogde expressie van het *Nt103-1/gusA* fusie-gen. De mutanten met een verhoogde expressie van beide fusie-genen zijn vermoedelijk veranderd in de signaal-transductie die leidt tot expressie van de *Nt103-1* promoter. Deze mutanten werden **g**lutathion *S*-transferase **up** (*gup*) mutanten genoemd. Enkele van deze mutanten werden nader gekarakteriseerd in **hoofdstuk 6**. Naast een uitgebreide analyse van de expressie van beide fusie-genen werden de *gup* mutanten genetisch gekarakteriseerd. Van twee mutaties werd de positie op het genoom bepaald. Het *GUP1-1* gen werd gelokaliseerd op het bovenste deel van chromosoom 1, terwijl *GUP2-1* op chromosoom 2 bleek te liggen. Ook de

morfologie van de mutanten werd bekeken. Opvallend was dat 7 van de 10 mutanten naar beneden hangende cotylen en bladeren hadden. Ook hadden de wortels van de helft van de onderzochte mutanten moeite om in de voedingsbodem door te dringen. Beide morfologische kenmerken werden reeds eerder omschreven als kenmerken die verband houden met veranderingen in de gevoeligheid voor - of in het niveau van - auxine. Naast een verhoogde expressie van de fusie-genen bleek in een aantal mutanten ook de expressie van het verwante arabidopsis gen, *At103-1a*, verhoogd te zijn.

In dit proefschrift werd de isolatie van mutanten met een veranderde expressie van auxine-induceerbare glutathion *S*-transferase genen beschreven. De mutanten hebben bovendien morfologische kenmerken die wijzen op veranderingen in de auxine-huishouding van de plant. Met de isolatie van deze mutanten hebben we aangetoond dat de methode om mutanten te isoleren via promoter/reporter fusie-genen succesvol kan zijn. Verdere karakterisering van de mutanten zal mogelijk helpen met het ophelderen van de signaal-transductie van auxine in planten.

List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ABP	auxin-binding protein
as-1	activating sequence 1
ASF-1	activating sequence 1 binding factor
At	*Arabidopsis thaliana*
AuxRE	auxin responsive element
bp	basepair
bZIP	basic leucine zipper
CaMv	cauliflower mosaic virus
CAPS	cleaved amplified polymorphic sequence
EMS	ethyl methane sulfonate
GA_3	gibberelic acid
gl1	glabrous1 genetic marker
GST	glutathione *S*-transferase
GUS	*ß*-glucuronidase
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
(I)PCR	(inverted) polymerase chain reaction
kb	kilobasepair
kDa	kiloDalton
NAA	1-naphthylacetic acid
nptII	neomycin phosphotransferase II
Nt	*Nicotiana tabacum*
ORF	open reading frame
p35S (CaMV 35S)	promoter region of the cauliflower mosaic virus 35S transcript
RFLP	restriction fragment length polymorphism
SA	salicylic acid
SAR	systemic acquired resistance
T-DNA	transferred DNA
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide
YAC	yeast artificial chromosome

Curriculum Vitae

Op 22 december 1965 ben ik geboren in Eindhoven. In 1984 behaalde ik het diploma Atheneum B aan het Bisschop Bekkers College in Eindhoven. In september van datzelfde jaar begon ik met de studie Plantenveredeling aan de Landbouwuniversiteit in Wageningen. In maart 1990 behaalde ik het doctoraal examen Plantenveredeling; vrije oriëntatie met als afstudeervakken Genetica (prof. dr.ir. M. Koornneef), Virologie (dr.ir. R. van der Vlugt en prof. dr. ir. R. Goldbach) en Plantenveredeling (dr. R. Visser en prof dr.ir. E. Jacobsen). Mijn praktijktijd Virologie volbracht ik als stagiaire bij MOGEN international nv. in Leiden (dr. A. Hoekema en dr. B. Cornellisen) Van april 1990 tot april 1994 was ik in dienst van het Centrum voor Fytotechnologie RUL/TNO. In die hoedanigheid was ik als assistente in opleiding verbonden aan het Instituut voor Moleculaire Plantkunde. Van april 1994 tot april 1995 was ik werkzaam op hetzelfde instituut.

Nawoord

Op deze plaats wil ik allereerst Edward en mijn ouders noemen. Zij hebben mij gestimuleerd om te gaan studeren en mijn verrichtingen met belangstelling gevolgd. De wetenschap dat jullie steeds achter me stonden betekende veel voor mij.
Met het afronden van dit proefschrift sluit ik een periode af waarin ik met plezier heb gewerkt op het Clusius laboratorium. Dit proefschrift kon alleen tot stand komen doordat ik bij iedereen terecht kon met vragen en voor suggesties. Eerst op het "*Agrobacterium* " en later op het "*Arabidopsis*" lab heb ik met plezier gewerkt met Amke, Tonny, Alice, Stephan, Teresa, Sander, Annette, Eric, Remco, Stephan, Daniel, Monique, Waldi, Cindy, Magda, Tomasz, Ellen, Barbara, Sylvia, Willem, Bas en Swiet. Met hen en anderen werden vaak discussies gevoerd die naast gezellig en stimulerend soms ook heftig en oeverloos waren. De labuitjes en de vele borrels hebben zeker ook bijgedragen aan de prettige werksfeer op het Clusius. De bijdragen van Bas Reichert en Monique Schuyer, die als doctoraalstudenten aan mijn project gewerkt hebben, zijn voor het in dit proefschrift beschreven resultaat van grote betekenis geweest. Hoewel arabidopsis een onkruid heet te zijn, was het vooral aan de kennis en toewijding van Bram en Elly dat de planten niet dood gingen onder minder optimale omstandigheden.
Buiten het laboratorium zorgde de kanovereniging "de Genneper Molen" voor de nodige (ont)spanning. Ook "Pyramidion" heeft hieraan steeds zijn/haar steentje bijgedragen, wat mij in staat stelde enkele thee/koffie en lunchpauzes van gespreksstof te voorzien.

Stellingen

Behorende bij het proefschrift: "Auxin signal transduction in *Arabidopsis thaliana*: a novel molecular genetic approach".

- Het is zeer onwaarschijnlijk dat de homologie die Walden *et al.* opmerken tussen een sequentie in een intron van het *axi 1* gen en (mogelijk) auxine-gereguleerde genen een reden is voor auxine-induceerbaarheid van het *axi 1* gen, aangezien deze homologie een "short interspersed repetitive element" betreft.

Walden *et al.* (1994) EMBO J 13: 4729-4736
Yoshioka *et al.* (1993) Proc Natl Acad Sci *USA* 90: 6562-6566
van der Zaal *et al.* (1991) Plant Mol Biol 16: 983-998

- De bewering van Takahashi *et al.*, dat het parB eiwit in tegenstelling tot het parA eiwit in het cytoplasma is gelokaliseerd, wordt niet ondersteund door de experimentele gegevens.

Takahashi *et al.* (1995) Planta: 111-117

- Ter voorkoming van interacties tussen promoters die leiden tot bijvoorbeeld cosuppressie, is in mutagenese experimenten, die als doel hebben de expressie van deze promoters te veranderen, het gebruik van heterologe promoters te verkiezen boven het gebruik van plant-eigen promoters.

Brusslan & Tobin. (1995) Plant Mol Biol 27: 809-813

- De kans dat het gebruik van de door Düring ontwikkelde transformatievector navolging zal vinden is klein, omdat deze vector geen *Agrobacterium* replicator bevat.

Düring. (1994) Transgenic Research 3: 138-140

- Het gebruik van een hogere bacteriedichtheid tijdens plantecel-transformaties teneinde een hogere transformatiefrequentie te verkrijgen, heeft alleen nut als deze hogere bacteriedichtheid geen verhoging van het aantal T-DNA inserties per cel ten gevolge heeft.

Lin *et al.* (1995) Focus 16:70-75

- Het bestaan van genetische kaarten van het genoom van arabidopsis welke onderling sterk verschillen, is niet bevorderlijk voor de ontwikkeling van arabidopsis als modelplant.

Hauge *et al.* (1993) Plant J 3: 745-754
Lister & Dean (1993) Plant J 4: 745-750

- Het aanvragen van vergunningen voor de teelt van gewassen, met een door genetische manipulatie verkregen resistentie tegen bestrijdingsmiddelen, is niet bevorderlijk voor de maatschappelijke acceptatie van genetische modificatie.

- Het bouwen van gespecialiseerde ruimten kan men het beste overlaten aan bedrijven die bewezen hebben hierin gespecialiseerd te zijn.

- Het instellen van vaarverboden op wildbeken in de Belgische Ardennen is niet ingegeven door het streven de natuur te ontzien, maar meer het gevolg van een actieve vissers lobby.

- 10 oktober 1995: een cijfer en zes miljoen problemen erbij.

- Opbouwende kritiek is vooral opbouwend tijdens de opbouw.

- In het verkeer staat links meestal rechts en rechts links.

- A.I.O. worden, wordt slecht voor de beurs.

- inter *nog* net *niet*

Dianne van der Kop