# Auxin-responsive genes:

# expression studies with the *AtARCA* gene

# and cDNA arrays

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# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| # | number |
| °C | degrees centigrade (Celsius) |
| µ | micro; $10^{-6}$ |
| 1-NAA | 1-naphthalene acetic acid |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 2-NAA | 2-naphthalene acetic acid |
| | |
| *A. thaliana* or At | *Arabidopsis thaliana* |
| ABA | abscisic acid |
| Acc. Nr. | Genbank accession number |
| ADP | adenosin-5'-diphosphate |
| $Amp^R$ | ampicillin resistance |
| ATG | start codon |
| ATP | adenosin-5'-triphosphate |
| | |
| BAC | bacterial artificial chromosome |
| Bn | *Brassica napus* |
| bp | base pairs |
| BY2 | bright yellow 2 suspension cells (Nagata *et al*., 1982) |
| | |
| $Ca^{2+}$ | calcium ion |
| cDNA | complement desoxyribonucleic acid |
| CHX | cyclohexamide |
| cpm | counts per minute |
| C-terminus | carboxy-terminus |
| CTP | cytidin-5'-triphosphate |
| | |
| DNA | desoxyribonucleic acid |
| Dm | *Drosophila melanogaster* |
| dNTP | 2'-desoxynucleotide-5'-triphosphate |
| | |
| *En1* | enhancer element; transposon from *Zea mays* |
| EST | expressed sequence tag |
| *et al*. | and co-authors |
| | |
| Fig. | figure |
| | |
| g | 1. gram; 2. gravitational force of acceleration |
| G | guanine |
| GST | glutathione-*S*-transferase |
| GTP | guanosine-5'-triphosphate |
| | |
| hr(s) | hour(s) |
| | |
| IAA | indole-3-acetic acid |
| IPTG | isopropyl-β-D-thiogalactoside |

| | |
|---|---|
| kb | kilo bases; $10^3$ bases |
| l | liter |
| liq. | liquid |
| m | milli; $10^{-3}$ |
| M | molar (mol/l) |
| *M. sativa* or Ms | *Medicago sativa* |
| min | minutes |
| mRNA | messenger ribonucleic acid |
| n | nano; $10^{-9}$ |
| *N. tabacum* or Nt | *Nicotiana tabacum* |
| NCBI | National Center for Biotechnology Information |
| N-terminus | amino-terminus |
| O/N | overnight |
| ORF | open reading frame |
| Os | *Oryza sativa* |
| p | plasmid |
| PCIB | 2-(p-chlorophenoxy)-2-methylpropionic acid |
| PCR | polymerase chain reaction |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| *S. cerevisiae*or Sc | *Saccharomyces cerevisiae* |
| SDS | sodiumdodecyl sulphate |
| sec | second |
| T | time point |
| TTP | thymidin-5'-triphosphate |
| U | units (enzyme activity measure) |
| UTR | untranslated region |
| UV | ultraviolet |
| v/v | volume per volume |
| w/ | with |
| w/o | without |
| w/v | weight per volume |
| WD(40) | tryptophan-aspartic acid repeat unit |
| wt | wildtype |
| X-Gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

**Amino acids**

| | | | | | |
|---|---|---|---|---|---|
| A | Ala | Alanine | M | Met | Methionine |
| C | Cys | Cysteine | N | Asn | Asparagine |
| D | Asp | Aspartic acid | P | Pro | Proline |
| E | Glu | Glutamic acid | Q | Gln | Glutamine |
| F | Phe | Phenylalanine | R | Arg | Arginine |
| G | Gly | Glycine | S | Ser | Serine |
| H | His | Histidine | T | Thr | Threonine |
| I | Ile | Isoleucine | V | Val | Valine |
| K | Lys | Lysine | W | Trp | Tryptophan |
| L | Leu | Leucine | Y | Tyr | Tyrosine |

# 1. INTRODUCTION

Auxins are plant growth regulators that control multiple processes in plant development, including cell division, cell elongation, cell polarity, and cell differentiation. Auxins have unique functions in different tissues. For example, they can stimulate stem elongation, promote lateral root formation, inhibit lateral bud outgrowth, or stimulate vascular strand differentiation. The functional diversity attributed to auxins suggests that plants have multiple mechanisms to sense and respond to these hormones and that different cells have certain subsets or amounts of the possible auxin sensing and/or responding components. However, we have only a poor understanding of these signaling pathways, and further investigation is needed to unravel the mechanisms of auxin action. Although evidence for auxin's effect on plant growth has been available since the time of Charles and Francis Darwin (1881), progress in unraveling the roles of auxin and the molecules it interacts with has been slow. For many years, the perception and transduction of the auxin signal has been relatively inaccessible. Moreover, our inability to visualize sources of auxin synthesis and routes of transport has impeded a molecular dissection of these important pathways in plants.

What is the conceptual molecular framework needed to interpret the activities of plant growth substances like auxin? Does auxin have fundamentally different signaling pathways when compared to signals acting in hormonal signaling systems of animals? Is it acting as a mitogen in the promotion of cell division and at other times (e.g. in the root) more like a morphogen? In other situations during plant development, it may act as an integrative or correlative signal when growing shoot tissues influence the development of the rest of the plant. It was argued that the basic functions of auxin and potentially of other major growth regulators were to allocate resources under poor growth conditions. The obvious structural similarity of indole-3-acetic acid with the amino acid tryptophan led to speculation about the origin of plant hormones from metabolite-mediated signaling systems.

These considerations indicate the difficulties in formulating a convincing, conceptual framework for auxin action. How will it be possible to strategically dissect the molecular pathways of auxin action? As the sequencing of the *Arabidopsis* genome is close to completion, molecular and genetic research in this field has provided better understanding of

these pathways. I will therefore first summarize some of the recent insights that resulted in the past few years from systematic application of molecular genetic approaches and leading to findings that shed light on auxin's many roles in plant growth and development. I will then discuss several approaches that have been used, besides molecular genetic approaches, to describe pathways in which genes are activated or repressed. Finally, as a result of these developments, I will discuss recently established technological advances that provide genome-scale information about gene expression patterns and their potential impact on describing auxin signaling pathways.

## 1.1 Regulation of gene expression

As the analysis of auxin-regulated genes will be the main topic of this thesis, I will first describe the major principles underlying the regulation of gene expression and then focus on what we know at present on auxin-regulated genes. Another chapter will then deal with strategies to further explore the complexity of auxin or other plant hormone regulated genes.

Normal development of the plant, as well as adaptation to changing environmental conditions, requires careful regulation of gene expression. For each response to changing factors (induction), early and late responding genes can be identified. Early genes are genes that are the genes to respond to the inductive stimuli and respond relatively fast (between 5 and 15 minutes after induction). Normally, these genes do not require other gene products and respond directly to the inducing agent (Guilfoyle, 1986; Theologis, 1986). In contrast, late genes respond relatively slow (from 15 minutes to several hours after induction). Late genes respond in an indirect manner, for these genes require other gene products for their expression (Guilfoyle, 1986; Theologis, 1986).

To acquire the optimal response, genes are regulated at multiple levels. Many early genes are primarily regulated at a transcriptional level. Transcriptional regulation takes place at the genomic DNA level (Finnegan *et al*., 1998; Ogbourne and Antalis, 1998; Ptashne and Gann, 1998). Post-transcriptional regulation can be seen as an additional level of regulation needed to increase the flexibility and speed of responses beyond that which can be achieved through transcriptional regulation alone. Such regulation occurs mainly through control of mRNA stability (Gallie, 1998; Gutiérrez *et al*., 1999). Additionally,

regulation of gene expression can occur at the translational or post-translational level. In the case of translational regulation, translational initiation is usually affected (see also Gradi *et al*., 1998; McCarthy *et al*., 1998; Preiss and Hentze, 1999; Sonenberg and Gingras, 1998), while post-translational control has been shown to involve proteolysis (Haas and Siepmann, 1998; Koepp *et al*., 1999; Gray and Estelle, 2000). In this thesis the rapid response to auxin exhibited by certain genes is examined on the presence of mRNA molecules, which does not include gene regulation on translation or post-translational levels.

## 1.2 Auxin responsive genes

To date, several genes and gene families have been identified whose expression level is changed in response to auxin. In many cases, these genes do not only respond to auxin but also to other inducing agents. On the other hand, changes in early gene expression may lead to physiological responses, making it difficult to discriminate between a change in late gene expression caused by auxin, and a change expression caused by the physiological response. Several groups of genes have been identified in the auxin response, and they respond in a direct or indirect manner. In the next chapters the following groups of genes will be discussed in more detail:

1. Genes encoding DNA-binding proteins
2. Glutathione *S*-transferase (GST)
3. ACC synthase
4. Genes encoding calcium-binding proteins
5. Genes involved in cell cycling
6. Genes encoding proteins involved in synthesis and modification of cell walls
7. Ubiquitin-related genes
8. Genes involved in wounding and pathogenesis response
9. Genes involved in secondary metabolism
10. Genes with unknown function

### 1.2.1 Genes encoding DNA-binding proteins

In a response to auxin, the first group of genes involved in the regulation of differential expression are those encoding for transcriptional activators. Since most of these genes respond quickly (often within several minutes after induction), a lot of research has focussed on these genes. For example, the *AUX/IAA* gene families are considered to be involved in the transcriptional activation of gene expression (Abel *et al*., 1994; Abel *et al*., 1995; Abel and Theologis, 1996). This reinforces the idea that auxin might influence cellular activities through the activity of DNA-binding proteins (Sitbon and Perrot-Rechenmann, 1997).

Another group of auxin-responsive DNA-binding proteins, but not belonging to the group of transcriptional activators, are histones (Sitbon and Perrot-Rechenmann, 1997). Histones are involved in DNA replication and cell division. This process is, in part, auxin-regulated; therefore, a histone response is not very surprising, although the response is induced by the effect of auxin on cell division rather than on the histones themselves (Reichheld *et al*., 1995).

### 1.2.2 Glutathione *S*-transferase (GST)

A number of enzyme-encoding genes have been found to be regulated by auxin. A particularly well-studied example is glutathione *S*-transferase. Glutathione *S*-transferases (GSTs) catalyze the transfer of reduced glutathione to electrophilic substances (Salinas and Wong, 1999). In plants, GSTs have been shown to function in herbicide detoxification and the protection against oxidative damage (Timmerman, 1989). An involvement in intracellular and transmembrane transport has been stated as well (Sitbon and Perrot-Rechenmann, 1997). The first auxin-induced gene to which a function could be assigned was *parB*, a GST homologue (Takahashi and Nagata, 1992).

Based on conserved amino acids and intron-exon placements, plant GSTs and GST-like proteins have been divided in three groups (Droog *et al*., 1995). GSTs belonging to groups I and III have been shown to be auxin-induced, while members of group II are regulated by ethylene (Marrs, 1996). However, other factors are also capable of regulating GST gene expression. These factors include non-auxin analogues, salicylic acid, heavy metal ions

and other stress conditions (Sitbon and Perrot-Rechenmann, 1997). Based on these observations, these genes were referred to as being stress-inducible rather than hormone-inducible.

### 1.2.3 ACC synthase

Auxin is a known inducer of ethylene production and regulates specific genes encoding ACC synthase, that are often regulated by other agents as well (Arteca and Arteca, 1999; Yi *et al*., 1999). ACC synthase is the key regulatory enzyme of ethylene biosynthesis and catalyses the conversion of *S*-adenosylmethionine into ACC, the immediate ethylene precursor. It is a short-lived cytosolic enzyme and is encoded by a multigene family in all higher plant species.

### 1.2.4 Genes encoding calcium-binding proteins

The changes in the concentration of cytosolic, free calcium have been intensively studied as an important link in the transduction of many signals (Trewavas and Gilroy, 1991; Shacklock *et al*., 1992; Gilroy and Jones, 1992; Neuhaus *et al*., 1993; Shishova *et al*., 1999). The cytosolic $Ca^{2+}$ concentration was found to increase in response to IAA, cytokinin and abscisic acid (ABA). Such an effect could be the result of the activation of calcium channels in the plasma membrane (calcium influx from the extracellular medium) and/or $Ca^{2+}$ release from intracellular compartments (Shishova *et al*., 1999). The fact that calcium is somehow involved in auxin signaling suggests that also calcium-binding proteins might be induced in response to auxin.

Calmodulin is a cellular, calcium-modulated protein which is thought to be the most important intracellular $Ca^{2+}$ receptor and is conserved among all eukaryotes. Moreover, calcium-bound calmodulin is able to bind to, and alter gene expression and the activity of other proteins (Rudd and Franklin-Tong, 1999). Auxin increases mRNA levels of different genes encoding calmodulin, which includes the calmodulin-related protein TCH3 (Sitbon and Perrot-Rechenmann, 1997). Furthermore, it has been found that triggers (e.g. auxin) increasing the cytosolic $Ca^{2+}$ concentration, have also an effect on calmodulin expression (Okamoto *et al*., 1995; Zielinski, 1998).

### 1.2.5 Genes involved in cell cycling

Several decades ago it has been discovered that the phytohormones auxin and cytokinin are very important in the growth and differentiation of plant tissues. Especially the balance between these hormones is of great importance (Skoog and Miller, 1957). Therefore, these hormones, either by themselves or in well-defined ratios, are used for the regulation of cell-division in *in vitro* cultures. This suggests that at least some components of the cell cycle are under hormonal control. As a matter of fact, the expression of *cdc2*, one of the key regulators of the cell cycle, has been shown to be influenced by both auxin (Hirt *et al*., 1991; Hemerly *et al*., 1993) and cytokinin (Hemerly *et al*., 1993; Miao *et al*., 1993). This family of cyclin-dependent kinases and their regulatory subunits, cyclins, have been isolated from all eukaryotes (Burssens *et al*., 1998). Binding of cyclins to *cdc2* activates the complex and contributes to the localization and substrate specificity (Pines, 1995). Cyclin transcript levels oscillate dramatically during different stages of the cell cycle and their protein level is subject to rapid ubiquitin-mediated proteolysis (King *et al*., 1996; Koepp *et al*., 1999). Cyclins are divided into mitotic cyclins and G1 cyclins, with further conserved subgroups within these classes (Renaudin *et al*., 1996). The mitotic cyclins are the regulating step of root growth and respond to auxin. The G1 cyclins are induced rapidly by cytokinin during the G1 phase of cells re-entering the cell cycle (den Boer and Murray, 2000).

### 1.2.6 Genes encoding proteins involved in synthesis and modification of cell walls

Plant cell elongation involves the absorption of solutes and water into the cell that, in combination with cell wall loosening, drives cell expansion (Okamoto-Nakazato *et al*., 2000). Since it is known that auxin is also involved in the modulation of membrane function (Napier and Venis, 1995), it might be not surprising that genes encoding proteins responsible for cell wall synthesis and modification are differentially expressed under the influence of auxin.

Three classes of structural cell-wall proteins have been identified: 1) hydroxyproline-rich glycoproteins (HRGPs), 2) glycine-rich proteins, and 3) proline-rich proteins (PRPs).

Several of the genes encoding these proteins have been shown to be differentially expressed in plant defense. HRGPs and PRPs have also been demonstrated to be auxin-induced (Sitbon and Perrot-Rechenmann, 1997).

### 1.2.7 Ubiquitin-related genes

Ubiquitins are important in the regulation of the cell cycle by targeting proteins for degradation. The formation of ubiquitin-protein conjugates requires 3 components: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) that functions in substrate recognition. Ubiquitin is then covalently attached to an $-NH_2$ group of a lysine residue within the substrate protein (Haas and Siepmann, 1998). Polyubiquitinated proteins are eventually degraded by the 26S proteasome (Koepp *et al*., 1999; Gray and Estelle, 2000). Several genes involved in the auxin signal transduction pathway have been cloned after mutant analysis and turned out to be components of the ubiquitin pathway (Leyser *et al*., 1993; Ruegger *et al*., 1998). In a model reviewed by Gray and Estelle (2000), it was suggested that ubiquitin may be involved in the regulation of repression of early auxin-response genes. As potential candidates for repressor genes that are regulated by this pathway, members of the *AUX/IAA* family and *SAR1* (see also Cernac *et al*., 1997) are mentioned.

### 1.2.8 Genes involved in wounding and pathogenesis response

The response of plants to pathogens is a complex one. The hypersensitive response of resistant plants to pathogens leads to localized cell death. Genes induced by pathogens are actually often downregulated by auxin (see for an example Pasquali *et al*., 1992).
The response to wounding is regulated by auxin as well. Genes involved in pathogen defense are often also induced by wounding. Some of the genes involved in this response have been shown to be repressed by auxin (e.g. β-1,3-glucanase reported by Mohnen *et al*., 1985; chitinase, described by Shinshi *et al*., 1987). Also, it has been found that IAA levels decline after wounding (Thronburg and Li, 1991).

### 1.2.9 Genes involved in secondary metabolism

Some aspects of plant secondary metabolism appear to be regulated by auxin. Since auxin seems to function as a switch between active cell division and secondary metabolite formation, two processes that have quite consistently been reported to be inversely related (Knobloch and Berlin, 1980; Sakuta and Komamine, 1987; Pasquali *et al*., 1992), the inhibiting effect of auxin on the gene expression of certain enzymes involved in secondary metabolism is not surprising. However, an interesting question is whether the pathway for auxin downregulation uses the same receptor involved in the auxin upregulation of genes (Palme *et al*., 1991).

Genes involved in terpenoid indole alkaloid biosynthesis, whose compounds not absolutely essential for the survival of individual cells but are thought to contribute to the overall fitness of the organism, are downregulated by auxin in different plant species, like carrot and *Catharanthus roseus* (Pasquali *et al*., 1992).

However, not all genes involved in secondary metabolism are repressed by auxin. Alcohol dehydrogenase (ADH) accelerates the ethanolic fermentation and is necessary for the removal of acetaldehyde and for tight cytoplasmic pH regulation (Roberts *et al*., 1984; Pereta and Alpi, 1991; Dolferus *et al*., 1997; Kato-Noguchi, 2000). The biological role for ADH is clear only under conditions in which the oxygen pressure is low. However, ADH is expressed under the influence of a number of environmental stresses (low oxygen, dehydration, low temperature). Gene expression is also induced by several plant hormones of which auxin seems to be the most active (Kato-Noguchi, 2000).

### 1.2.10 Genes with unknown function

A large number of genes induced by auxin belong to the group of genes with unknown function. Some of the genes belonging to this group are among the first auxin-inducible genes isolated, such as *gh3* (Hagen *et al*., 1984) and *saur* (McClure and Guilfoyle, 1987). An interesting member of this class of genes is the *ARCA* gene. This gene was originally isolated from tobacco suspension cultures (Ishida *et al*., 1993), and several homologues have been isolated from other plants since then (Iwasaki *et al*., 1995; Kwak *et al*., 1997; McKhann *et al*., 1997; Vahlkamp and Palme, 1997; Kiyosue and Ryan, 1999; Perennes *et al*., 1999). Most members of this family are induced by auxin, not by other plant

hormones. An exception to this rule is the *ARCA* gene from *Medicago sativa*: it is induced by cytokinin, whereas induction by auxin could not be shown (McKhann *et al*., 1997). Since part of the research described in this thesis concerns the study on the *Arabidopsis* homologue of *ARCA*, this family of genes will be discussed in more detail in the next chapter.

## 1.3 ARCA belongs to the family of WD40 repeat proteins

ARCA belongs to the large WD40 repeat protein family (more than 140 members identified so far) whose family members have very diverse functions but all contain 4-16 copies of a conserved motif: the Trp-Asp (WD) repeat. Several of these proteins have been shown to be involved in signal transduction (see also Neer *et al*., 1994 and Smith *et al.*, 1999 for reviews).

Typical members of the WD40 repeat protein family are the β subunits of heterotrimeric G proteins, the subunits of phosphatase, and the anchors for protein kinase C. However, WD40 repeat proteins are also involved in other processes, such as RNA-processing, transcriptional regulation, cytoskeleton assembly, mitotic-spindle formation, vesicle formation and trafficking, and the control of various aspects of cell division (Smith *et al*., 1999).

Depending on the structure and sequence of the WD repeats, the family has been divided in several subfamilies. The typical motif structure of these subfamilies is shown in Figure 1-1. Each WD repeat consists of a conserved core of about 40 amino acids and a variable region of 7-11 amino acids at the N-terminus. The core typically starts with Gly-His (GH) and ends with Trp-Asp (WD) (Garcia-Higuera *et al.*, 1998).

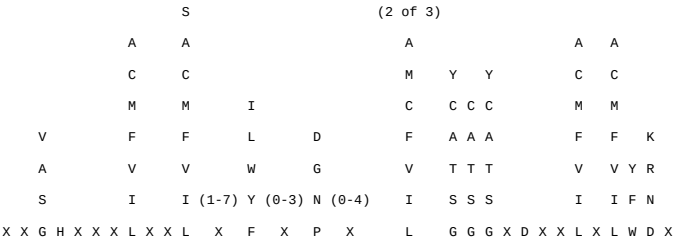



**Figure 1-1: Consensus sequence of WD repeat proteins.** In this table, X is any amino acid. Underlined regions are beta sheets. The second beta sheet starts at the last amino acid of this group (0-4 possible). Adapted from Neer *et al.* (1994).

The crystal structure of one family member has previously been determined: Gβ (Wall *et al.*, 1995 and Sondek *et al.*, 1996). This β subunit of heterotrimeric proteins consists of an N-terminal α-helix followed by seven WD repeats. These WD repeats form a ring of seven antiparallel β-sheets to make up a propeller with seven blades. It is likely that all family members of the WD family create such a propeller structure, although with varying numbers of blades, depending on the number of WD repeats (Smith *et al*., 1999).

The *ARCA* gene from plants is homologous to the *RACK* (Receptor for Activated C Kinase) gene which was initially isolated from rat brain (Ron *et al*., 1994). *In vivo*, RACKs are thought to anchor translocated, active protein kinase C (PKC) to specific membrane domains proximal to appropriate substrate proteins and consequently promote substrate phosphorylation (Mochly-Rosen and Gordon, 1998). Although there are some reports on cross-reactivity of plant extracts with antibodies from animal systems, which indicate that PKC might be present in plants (Kuo *et al*., 1995), no genes for PKC have so far been isolated. Since recent results suggest that PKC may be a conserved regulator for cell cycle events that links signal transduction pathways with the cell-cycle machinery (Livneh and Fishman, 1997), the study on regulators of PKC activity has become more intense. The fact that *ARCA* might share a common function between animal and plant systems and might provide a direct link between auxin response and cell-cycle regulation makes this gene a very interesting subject for studies on auxin signal transduction. Analysis of the pathways the *ARCA* gene from plants is involved in may give a better insight in the way auxin acts at a molecular level. The molecular response of auxin is studied by the analysis of differential

expression of auxin-responsive genes. Several methods have been developed to study these genes.

## 1.4 Characterization of the auxin response

For the dissection of auxin-regulated pathways on the molecular level, different approaches can be chosen. The analysis of mutants can be used to study different effects of the interruption of the response pathways and thereby isolate the mutated genes and find the chronological order of response. A combination of molecular genetic and microscopic approaches can be used to analyze promoter activity and to study the effects of auxin at a subcellular level. Finally, different molecular-genetic techniques have been developed for the isolation and characterization of auxin-responsive genes.

### 1.4.1 Mutant analysis and auxin action

Although many auxin-responsive genes have been identified, the precise biological and biochemical function of their gene products remains unclear. One of the methods used to shed light on components required for auxin response is the analysis of mutants. In an attempt to identify the function of all genes in *Arabidopsis*, large-scale insertional mutagenesis projects have become very important. Vast resources of gene knockouts can be subjected to different types of reverse genetic screens to deduce the function of genes sequenced in the *Arabidopsis* sequencing project (Martienssen, 1998; Parinov and Sundaresan, 2000).

A wide range of mutants in auxin response have been identified in *Arabidopsis*. These all define genes required for normal auxin signaling and can be divided in two groups: 1) those that are auxin-resistant (*axr*) and 2) those that exhibit resistance to auxin transport inhibitors (*tir*). The genes encoding AXR1 and TIR1 have been cloned and have been shown to be components of the ubiquitin pathway (Leyser *et al*., 1993; Ruegger *et al*., 1998). The *AXR1* gene encodes a protein with similarity to the amino-terminal half of the ubiquitin-activating enzyme E1, which catalyzes the first step in the pathway leading to the conjugation of ubiquitin to specific target proteins. Ubiquitination can target proteins for degradation, and regulated degradation has been shown to be an important mechanism for

signal transduction in many systems such as cell cycle progression in yeast (Leyser and Berleth, 1999). The hypothesis that AXR1 functions in a ubiquitin pathway has greatly been strengthened by the characterization of the *TIR1* gene in *Arabidopsis*. Mutations in *TIR1* result in an auxin-resistant phenotype that is similar but less severe than *axr1* (Leyser and Berleth, 1999). *TIR1* encodes an F-box protein that is part of the ubiquitin-protein ligase complex E3 (Walker and Estelle, 1998; Leyser and Berleth, 1999). These data suggest that regulated protein degradation is one of the main pathways involved in auxin signal transduction. To identify target proteins for auxin-regulated protein degradation, genetic screens have been used to identify mutants that can suppress the phenotype of *axr1*. The best characterized of these are in the Suppressor of Auxin Resistance (*SAR1*) locus (Cernac *et al*., 1997). The *sar1* mutation suppresses the auxin resistance in an *axr1* background, and the gene was shown to act downstream of the *AXR1* gene in auxin response (Cernac *et al*., 1997). This could be explained if SAR1 is a target for AXR1-mediated degradation.

Members of the IAA/AUX family of proteins have been identified as possible targets for auxin-regulated protein degradation through a variety of methods (Leyser and Berleth, 1999). It has been shown by Ulmasov *et al*. (1997) that members from this family repress their own transcription. Combined with the evidence that a rapid turn over of the repressor of *IAA/AUX* gene transcription exists, it may be possible that the transcription of these genes is regulated by a negative feedback cycle temporarily broken by auxin-accelerated degradation of the encoded proteins (Leyser and Berleth, 1999).

The *pin1* mutant shows impaired auxin transport in stem segments and structural similarity to bacterial membrane transporters (Gälweiler *et al*., 1998). The precise regulation of auxin transport and redistribution has been implicated in many aspects of development, such as the establishment of embryonic axis, the continuous differentiation of vascular tissues and tropic growth responses (e.g. gravitropism, Leyser and Berleth, 1999). The *PIN1* gene product has been identified as a basally localized plasma membrane protein involved in auxin efflux across the apical-basal axis.

### 1.4.2 Elucidation of gene function by non-genetic methods

The functional analysis of a gene of interest implicates the use of reporter genes fused to its promoter, or to the gene of interest using a strong promoter, to visualize the spatial and temporal regulation of its expression (reviewed by Godde *et al.*, 1999). Ideal reporter gene expression should be easily detectable and reflect promoter activity as well as the (sub)cellular localization of the gene product. The most widely used reporter genes are those encoding for the green fluorescent protein (GFP), aequorin, luciferase and β–glucuronidase (GUS) (Godde *et al*., 1999).

To improve the repertoire of methods used to study molecules in living cells and to overcome some of the limitations observed with the frequently used reporters, several new approaches have been developed (Tsien and Miyawaki, 1998). One of these approaches has been the development of a new protein labeling technique, which makes use of a small peptide motif (Griffin *et al*., 1998). The advantage of this is that fusion to these small motifs does not interfere with the labeled protein properties, a phenomenon observed with the bulky reporters used so far. Another approach has been the development of the β–lactamase reporter assay (Zlokarnik *et al*., 1998). This technique makes use of the catalytically active β–lactamase as a reporter, for cleavage of a dye (CCF2) which causes a color change. The advantage of this technique is the sensitivity and the short half-life of β–lactamase.

Important tools used for qualitatively assessing these reporter systems in living cells are several, new developed microscopic techniques (e.g. fluorescence resonance energy transfer microscopy (FRET), confocal laser scanning microscopy (CSLM), two-/multi-photon laser scanning microscopy (MPLSM), digital imaging microscopy (DIM), and fluorescence lifetime imaging microscopy, Godde *et al*., 1999). Additionally, these different applications can be combined in several ways, dependent of the special tasks.

Another approach is the use of new fluorescent indicator-dyes to monitor spatial and temporal changes in physiological parameters (e.g. $Ca^{2+}$ concentration, pH, or membrane potential) and the methods for their delivery into the cell (Godde *et al*., 1999). The visualization of the physiological responses is an important tool for the elucidation of gene function. Once a gene sequence has been identified, the study of its function is facilitated. A first indication on gene function can be determined on the basis of its involvement in

certain physiological responses to different factors. The methods described in the next chapters will give an insight in the molecular methods that can be used in these studies.

### 1.4.3 Isolation of differentially expressed genes

The study on differential expression of genes under the influence of various biotic and abiotic factors has been a main research topic in the last few decades. Among the first methods that were applied is subtractive hybridization of cDNA libraries, in which phage libraries are hybridized with the various samples and plaques that show differential hybridization are isolated (St. John and Davis, 1979). The second method that was used for many years is subtractive hybridization of cDNA with mRNA, in which cDNA of one sample is mixed with mRNA of the other sample and hybrids are removed by chromatography on hydroxyapatite and discarded (Zimmermann *et al*., 1980). The difficulties with these techniques were the complexity, the labor intensity and the fact that they were unreliable (Wolff, 1999). After development of PCR, the methods for the isolation of differentially expressed genes were improved. One of the first techniques that was used broadly was the differential display reverse transcription PCR (DDRT-PCR) or differential display (Liang and Pardee, 1992). In this method, partial cDNA sequences are amplified by reverse transcription and PCR by random priming. Differentially expressed genes are identified by analysis on polyacrylamide gels. An adaptation of this method was actually used to isolate new differentially expressed genes responding to auxin in tobacco (Roux *et al*., 1998).

In general, two main groups of methods for the isolation of differentially expressed genes can be recognized: 1. DDRT-PCR-based methods and 2. PCR-based methods. The DDRT-PCR-based methods rely on the use of polyacrylamide gels for the identification of differentially expressed genes. Two samples are compared on one gel and fragments can be isolated. Apart from differential display, also the method of cDNA-AFLP belongs in this group. The method of AFLP (Amplified Fragment Length Polymorphism) was originally established for the analysis of genomic DNA (Vos *et al*., 1995). However, as was shown later (Bachem *et al*., 1996; Money *et al*., 1996), this method could also be adapted for differential display analysis of genes.

For PCR-based methods the genes that show no difference in expression are eliminated in a series of subtractive hybridizations followed by PCR. Only those genes that show

differential expression can be amplified. A typical member of this group is the cDNA-RDA. Representational Difference Analysis (RDA) was originally developed for analysis of genomic DNA (Lisityn *et al*., 1993) and later adapted for the identification of differentially expressed genes (Hubank and Schatz, 1994). In this method only two different samples can be compared. The technique of suppression subtractive hybridization (SSH) (Diatchenko *et al*., 1996; Akopyants *et al*., 1998) is based on the RDA method and is supposed to lead to a smaller number of false positives. SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress non-target DNA amplification.

The advantage of all of these techniques compared to the original protocols were the reliability, the simplicity of an one-tube method, and the fact that, since very small amounts of total RNA are needed, these methods are very advantageous in situations where the availability of RNA is limited (Kozian and Kirschbaum, 1999; Terryn *et al*., 1999). However, all of these techniques share some major drawbacks. The main drawbacks are the high number of false positives that is isolated by these techniques, upto 70% of all isolated clones in the case of differential display (Sun *et al*., 1994), and the isolation of partial sequences which may not lead to gene identification. Also, the DDRT-PCR-based methods have the disadvantage that only redundant mRNA species can be isolated (Bertioli *et al*., 1995) and that the amplified sequences mainly contain the 3' untranslated region, which makes further analysis of the gene identity difficult (Bachem *et al*., 1996). In the PCR-based techniques the isolation of rare transcripts is possible (O'Neill and Sinclair, 1997), although this is more a matter of luck (Karrer *et al*., 1995). But also the fact that differential expression could be monitored to some extend, but not quantified, led to the development of other techniques. One of these techniques was the serial analysis of gene expression (SAGE) (Velculescu *et al*., 1995).

In this method, cDNAs are digested with a number of different enzymes to create small fragments of about 10-13 bp in length, each so-called tag representing one mRNA. These tags are then ligated together to concatamers that are cloned and sequenced. The original protocol has been optimized for increased concatemer length, resulting in an average size of 67 tags in comparison with 22 tags in the original protocol (Kenzelmann and Mühlemann, 1999). The frequencies of certain fragments in the chimeric sequences is a measure for the redundancy of certain mRNAs in the RNA population. The main advantage comes from the fact that differential expression is monitored by the abundance

of certain tags in the sample, and that measures can be more exact. Also, all genes can be monitored in one experiment in a relatively short period of time (Harmer and Kay, 2000). Unfortunately, the length of the tags makes this technique not appropriate for studies on organisms for which no large databases of ESTs are available. Also, rare transcripts are likely to be missed by this technique (Harmer and Kay, 2000).

A relatively new method for the analysis of differential gene expression that shows a huge potential is the use of high density arrays or DNA arrays. These arrays have the main advantage in that they are all well-suited for the analysis of gene expression in high throughput projects with a relatively small amount of effort. In general, these arrays have clearly identified genes or primers spotted in a very high density on a solid support. This allows for the characterization of large numbers of genes in a small set of experiments. Furthermore, the use of arrays makes it possible to quantify the differential expression and to select for differentially expressed genes according to clear measures (e.g. only those genes that show a difference of more than 3-fold, or 5-fold, or 10-fold). In the next chapter, the different types of DNA arrays will be described in more detail and their advantages and disadvantages when compared to each other and the methods described above will be discussed.

### 1.4.8 DNA arrays

Three types of DNA arrays can be distinguished: 1) oligonucleotide arrays, 2) cDNA arrays on glass support, and 3) cDNA arrays on nylon. The use of either type of arrays (or chips) has its advantages and disadvantages (for an overview see Duggan *et al*., 1999). At the end of this chapter, in Table 1-1, an overview of the specifics of the three types of arrays will be given.

On oligonucleotide arrays, series of gene-specific oligonucleotides are synthesized on a glass chip by a process of photolithography (Fodor *et al*., 1993; Lipschutz *et al*., 1999). This method has been the basis of the method used by Affymetrix (Santa Clara, California) and has also been described extensively by Meier-Ewert *et al*. (1998). For each gene monitored on these chips, a set of 5-8 gene-specific oligonucleotides is used. Also, a set of

5-8 gene-specific false-positive (mismatching) oligonucleotides is synthesized as a negative control.

The chips are hybridized with two fluorescent probes simultaneously. In general these probes are made by reverse transcription of poly$A^+$ purified mRNA in the presence of *Cy3*- or *Cy5*-dUTP. This will result in either green (*Cy3*) or red (*Cy5*) fluorescence. These probes can be mixed in the same hybridization solution and scanned simultaneously. A red signal indicates a stronger expression in the sample labeled with *Cy5*, while a green signal indicates a stronger expression in the sample labeled with *Cy3* and a yellow signal indicates equal expression in both samples. At present, new labeling dyes are being developed to increase the number of samples studied per hybridization.

These chips use specific oligos, which requests the specification of the target genes, thereby eliminating the possibility of identifying new cDNA species. However, in the light of the genome sequencing projects (yeast, human and *Arabidopsis*), in which many putative open reading frames are identified, this type of chips is very useful, since the use of specific oligonucleotides does not require the cDNA isolation of potential genes. Unfortunately, the use of 10-16 oligos per gene on the chip, decreases the number of genes that can be spotted drastically, which leads to high costs.

The second type of arrays used is the cDNA (micro)array on glass support. In this type of arrays, PCR fragments of cDNAs, are spotted in high density on a glass slide coated with special chemicals (for an overview see Duggan *et al*., 1999; Kehoe *et al*., 1999; Schaffer *et al*., 2000). Selected genes are used as negative controls, and the hybridization is as described for the oligonucleotide arrays. Compared to the oligonucleotide-based chips, the main advantage is flexibility because of the use of cDNAs. Although different genes within gene families cannot be distinguished, genes spotted on the chip do not have to be pre-selected or even pre-sequenced, which makes this type of arrays more flexible. Furthermore, since simpler chemistry is used for DNA attachment on the glass, this method is cheaper and can be performed in normal laboratories.

The type of array that is simple and therefore used very often, is the cDNA array on nylon support. As with the cDNA arrays on glass support, PCR fragments of cDNAs are spotted by robot on the support. Compared to the glass chips, the chemistry is very simple and is described in more detail later in this thesis. The probes are labeled using radioactivity (in general $^{33}$P-αdCTP). Per hybridization, therefore only one sample can be tested, although the array can be used at least 8-10 times. Since the volume used in these hybridizations is

much larger, part of the sensitivity is lost because of probe dilution. However, the amount of sample used for the probe is still lower than with fluorescence. The sample quality does not have to be extremely high (total RNA can be used, in contrast of the both other types of arrays, that need high quality purified mRNA). The main advantage of this type of array compared to the other arrays is that the methods used are relatively simple and are based on standard laboratory methods as described by Sambrook *et al*. (1989). As with the cDNA arrays on glass support, the disadvantage when compared to oligonucleotide arrays is that the fact that it is often impossible to discriminate between different members of the same gene family.

| *Parameters* | *Oligonucleotide arrays* | *cDNA on glass* | *cDNA on nylon* |
|---|---|---|---|
| Targets | 20-mer oligo's | PCR products | PCR products |
| Detection limit | 1 in 300,000 | 1 in 100,000 | 1 in 10-50,000 |
| Specificity* | High | Moderate | Moderate |
| # of elements/array | 8,000 | >20,000 | >20,000 |
| Cost | Very high | High | Low |
| Element quality | Essentially high | Good source quality | Good source quality |
| Probe quality | High | High | Moderate |
| Probe | 2-5 µg mRNA | 2-5 µg mRNA | 20-30 µg total RNA |
| Labeling method | Fluorescence | Fluorescence | Radioactivity |
| Hybridization volume | 10 µl | 10 µl | 10-20 ml |
| # of experiments/array | 1 per color | 1 per color | >8 per array |

**Table 1-1: Typical features of different types of arrays**. *: The specificity in the discrimination between different members of the same gene family; #: number; /: per. This table is based on results from Bertucci *et al*., 1999; Schaffer *et al*., 2000.

In conclusion it can be stated that the DNA arrays have many advantages over the methods described in the previous chapter. The main advantage lies in the relatively short amount of time that is necessary to obtain quantified data on a large set of genes and the small number of false positives. The type of array that is best-suited is dependent on the question that is asked. The oligonucleotide arrays are very specific, which makes them very suitable for the comparison of different members of the same gene family or the detection of SNPs. The use of cDNA arrays allows for a semi-blind approach in which not all genes on the

array have been sequenced, is simple, can be home-made and is more cost-effective than the oligonucleotide arrays. The use of glass-based cDNA arrays has the advantage of the direct comparison between two (or even more in the near future) samples, eliminating thereby the effect of hybridization differences. However, this type of array is less suitable for running time course experiments than the radioactivity-based arrays. The latter have also the advantage of being very cost-effective and providing researchers with a simple but extremely powerful method for the investigation of differential expression of genes.

Although the application of cDNA arrays is a hot topic at the moment in literature, only few research papers have been published so far, especially in plant research. Still, some research has been done, even on *Arabidopsis*, using small subsets of genes (Gress *et al*., 1992; Desprez *et al*., 1998; Giegé *et al*., 1998; Ruan *et al*., 1998; Spellman *et al*., 1998; van Hal *et al*., 2000). However, the power of this novel method becomes clear from these publications. The best overview on the possibilities of this technique comes from a recent publication by Hughes *et al*. (2000), in which cluster analysis of expression profiles from all open reading frames of *S. cerevisiae* after 300 different treatments and gene mutations was studied. By using this method it became possible to determine the cellular pathways affected by pattern matching, even among very subtle profiles.

## 1.5 Experimental goal

The goal of the work described in this thesis was the analysis of differential gene expression in response to auxin. These studies should be based on the characterization of the *Arabidopsis* homologue of the *ARCA* gene from tobacco. This gene shows very high homology to animal counterparts that are involved in the regulation of key regulator of cell division in animal cells, protein kinase C, which so far has not been isolated from plant systems. Upon auxin induction in suspension cells, the *ARCA* expression is induced, which may provide a direct link between auxin and cell division. Since *Arabidopsis* is a well-studies plant species and the genetic tools available for the analysis of this plant are established in our institute, it was decided to characterize this auxin-responsive gene and the pathways it is involved in, in this plant. The analysis should lead to the following flow chart:

1. Identification of the *Arabidopsis thaliana ARCA* homologue and gene analysis.
2. Characterization of the *AtARCA* expression pattern in a suspension cell system by northern (dot) blots.
3. Identification of a *ARCA* knockout line (*En1*-library) and phenotypical analysis of the line.
4. Study of the differential gene expression profile between wildtype and ARCA knockout suspension cells, with and without auxin treatment.
5. For the analysis described in point 4 the establishment of cDNA arrays will be necessary.

# 2 MATERIALS

## 2.1 Organisms

### 2.1.1 Plants

*Arabidopsis thaliana* (L.) Heynh., ecotype Columbia 0

*Arabidopsis thaliana* (L.) Heynh., ecotype Columbia 0, suspension culture line At7 (provided by Dr. I. Sommsich)

### 2.1.2 Bacteria

*Escherichia coli*

DH5 α F$^-$, φ80d*lac*Z ΔM15, Δ(*lac*ZYA-argF), U169, *end*A1, *rec*A1, *hsd*R17, ($r_k^-$-$m_k^+$), *deo*R, *thi*-1, *sup*E44, λ-, *gyr*A96, *rel*A1 (Hanahan, 1983).

## 2.2 DNA

### 2.2.1 Libraries

EST library Ohio State University *Arabidopsis thaliana* EST library

### 2.2.2 Plasmids and vectors

| Vector | Selection | Reference; | GeneBank Accession |
|---|---|---|---|
| pUC18 | $Amp^R$ | Vieira and Messing (1982) | L08752 |
| pBluescript SK(-) | $Amp^R$ | Short *et al.* (1988) | X52330 |

### 2.2.3 Oligonucleotides

| Oligo | Sequence 5'-3' | Ann.ealing temperature |
|---|---|---|
| T7 | GTAATACGACTCACTATAGGGC | 55 °C |
| T7-SHORT | ACGACTACTATAG | -- |
| EST-FW | CCCAGTCACGACGTTGTAAAACG | 63 °C |
| EST-RV | AGCGGATAACAATTTCACACAGG | 63 °C |
| AAEnFW | ATGGCGGAAGGACTCGTTTTGAAG | 60 °C |
| AAEnRV | GACCAATACCCCAAACTCTAATGA | 60 °C |
| AA5' | CGATTGCCGTCACCATGTCAGTGTGT | 60 °C |
| AA3' | CTTCCCGCGACAAATCCATCATTTTG | 60 °C |
| AA5'-NEST | CGCATGGTGCCCTTCAAAACGAGTCC | 65 °C |
| AA3'-NEST | GCGTCTCACTGGTCACTCTCACTTCG | 65 °C |

## 2.3 Chemicals

| | |
|---|---|
| β-mercaptoethanol | J.T. Baker Chemicals, Deventer |
| [α-$^{32}$P] dCTP | Amersham Buchler, Braunschweig |
| [γ-$^{33}$P] ATP | Amersham Buchler, Braunschweig |
| [α-$^{33}$P] dCTP | Amersham Buchler, Braunschweig |
| 1-NAA (1-naphthalene acetic acid) | Biomol, Hamburg |
| 2,4-D (2,4-dichlorophenoxyacetic acid) | Ducheva, Haarlem |
| 2-NAA (2-naphthalene acetic acid) | Sigma, München |
| Agar | Gibco BRL, Gaithersburg |
| Agarose | Gibco BRL, Gaithersburg |
| Amino acids for media | Sigma, München |
| Ampicillin | Biomol, Hamburg |
| Aphidicolin | Sigma, München |
| Bacto peptone | Difco Laboratories, Detroit |
| Bacto tryptone | Difco Laboratories, Detroit |
| BAP ($N^6$-Benzyladenine) | Ducheva, Haarlem |

| | | |
|---|---|---|
| Colchicine | | Sigma, München |
| CTAB (Hexadecyltrimethyl-ammoniumbromide) | | Sigma, München |
| Cycloheximide | | Sigma, München |
| DEPC (Diethylpyrocarbonate) | | Sigma, München |
| dNTP's: | for PCR on EST library | Roth, Karlsruhe |
| | for most other purposes | Roche Mol. Biochem., Mannheim |
| DTT (Dithiothreitol) | | Gibco BRL, Gaithersburg |
| Ethanol | | J.T. Baker Chemicals, Deventer |
| Ethidium bromide | | Sigma, München |
| Fish sperm DNA | | Roche Mol. Biochem., Mannheim |
| Glycogen (10 µg/µl) | | Roche Mol. Biochem., Mannheim |
| IAA (Indole-3-acetic acid) | | Sigma, München |
| IPTG (Isopropyl-β-D-thiogalacto-pyranoside) | | Biomol, Hamburg |
| Mineral oil | | Sigma, München |
| MOPS (3-(N-Morpholino)-propanesulphonic acid) | | Serva Feinbiochemika, Heidelberg |
| MS basal salt mixture | | Sigma, München |
| Oligo(dT)$_{12-18}$ primer (500 ng/µl) | | Pharmacia Biotech, Uppsala |
| Oligonucleotides | | MWG Biotech, Ebersberg |
| PCIB (2-(p-chlorophenoxy)-2-methylpropionic acid) | | Sigma, München |
| Phage λ DNA (500 ng/µl) | | New England Biolabs, Schwalbach |
| Propyzamid | | Serva Feinbiochemika, Heidelberg |
| PVP (Polyvinylpyrrolidon) | | Fluka, Neu-Ulm |
| RNA size marker | | Gibco BRL, Gaithersburg |
| Sarcosyl solution 30% | | Serva Feinbiochemika, Heidelberg |
| SDS (Sodium dodecylsulphate) | | Serva Feinbiochemika, Heidelberg |
| Tris(hydroxymethyl)aminomethan | | Gibco BRL, Gaithersburg |
| Tween-20 | | J.T. Baker Chemicals, Deventer |
| X-Gal | | Biomol, Hamburg |
| Yeast extract | | Gibco BRL, Gaithersburg |
| Yeast nitrogen base | | Difco Laboratories, Detroit |
| Yeast tRNA | | Roche Mol. Biochem., Mannheim |

If not mentioned in this list, chemicals were pure or p.a. chemicals from Merck, Darmstadt and Roth, Karlsruhe.

## 2.4 Enzymes

| | |
|---|---|
| *Pfu* DNA polymerase (recombinant) | Stratagene, La Jolla |
| Restriction enzymes | New England Biolabs, Schwalbach |
| | Roche Mol. Biochem., Mannheim |
| Reverse transcriptase *Superscript™ II* (200 u/µl) | Gibco BRL, Gaithersburg |
| RNase A | Sigma, München |
| SAP (Shrimp alkaline phosphatase) | USB™, Cleveland |
| T4 DNA ligase | Roche Mol. Biochem., Mannheim |
| T4 DNA polymerase | BRL Life Science |
| | New England Biolabs, Schwalbach |
| T4 polynucleotide kinase | New England Biolabs, Schwalbach |
| *Taq* DNA polymerase | Gibco BRL, Gaithersburg |

## 2.5 Disposables

| | | |
|---|---|---|
| 3MM paper | | Whatman, Maidstone |
| Nylon membranes | *Hybond N* | Amersham Buchler, Braunschweig |
| | *Hybond N*$^+$ | Amersham Buchler, Braunschweig |
| | *BioDyne B* | Pall, Portsmouth |
| | *BioDyne H* | Pall, Portsmouth |
| Filters colony lift | *HA filters 0.45 µm* | Millipore, Molsheim |
| Miracloth | | Calbiochem, La Jolla |
| Sterile filter units: | *CN(150 ml/0.2 µm)* | Nalgene, Rochester |
| | 0.2 µm and 0.45 µm | Sartorius, Göttingen |
| Kits: | *Qiaquick Spin Mini Kit* | Qiagen, Hilden |
| | *QIAprep Midi Kit* | Qiagen, Hilden |

| | | |
|---|---|---|
| | *Rediprime DNA labeling system* | Amersham Buchler, Braunschweig |
| | *High Pure PCR Purification Kit* | Roche Mol. Biochem., Mannheim |
| | *RNeasy Plant Mini Kit* | Qiagen, Hilden |

## 2.6 Equipment

| | | |
|---|---|---|
| Centrifuges: | *5417C tabletop* | Eppendorf, Engelsdorf |
| | *Biofuge A* | Heraeus Sepatech, Düsseldorf |
| | *J2-21*, rotors *JA-14*; *JA 20* | Beckman Instruments, München |
| | *Varifuge 3.2 RS* | Heraeus Sepatech, Düsseldorf |
| Computer based imaging system | | Intas, Göttingen |
| Electroporator | *Gene pulser* | BioRad, München |
| Homogenizer | *RW18* | IKA Labortechnik, Staufen i. Br. |
| Hybridization ovens | *Herahybrid 6* | Heraeus, Hanau |
| | *7601* | GFL, Burgwedel |
| Liquid scint. counter | *LS5000TD* | Beckman Instruments, München |
| | *LS6500* | Beckman Instruments, München |
| PCR machines: | *PTC DNA Engine Tetrad* | MJ Research, Watertown |
| | *Tri-Thermoblock* | Biometra, Göttingen |
| | *GeneAmp™ PCR System 9600* | Perkin Elmer, Weiterstadt |
| Phosphor screens | | Molecular Dynamics, Sunnyvale |
| PhosphorImager | *Storm 860* | Molecular Dynamics, Sunnyvale |
| Spectrophotometer | *UV/VIS Lambda 20* | Perkin Elmer, Weiterstadt |
| Spotting robot | *BIOGRID* | Biorobotics |
| Thermo blocks | *Thermostat 5320* | Eppendorf, Engelsdorf |
| | *Dri-block DB-3* | Techne, Duxford |
| Thermo shaker | *Thermomixer 5436* | Eppendorf, Engelsdorf |
| UV cross-linker | *Stratalinker 2400* | Stratagene, La Jolla |

## 2.7 Electronic data management

### 2.7.1 Hardware

| | |
|---|---|
| Power Macintosh 6500/250 | Apple |
| Power Macintosh 8100/100 | Apple |
| Power Macintosh 8500/150 | Apple |
| Power Macintosh 9600/233 | Apple |
| Precision 410 PentiumIII/500 | Dell Computer Corporation |

### 2.7.2 Software

| | |
|---|---|
| Adobe Photoshop 4.0 | Adobe Systems |
| ArrayVision 4.1.7/5.1 | Imaging Research Inc. |
| Canvas 5.0.2 | Deneba Systems |
| ClarisDraw | Claris |
| DNASTAR | Lasergene |
| ImageQuant 2.0 | Molecular Dynamics |
| Microsoft® Excel 97 (PC) & 98 (Mac) | Microsoft Corporation |
| Microsoft® Visual Basic for Excel 97 | Microsoft Corporation |
| Microsoft® Word 6.0.1 & 98 | Microsoft Corporation |
| Netscape Navigator 3.0-4.7 | Netscape |
| Oligo 4.0-s | National Bioscience |

### 2.7.3 WWW-sites

| | |
|---|---|
| *Arabidopsis* Gene Hunter | http://genome-www.stanford.edu/cgi-bin/ |
| BLAST | http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/ |
| Entrez | http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/ |
| MOTIF | http://www.motif.genome.ad.jp/ |
| OVID | http://ovid.gwdg.de/ |
| PLACE | http://www.dna.affrc.go.jp/htdocs/PLACE/ |
| PSORT | http://psort.nibb.ac.jp/form.html |
| Genemark | http://dixie.biology.gatech.edu/Genemark/eukhmm.cgi |

## 2.8 Culture media

All media were autoclaved for 20 min, unless stated different. If plates were made, for bacteria 15 g/l agar was added, for yeast 20 g/l.

### 2.8.1 Bacterial media

| | | | | |
|---|---|---|---|---|
| LB | 1 | % (w/v) | Bacto tryptone | |
| | 0,5 | % (w/v) | Yeast extract | |
| | 0,5 | % (w/v) | NaCl | pH 7.5 |

| | | | | |
|---|---|---|---|---|
| 2YT | 1,6 | % (w/v) | Bacto tryptone | |
| | 1 | % (w/v) | Yeast extract | |
| | 1 | % (w/v) | NaCl | |
| | 1 | mM | $MgSO_4$ | |
| | 100 | mM | Tris | pH 7.5 |

### 2.8.2 Plant media

| | | | |
|---|---|---|---|
| *Arabidopsis* At7 medium | 0,23% (w/v) | MS basal salt mixture | |
| | 3 % (w/v) | saccharose | pH 5.7 |
| (after autoclaving) | 1 % (v/v) | 100 x B5 vitamins | |
| | 1 mg/l | 2,4-D | |
| *At* liquid culture medium | 0.,23% (w/v) | MS basal salt mixture | |
| | 3 % (w/v) | saccharose | pH 5.7 |
| | 0,1 ‰(w/v) | myo-inositol | |
| | 0,5 ‰(w/v) | MES | |

### 2.8.3 Stock solutions for antibiotics, hormones and vitamins

| | **Concentration stock** | **Solvent** | **End concentration** |
|---|---|---|---|
| 1-NAA | 1 mg/ml | $NaOH/H_2O$ | 1 mg/l |
| 2-NAA | 1 mg/ml | $NaOH/H_2O$ | 1 mg/l |
| 2,4-D | 1 mg/ml | Ethanol | 1 mg/l |
| Ampicillin | 100 mg/ml | $H_2O$ | 100 mg/l |
| Aphidicolin | | | |
| B5 vitamins (100x) | 0.1‰ (w/v) Nicotinic acid | | |
| (Gamborg *et al*., 1968) | 0.1‰ (w/v)Pyridoxine-HCl | | |
| | 0.1% (w/v) Thiamine-HCl | $H_2O$ | 1 x |
| | 0.1‰ (w/v)Glycine | | |
| BAP | 1 mg/ml | $NaOH/H_2O$ | 1 mg/l |
| Colchicine | | | |
| Cycloheximide | 50 mg/ml | $H_2O$ | 50 µg/ml |
| IAA | 1 mg/ml | $NaOH/H_2O$ | 1 mg/l |
| IPTG | 100 mM | $H_2O$ | 0.5 mM |
| PCIB | 5 mg/ml | $NaOH/H_2O$ | 1-5 mg/l |

All watery solutions were sterile filtered (0.2 µm). Storage of all solutions at –20 °C.

# 3 METHODS

## 3.1 General methods in molecular biology

Most of the methods used in this thesis are standard methods in molecular biology and described in Sambrook *et al.* (1989). I will not mention most of these methods. In the cases that a standard method was modified, this will be stated. Standard enzymatic reactions on DNA (restriction analysis, ligation, dephosphorylation, etc.) were done according to the supplier's protocols.

## 3.2 Southern blotting

Southern blotting was performed as described previously by Sambrook *et al*. (1989). A total of 3 µg of genomic DNA from *Arabidopsis* wildtype plants was used per digestion. For stringent washing conditions, blots were washed in 0.1x SSC, 0.1% SDS for 30 min at 65 °C.

## 3.3 Isolation of high quality genomic DNA from plant tissue

This method to isolate high quality genomic DNA from plant tissue is based on the protocol from Rogers and Bendich (1994). About 1 g of leaf tissue was ground under liquid nitrogen in a mortar. The ground tissue was transferred to a 50 ml reaction tube and 15 ml of pre-warmed (65 °C) 2x CTAB buffer was added. This mixture was incubated for 20 minutes at 65 °C. After incubation, 15 ml chloroform were added and after careful mixing centrifugation for 10 minutes at 5000 g was performed. The nucleic acids were then isolated by ethanol precipitation and taken up in 1 ml $H_2O$. RNA was removed by RNase treatment (5 µl; 1 mg/µl). After chloroform/isoamylalcohol (24:1) purification followed by ethanol precipitation the genomic DNA was taken up in 200 µl $H_2O$.

2x CTAB buffer: 2% CTAB (w/v); 100 mM Tris; 20 mM EDTA; 1.4 M NaCl; 1% PVP (w/v); pH 8.0

## 3.4 Isolation of genomic DNA from plant tissue for PCR

This method for the isolation of genomic DNA from plant tissue for PCR purposes is based on the protocol described by Edwards *et al.* (1991). One leaf was frozen in liquid nitrogen in a 1.5 ml reaction vial. After addition of 400 µl of lysis buffer the leaf was homogenized using a rotating glass pestle. The cell debris was pelleted after centrifugation for 1 minute. Nucleic acids were isolated from the supernatant by isopropanol precipitation and taken up in 50 µl $H_2O$. 4 µl of this template were used per PCR reaction.

Lysis buffer: 200 mM Tris; 250 mM NaCl; 25 mM EDTA; 0.5% SDS; pH 8.0

## 3.5 Radioactive labeling of DNA

For labeling of DNA probes for Northern and Southern blots with $^{32}P$ the *Rediprime DNA labeling system* based on the random labeling method was used according to manufacturer's protocols. Purification of the radioactive probe was done using the *High Pure™ PCR Purification Kit*. The activity of the sample was measured using a SL6500 liquid scintillation counter from Beckman. Only probes with a specific activity of more than $10^7$ cpm were used. Probes were denatured for 5 min at 95 °C and chilled on ice immediately before use.

## 3.6 DNA sequencing

All DNA sequencing was performed by the ADIS service unit at the MPI für Züchtungsforschung.

## 3.7 Cloning of cDNA's

In general, cDNA's were made compatible for cloning into different vectors by addition of restriction sites at the C- and N-terminus of the ORF by PCR. Primers used for these PCR's are listed above.

## 3.8 Isolation of plasmid DNA from *E. coli*

Different methods for the isolation of plasmid DNA from *E. coli* were used, based on the following experiments. The standard method for isolation of plasmid DNA was alkaline lysis. Of an O/N bacterial culture 1.5 ml were centrifuged briefly. The bacterial pellet was resuspended in 300 µl of TENS buffer. To the suspension, 150 µl of 3 M sodium acetate (pH 5.2) were added. After carefully mixing the suspension was centrifuged 10 min at full speed. The pellet consisting of cell debris was removed with a toothpick. Nucleic acids were precipitated from the supernatant using ethanol. The pellet was then dissolved in 39 µl of $H_2O$. To this 1 µl of RNase A (1 mg/ml) was added.
If very pure DNA was needed in the following experiments (sequencing), plasmid DNA was isolated using the *QIAquick Plasmid Spin Mini Kit* or for larger amounts of DNA the *Qiaprep Plasmid Midi Kit* according to the manufacturer's protocol.

TENS buffer: 10 mM Tris; 1 mM EDTA; 0.1 mM NaOH; 0.5% SDS (w/v); pH 8.0

## 3.9 PCR

PCR was performed with different programs, on different machines, in different volumes, with different polymerases. Therefore, only a general protocol can be given. In case the DNA was used for further cloning, always *Pfu* polymerase was used, because of the proofreading capabilities of this enzyme. The annealing temperature was chosen based on the primers (see

above). Changes in incubation time of the different steps in the reaction were optimized for the length and the complexity of the template (for instance longer annealing time when the template is genomic DNA).

The standard reaction protocol was:

Denaturation (5 min 94 °C) followed by 30-40 cycles consisting of denaturation (30 sec-1 min 94 °C), annealing (30 sec-1 min 55-65 °C) and elongation (1-2 min 72 °C). After these cycles a final elongation step of 5 min 72 °C was used.

For methods that make use of PCR, the different conditions will be given (i.e. the machine on which the reactions were performed, the volume of the reaction and the polymerase that was used).

## 3.10 Inverse PCR

2.25 µg of high quality genomic DNA was digested with 50 units of a restriction enzyme that has a site in the gene of interest. Digestion was done in a volume of 100 µl for 5 h. The DNA was then purified using the *High Pure™ PCR Purification Kit* according to the manufacturer's protocol. After purification the DNA was ligated in a total volume of 100 µl, in the presence of 2 units of T4 DNA ligase. Incubation was O/N at 16 °C.

A first set of PCR primers was developed. The first primer is located on the 5' part of the gene, at some distance from the ATG, pointing towards the promoter. The second primer is located near the restriction site, pointing in 3' direction. After PCR (in 50 µl on the Perkin Elmer 9600 using program 1 using a 1:1 mix of *Pfu* and *Taq* DNA polymerase) using 2 µl of ligated DNA as template DNA, the product was purified using the same kit as mentioned above.

A second set of (nested) primers was developed. PCR was performed as described earlier. After PCR products were analyzed on an agarose gel. Single bands were isolated and purified. Some DNA was sent off for sequencing, the rest was used for blunt end cloning into pUC18.

## 3.11 Isolation of total RNA from plant tissues

Plant tissue was ground with a mortar and pestle under liquid nitrogen. The isolation of total RNA was done using the *RNeasy Plant Mini Kit* (Qiagen, Hildesheim) according to the manufacturer's protocol.

## 3.12 Dot Blotting of RNA using a Vacuum Manifold

Of all samples 1 µg of RNA from each sample was loaded on an agarose gel to confirm the quality. For dot blotting of RNA on Hybond filters, 5 µg of total RNA, as determined by spectrophotometric analysis, was used in equal volumes. To this RNA 3 volumes of a formamide mix were added. This RNA mix was then incubated at 65 °C for 5 minutes and chilled on ice. To this mixture 1 volume of cold 20x SSC was added.
A prewetted Hybond membrane on 2 sheets of Whatman 3MM paper soaked in 10x SSC was casted in the dot blotting apparatus. The samples were applied in aliquots of a maximum volume of 200 µl, the remaining slots were filled with 10x SSC. A gentle vacuum was applied until all liquid ran through the filter (about 15 minutes). Then the membrane was dried on room temperature followed by cross-linking of the RNA with UV.

Formamide mix: formamide : formaldehyde 37% : 10x MOPS = 500 : 162 : 100
10x MOPS: 200 mM MOPS; 80 mM NaAc; 10 mM EDTA; pH 7.0
10x SSC: 1.5 M NaCl; 150 mM sodium citrate

## 3.13 cDNA first strand synthesis with $^{33}$P labeling

To 10 µl (25-30 µg) of total RNA, 1 µl (500 ng) oligo $dT_{12-18}$ primer was added. This was incubated for 10 min at 70 °C, then equilibrated at 43 °C for several min. RT cocktail was added and after mixing, incubation was done at 43 °C for 1 h. After incubation the RNA was hydrolyzed by addition of 1 µl 1% SDS, 1 µl 0.5 M EDTA, 3 µl 3 M NaOH and incubation at

65 °C. After 30 min the sample was transferred to RT and incubated another 15 min. Then neutralization was performed by adding 10 µl 1 M Tris-HCl pH 5.3 and 3 µl 2 N HCl. The total activity in cpm was measured at this point for later calculation of the incorporation efficiency.

To purify the cDNA, an isopropanol precipitation was done, using 5 µl (1 mg/ml) yeast tRNA as a carrier. Before centrifugation, the isopropanol mixture was incubated at –20 °C for 30 min to increase the efficiency of precipitation. The cDNA was then dissolved in 100 µl $H_2O$. The activity of the sample in cpm was measured and the incorporation efficiency was calculated. When the efficiency was higher than 30% incorporation, the sample was used in further experiments.

RT cocktail:

| | |
|---|---|
| 6 µl | 5 x RT buffer |
| 3 µl | 0.1 M DTT |
| 3 µl | 10 mM dAGT mix (dNTPs w/o dCTP) |
| 3 µl | 50 µM dCTP |
| 3 µ | l[α-$^{33}$P]-dCTP (30 µCi) |
| 6 µl | $H_2O$ |
| 1 µl | Superscript RT (200 u/µl) |

## 3.14 Filter hybridization of high density arrays

Hybridizations of high density arrays were not much different than standard Southern hybridizations as described in Sambrook *et al.* (1989). However, some minor changes were made. Prehybridization and hybridization were done at 65 °C in Church buffer containing 0.2 mg/ml fish sperm DNA. Per hybridization flask (diameter 76 mm) two filters separated by a nylon mesh (100 µm pore size) were incubated. After hybridization O/N, the filters were washed twice with washing buffer at 65 °C for 30 min. The filters were wrapped in Saran wrap, removing all air bubbles, because these would interfere with the signal detection. Exposure of phosphor screens was done O/N. Scanning of the screen was done at a pixel size

of 50 µm. Then, the filters were regenerated by incubation in a large volume of regeneration buffer for 30-60 min at 85 °C, followed by cooling down until the buffer reached RT. To see whether the filter was regenerated properly, exposure on a phosphor screen was done O/N.

Church buffer: 0.5 M sodium phosphate buffer pH 7.2; 7% SDS (w/v); 1 mM EDTA
Washing buffer: 40 mM sodium phosphate buffer pH 7.2; 0.1% SDS (w/v)
Regeneration buffer: 5 mM sodium phosphate buffer pH 7.2; 0.1% SDS (w/v)

## 3.15 Oligonucleotide labeling using ($\gamma$-$^{33}$P)-ATP

To 20 pmol of oligonucleotides, 1 µl 10x PNK buffer, 1 µl T4 polynucleotide kinase, 1 µl (10 µCi) ($\gamma$-$^{33}$P)-ATP and $H_2O$ to reach a total volume of 10 µl were added. This mix was incubated for 1 h at 37 °C. After incubation, the total activity of the sample in cpm was measured, for later calculation of the incorporation efficiency. Ethanol precipitation was done in the presence of 10 mM $MgCl_2$ to increase the efficiency of precipitation of small DNA fragments. Before centrifugation at 4 °C the sample was pre-incubated for 30 min at –80 °C. The pellet was dissolved in 100 µl of $H_2O$ and the radioactivity of the sample was measured. If the incorporation efficiency was higher than 30%, the probe was used in further experiments.

## 3.16 Hybridisation of high density arrays with oligonucleotides

Prehybridization and hybridization were done in pre-cooled SSARC buffer containing 0.2 mg/ml fish sperm DNA at 15 °C. Per hybridization flask (diameter 76 mm) two filters separated by a nylon mesh (100 µm pore size) were incubated. After hybridization O/N the filters were washed for 5-10 min at 15 °C in pre-cooled SSARC buffer. Further treatment of the filters was equal to the normal hybridization as described above.

SSARC buffer: 0.6 M NaCl; 60 mM sodium citrate; 7.2% sodium sarcosyl

## 3.17 Spotting of PCR products on nylon membranes using a spotting robot

Before the spotting, the nylon membranes (22.2 x 22.2 cm) were equilibrated for at least 20 min in denaturation buffer. Three layers of Whatman 3MM paper of the appropiate size were soaked in denaturation buffer and placed in Genetix trays (24 x 24 cm). Excess fluid was removed and the equilibrated membranes were placed on the Whatman paper.
Spotting was done using a BIOGRID spotting robot with the appropriate software using a 384 well pin tool with a pin diameter of 0.4 mm. The pattern used was a 4 x 4 grid with double offset. DNA was spotted 10 times per spot.
After spotting the membranes were laid on three layers of Whatman 3MM paper soaked in neutralization buffer and incubated for 15-30 min. Then the membranes were dried O/N on dry Whatman 3MM paper. Fixing of the DNA on the membranes was done using UV cross-linking (0.12 J/cm$^2$).

Denaturation buffer: 1.5 M NaCl; 0.5 M NaOH
Neutralization buffer: 1.5 M NaCl; 0.5 M Tris; pH 7.2

## 3.18 Cultivation of *Arabidopsis* suspension cells

*Arabidopsis* At7 suspension cells were weekly subcultured by inoculation of 40 ml of *Arabidopsis* At7 medium (see chapter 2.8.2) in a 200 ml erlenmeyer flask containing 3.0-3.5 g of cells. The culture was cultivated under shaking conditions (105 rpm) at 27 °C in the dark.

## 3.19 Sterilization of *Arabidopsis* seeds

Seeds were put in an Eppendorf reaction vial and 1 ml of sterilization solution was added. The seeds were incubated in this solution for 5-10 min while mixed constantly. The sterilization fluid was then removed and seeds were washed three times with 95% ethanol. Seeds were dried O/N in the fume hood.

Sterilization solution: 5% Ca-hypochloride; 0.1% Tween-20; 90% ethanol

## 3.20 Liquid culture of *Arabidopsis* seedlings and auxin induction

Ten to 20 sterilized *Arabidopsis* seeds were added to 40 ml *At* liquid culture medium (see chapter 2.8.2). After 10 days of growth, seedlings were induced using 10 µM IAA dissolved in medium. As mock control the same volume of medium (with the same pH) was added to controls.

## 3.21 Auxin induction of suspension cells

*Arabidopsis* At7 suspension cells were subcultured as described above. After 2 days of relaxation, cells were transferred to new flasks with medium lacking auxin. The cells were incubated for 2-3 days in this medium to starve them for auxin. After starvation, inducers (hormone or mock control) were added. Then, cells were harvested at different time points by vacuum filtration over Whatman filters and frozen in liquid nitrogen. The cells were stored at –80 °C until further use.

# 4 RESULTS

Although research in the field of auxin is extensive, much remains unknown about its signal transduction pathways. One way to investigate auxin signal transduction pathways is to examine genes that are responsive to auxin. In our studies, the auxin response of the *Arabidopsis* homologue of *ARCA* from tobacco (Ishida *et al.*, 1993) was examined. The suspension cell system used to characterize the expression profile of this gene and the identification of an *En-1*-insertional line will be described. This thesis will also go into detail on the establishment of cDNA arrays as a novel tool to study insertional mutants in their response to auxin.

## 4.1 Isolation and sequence analysis of *AtARCA*

The nomenclature for the different *ARCA* homologues used here is the three letter gene name, as proposed by the plant gene nomenclature commission, (http://www.arabidopsis.org/nomencl.html). The gene name will be preceded by two letters for the species identity. In the case of the tobacco gene, it would be written *NtARCA*.

In tobacco suspension cells, an auxin-regulated gene, *NtARCA* (GenBank Accession D17526), was described that showed identities of around 60-70% at the protein level to homologues isolated from animals (Ishida *et al*., 1993). The EST database at the National Center for Biotechnology Information was searched for ESTs from *Arabidopsis thaliana* which showed homology to *NtARCA*. EST clone 166F17T7 was selected and sequenced, and it was also shown to have a full-length open reading frame of 984 base pairs (see Figure 4-1). Examination of the *AtARCA* sequence (GenBank Acc. U77381) revealed that the 5' untranslated region consists of 56 nucleotides. The deduced protein has a length of 384 amino acids and an estimated molecular weight of 35,784 Dalton.

The strong conservation between AtARCA, related proteins from this family, and an unrelated WD40 repeat protein (SEC13) (previously described by Guillemot *et al*., 1989; Schloss, 1990; Ishida *et al*., 1993; Iwasaki *et al*., 1995; Müller *et al*., 1995; McKhann *et al*., 1997; Kwak *et*

*al*., 1997) is illustrated in Table 4-1. AtARCA contains seven WD40 repeats and has no extended N-terminus, which has also been observed for other ARCA homologues. In true G protein β subunits, the binding specificity to the corresponding subunit is determined by the WD repeat region, while the extended N-terminus is necessary for the binding to the membrane (Garcia-Higuera *et al*., 1996a; Garcia-Higuera *et al*., 1996b). The AtARCA protein was analyzed by using the PSORT program (http://psort.nibb.ac.jp/form.html), which gives an indication for the cellular localization of proteins based on the conservation of targeting signals. Localization of the AtARCA protein was predicted to be in the cytoplasm or in microsomes (peroxysomes).

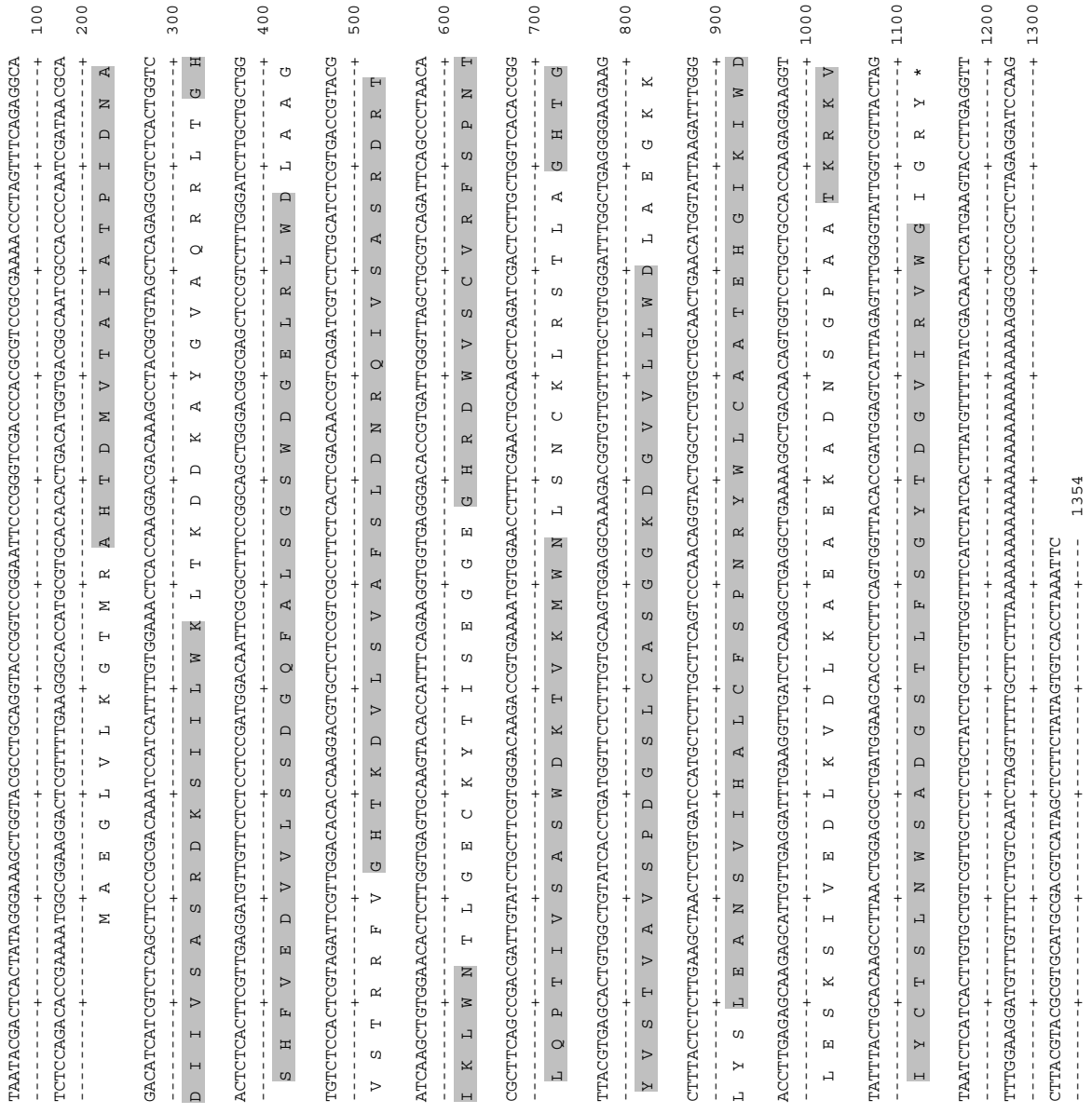


**Figure 4-1: Complete cDNA sequence including the putative translation product of AtARCA.** WD40 repeats are marked in grey.

| | AtARCA | BnARCA | MsARCA | NtARCA | OsARCA | C12.3 | Cblp | DmRACK1 | Cpc-2 | Sec13 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | |
| AtARCA | *** | 95.7 | 84.3 | 78.0 | 70.4 | 64.4 | 67.0 | 61.3 | 60.8 | 13.8 |
| BnARCA | 4.3 | *** | 83.4 | 78.0 | 70.6 | 63.4 | 66.4 | 61.3 | 60.8 | 12.5 |
| MsARCA | 13.9 | 15.1 | *** | 84.0 | 72.6 | 65.9 | 69.8 | 60.7 | 59.2 | 12.8 |
| NtARCA | 19.4 | 19.4 | 15.7 | *** | 70.6 | 65.0 | 67.0 | 58.8 | 58.2 | 11.8 |
| OsARCA | 23.5 | 23.2 | 20.7 | 23.2 | *** | 62.8 | 68.2 | 60.1 | 59.2 | 13.5 |
| C12.3 | 33.4 | 34.2 | 31.5 | 33.0 | 32.7 | *** | 71.6 | 75.4 | 70.9 | 13.5 |
| Cblp | 29.6 | 29.9 | 27.2 | 30.0 | 27.0 | 25.3 | *** | 64.8 | 66.1 | 14.5 |
| DmRACK1 | 35.6 | 35.4 | 35.9 | 37.7 | 35.8 | 23.7 | 31.1 | *** | 65.5 | 12.8 |
| Cpc-2 | 37.1 | 36.9 | 37.7 | 39.2 | 35.4 | 29.1 | 30.4 | 33.5 | *** | 12.8 |
| Sec13 | 81.3 | 80.9 | 80.4 | 79.6 | 81.9 | 77.0 | 80.2 | 79.1 | 79.3 | *** |
| | | | | | | | | | | |
| Percent Similarity in upper triangle | | | | | | | | | | |
| Percent Divergence in lower triangle | | | | | | | | | | |

**Table 4-1: Identical amino acids between different WD40 repeat proteins in %.** Calculations were done using the DNAStar program MegAlign. AtARCA: *Arabidopsis thaliana* ARCA (U77381); BnARCA: *Brassica napus* ARCA (Z33643); MsARCA: *Medicago sativa* ARCA (Y08678); NtARCA: *Nicotiana tabacum* ARCA (D17526); OsARCA: *Oryza sativa* ARCA (Iwasaki *et al*., 1995); C12.3: chicken RACK homologue C12.3 (M24193); Cblp: *Chlamydomonas* ARCA homologue Cblp (Schloss, 1990); DmRACK1: *Drosophila melanogaster* RACK1 (U96491); Cpc-2: *Neurospora crassa* ARCA homologue cpc-2 (X81875); Sec13: *Saccharomyces cerevisiae* SEC13 (Q04491).

## 4.2 Southern blot analysis

A Southern blot was performed in order to investigate the possible presence of similar genes in the *Arabidopsis* genome. Genomic DNA from *Arabidopsis* wildtype plants was digested with either *Bam*HI, *Eco*RI or *Eco*RV and analyzed on a Southern blot. A *Sal*I/*Hin*dIII-digested

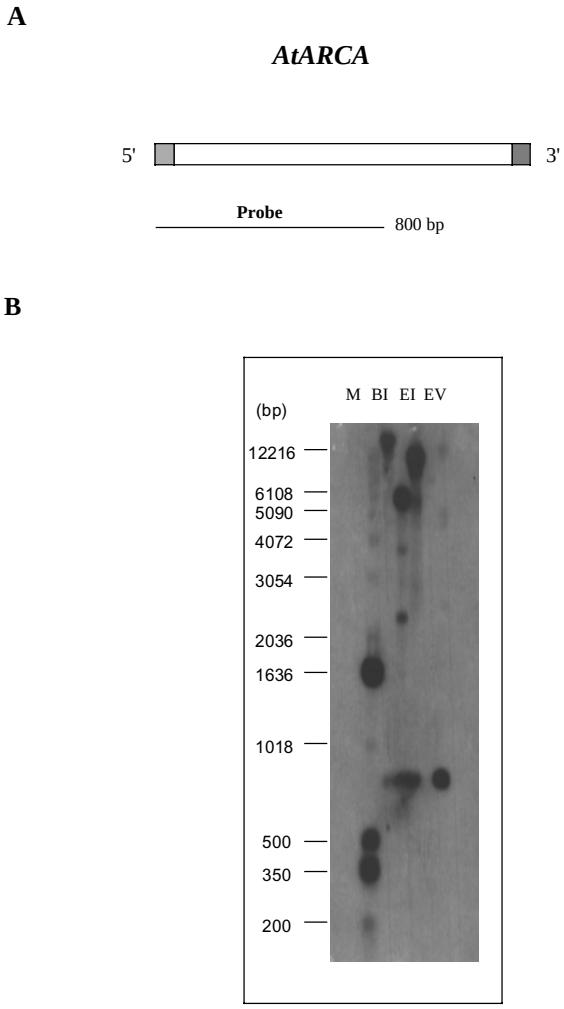



**Figure 4-2: Genomic Southern blot analysis of *AtARCA*.** A: Schematic representation of the *AtARCA* cDNA with the position of the restriction sites and the probe used for the hybridization. B: Autoradiography after hybridization with the radioactive probe. For the analysis, 3 µg of genomic DNA was digested by *Bam*HI (BI), *Eco*RI (EI) and *Eco*RV (EV).

fragment from EST 166F17T7 was used as a probe, which contains the first 800 base pairs of the *AtARCA* cDNA. The results of the lower stringency washing conditions are shown in

Figure 4-2. A strong hybridizing fragment of more than 12 kb is visible after *Bam*HI digestion. The second hybridizing band of approximately 800 base pairs appears to be a background-band, since it is visible in all lanes. Digestion with *Eco*RI gives rise to another hybridizing fragment of ~6 kb and two weaker signals ( 2.2 and 3.8 kb in size). In the case of *Eco*RV, a hybridization signal of 11 kb was identified with a weaker signal of ~6 kb underneath. Therefore, it may be that apart from the *AtARCA* gene another homologue is present in the *Arabidopsis* genome.

## 4.3 *AtARCA* promoter isolation and comparison with the promoter of *NtARCA*

The isolation of the gene's promoter can give more insight in the possible factors involved in the gene regulation. Since the *NtARCA* promoter has been investigated for the regions involved in the regulation of the gene (Ishida *et al.*, 1996), a comparison of the regulating elements conserved between the *NtARCA* and *AtARCA* promoters may give useful information about the importance of certain motifs.

To isolate the promoter of *AtARCA*, inverse PCR was performed on *Arabidopsis* genomic DNA, which was digested with *Sac*I and religated. Two primers, AA5' and AA3', were designed: the first primer, directed towards the promoter, was located near the ATG start site, while the second primer, pointing downstream, was designed close to the *Sac*I site. The resulting PCR product was then used as a template for another PCR using the nested primers AA5'-NEST and AA3'-NEST. The final PCR product was isolated and sequenced.

A total of 1687 bp of unknown promoter sequence was identified. The promoter sequence was analyzed by using the PLACE promoter analysis program currently available on the internet (http://www.dna.affrc.go.jp/htdocs/PLACE/). This analysis revealed several conserved motifs present in both the *AtARCA* and the *NtARCA* promoter (see Figure 4-3). Since it is known which part of the *NtARCA* promoter is responsible for the auxin response (Ishida *et al.*, 1996), the aim was to identify conserved elements within this region. In particular, the MYB binding sites are of interest, since MYB proteins have been shown to respond to different inducing agents (Kirik *et al.*, 1998; Kranz *et al.*, 1998).

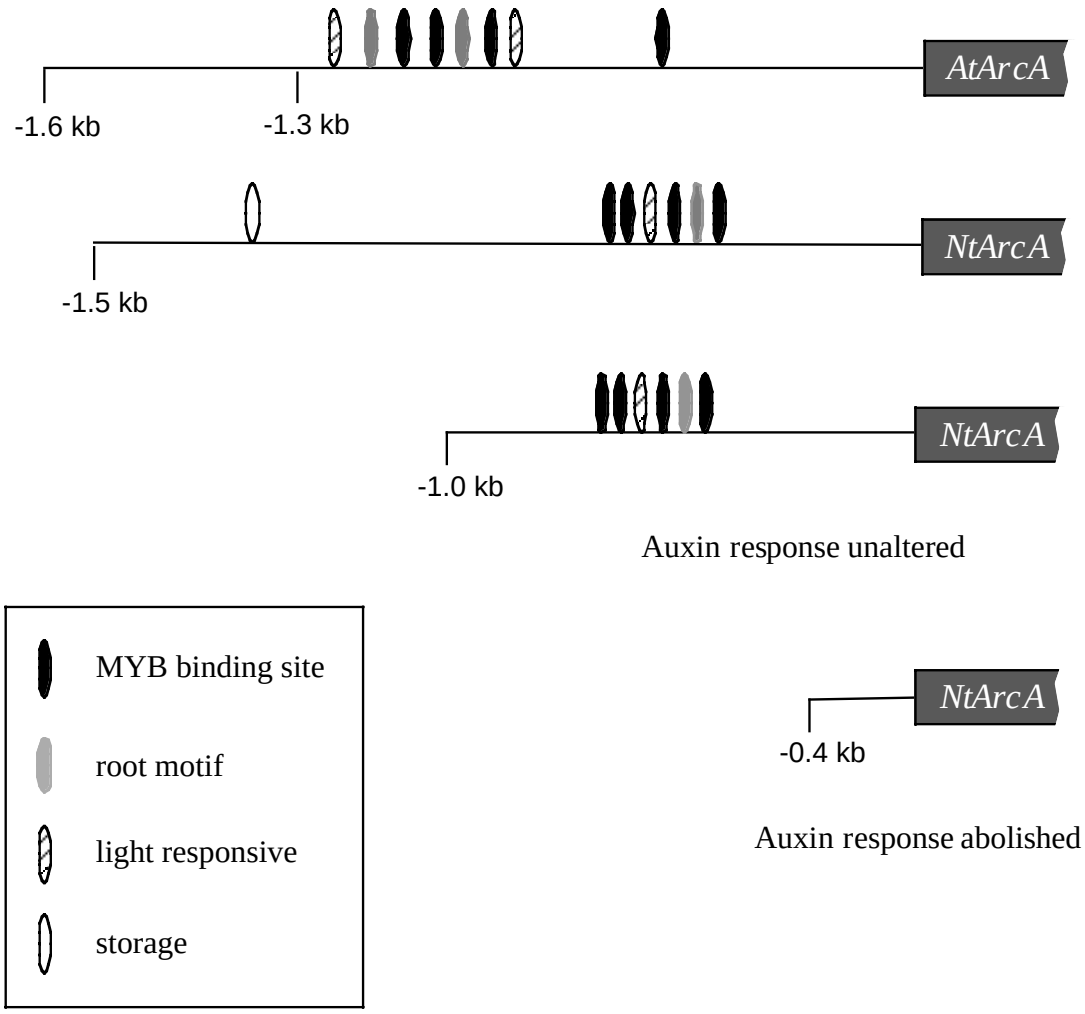



**Figure 4-3: Positions of different conserved motifs in the *AtARCA* and the *NtARCA* promoter.** The deletion mutants of the *NtARCA* promoter have been described by Ishida *et al*. (1996). Motifs that are conserved between both promoters are shown.

## 4.4 Isolation of *AtARCA* genomic DNA

On the basis of the cDNA sequence, primers AAEnFW and AAEnRV were designed for PCR amplification of the genomic DNA of *AtARCA*. Genomic DNA from wildtype *Arabidopsis* plants was used for PCR, and the resulting amplicon was sequenced. This revealed that the *AtARCA* gene contains a single intron of about 600 base pairs (Figure 4-4).

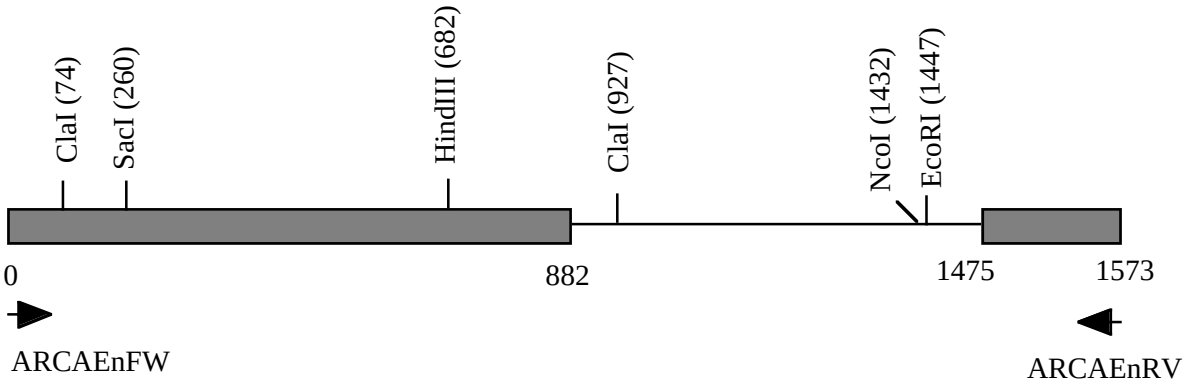



**Figure 4-4: Schematic representation of the *AtARCA* genomic structure**. The gene contains a single intron of 600 base pairs at position 882 of the cDNA sequence.

The genomic sequence of *AtARCA* was used in a BLAST (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/) search to obtain more information about the chromosomal localization of the gene. The partial sequence of the gene was found on BAC F20L2TF, which was mapped to chromosome I.

## 4.5 Characterization of the *AtARCA* expression pattern

The response of organisms to different factors can be measured in various ways. Analysis of gene expression by quantitative mRNA analysis can give insight into genes and pathways

involved in the response to different biotic and abiotic factors. To study the function of *AtARCA*, different treatments have been used.

### 4.5.1 Tissue specific expression of *AtARCA*

To study the tissue-specific expression pattern of *AtARCA* in the plant, a northern blot with different tissues from wildtype *Arabidopsis* plants (obtained from C.H. Guan) was probed with a *Hin*dIII-digested fragment of approximately 500 bp from EST 166F17T7. This fragment contains the 3' UTR for increased specificity. *AtARCA* mRNA could be detected in all tissues, although the expression level in roots seemed to be slightly higher. On the other hand, the expression level in leaves (rosette and particularly stem) appeared to be very low when compared to other tissues (Figure 4-5).

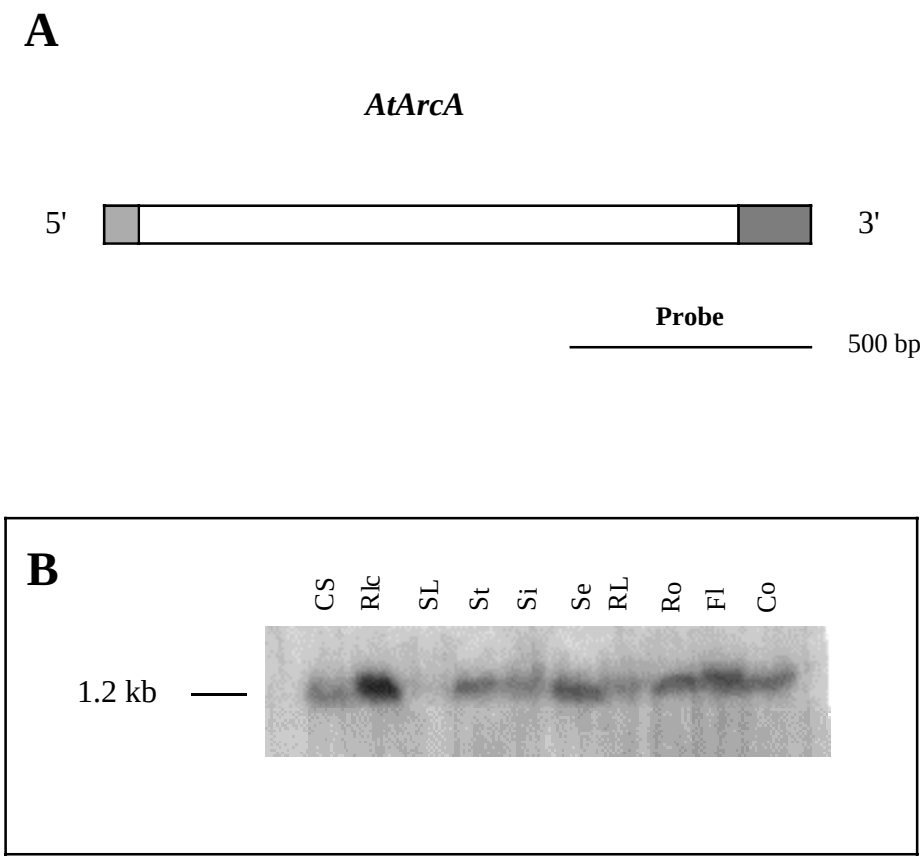



**Figure 4-5: Tissue specific expression of the *AtARCA* gene.** A: Schematic representation of the *AtARCA* cDNA with the position of the restriction sites and the probe used for the hybridization. B: Autoradiograph of the Northern hybridization. CS = Cell Suspension; Rlc = Root liquid culture; SL = Stem Leaves; St = Stem; Si = Siliques; Se = Seedlings; RL = Rosette Leaves; Ro = Roots; Fl = Flowers; Co = Cotelydons. Loading controls performed by Guan (not shown) showed similar loading of the RNA.

### 4.5.2 RNA dot blots of *AtARCA*

As is shown by the northern blot analysis, *AtARCA* was expressed in suspension cells. These cells can easily be subjected to a wide variety of alterations in culture conditions, hormones and other drugs. Therefore, in order to characterize the response of *AtARCA* to different external factors, *Arabidopsis* culture cells were induced in different ways. Dot blot analysis was used to allow for a more precise quantification, in contrast to normal northerns. Every experiment was repeated two to four times and done in duplicate; the graphs display normalized data as a ratio of the controls from the same experiment. The results presented here are median values of all experiments, since the impact of the extremes is lower in median values than with averages. Since it was impossible to find proper controls for loading, the spectrophotometric evaluation of RNA concentration was used instead.

From previous experiments (Ishida *et al*., 1993), it was shown that *NtARCA* responds to different auxins after several days of starvation. To analyze the response of *AtARCA* to auxin treatment, the experimental setup as described in chapter 3.21 was chosen, using the same probe as described for the tissue-specific expression. In Figure 4-6 it is shown that already after two hours, a clear response of *AtARCA* to the synthetic auxins 1-NAA and 2,4-D can be observed. A pretreatment with known auxin inhibitors (e.g. 2-NAA) does not have a significant effect on the response. On the other hand, pretreatment with cycloheximide blocks the auxin response of *AtARCA*, indicating that *de novo* protein synthesis is necessary for its expression. The *AtARCA* mRNA level remains relatively stable over several hours, suggesting that *AtARCA* mRNA is quite stable.

The absolute values in the different experiments varied clearly, although the overall effect of auxin induction after starvation is significant. It was found that the variability was higher between two different experiments than between two cultures treated similar within the same experiment. This effect may be caused by a difference in starvation efficiency.

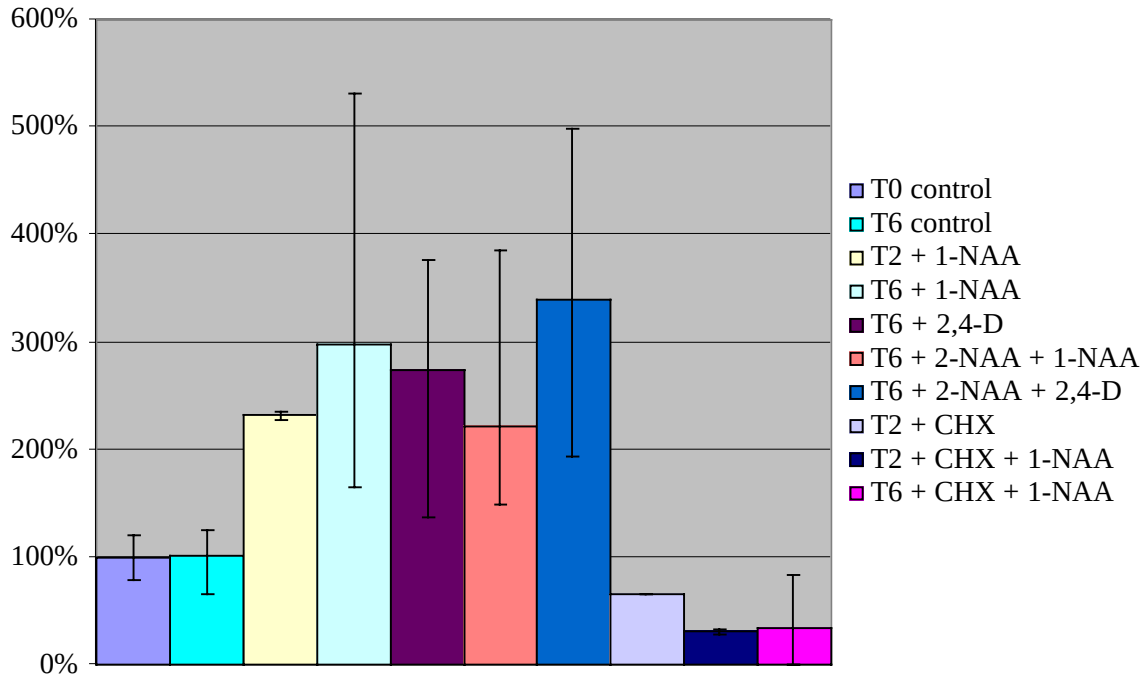



**Figure 4-6: *AtARCA* expression after starvation and re-addition of auxin.** Suspension cells were starved for two days for auxin. After induction with different agents, the expression level of *AtARCA* was measured by dot blot at different timepoints. T = Timepoint in hours after induction. The controls are mock-induced. The timepoint two hrs after induction with cycloheximide (T2 + CHX) is based on the value of a single experiment. Error bars indicate the standard deviation. When two compounds are divided by a "+", this indicates a pretreatment with the first compound.

To investigate *AtARCA* expression further, different synthetic auxins and auxin inhibitors (2-NAA and PCIB) were added to suspension cells that were grown in the presence of auxin. The use of the inactive auxin analogue 2-NAA was chosen for it may compete for the same binding sites as 1-NAA. The auxin antagonist PCIB was selected because of the clear inhibition of auxin response in various experiments (Heupel and Stange, 1995; Bellamine *et al.*, 1998). Dot blot analysis revealed that the expression level of *AtARCA* cannot be influenced by the addition of auxins without a preceding starvation for auxin (Figure 4-7). The expression level of *AtARCA* was similar in all samples. Also application of auxin inhibitors

did not show a significant effect. However, addition of cycloheximide in these experiments also showed a clear decrease in *AtARCA* expression.

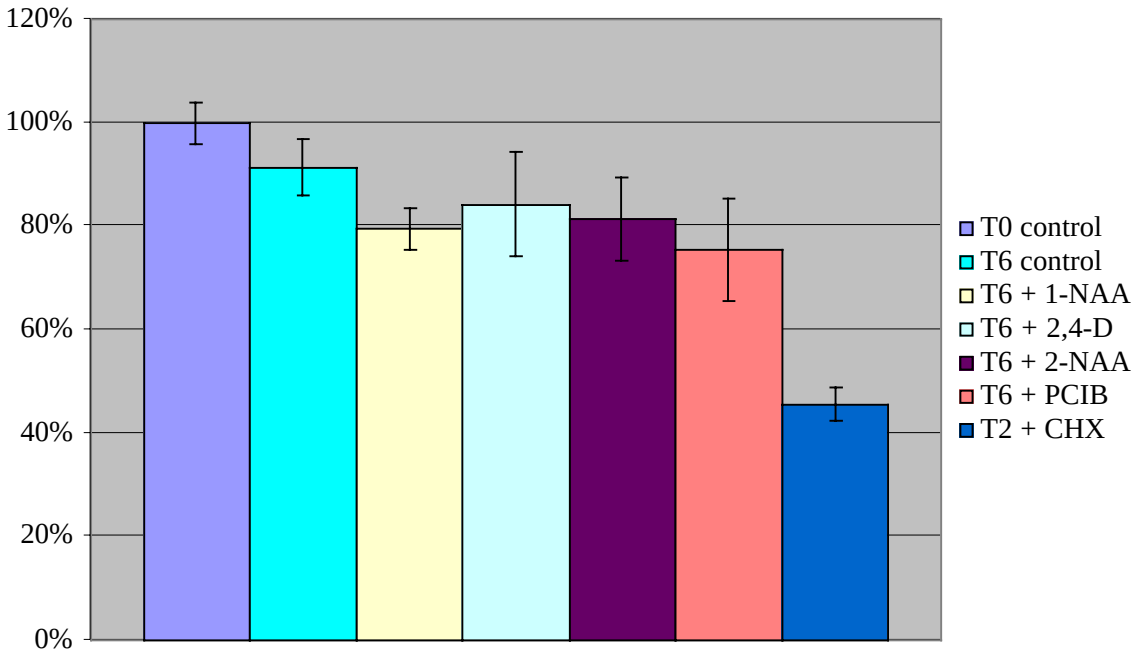



**Figure 4-7: Effect of different agents on *AtARCA* expression of cells grown in the presence of auxin.** Untreated cells were induced with different inducing agents. The effect on the *AtARCA* expression was measured by dot blot. T = Time point in hours after induction. The controls are mock-induced. Error bars indicate the standard deviation.

The effect of the auxin-starvation and aging on the expression of the *AtARCA* gene expression was examined. *AtARCA* gene expression in one-week-old cells was compared to that of fresh cells (Figure 4-8). The starvation of suspension cells causes a severe decrease in *AtARCA* gene expression. The expression of AtARCA in 1-week-old cells was also significantly lower than in fresh cells. Results from this experiment suggest that aging has a significant effect on *AtARCA* gene expression, although not as strong as auxin starvation.


**Figure 4-8: The effect of auxin starvation and aging on the *AtARCA* gene expression**. Error bars indicate the standard deviation of the signal intensity.

## 4.6 Search for an *En-1* knockout line for *AtARCA*

It is useful to analyze the phenotype of plants lacking a functional *AtARCA* gene or plants in which normal *AtARCA* gene expression is extremely weak. The phenotype of these mutants may give better insight into the function of the gene and the pathways it is involved in. In our institute, an *Arabidopsis* library saturated with the *En/Spm* transposon from *Zea mays* is available (Wisman *et al*., 1998) and has been analyzed for the presence of an *En-1*-insertion in the *AtARCA* gene.

A systematic PCR screening using primers specific for *AtARCA* and *En-1* was performed as described by Wisman *et al.* (1998). Two positive candidates were identified, 6AAR74 and 6W35. The PCR products of both lines were sequenced to determine the exact position of

insertion. It was observed for both lines that the insertion was located in the *AtARCA* promoter region. In the case of line 6W35, the insertion site was about 1.3 kb upstream of the start codon; in line 6AAR74, the *En-1* inserted 831 bases in front of the start ATG. In Figure 4-9, a schematic representation of the *En-1*-insertion in line 6AAR74 is shown.

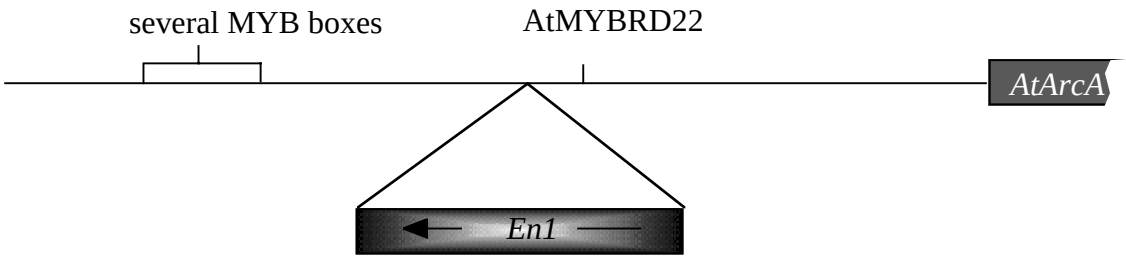



**Figure 4-9: Schematic representation of the *En-1*-insertion in line 6AAR74.** The *En-1* has inserted in between 2 MYB binding boxes, 831 bases upstream of the ATG start codon.

Comparison of the *AtARCA* promoter sequence with that of *NtARCA* (Ishida *et al.*, 1996, see also chapter 4.3), as well as analysis of both promoters via the PLACE promoter analysis program lead to the hypothesis that the auxin response in line 6AAR74 may be corrupted, which as of yet has not been confirmed. Additionally, the next generation of line 6AAR74 is currently being screened for the presence of the *En-1*-insertion within the *AtARCA* coding region. This is done due to concern that the insertion within the *AtARCA* promoter may not generate a valid knockout for the purpose of our experiments.

## 4.7 Isolation of *AtARCA2*

In the search for the chromosomal localization of *AtARCA*, a homologous *Arabidopsis* gene was also identified. This *AtARCA2* gene was identified on BAC MRC8 (GenBank Accession AB020749) at position 41353 to 40334. This BAC has been mapped to chromosome 3 and contains the entire genomic *AtARCA2* sequence. Results from the alignment of the genomic sequences of both *AtARCA* and *AtARCA2* and from the internet intron prediction program

Genemark (http://dixie.biology. gatech.edu/Genemark/eukhmm.cgi), led to the elucidation of the putative *AtARCA2* open reading frame. As for the *AtARCA* gene, a single intron could be identified.

The alignment of the predicted proteins of *AtARCA* and *AtARCA2* (Figure 4-10) reveals an identity of 84%. The main difference between the two proteins lies in the carboxy terminus, in the beginning of the last WD repeat. The AtARCA2 protein shows a nine amino acid extension in this region. Like for the AtARCA protein, this protein was also predicted by the PSORT program to be localized either in the cytoplasm or in microbodies.

```
  1 MAEGLVLKGTMRAHTDMVTAIATPIDNADIIVSASRDKSIILWKLTKDDK 50
    ||||||||| |||||| |||||||||| |||| ||||||||||||||||
  1 MAEGLVLKGIMRAHTDIVTAIATPIDNSDIIVTASRDKSIILWKLTKDDK 50

 51 AYGVAQRRLTGHSHFVEDVVLSSDGQFALSGSWDGELRLWDLAAGVSTRR 100
     |||||||||||||||||||||||||||||||||||||||| |  |||
 51 SYGVAQRRLTGHSHFVEDVVLSSDGQFALSGSWDGELRLWDLATGETTRR 100

101 FVGHTKDVLSVAFSLDNRQIVSASRDRTIKLWNTLGECKYTISEGGEGHR 150
    ||||||||||||| |||||||||||||||||||||||| | ||
101 FVGHTKDVLSVAFSTDNRQIVSASRDRTIKLWNTLGECKYTISE.GDGHK 149

151 DWVSCVRFSPNTLQPTIVSASWDKTVKMWNLSNCKLRSTLAGHTGYVSTV 200
     |||||||||||| |||||||||||| ||| |||||  | || ||  ||
150 EWVSCVRFSPNTLVPTIVSASWDKTVKVWNLQNCKLRNSLVGHSGYLNTV 199

201 AVSPDGSLCASGGKDGVVLLWDLAEGKKLYSLEANSVIHALCFSPNRYWL 250
    |||||||||||||||| |||||||||||||| | || ||||||||||
200 AVSPDGSLCASGGKDGVILLWDLAEGKKLYSLEAGSIIHSLCFSPNRYWL 249

251 CAATEHGIKIWDLESKSIVEDLKVDLKAEAEKADNSGPAATKRK------ 294
    |||||  | |||||||| ||||||||| ||||           |
250 CAATENSIRIWDLESKSVVEDLKVDLKSEAEKNEGGVGTGNQKKVRHYLM 299

295 ---VIYCTSLNWSADGSTLFSGYTDGVIRVWGIGRY 327
       |||||||||||||||||||||| ||||||||
300 ISLVIYCTSLNWSADGSTLFSGYTDGVVRVWGIGRY 335
```

**Figure 4-10: Alignment of both ARCA proteins from *Arabidopsis thaliana*.** Top: AtARCA, bottom: AtARCA2. Identical amino acids are connected by bars.

In the first part of this thesis, the isolation of two novel genes from *Arabidopsis thaliana*, *AtARCA* and *AtARCA2*, has been described. These genes bear homologies to genes from other plants, but also animals, of upto 94% identity on protein basis. The expression pattern of *AtARCA* has been characterized by differential treatment of suspension cells. Furthermore, an *En-1*-insertion has been identified in the promoter region of this gene. The putative function will be discussed in the first part of the discussion.

## 4.8 cDNA arrays

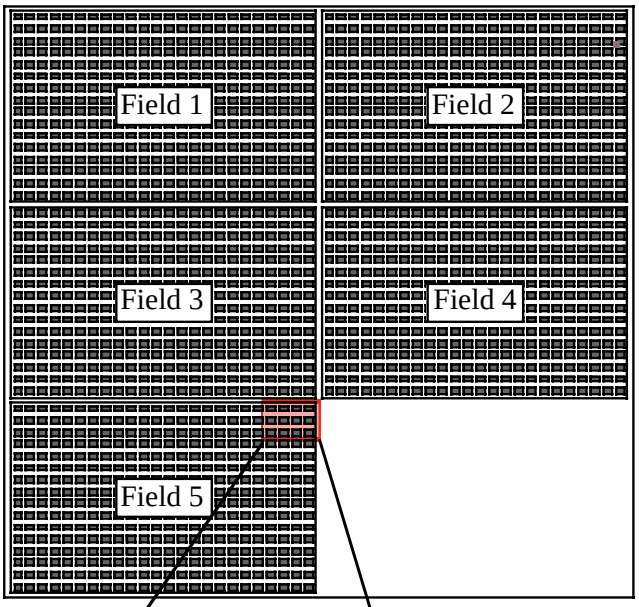
When the expression of a gene is altered, this does not necessarily lead to a phenotype. Also, if a phenotype can be observed, this may not improve the knowledge about the gene's function. Therefore, cDNA arrays were utilized to address this concern. The use of cDNA arrays enables the expression of a large number of genes under different conditions to be observed simultaneously. Examining the response to auxin by wildtype plants and *AtARCA* knockout plants may result in important information on the molecular pathway in which *AtARCA* is involved. This information is very useful in subsequent studies on the process of auxin perception and cell division. Since the technique has not yet been established at our institute, we decided to make use of methods and equipment already available in our laboratory. For this reason, the use of cDNA arrays on nylon membranes was chosen. The techniques used for this type of array are, in general, based on Southern hybridizations, although some optimizations are necessary. The equipment for the analysis is the same as is used for other methods based on radioactive hybridizations. Furthermore, the *Arabidopsis thaliana* EST library from Ohio State University was used. This library has the advantage that it contains 12672 clones from which partial sequences have been obtained. The identity of responding genes can therefore easily be determined, leading to easier and faster data evaluation. The library has also been filtered for overlapping sequences, which reduces its redundancy.
The most important steps that were optimized for the establishment of this technique for our laboratory will be shown in the following chapters. Additionally, results from experiments using this technique for the study of auxin-responsive genes will be given.

### 4.8.1 Preparation of PCR products

Since all ESTs in the library from Ohio State University have been cloned in plasmids that contain the M13 universal and reverse priming sites, a longer version of these primers, EST-FW and EST-RV, as descibed by Stracke (1998) has been used for PCR amplification. These primers have the advantage of being specific for all plasmids used in EST libraries. PCR was performed in 100 µl reaction volumes in 96-well plates. The quality of each reaction was confirmed by analysis on agarose gels.

### 4.8.2 Spotting of cDNA

One advantage in the use of nylon-based cDNA arrays is the fact that the PCR products are not necessarily purified before spotting. Spotting of the cDNA is normally done in double offset patterns (Figure 4-11). On a nylon membrane of 22.2 x 22.2 cm, five fields (panel A) are spotted. On each of these fields, a pattern of 384 (16 x 24, panel B) grids of 4 x 4 (panel C) spots is made. The positions of the plates are chosen in such a way that it is possible to recognize from hybridizing doubles which original plate they belong to (panel D).
*INF1* and *INF2A* are genes encoding fungal elicitors that do not show any cross-hybridization with plant DNA (Kamoun *et al.*, 1997a; Kamoun *et al.*, 1997b). In these experiments, the *INF2A* was used for local background determination, since this control contains a poly(A)-tail. The poly(A)-tail is known to cause cross-hybridization with cDNA that is reverse-transcribed using the oligo-dT primer. INF1 and the other controls CTL4A (Dariavach *et al.*, 1988) and BPI (Sawamura *et al.*, 1991) were used as negative controls and presented among the ESTs on the filter. The type of membranes and the amount of spotted DNA have been shown to exert a strong influence on the array quality. The amount of DNA that is spotted can be optimized by the number of replications per spot and the diameter of the pins used.

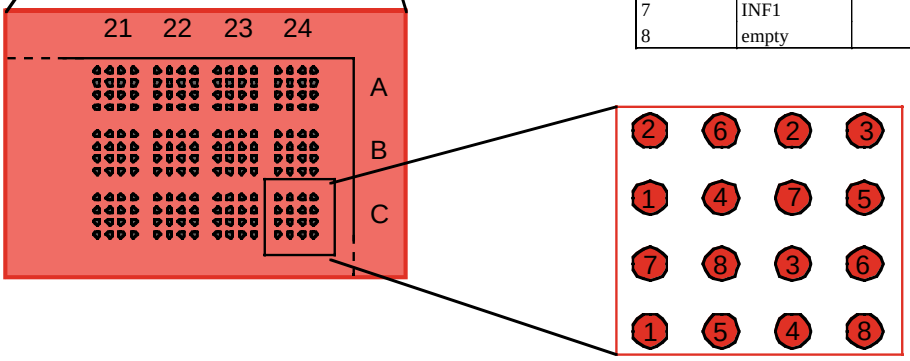



A

| Position | FIELD 1 Plate | FIELD 2 Plate |
|---|---|---|
| 1 | INF2A | 8 |
| 2 | 1 | INF2A |
| 3 | 2 | 9 |
| 4 | 3 | 10 |
| 5 | 4 | 11 |
| 6 | 5 | 12 |
| 7 | 6 | 13 |
| 8 | 7 | 14 |

| Position | FIELD 3 Plate | FIELD 4 Plate |
|---|---|---|
| 1 | 15 | 22 |
| 2 | 16 | 23 |
| 3 | INF2A | 24 |
| 4 | 17 | INF2A |
| 5 | 18 | 25 |
| 6 | 19 | 26 |
| 7 | 20 | 27 |
| 8 | 21 | 28 |

| Position | FIELD 5 Plate |
|---|---|
| 1 | 29 |
| 2 | 30 |
| 3 | 31 |
| 4 | 32 |
| 5 | INF2A |
| 6 | 33 |
| 7 | INF1 |
| 8 | empty |




B

C

D

**Figure 4-11: Spotting pattern of EST arrays.** (A). On a 22.2 x 22.2 cm filter, 5 fields of 24 x 16 grids of 4 x 4 spots are spotted. The position in the field is determined by using the position from (B), the plate number as is listed in (C) and the identity of the spot in this plate as is shown in (D).

Three different membranes were tested for their suitability of high density spotting of DNA. The membranes were tested with 5 and 10 replications per spot (the PCR products were 5 or 10 times spotted in the same position on the membrane). Nylon membranes tested were Amersham Hybond $N^+$, PALL Biodyne H and Genetix. The Genetix membranes were cheaper than the other membranes, while the PALL Biodyne H membranes were supposed to be optimized for high density spotting.

The probe used for these experiments was radioactively labeled T7 primer (for oligo hybridization, see chapter 4.8.3). As can be seen in Figure 4-12 the Genetix membranes were not very suitable for high density spotting. The highest quality was observed with the PALL Biodyne H membrane, although in comparison to the Hybond $N^+$ membrane, no significant difference in quality was seen. In further experiments it was found that PALL Biodyne B membranes were of equal quality as Amersham Hybond $N^+$ membranes (data not shown), a result observed also in other laboratories.

A pintool with a pin diameter of 0.4 mm was used to transfer DNA. The replication of five times per spot was shown to be insufficient. This was caused by PCR reactions with lower DNA concentrations leading to an amount of DNA on the membrane that was too low for further experiments. 20 nl were spotted per transfer, resulting in a final amount of 0.2 µl per spot after 10 replications.

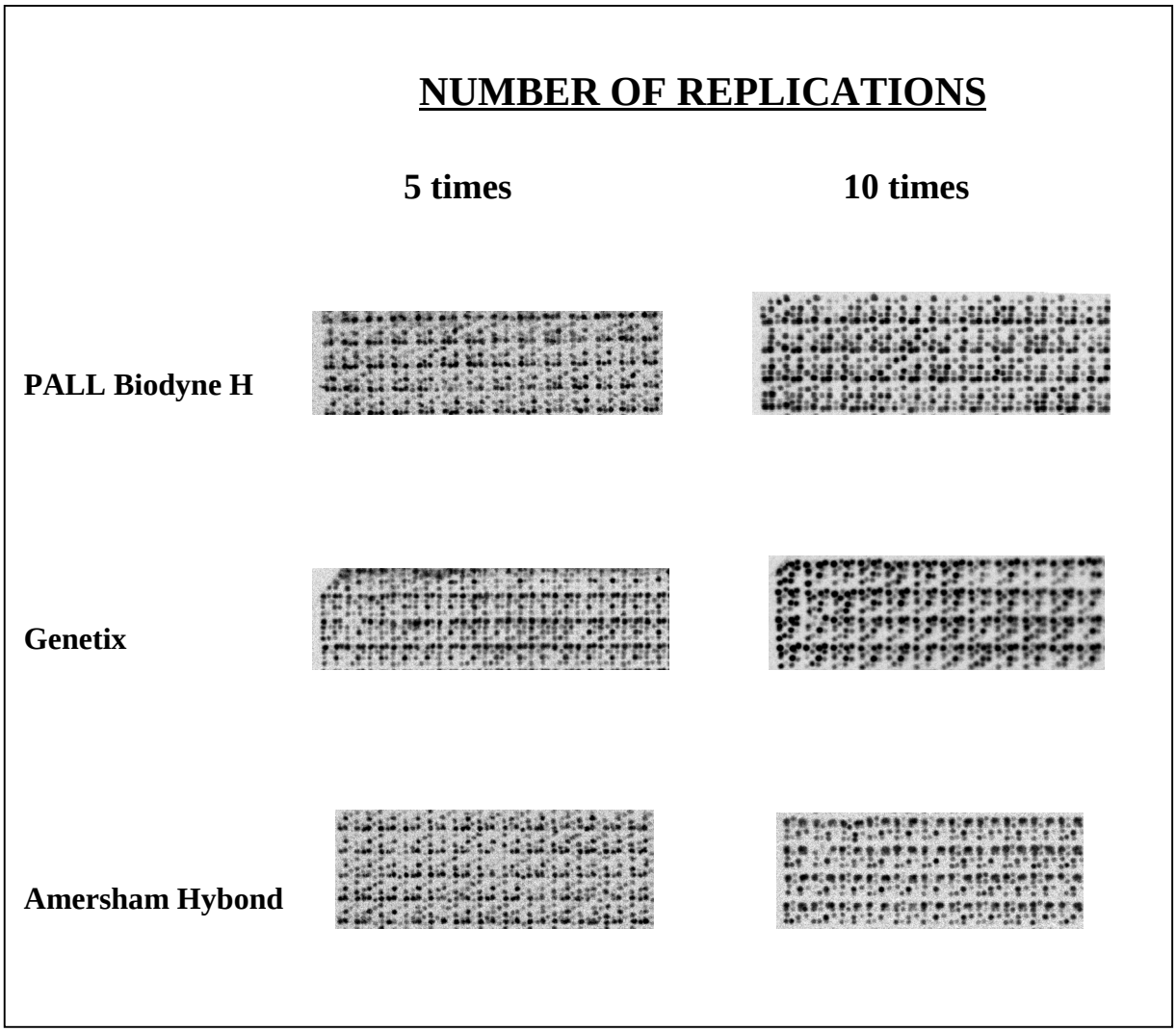



**Figure 4-12: Comparison of different membranes and different spotting replications.** The results shown in this figure are derived from oligo hybridizations using the T7 primer. This type of hybridizations will be discussed in the next chapter. The Genetix membrane shows blurring of the spots after 10 replications.

### 4.8.3 Oligonucleotide hybridizations

Before the spotted arrays could be used in experiments to determine differential expression of genes, it was necessary to confirm the quality of the arrays. The easiest way to confirm the quality by using of hybridizations with radioactively labeled oligonucleotides. The oligonucleotides used in the hybridization were labeled with $\gamma^{33}$P-dATP using T4 polynucleotide kinase. The efficiency of the labeling reaction was measured by calculation of $^{33}$P incorporation. When this incorporation efficiency was higher than 30%, the labeled nucleotides were used in further experiments. Hybridization was performed overnight at 15 °C in SSARC buffer. This buffer contains sarcosyl instead of SDS to avoid precipitation. After hybridization, the filters were washed briefly in SSARC buffer and then wrapped in Saran wrap. It was found that in this step, all air bubbles had to be removed because of the risk of signal blurring. The membranes were exposed on PhosphoImager screens overnight at room temperature. Afterwards, the screens were developed using a PhosphoImager scanner at a pixel size of 50 µm which could then be evaluated.

Since the PCR reactions were not purified to remove free primers before spotting, the primers were tested for their ability to exhibit a background signal after hybridization. As is shown in Figure 4-13, a hybridization with the original PCR primers resulted in a signal in every spot, even if no PCR product was present. Indeed, if the same filter was hybridized using the T7 primer, which is present as a nested primer in each PCR product originating from the EST-FW and EST-RV primers, the result was quite different. Only those samples that had a PCR product showed a hybridization signal with the T7 primer. In Figure 4-13 the same part of the filter after hybridization with either oligonucleotide is shown.
An important problem that occurred after oligo hybridization, was the inability to remove oligos of more than 17 nucleotides from the filter. For efficient removal of hybridizing oligonucleotides, a short version of the T7 primer (T7-SHORT) was used in further experiments (data not shown).

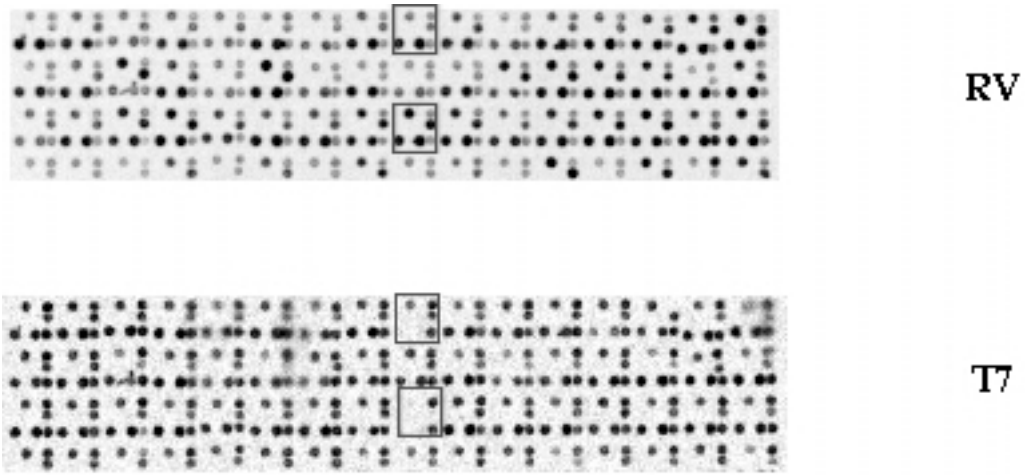



**Figure 4-13: Comparison of oligonucleotide hybridizations with the EST-RV primer and the T7 primer.** A four by four grid was spotted using three plates in double offset. This filter was subsequently hybridized with the EST-RV primer, stripped and hybridized with the T7 nested primer. As can be seen in the grids in the red boxes, use of the EST-RV primer gives a signal of all spots, in contrast to the T7 primer. This is probably caused by the presence of the primers used in the PCR reaction on the filter. The diffuse spots on the membrane hybridized with the T7 primer are caused by air bubbles.

For further comparative analysis, it is important that the reproducibility of the spotting experiments is very high. When the data was evaluated, the comparison of the duplicates on the same membrane was used to investigate the effect of contaminating signals. These are part of the normal problems of Southern hybridization, but it may also be caused by strong signals from neighboring spots. To eliminate false positives caused by these contaminants, only those data that show the same effect for both duplicates were analyzed. Therefore, the reproducibility of the spotting was confirmed by oligonucleotide hybridization and subsequential comparison of duplicate spots on the same membrane. This comparison of spots "A" and "B" (Figure 4-14) shows clearly that the reproducibility is very high.

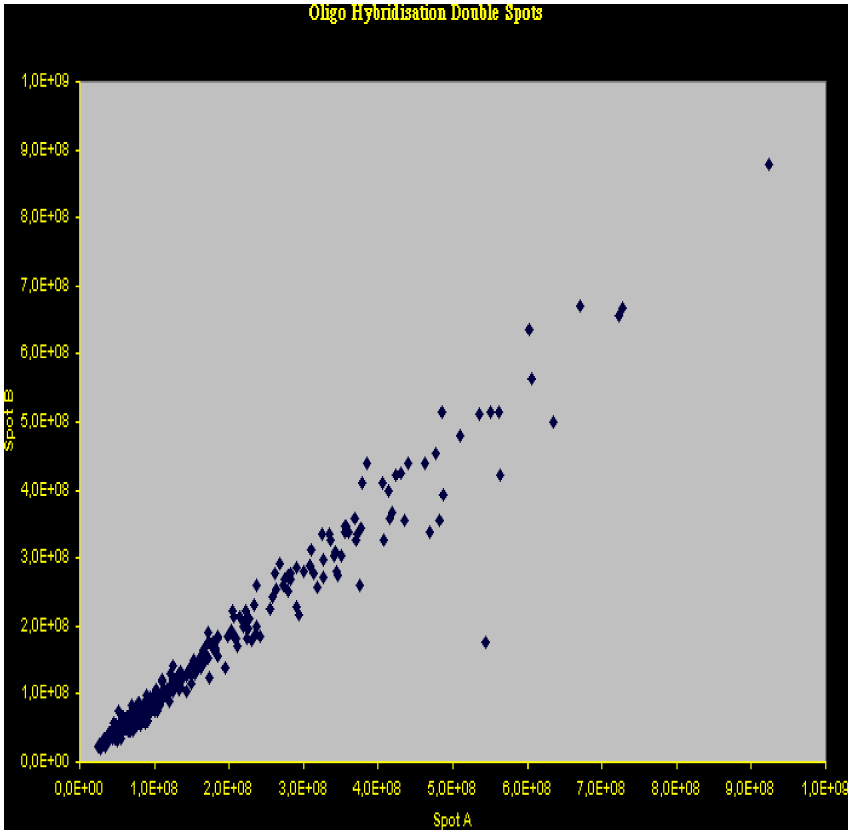



**Figure 4-14: Comparison of duplicate spots using oligonucleotide hybridizations.** On the X-axis the spot intensity of spot "A" is shown, on the Y-axis the spot intensity of spot "B". The reproducibility of spotting is very high .

### 4.8.3 Hybridization with complex probes

*4.8.3.1 Probe preparation*

For hybridization, total RNA was isolated by the RNeasy Plant Mini Kit from Qiagen (Hildesheim). The total RNA concentration was measured for each sample using a spectrophotometer and the quality of the RNA was confirmed by agarose gel electrophoresis. For the complex probe, cDNA was synthesized by reverse transcription using an oligo(dT)-primer in the presence of $\alpha^{33}$P-dCTP. Probes with an incorporation efficiency higher than 20-30% were used in further experiments. The filters were treated further as described in chapter 4.8.2 for the oligonucleotide hybridizations.

*4.8.3.2 Data evaluation*

The evaluation was done by using the ArrayVision program from Imaging Research. This program is useful for semi-automatic grid determination. However, the version used for data evaluation in the main part of this thesis (4.1.7) is not suited for further evaluations because of the lack of good statistical tools. For this reason, macros were written in Visual Basic for further data analysis in Microsoft Excel.

The macros were used to calculate the median value for duplicate experiments (for each single spot). The median values for different experiments were then used for further comparisons. The second step in the evaluation was the calculation of the difference between the duplicate spots. In the experiments described here, the duplicate spots with median values that differed more than 20% were removed from the data set. The average value of the remaining duplicates was determined so as to be used in further calculations. The preparation of the data sets was then followed by the comparison of different treatments. The differences and ratios were calculated. In these experiments, only genes that showed a difference of 1/3 of the average spot intensity of all spots and a 2.5-fold induction or repression were considered differentially expressed and analyzed further.

### 4.8.4 Treatment of *Arabidopsis thaliana* seedlings with IAA

In the experiments described in chapter 4.5.2 it was shown that the variability between different experiments using suspension cells starved for auxin was high. Thus the use of suspension cells, but also, because of the strong influence of outside factors (e.g. sunlight, pathogens, watering, soil composition), plants grown in the greenhouse were excluded for experiments using cDNA arrays. Since the application of auxin to plants grown on sterile, solid medium is difficult and induces further stress, it was decided to use seedlings grown *in vitro* with a 16-hour light period for 10 days in liquid medium.

The flasks were shaken in a climatized culture chamber in which light came from one side. This had the effect that flasks had different amounts of light. Between flasks on the front and on the back, the difference in LUX was doubled. To reduce this effect, the flasks were chosen in such a way that the average light to three flasks for every condition would be similar.

The seedlings were induced with 10 µM IAA or mock–treated with the same volume in solvent (with an identical pH). The samples were harvested after three hours, since it has been shown for *AtARCA* that a very strong response to auxin can be expected between two and six hours after auxin application (Chapter 4.5.2). Two independent hybridizations per treatment with mixtures of three different RNA samples were used. Each hybridization was performed using two membranes separated by a nylon mesh. All differentially expressed ESTs have been listed in Appendix A. However, since only those ESTs that have an assigned identity can be used in the analysis, the following table shows only an overview of these.

In Table 4-2 the differentially expressed ESTs have been sorted into functional groups for easier evaluation. These groups are mainly based on the review from Sitbon and Perrot-Rechenmann (1997) which divides auxin-responsive genes in these groups. However, I decided to add two more groups: 1. genes involved in auxin-related functions and 2. light-responsive genes. The latter group had to be added because a noise caused by light-responsive genes could be observed.

The response of the different ESTs has been given as a ratio of the mock-treated value to the difference between both values. In Formula 4-1, the calculations used for the evaluation of the data obtained from the arrays are shown. The normalized intensity of the spots refers to the

median intensity after background subtraction. |D| indicates to the absolute value of the difference after normalization. In Table 4-2, a ratio of >> indicates that AM (average value of the mock-treated sample) = 0.000.

```
(IMa + IMb) / 2 = AM

(IIa + IIb) / 2 = AI

AM – AI = D

|D| / AM = R

If R < 1 then Rr = 1 / R
If R > 1 then Ri = R

IMa: Normalized intensity of spot a (median), mock-treated
IMb: Normalized intensity of spot b (median), mock-treated
IIa: Normalized intensity of spot a (median), IAA-treated
IIb: Normalized intensity of spot b (median), IAA-treated
AM : Average value mock-treated
AI : Average value IAA-treated
D  : Difference
R  : Ratio
Rr : Ratio of repressed genes
```

**Formula 4-1: Formulas used for the evaluation of array data.**

## T3 mock-treated/T3 IAA-treated

| | *Ind.* | *Repr.* | *Diff.* |
|---|---|---|---|
| **Auxin-related function** | | | |
| IAA-amino acid hydrolase homologue (ILL1) | | 5.5 | 0.406 |
| PIN4 homologue | 3.5 | | 2.216 |
| **RNA/DNA-binding** | | | |
| 18S ribosomal protein | | 3-133 | 0.629-0.694 |
| 23S ribosomal protein | | 3.5 | 0.825 |
| 26S ribosomal protein | | 3.5 | 0.472-0.694 |
| 30S ribosomal protein | | 3 | 0.676 |
| 5.8S ribosomal protein | | 2.5 | 1.927 |
| AP2 domain containing protein RAP2.8 (transcription factor) | 4 | | 2.087 |
| DNA repair protein homologue (XPBara) | 3 | | 0.424 |
| eIF4E protein | 7.5 | | 0.377 |
| ESCAROLA (ESC) | | 2.5 | 0.723 |
| IAA1 | 3 | | 0.651 |
| ring-box protein 1 (mammalian) homologue | | 2.5 | 0.396 |
| RNA-binding protein | | 2.5 | 0.514 |
| rRNA repeat unit (most frequent IGR type) | | 4 | 1.760 |
| **GST** | | | |
| GST | 4.5 | | 9.604 |
| **ACC-synthase** | | | |
| **Calcium-binding** | | | |
| calmodulin-2 (CAM2) | | 2.5 | 0.517 |
| **Cell cycling** | | | |
| annexin-like protein | | 2.5 | 0.679 |
| MAP4K alpha 1 | | 2.5 | 0.333 |
| protein phosphatase 2C | 3 | | 11.673 |
| **Cell wall modification** | | | |
| actin 2 | 11 | | 29.591 |
| cellulose synthase catalytic subunit (RSW1) | 11 | | 8.925 |
| fatty acid elongase 3-ketoacyl-CoA synthase 1 (KCS1) | 5.5 | | 3.032 |
| GDP-mannose pyrophosphorylase (GMP1) | 5.5 | | 8.434 |
| membrane-associated acyl-CoA binding protein (ACBP1) | 12 | | 24.421 |
| non-specific lipid transfer protein LTP1 homologue | | 2.5 | 2.794 |

| | | | |
|---|---|---|---|
| **Ubiquitin pathway/protein degradation** | | | |
| shaggy-like kinase etha | | 4.5 | 0.926 |
| SKP1P homologue | | 3.5 | 0.359 |
| ubiquitin conjugating enzyme E2 homologue | | 11.5 | 0.339 |
| **Wounding/defense/stress** | | | |
| ATHP1 | | 6.5 | 0.745 |
| Dr4 | | 4.5 | 0.359 |
| gamma tonoplast intrinsic protein 2 (TIP2) | | 2.5 | 2.748 |
| lipoxygenase (LOX2) | 3 | | 0.403 |
| methionine sulfoxide reductase | >> | | 1.808 |
| myrosinase-associated protein homologue | | 2.5 | 0.504 |
| peroxidase PRXR1 | | 2.5 | 0.887 |
| peroxidase PRXR3 | | 2.5 | 0.816 |
| phenylalanine ammonia-lyase (PAL2) | | 3 | 21.017 |
| thioglucoside glucohydrolase | | 6 | 0.346 |
| wounding/stress induced gene | | 2.5 | 0.813 |
| **Secondary metabolism** | | | |
| 10-formyltetrahydrofolate synthetase (THFS) | | 2.5 | 7.877 |
| ADH homologue | 3 | | 0.561 |
| cobalamin-independent methionine synthase (ATCIMS) | | 3.5 | 1.267 |
| sulfate transporter | | 4 | 1.795 |
| **unknown function or expression pattern** | | | |
| alpha II mitochondrial processing peptidase homologue | 3.5 | | 1.385 |
| beta-glucosidase (psr3.2) | | 3 | 1.116 |
| dormancy-associated protein (DRM1) | | 4 | 0.375 |
| ERD14 | 9 | | 10.712 |
| K efflux antiporter (KEA1) | 3 | | 0.354 |
| receptor-like protein kinase | | 3 | 0.391 |
| Ser/Thr protein phosphatase | | 3 | 0.765 |
| TCTP-like protein | | 8 | 1.743 |
| **light-responsive/plastid localized** | | | |
| chlorophyll a/b binding protein (CAB4) | | 2.5-3 | 0.988-6.462 |
| ferrodoxin-NADP oxidoreductase | 2.5 | | 1.822 |
| IPP2 | | 2.5 | 0.387 |
| plastid protein J8 | | 4 | 1.323 |
| PORB | | 8.5 | 0.477 |
| PS II 10 kDa polypeptide | | 3.5 | 0.446 |
| PS II chlorophyll a/b binding protein (LHB1B1) | | 3 | 23.334 |
| ribosomal protein L12 (chloroplast) | 11 | | 1.687 |
| RNA polymerase A beta subunit | 10 | | 16.395 |
| rubisco small subunit | | 3.5 | 1.195 |
| TRAP | | 7.5 | 0.382 |

**Table 4-2 (previous page): Comparison of mock-treated and IAA-treated seedlings after three hrs.** Ind. = induced; Repr. = repressed; Diff. = difference between the spots as a ratio of the average value of all spots on the filter. >> = spots that do not show a signal in the mock-treated sample (0.000), but do show a signal in the IAA-treated sample.

# 5 DISCUSSION

The aim of this work was the characterization of *AtARCA* gene function, primarily by analysis of the gene expression pattern, the identification of a gene knockout line and the establishment of cDNA arrays in order to analyze the auxin response of this knockout line in comparison to wildtype.

## 5.1 *AtARCA*

### 5.1.1 Structure of the *AtARCA*

***DNA isolation and analysis of the putative translation product***

Two genes have been isolated from *Arabidopsis thaliana*, *AtARCA* and *AtARCA2*, based on homologies with *ARCA* genes from other organisms. The alignment of the two predicted proteins showed 84.8% identical amino acids. Most of the divergence was found in the C-terminal part of the protein. Both proteins consist of seven WD40 repeat units. In the C-terminal part of the protein, the AtARCA2 protein contains an eight amino acid extension in the last WD-repeat, preceded by a stretch of 11 amino acids that bear no identity with the AtARCA protein. It is also in this region, that most of the divergence between plant homologues can be found, although other differences between the plant ARCA's are spread randomly over the proteins. This correlates very well with the finding that for members of the related RACK family from animals the most variable WD40 repeats are three C-terminal repeats, which are thought to be involved in binding of the partners (Csukai *et al*., 1997; Liliental and Chang, 1998).

WD repeats have been found in proteins from all eukaryotes and are implicated in a wide variety of crucial functions. Such WD40 proteins have been shown to be involved in the regulation of vesicular traffic, gene expression, secretion, RNA splicing, neurogenesis, photomorphogenesis of plants and cell cycle progression (reviewed by Neer *et al*., 1994; Smith *et al*., 1999).

The three-dimensional structure of one WD repeat protein, a G protein β subunit, has been published (Wall *et al*., 1995; Sondek *et al.*, 1996; see figure 5-1 panel A). Each WD40 repeat unit has been shown to make up a blade of a typical propeller structure. It is assumed that this propeller structure will be common among all members of the family (Smith *et al*., 1999). Based on this structure a schematic representation of the protein structure has been made (figure 5-1 panel B).

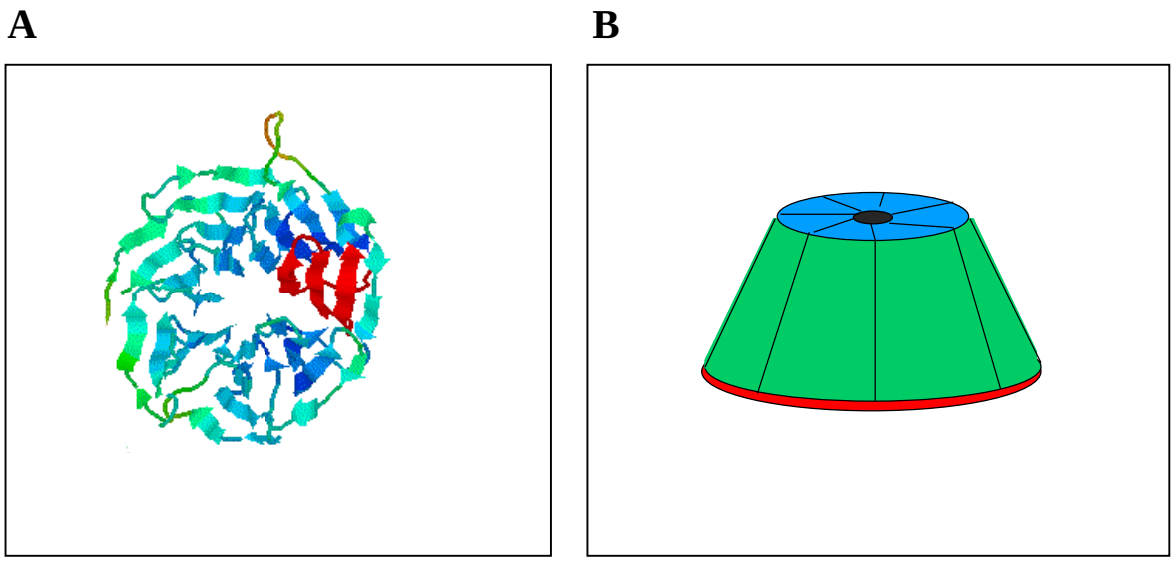



**Figure 5-1: Structure of WD40 repeat proteins.** Panel A: Top view of the propeller structure of WD40 repeat proteins, based on the structure of a G protein β subunit. In red one WD repeat has been indicated. The N-terminal extension typical for Gβ proteins has been removed. Panel B: Schematical representation of the three-dimensional structure of WD40 repeat proteins with 7 repeats. The figure is based on Smith *et al*. (1999).

This closed circular structure is extremely rigid. The common function for the propeller structure could be that it creates a stable platform that can form reversible complexes with several other proteins, thus coordinating sequential and/or simultaneous interactions involving several sets of proteins (Garcia-Higuera *et al*., 1996b; Smith *et al*., 1999).

***Analysis of the AtARCA promoter***

A comparison of the *NtARCA* promoter (Ishida *et al*., 1996) with the *AtARCA* promoter (see Chapter 4.3), revealed several conserved motifs. In the region of the *NtARCA* promoter that has been shown to contain the auxin-responsive motifs (Ishida *et al*., 1996), several MYB binding sites were conserved between *Arabidopsis* and tobacco.

In animals only few MYB-like transcription factors were identified, but from plants like *Arabidopsis thaliana* more than 100 MYB-like factors have been isolated (Martin and Paz-Ares, 1997; Kranz *et al*., 1998). This suggests that MYB transcription factors are functional in the regulation of many plant-specific processes (Kirik *et al*., 1998). For some members of the *MYB* gene family it has been shown that they respond to plant hormones (for an overview see Krantz *et al*., 1998).

Analysis of the *En-1*-mutagenized *Arabidopsis* library (Wisman *et al*., 1998) led to the isolation of a plant line in which the *En-1* transposable element inserted between the MYB binding sites and the start codon (see Chapter 4.6). However, it has been shown in promoter deletion mutants from tobacco, that shorter versions of the *NtARCA* promoter were still capable of maintaining a basic expression level, although this expression could not be increased by auxin treatment (Ishida *et al*, 1996). It can therefore be hypothesized that the identified *En-1*-insertional mutant will show low *AtARCA* expression, leading to a moderate phenotype.

### 5.1.2 *AtARCA* gene expression

The study of the expression profiles in the northern dot blots (see chapter 4.5.2) lead to the hypothesis that *AtARCA* might not be responding to auxin in a direct manner, but rather to the processes evoked by subsequential starvation and re-addition of auxin. It might very well be that *AtARCA* is involved in the secondary process of cell division itself, which is in part auxin regulated. This hypothesis is based on the following observations:

- The *AtARCA* gene is responding to auxin in suspension cells after starvation and re-addition of auxin. Pre-incubation with cycloheximide inhibited this effect. This indicates that the induction of *AtARCA* gene expression is secondary and that continued *de novo* protein synthesis is required for the response to auxin. The treatment with cycloheximide alone did not lead to an increase of *AtARCA* gene expression. This further supports the idea that the response of *AtARCA* to auxin is secondary.
- The pre-incubation of starved cells with either an inactive auxin or an auxin inhibitor did not lead to an inhibition of the auxin response of *AtARCA*. Therefore, the assumption lies near that the *AtARCA* gene might not be involved in auxin perception itself, but rather in processes further downstream of the response. Apparently, the inhibition of the auxin effect does not interfere with the pathway leading to an induction of *AtARCA*.
- The auxin-specific response could not be shown using cells that were not starved for auxin. This indicates that *AtARCA* does not respond to auxin itself, but rather to a process evoked by auxin application to starved cells.

In literature, only indirect support was found for the original finding that *ARCA* genes are involved in the auxin-response itself. The response to auxin after starvation was found for the original *NtARCA* as described by Ishida *et al*. (1996). In these experiments it was also shown by application of cycloheximide that the response of *NtARCA* is secondary. It has also been shown in literature that starvation for auxin leads to a cell cycle arrest in both G1 and G2 (Ferreira *et al*., 1994). In *Catharanthus roseus* it has been shown that re-addition of auxin to auxin-starved suspension cells lead to the synchronous division of cells (Nishida *et al*., 1992).

This indicates that the subsequential starvation and re-addition of auxin involves severe physiological changes. Therefore, the response of a gene in such a situation may very well be caused by physiological changes resulting from auxin application to auxin-starved cells and less by the auxin application itself.

Also other homologous genes have been shown to respond to different agents. The homologue from *Medicago sativa* (*MsARCA*) does not show an auxin response as well (McKhann *et al*., 1997). The expression of *MsARCA* was highest in dividing tissue. Application of 2,4-D to (not starved) root tissue did not lead to *MsARCA* induction. The response to 2,4-D in auxin-starved suspension cells was only very weak. The authors explain this in part by the difficulty to starve alfalfa suspension cells for auxin. The *MsARCA* gene was shown to be induced upon treatment of root culture with synthetic cytokinin, which has originally been isolated as stimulant of cell division (see Palme *et al*., 1996 for an overview). The expression of tobacco *NtARCA3*, a gene that differs from the original *NtARCA* isolated by Ishida *et al*. (1993) only in the 3' untranslated region, is not induced auxin itself, but rather by the entrance into the cell cycle (Perennes *et al*., 1999). This is in contrast to *NtARCA*, which was shown to be independent of cell cycling (Ishida *et al*., 1996). *NtARCA3* expression was induced upon treatment with UV light, whilst treatment with salicylic acid prevents gene induction (Perennes *et al*., 1999). Two homologous genes from tomato were shown to be upregulated in lines that overexpresssed systemin, a wound-induced defense signal (Kiyosue and Ryan, 1999). In these lines, many genes involved in the response to wounding were found to be upregulated. This would implicate a role for these *ARCA* genes in the wound response.

The observations in this thesis combined with the studies on homologous genes in other plant species lead to the hypothesis that *ARCA* genes are involved in the secondary response to various inducing agents. Different hormones seem to play a role in the regulation of some of these genes (e.g. auxin, cytokinin, salicylic acid), but also plant defense seems to play a role. The isolation, and the further characterization by cDNA arrays, of *AtARCA* knockout mutants may provide further insights in this complex process. Since it has been described by several authors (Machida *et al*., 1997; Ito *et al*., 1999) that transposition often occurs into adjacent sites, the analysis of the offspring of line 6AAR74 might lead to the isolation of a complete knockout of *AtARCA*.

## 5.2 The use of cDNA arrays in the isolation of auxin-responsive genes

### 5.2.1 Establishment of the technique

In the presented work a set of protocols for the generation and analysis of cDNA arrays (derived from J. Hoheisel, Heidelberg) has been optimized. The following chapter will address the most crucial steps of this procedure.

***EST library***

Although it is possible to use plasmid DNA or bacteria in arrays for certain experiments (i.e. analysis of insertions in genes or gene localization on BACs), this is not suitable for expression analysis. The reason for this is that the amount of DNA spotted will differ from array to array. Also the background caused by the bacterial plasmid in hybridizations leads to problems in the evaluation of the results. Therefore, the DNA used for spotting is normally derived from PCR reactions.

The quality of the EST library used for these experiments is not optimal (R. Stracke, personal communication). After sequence analysis of 150 random picked clones through the whole library, it turned out that about 4% of all clones did not grow. About 12% of the clones showed the wrong identity, caused by cross-contamination. Furthermore, 26.5% gave rise to more than 1 PCR product or didn't result in any product at all and could therefore not be sequenced. In the end, 57.5% of the clone identities could be confirmed. The exchange of 4000 of the clones only in part improved this. In the case of missing PCR products or the presence of empty clones no hybridization signal will be obtained. However, the fact that several clones show contamination with other clones may lead to artefacts, hence the results from the hybridizations may show an expression profile with a wrong identity for these clones. Furthermore, reports from TIGR (http://www.tigr.org) indicated a redundancy of the uniset EST library of 2-3, based on alignments of the ESTs with genomic DNA.

Although the cross-contamination and redundancy of the EST library may reduce its quality, it is still the library of choice when investigating complex expression patterns by cDNA arrays in *Arabidopsis thaliana*. The main advantage lies in the partial sequences that are available for all clones. Until new libraries have been developed, filtered for redundancy and gene identities have been established, the EST library remains the best choice.

A large number of ESTs (approximately 50%) have no homologies to genes with a known function (see Appendix A). Some of these ESTs show a strong expression difference and therefore become more interesting for further research. Expression analysis on arrays followed by clustering might in the end lead to a function for these ESTs. It has been described recently that cluster analysis led to the identification of the function for some open reading frames in yeast (Hughes *et al*., 2000).

***Spotting of arrays***

Several steps in the spotting procedure have to be performed carefully, since improper spotting may lead to arrays that could not be evaluated anymore. The most typical of these steps were:

- *Moisture of the membrane.* Spotting on membranes that are too wet leads to diffuse spots or even diffuse patterns because the PCR product is not concentrated on a single position.
- *The amount of spotted DNA.* When too much PCR product is spotted, this leads to overlapping spots or signals. When too little PCR product is spotted the signal will be too weak for good evaluation. This often occurs when the wrong combination of pin diameter and spotting replications is chosen.
- *The choice of the membrane.* The use of membranes with lower quality may lead to diffuse spots as was shown in the case of the Genetix membranes. These membranes might be very good for other procedures (for instance bacterial high density membranes for clone identification) but are not suitable for cDNA spotting.

The best quality of arrays was obtained using precut Amersham Hybond $N^+$ or PALL Biodyne B membranes in combination with a 10-fold spotting replication per spot. The grid that seems

to lead to the best results is the 4 x 4 pattern which leaves enough space between the spots to avoid too much influence of neighboring spots after hybridization.

A typical array can be used up to 10 times. Prior the first hybridization the array was submitted to a cold hybridization followed by a normal recycling procedure because the signal from the first hybridization would be much stronger when compared to the following hybridizations. The second hybridization is then the oligonucleotide hybridization. This leaves 8 hybridizations with complex probes before the array becomes too old. This makes the membrane based arrays quite cost-efficient and attractive to use.

One of the most important problems of these experiments is the sensitivity of the method and the need of standardized, controlled growth conditions. In these experiments an artificial setup was chosen to keep the growth conditions optimal. Still, an influence of light was observed. If plants are grown in the greenhouse, even more factors would have an effect. Factors such as soil composition, light, water, pathogens and time of harvest will be among the most important triggers influencing gene expression. Even comparison of plants standing next to each other in the same greenhouse will be difficult. During the evaluation of the data, it also became clear that quite a number of replications is necessary for reliable results. Furthermore, it is necessary to confrim the expression profiles of some genes obtained by these novel high density DNA arrays using more traditional methods such as northern blots.

### 5.2.2 Evaluation of the differential expression of ESTs under the influence of auxin

After optimization of the technique the arrays were used to analyze the differential expression of genes after auxin induction. Since it was found that the *ARCA* expression level has a peak between 1 and 6 hours after auxin induction (chapter 4.5.2 and Ishida *et al.*, 1996) it was decided to use the three-hour time point for auxin induction to study the response in wild type plants. In the following chapter some abbreviations will be used, T3 for the mock-induced sample harvested after 3 hours and T3 IAA for the IAA-induced sample harvested after 3 hours. Since the results were not confirmed as yet by either northern analysis or sequence determination, they have to be treated with care.

An important factor for the calculations is not only the ratio of the values, but also the difference between them. This is important for the removal of genes showing only background expression (an increase from 0.001 to 0.050 would be a 50-fold increase, although both values are considered background) and is necessary for better comparison. In fact, ESTs showing variations between duplicate spots of more than 20% were removed for noise correction. Some ESTs that have homologies to known auxin-responsive genes may have been removed by this operation. This variation between duplicate spots has been used to eliminate those ESTs that have too much noise. This can be caused by differences during the spotting, but also by overlapping radiation of strong neighboring spots or the presence of contaminant 'sprinkles' common to the Southern blot procedure. This noise correction has more influence on weak signals than on strong signals because of the use of percentages.
In the following chapter, the auxin responsive elements will be discussed in the groups that have been used in Table 4-2 as well. However, two exceptions were made. Since ACC-synthase was not shown to respond in these experiments, this group was removed. The group of light-responsive genes, originating from noise caused by the experimental setup, is not discussed further in this chapter.

*Genes involved in auxin-related functions*

Some of the genes responding to auxin are involved in auxin-related functions in the cell. Among these genes is the *PIN4* gene, a putative auxin transporter, which shows an 4-fold induction (Chen *et al*., 1998; Gälweiler *et al*., 1998; Luschnig *et al*., 1998; Müller *et al*., 1998). The fact that the expression of *PIN4* is apparently regulated by auxin concentration, which was not published until now, indicates that an increased IAA concentration may promote auxin transport by PIN4.
Another gene that was found to be differentially expressed is the IAA-amino acid hydrolase ILL1, which seems to be down-regulated under the influence of auxin. This enzyme is responsible for the cleavage of IAA that is conjugated to amino acids and regulation of the endogenous IAA concentration (Davies *et al*., 1999). The IAA-amino acid conjugates have been suggested to serve as transportable or inactive forms of the hormone on pathways to IAA catabolism (Bandruski *et al*., 1995; Davies *et al*., 1999). A direct influence of the IAA

concentration in the cell on the gene expression of this enzyme has not been described as yet. However, a feedback mechanism seems logical for this enzyme.

*Genes encoding DNA-binding proteins*

Among the most investigated genes that are expressed under the influence of auxin are members of the *IAA/AUX* family. One gene of this family is *IAA1*, which is up regulated after auxin application in the experiments described here. There are several indications that the gene products of *AUX/IAA* genes bind DNA. Proteins of the family of *IAA* and *AUX* genes are considered to be nuclear localized regulators for gene expression, based on three observations. First, most IAA/AUX proteins are nuclear localized and contain conserved targeting signals that are essential for nuclear transport (Abel *et al*., 1994; Abel and Theologis, 1995; Abel and Theologis, 1996). Also, it has been shown that expression of *PS-IAA4/5* and *PS-IAA6* is stringently regulated at multiple levels (Abel *et al*., 1994). This is typical for genes encoding regulatory proteins. Finally, AUX/IAA polypeptides contain five invariant hydrophobic amino acids that are predicted to form an amphipathic helix. This helix contributes to a putative βαα-fold with similarities to the DNA-binding domain of prokaryotic repressor proteins (Abel *et al*., 1994; Abel and Theologis, 1996).

The gene encoding the RAP2.8 transcription factor was induced in the IAA-treated sample. RAP2.8 is a member of the AP2 domain containing protein family, of which responses to, amongst others, stress and hormones have been shown before (Okamuro *et al*., 1997). All members of this family have been shown to be nuclear transcription factors in plants (Okamuro *et al*., 1997). The RAP2.8 protein belongs to the subfamily of ethylene responsive element binding proteins (EREBPs) (Okamuro *et al*., 1997). It has been reported previously that exogenous application of IAA can induce ethylene production (Abeles, 1966; Burg and Burg, 1966). Plants overproducing IAA have also been shown to have an enhanced ethylene production (Sitbon *et al*., 1999), which would correlate also with the induction of the ethylene precursor synthesizing enzyme ACC-synthase in auxin-treated cells that has been reported previously (Arteca and Arteca, 1999; Yi *et al*., 1999). This is the first publication in which involvement of the RAP2.8 protein in the auxin response has been shown.

*Glutathione-S-transferase*

Under the influence of auxin the induction of a few GSTs was observed. This has been found by several other researchers as well (see for an overview also Sitbon and Perrot-Rechenmann, 1997). Using photoaffinity labeling, GSTs encoded by the *Hmgst-1* and *Atpm24* genes have been identified as auxin-binding proteins (Bilang *et al*., 1993; Zettl *et al*., 1994; Sitbon and Perrot-Rechenmann, 1997). It was proposed that auxin binds to a non-catalytic site and is conjugated to the GST substrate glutathione (GSH) (Bilang and Sturm, 1995; Sitbon and Perrot-Rechenmann, 1997). Although *Atpm24* was not able to catalyze the formation of IAA-GSH *in vitro* (Zettl *et al*., 1994), the possibility still excists that IAA-GSH conjugates are formed, thus influencing auxin activity or transport at the same time as auxins play a role (Marrs, 1996; Sitbon and Perrot-Rechenmann, 1997). This result would actually agree with the reduced expression of the IAA-amino acid hydrolase homologue *ILL1*. It would seem likely that the auxin concentration in the cell acts directly on the genes encoding proteins that regulate storage of auxin. More auxin may be stored in an inactive, bound form, and less auxin retrieved from this storage.

*Genes encoding ubiquitin-related proteins*

Subunits of the ubiquitin E2 and E3 complex (ubiquitin conjugating enzyme E2 and SKP1P) were downregulated after auxin induction. The ubiquitin pathway has previously been reported to be involved in the primary response to auxin, as has also been described in chapter 1.2.7. For one of the putative targets, the group of IAA proteins, it has been shown that these are very strictly regulated and that the gene expression declines after a peak expression level between 30 and 120 minutes after auxin induction (Abel *et al*., 1995). The apparent drop in the mRNA concentration of ubiquitin-related genes three hours after auxin induction might therefore be caused by the normal variation caused by the setting of the new equilibrium.

*Genes encoding calcium-binding proteins*

In the experiments described here, a calmodulin-encoding gene, *CAM2*, was shown to be repressed. Although no studies on the differential expression of genes encoding calcium-binding proteins under influence of auxin have been reported, there are some indications that

these genes might respond to auxin indirectly, caused by an increase in the cytosolic calcium concentration induced by auxin. Although this increase in calcium concentration has been observed in the response to many different factors (e.g. light, auxin, ABA, touch), not much is known about the exact regulation of this pathway.

*Genes encoding proteins involved in synthesis and modification of cell walls*

The differential expression of genes encoding proteins involved in synthesis and modification of cell walls was observed as well. One of the genes induced by auxin was *actin-2*. Since the *actin-2* gene is supposed to be expressed at a constant level in vegetative tissues of the plant (An *et al*., 1996), it is often used as a loading control for northern analysis. In the experiments performed in this thesis it has been found that actin-2 is actually differentially expressed under the influence of auxin and therefore not always suitable as a loading control. Attempts have been made to use *actin-2* as a loading control in the *AtARCA* expression studies using auxin-starved suspension cells (data not shown). The use of this gene as a control was not possible in these experiments, since it turned out that *actin-2* also responded to the starvation and re-addition of auxin. This is not really a surprise if one considers the function of actin. Actins are involved in cytoskeletal processes, including cell division, cell elongation and cell wall deposition (Gilliland *et al*., 1998). It has also been found that some actins respond to auxin: the longitudinal actin microfilaments become loosened in response to auxin (Wang and Nick, 1998). Since apparently actins are involved in cell elongation, a process that is stimulated by auxin, differential expression of these genes would be expected, although the differential expression of *actin-2* under the influence of auxin was not reported as yet.

*Genes involved in wounding and pathogenesis response*

The down-regulation of the peroxidases *PRXR1* and *PRXR3* by auxin is interesting. Peroxidases are involved in the lignification of secondary cell walls. Therefore, the response of the genes are often correlated with plant defense and stress response (Klotz and Lagrimini, 1996). This type of peroxidases is down-regulated by auxin. Other peroxidases however, are important in the oxidation of IAA and their expression is upregulated by exogenous auxin

application (Savitsky *et al*., 1999). The repression of the peroxidases *PRXR1* and *PRXR3* by IAA seems to point to a function of these particular genes in plant defense.

*Genes involved in secondary metabolism*

Alcohol dehydrogenase gene expression was induced by auxin. This has been reported previously (Kato-Noguchi, 2000). Of all plant hormones capable of induction of ADH gene expression, auxin seems to be the most potent of all.

A gene involved in the methionine synthesis, *ATCIMS*, is inhibited after IAA treatment. Methionine is the immediate precursor of S-adenoylmethionine, which has been implicated to be linked to cytokinin and auxin action (Ravanel *et al*., 1998). The expression of cystathionine gamma-synthase, an enzyme involved in methionine biosythesis has previously been shown to be inhibited under influence of auxin (Marty *et al*., 2000), indicating that auxin could have a repressive effect on this pathway, as was confirmed by these experiments. Methionine is synthesized in several steps from aspartate (Asp), as is also the case for the essential amino acids lysine and threonine. These both essential amino acids are considered to be amongst the most important amino acids, since they are the seriously limited (Bright and Shewry, 1983).

Plants produce conjugates when exposed to auxin (e.g. IAA-Asp, IAA-Ala , and IAA-Glu; Andreae and Good, 1955; Venis, 1972; Catala *et al.*, 1992; Riov and Bangerth, 1992; Barratt *et al*., 1999). Most of the plant's endogenous IAA is conjugated to aspartate. If therefore the concentration of auxin increases, the concentration of free aspartate available for synthesis of lysine, threonine and methionine is decreases. It seems likely that the synthesis of methionine will be inhibited first, since the limitation in the cell for methionine is not as serious as for the both other amino acids. This hypothesis correlates with the decreased expression of *ILL1*, responsible for the cleavage of IAA-amino acid conjugates, and with the increased expression of *GST*, which is also involved in IAA storage. This indicates that plant cells store IAA after external application of auxin and that the auxin concentration may directly regulate this process.

## 5.3 Future prospects

For further studies on *AtARCA* a functional knockout has to be isolated. For this purpose, the offspring of line 6AAR74 will be analyzed for mutants in which the *En-1* transposed to an exon. A T-DNA tagged *Arabidopsis thaliana* library has become available for our institute recently. This library will be screened for *AtARCA* knockout lines as well. These knockout lines may give a better insight in the way auxin and cell division are linked and how the cell division process is regulated.

Furthermore, it would be interesting to see which binding partners might be involved in the action of AtARCA. No PKC has been isolated from plants so far. Because of the high conservation the PKC receptor in animals and the homologue in plants, the isolation of binding partners of the plant homologues may lead to further insight into the conservation of the PKC regulated pathways as well. The isolation of binding partners for ARCA proteins could be performed by the yeast two hybrid system. Another method would be the cross-linking of *in vitro* transcribed and translated ARCA in a crude extract of *Arabidopsis thaliana* suspension cells, similar to the experiments that have been described for the isolation of partners of the signal recognition complex in *Escherichia coli* (Valent *et al*., 1997). However, the latter method would require the use of good antibodies against (recombinant) ARCA, which are not available so far, and will not allow for easy gene identification, in contrast to the yeast two hybrid system. Finally, the recent progress in the development of protein and peptide arrays (for an overview see Emili and Cagney, 2000) makes this type of analysis an interesting alternative. In addition, one could emphasize the study on binding partners of both *Arabidopsis* ARCA proteins combined with swapping experiments, in which the seventh WD40 repeat is exchanged between both proteins, thus creating protein hybrids. This may give more insight into the question if this region is important for the specificity in binding of partners.

The difference between the both *ARCA* genes and their regulation in *Arabidopsis* is an interesting factor. The use of promoter-GUS fusions in the analysis of promoter action of both genes may be used in this respect. Also, it would be interesting to see if *AtARCA* and

*AtARCA2* show differences in the regulation by plant hormones. It may be possible that different *ARCA* genes are regulated by different factors (e.g. auxin, cytokinin, UV-damage).

With the establishment of the cDNA arrays an important and powerful method has become available. This method can be used in the identification of genes involved in certain signal transduction pathways. Once these have been identified, other techniques will be used for further research. The major advantage is the possibility to obtain a broad view on all genes that are involved in the response to certain triggers. When these data are combined in large databases, possibilities for comparisons of different hybridizations and sunsequential cluster analysis of genes is possible. As was shown for *Saccharomyces cerevisiae*, cluster analysis can eventually lead to the identification of functional pathways unknown genes are involved in (Hughes *et al*., 2000).

Although the technical development of the experiments with cDNA arrays has been established, the data analysis is not established completely. For the work in this thesis, all EST identities had to be confirmed by manual BLAST analysis. The need for good bioinformatic assistance is therefore indispensable. The comparison with other techniques for the isolation of differentially expressed genes often leads to the idea that the experiments with DNA arrays are taking relatively little time. On the other hand, data evaluation is, because of the huge amount of data, very time consuming.

# 6 SUMMARY

The goal of the work described in this thesis was the analysis of differential gene expression in response to auxin. These studies should be based on the characterization of the *Arabidopsis* homologue of the *ARCA* gene from tobacco. This gene shows very high homology to animal counterparts that are involved in the regulation of key regulator of cell division in animal cells, protein kinase C, which so far has not been isolated from plant systems. Upon auxin induction in suspension cells, the *ARCA* expression is induced, which may provide a direct link between auxin and cell division.

Two genes homologous to *NtARCA* were isolated from *Arabidopsis thaliana*. The characterization of the expression pattern of one of these genes (*AtARCA*) was studied by northern dot blots of RNA isolated from *Arabidopsis* suspension cells that were treated in different ways. It was found that the response of *AtARCA* is dependent on *de novo* protein synthesis and only takes place after starvation of the cells for auxin. The auxin that is used in the induction does not make a difference and the response can not be inhibited by pre-incubation with an inactive auxin, 2-NAA, or an anti-auxin, PCIB. It was also found that the expression level is lower in older cells. Therefore, it was concluded that the response of *AtARCA* might be a secondary effect of the cellular response to auxin.

For further studies on the pathways *AtARCA* may be involved in, an *Arabidopsis thaliana En-1* insertional library was screened for the presence of a knockout line in which this gene is corrupted. Although one line was identified in which the *En-1* element inserted 830 base pairs in front of the start codon, no lines with insertions into the coding region could be identified as yet. Such a knockout will be used to study the response to auxin in a wide expression pattern by the relatively novel technique of cDNA arrays. The establishment of this technique based on nylon membranes has been described.

In first studies after optimization of this technique, ten-day-old seedlings grown in liquid cultures were induced with 10 µM IAA. The differential response of 12672 ESTs derived from the Ohio State EST library after 3 hours of IAA-induction was investigated. The response to auxin of some of the genes corresponding to the ESTs was described previously, which indicated that the auxin induction worked well. Furthermore, the response to IAA of several

genes was described for the first time. Moreover, based on these results it could be concluded that upon external IAA application cells apparently store auxin as conjugates that may serve as transportable or inactive forms of the hormone on pathways to IAA catabolism.

# 7 ZUSAMMENFASSUNG

Das Ziel dieser Arbeit ist die Analyse differentieller Genexpression im Hinblick auf eine Auxinantwort in *Arabidopsis thaliana*. Dabei bezieht man sich auf die Charakterisierung des *Arabidopsis* homologen *ARCA*-Gens aus Tabak. Es zeigt sich eine sehr große Homologie zu den entsprechenden tierische Analoga, die eine Rolle bei der Regulation einer der Schlüsselfaktoren in der tierische Zellteilung, Protein Kinase C, die bisher in Pflanzen noch nicht isoliert werden konnte, spielen. Tatsächlich ist nach Auxin-Induktion in pflanzlichen Suspensionskulturen die ARCA-Genexpression erhöht, was auf eine direkte Beziehung zwischen Auxin und Zellteilung hinweisen könnte.

Es wurden zwei Homologe zu *NtARCA* aus *Arabidopsis thaliana* isoliert. Die Charakterisierung des Expressionsmusters einer diese Gene, *AtARCA*, erfolgte anhand von RNA isoliert aus *Arabidopsis* Suspensionszellen, die mit unterschiedlichen Substanzen behandelt wurden. Es wurde gezeigt daß *AtARCA*-Induktion abhängig ist von *de novo* Proteinsynthese und nur nach Auxindepletion des Mediums stattfindet. Dabei spielte es keine Rolle welches synthetisches Auxin verwendet wurde. Außerdem konnte die Vorinkubation mit einem inaktiven Auxin, 2-NAA, oder auch mit einem Anti-Auxin, PCIB, keine Hemmung der Auxinantwort des *AtARCA*-Gens bewirken. In älteren pflanzlichen Zellen wurde eine geringere *AtARCA*-Expression gefunden. Auf Grund dieses indirekten Beweises und ähnlicher Beobachtungen für andere heterologen pflanzlichen Gene kann die Auxinantwort des *AtARCA*-Gens auf einen sekundären Effekt zurückgeführt werden und somit in den Zellteilungsprozeß involviert sein.

Darüberhinaus wurde eine *Arabidopsis thaliana En-1* Insertionsbank auf eine *knockout* Linie hin durchgemustert um Stoffwechselwege zu charakterisieren bei denen *AtARCA* eine Rolle spielt. Eine Linie, die eine En-1-Insertion 830 Basen vor dem Start-Codon aufwies konnte isoliert werden, aber keine die eine Insertion innerhalb der kodierenden Region besaß. Ein solcher *Knockout* würde dann die Analyse in Bezug auf eine Auxinantwort mit einer sehr große Expressionbreite mittels der relativ neuen *cDNA-Array* Methode ermöglichen. In dieser Arbeit wurde die *cDNA-Array* Methode basierend auf die Anwendung von Nylonmembranen etabliert. Dabei wurden die besten Ergebnisse mit vorgeschnittenen Amersham Hybond $N^+$

oder PALL Biodyne B Membrane in Kombination mit 10-facher Wiederholung des *Spottens* erzielt. Ein 4 x 4 Muster ergibt die eindeutigsten Signale, sodaß diese sich nach der Hybridisierung nicht gegenseitig beeinflussen. In ersten Versuchen nach der Optimierung dieser Technik wurden 10 Tage alte Keimlinge in Flüssigkultur mit 10 µM IAA induziert. Die differenzielle Expression von 12672 ESTs aus der Ohio State University EST Bank wurde 3 Stunden nach IAA-Zugabe untersucht. Obwohl die Qualität dieser Bank nicht optimal ist, liegt der Vorteil darin, daß partielle Sequenzen für alle Klone vorhanden sind.
Die Auxinantwort für einige Gene die als EST in dieser Bank vorliegen ist vorher beschrieben worden, womit eine funktionierende Auxin-Induktion bestätigt werden konnte. Weiterhin wurde die differenzielle Expression einiger Gene zum ersten Mal beschrieben. Hierbei konnte gezeigt werden daß nach einer externen Auxinzugabe die Keimlinge offensichtlich Auxin als Konjugate speichern, die als transportable oder inaktive Form des Hormons zum IAA-Katabolismus dienen könnten. Die Stimulierung des Auxintransporters PIN4 deutet auf einen direkten Einfluß der Auxinkonzentration auf den Auxintransport hin.

Die Anwendung von Arrays ergab ebenfalls einige Hintergrundsignale in Bezug auf Lichtregulierte Gene, verursacht durch den Aufbau des Experiments. Dies bedeutet daß diese Methode sehr sensitiv ist und somit Probenbereitung und –auswahl sowie deren Kontrolle sehr wichtig ist. Außerdem ist eine adäquate Anzahl von Kontrollen zu berücksichtigen und einige Wiederholungen sind zu vollziehen, die die interne Variationen bediungt durch den Versuchsaufbau einschränken. Die Ergebnisse erzielt mit *cDNA-Arrays* müssen weiterhin im traditionellen Northern Blot bestätigt werden.

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# 9 APPENDIX

| Position | | Gene identity | - | + | Ratio |
|---|---|---|---|---|---|
| 1 | A - 24 | unknown | 0,531 | 0,157 | -3,5 |
| 1 | A - 9 | ERD14 | 1,360 | 12,072 | 9,0 |
| 1 | B - 4 | GST | 2,789 | 12,393 | 4,5 |
| 1 | D - 2 | cellulose synthase catalytic subunit (RSW1) | 0,839 | 9,764 | 11,5 |
| 1 | D - 3 | eIF4E protein | 0,060 | 0,437 | 7,5 |
| 1 | G - 11 | chlorophyll a/b binding protein (CAB4) | 4,835 | 1,844 | -2,5 |
| 1 | J - 24 | unknown | 2,922 | 1,027 | -3,0 |
| 2 | B - 3 | unknown | 10,303 | 30,543 | 3,0 |
| 2 | B - 4 | GDP-mannose pyrophosphorylase (GMP1) | 1,929 | 10,363 | 5,5 |
| 2 | B - 5 | unknown | 2,779 | 24,127 | 9,0 |
| 2 | B - 9 | membrane-associated acyl-CoA binding protein (ACBP1) | 2,009 | 26,430 | 13,0 |
| 2 | C - 1 | unknown | 1,327 | 5,333 | 4,0 |
| 2 | C - 2 | alpha II mitochondrial processing peptidase homologue | 0,552 | 1,937 | 3,5 |
| 2 | C - 3 | unknown | 0,385 | 1,429 | 3,5 |
| 2 | D - 2 | unknown | 0,462 | 1,399 | 3,0 |
| 2 | F - 2 | unknown | 0,692 | 2,053 | 3,0 |
| 2 | J - 23 | unknown | 0,588 | 0,198 | -3,0 |
| 2 | N - 14 | unknown | 0,329 | 0,894 | 2,5 |
| 2 | O - 22 | unknown | 0,522 | 0,165 | -3,0 |
| 3 | A - 13 | ferrodoxin-NADP oxidoreductase | 1,176 | 2,998 | 2,5 |
| 3 | A - 2 | unknown | 3,995 | 20,709 | 5,0 |
| 3 | A - 20 | unknown | 2,084 | 0,828 | -2,5 |
| 3 | A - 4 | RNA polymerase A beta subunit (chloroplast) | 1,963 | 18,358 | 9,5 |
| 3 | A - 6 | AP2 domain containing protein RAP2.8 | 0,724 | 2,811 | 4,0 |
| 3 | B - 3 | unknown | 0,382 | 1,480 | 4,0 |
| 3 | B - 5 | unknown | 2,436 | 39,316 | 16,0 |
| 3 | B - 7 | ADH homologue | 0,340 | 0,901 | 2,5 |
| 3 | C - 2 | actin-2 | 2,820 | 32,411 | 11,5 |
| 3 | C - 3 | unknown | 0,400 | 1,060 | 2,5 |
| 3 | C - 5 | unknown | 2,347 | 13,764 | 6,0 |
| 3 | D - 3 | gamma tonoplast intrinsic protein (TIP2) | 4,478 | 1,729 | -2,5 |
| 3 | E - 14 | unknown | 0,729 | 0,291 | -2,5 |
| 3 | I - 6 | peroxidase (PRXR3) | 1,300 | 0,484 | -2,5 |
| 3 | J - 20 | unknown | 0,038 | 0,452 | 12,0 |
| 3 | J - 23 | unknown | 0,662 | 0,256 | -2,5 |
| 3 | M - 22 | annexin-like gene | 1,131 | 0,452 | -2,5 |
| 3 | M - 23 | chlorophyll a/b binding protein (CAB4) | 10,234 | 3,773 | -2,5 |
| 3 | N - 19 | unknown | 0,701 | 0,236 | -3,0 |
| 3 | O - 11 | MAP4K alpha 1 | 0,520 | 0,187 | -3,0 |
| 3 | O - 22 | unknown | 1,029 | 0,346 | -3,0 |
| 3 | O - 3 | unknown | 1,608 | 0,441 | -3,5 |
| 4 | A - 2 | lipoxygenase (LOX2) | 0,224 | 0,627 | 3,0 |
| 4 | G - 24 | peroxidase (PRXR1) | 1,426 | 0,538 | -2,5 |

| | | | | | |
|---|---|---|---|---|---|
| 4 | J - 24 | plastid protein J8 | 1,783 | 0,460 | -4,0 |
| 4 | N - 18 | chlorophyll a/b binding protein (CAB4) | 1,488 | 0,500 | -3,0 |
| 4 | P - 20 | ring-box protein 1 (mammalian) homologue | 0,634 | 0,239 | -2,5 |
| 5 | A - 2 | unknown | 3,353 | 20,660 | 6,0 |
| 5 | B - 4 | fatty acid elongase 3-ketoacyl-CoA synthase (KCS1) | 0,703 | 3,735 | 5,5 |
| 5 | D - 2 | unknown | 0,373 | 1,243 | 3,5 |
| 5 | F - 18 | catalase homologue | 0,811 | 0,314 | -2,5 |
| 5 | H - 8 | chlorophyll a/b binding protein (CAB4) | 1,939 | 0,714 | -2,5 |
| 5 | J - 18 | unknown | 1,152 | 0,372 | -3,0 |
| 5 | J - 22 | unknown | 0,628 | 0,218 | -3,0 |
| 5 | N - 24 | ESCAROLA (ESC) | 1,127 | 0,404 | -3,0 |
| 5 | O - 2 | unknown | 0,755 | 0,281 | -2,5 |
| 6 | A - 12 | unknown | 0,214 | 1,010 | 4,5 |
| 6 | A - 2 | PIN4 homologue | 1,011 | 3,227 | 3,0 |
| 6 | B - 12 | unknown | 0,328 | 0,987 | 3,0 |
| 7 | A - 4 | 10-formyltetrahydrofolate synthetase (THFS) | 12,558 | 4,680 | -2,5 |
| 7 | A - 9 | unknown | 0,222 | 0,611 | 3,0 |
| 7 | B - 2 | ribosomal protein L12 (chloroplast) | 0,161 | 1,848 | 11,5 |
| 7 | B - 24 | unknown | 3,619 | 1,191 | -3,0 |
| 7 | B - 3 | unknown | 0,528 | 2,163 | 4,0 |
| 7 | B - 4 | protein phosphatase 2C | 6,295 | 17,968 | 3,0 |
| 7 | B - 6 | unknown | 0,271 | 0,750 | 3,0 |
| 7 | C - 24 | phenylalanine ammonia-lyase | 30,843 | 9,826 | -3,0 |
| 7 | E - 24 | chlorophyll a/b binding protein (LHCP AB 140) | 39,258 | 13,440 | -3,0 |
| 7 | J - 1 | unknown | 0,518 | 0,132 | -4,0 |
| 7 | K - 22 | wounding/stress-induced gene | 1,337 | 0,525 | -2,5 |
| 7 | M - 1 | Dr4 | 0,466 | 0,107 | -4,5 |
| 7 | M - 22 | shaggy-like kinase etha | 1,194 | 0,269 | -4,5 |
| 7 | N - 3 | rRNA repeat unit (most frequent IGR type) | 2,328 | 0,568 | -4,0 |
| 7 | N - 4 | unknown | 1,506 | 0,463 | -3,5 |
| 7 | O - 7 | unknown | 0,698 | 0,262 | -2,5 |
| 7 | P - 14 | non-specific lipid transfer protein LTP1 homologue | 4,357 | 1,563 | -3,0 |
| 7 | P - 17 | Ser/Thr protein phosphatase | 1,116 | 0,351 | -3,0 |
| 7 | P - 22 | unknown | 2,825 | 0,784 | -3,5 |
| 7 | P - 24 | unknown | 4,198 | 1,627 | -2,5 |
| 8 | D - 22 | light-dep. NADPH:protochlorophylline oxidoreductase (PORB) | 0,541 | 0,064 | -8,5 |
| 9 | M - 1 | DNA repair protein homologue (XPBara) | 0,235 | 0,659 | 3,0 |
| 10 | C - 21 | myrosinase-associated protein | 0,757 | 0,271 | -3,0 |
| 11 | E - 22 | unknown | 1,679 | 0,526 | -3,0 |
| 11 | F - 22 | IAA1 | 0,370 | 1,021 | 3,0 |
| 11 | G - 22 | cobalamin-independent methionine synthase (ATCIMS) | 1,727 | 0,460 | -4,0 |
| 12 | B - 11 | IAA-amino acid hydrolase (ILL1) | 0,492 | 0,086 | -5,5 |
| 12 | E - 19 | receptor-like protein kinase | 0,603 | 0,212 | -3,0 |
| 12 | F - 7 | IPP2 * | 0,628 | 0,241 | -2,5 |
| 12 | O - 12 | unknown | 0,812 | 0,274 | -3,0 |
| 13 | E - 14 | unknown | 0,485 | 0,105 | -4,5 |

| | | | | | |
|---|---|---|---|---|---|
| 13 | I - 18 | unknown | 0,723 | 0,267 | -2,5 |
| 14 | B - 11 | unknown | 0,694 | 0,221 | -3,0 |
| 14 | C - 12 | unknown | 0,827 | 0,324 | -2,5 |
| 14 | I - 14 | photosystem II 10 kDa polypeptide | 0,636 | 0,191 | -3,5 |
| 14 | N - 11 | unknown | 0,451 | 0,014 | -31,5 |
| 14 | P - 18 | unknown | 0,690 | 0,235 | -3,0 |
| 15 | I - 15 | 18S ribosomal protein | 0,634 | 0,005 | -133,5 |
| 16 | L - 23 | 5.8S ribosomal protein | 3,172 | 1,245 | -2,5 |
| 16 | M - 6 | calmodulin-2 (CAM2) | 0,857 | 0,340 | -2,5 |
| 16 | P - 10 | unknown | 0,853 | 0,220 | -4,0 |
| 17 | A - 7 | TRAP | 0,439 | 0,057 | -7,5 |
| 17 | K - 8 | ATHP1 | 0,887 | 0,142 | -6,5 |
| 17 | N - 15 | unknown | 0,460 | 0,097 | -5,0 |
| 17 | N - 24 | dormancy-associated protein (DRM1) | 0,503 | 0,129 | -4,0 |
| 17 | P - 22 | unknown | 0,000 | 1,962 | >> |
| 18 | O - 16 | unknown | 0,766 | 0,216 | -3,5 |
| 18 | O - 24 | unknown | 0,583 | 0,073 | -8,0 |
| 19 | D - 24 | methionine sulfoxide reductase | 0,000 | 1,808 | >> |
| 19 | E - 18 | thioglucoside glucohydrolase | 0,418 | 0,072 | -6,0 |
| 19 | O - 24 | unknown | 0,405 | 0,053 | -7,5 |
| 20 | E - 23 | unknown | 1,973 | 0,432 | -4,5 |
| 20 | G - 8 | unknown | 0,446 | 1,527 | 3,5 |
| 20 | H - 12 | unknown | 2,133 | 0,707 | -3,0 |
| 21 | M - 6 | sulfate transporter | 2,363 | 0,569 | -4,0 |
| 22 | E - 17 | unknown | 0,076 | 0,617 | 8,0 |
| 22 | F - 11 | 26S ribosomal protein | 0,993 | 0,299 | -3,5 |
| 22 | G - 23 | unknown | 1,825 | 0,488 | -3,5 |
| 22 | L - 12 | unknown | 0,610 | 0,210 | -3,0 |
| 22 | P - 22 | photosystem II type I chlorophyll a/b binding protein (LHB1B1) | 35,785 | 12,452 | -3,0 |
| 23 | N - 21 | beta-glucosidase (PSR3.2) | 1,658 | 0,543 | -3,0 |
| 24 | J - 19 | unknown | 1,040 | 0,220 | -4,5 |
| 24 | N - 24 | 18S ribosomal protein | 1,074 | 0,381 | -3,0 |
| 25 | D - 19 | 30S ribosomal protein | 1,052 | 0,377 | -3,0 |
| 25 | L - 8 | TCTP-like protein | 1,997 | 0,254 | -8,0 |
| 26 | B - 10 | rubisco small subunit | 1,700 | 0,505 | -3,5 |
| 26 | K - 15 | SKP1P homologue | 0,517 | 0,158 | -3,5 |
| 26 | L - 20 | ubiquitin-conjugating enzyme E2 homologue | 0,372 | 0,033 | -11,5 |
| 27 | K - 23 | unknown | 0,452 | 0,088 | -5,0 |
| 27 | M - 24 | RNA-binding protein | 0,806 | 0,292 | -3,0 |
| 28 | I - 13 | 23S ribosomal protein (chloroplast) | 1,134 | 0,309 | -3,5 |
| 28 | I - 24 | unknown | 2,114 | 0,652 | -3,0 |
| 28 | J - 24 | unknown | 0,906 | 0,359 | -2,5 |
| 28 | O - 10 | $K^+$ efflux antiporter (KEA1) | 0,207 | 0,561 | 2,5 |
| 30 | G - 21 | 26S ribosomal protein | 0,668 | 0,196 | -3,5 |
| 32 | C - 4 | unknown | 1,840 | 0,394 | -4,5 |
| 32 | E - 2 | unknown | 1,223 | 0,453 | -2,5 |

*: IPP2 = isopentenyldiphosphate:dimethylallyl diphosphate isomerase 2

Position :Plate number followed by position number
- : untreated
+ : IAA-treated
Ratio : A value greater than 0 is induced; a value smaller than 0 is repressed. A value of >> indicates that the untreated sample had only background intensity (0,000). Values are rounded to multiples of 0.5.

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## ERKLÄRUNG

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde.

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Ich versichere, daß ich alle Angaben wahrheitsgemäß nach bestem Wissen und Gewissen gemacht habe, und verpflichte mich, jede die obrigen Angaben betreffende Veränderung dem Dekanat unverzüglich mitzuteilen.

Teilpublikationen:

Vahlkamp L, Palme K (1997): *AtArcA* (Acc. No. U77381), the *Arabidopsis thaliana* homolog of the tobacco *arcA* gene. *Plant Physiol.* **115**: 863.

# LEBENSLAUF

## Persönliche Daten

| | |
|---|---|
| Name: | Lars Vahlkamp |
| Geburtsdatum: | 21.09.1969 |
| Geburtsort: | Amsterdam |
| Familienstand: | ledig |
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## Ausbildung

| | |
|---|---|
| 1974-1981 | Grundschule *Openbare basisschool De Willespoort*, Wilnis. |
| 1981-1988 | Atheneum/Gymnasium *Alkwin Kollege*, Uithoorn. |
| 1988-1993 | Studium der Biologie an der *Vrije Universiteit Amsterdam* |
| 1991-1992 | Erster "Stage" in der Abteilung Pflanzengenetik, *Vrije Universiteit Amsterdam* von Prof. Dr. H.J.J. Nijkamp unter Anleitung von Dr. M.J.J. van Haaren mit dem Thema: "Cloning of strong promoters and an enhancer element in the Ds transposable element to be used in new transposon tagging strategies and the development of melanin as a phenotypic marker in plant transformation studies". |
| 1992-1993 | Zweiter "Stage" am *Center for Engineering Plants for Resistance Against Pathogens* (CEPRAP) in Davis (USA) unter Anleitung von Dr. L. Yu und Prof. Dr. D.G. Gilchrist in Zusammenarbeit mit Dr. J. Hille. Thema der Arbeit: "Solubilization of an insoluble fusion protein and fluorescent labeling of an elicitin from *Phytophthora cryptogea*". |
| 1993 | Abschluß in der Biologie: Master of Science, *Vrije Universiteit Amsterdam*. |
| 1994 | Wissenschaftlicher Mitarbeiter an der *Vrije Universiteit Amsterdam* in der Abteilung Molekulare Mikrobiologie. |
| 1995 | Wissenschaftlicher Mitarbeiter am IPO-DLO, Wageningen. |
| 1996 | Technischer Angestellter am Niederländischen Krebsinstitut (NKI) Amsterdam in der Abteilung Immunologie. |
| 1996-2000 | Doktorand am Max-Delbrück-Laboratorium in der Max-Planck-Gesellschaft in Köln, Anfertigung der vorliegende Dissertation unter Anleitung von Priv.-Doz. Dr. K. Palme mit dem Thema: "Auxin-responsive genes: expression studies with the *AtARCA* gene and cDNA arrays". |