Isolation and Characterisation of Novel *AtPIN* Genes from *Arabidopsis thaliana L.*

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

by

Jirí Friml

aus Nedakonice

Köln 2000

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Abbreviations and symbols

A	Absorbance
aa	Amino acids
ABI	*Applied Biosystems Inc.*
ABP	Auxin binding protein
Amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammoniumperoxodisulfate
A.thaliana	*Arabidopsis thaliana*
A.tumefaciens	*Agrobacterium tumefaciens*
ATP	Adenosin-5'-triphosphate
BAC	Bacterial artificial chromosome
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
bp	Base pair
BSA	Bovine serum albumin
c.1	First columella tier
c.2	Second columella tier
c.i.	Columella initials
cDNA	Complementary DNA
CHX	Cycloheximide
Ci	Curie
4-Cl-IAA	4-Chloriondole-3-acetic acid
cM	CentiMorgan
Col-0	*Arabidopsis thaliana* L., ecotype Columbia 0
CTAB	Cetyldimethylethylammonium bromide
C-terminal	Carboxyterminal
2,4-D	2,4-Dichlorophenoxyacetic acid
Da	Dalton
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
dCTP	Deoxycytidine triphosphate
DIG	Digoxigenin
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP's	2'-Deoxyribonucleotidtriphosphate
DR5rev	Auxin responsive promoter DR5rev
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetate
EGTA	Ethylenglykol-(b-aminoethylether)-N,N,N',N'-tetraacetate
ER	Endoplasmatic reticulum
Etbr	Ethidiumbromide
EtOH	Ethanol
FITC	Fluorescein-Isothiocyanat
g	Gram

GTC	Guanidiumthiocyanat
GUS	β-Glucuronidase
HAc	Acetic acid
h	hour
HEPES	N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IgG	Immunoglobulin G
IPTG	Isopropylthiogalactoside
KAc	Potassiumacetate
kb	Kilobase
kDa	Kilodalton
l	Liter
M	Molar
mA	Milliampere
MeOH	Methanol
Mes	2-(N-Morpholino)ethansulfonic acid
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	messenger" RNA
MOPS	3-(Morpholino)propanesulfonic acid
MS medium	Murashige and Skoog Basal-salt-mixture medium
1-NAA	1-Naphtylacetic acid
NaAc	Natriumacetate
NBT	Nitro Blue Tetrazolium chloride
1-NPA	1-N-naphthylphthalamic acid
Nr.	Number
N-terminal	Amino terminal
o/n	over night
PAA	Polyacrylamide
PAB	Polyclonale antibodies
PAGE	Polyacrylamide gel electrophoresis
pBSK	pBluescript
PCR	Polymerase Chain Reaction
PEG	Polyethylenglykol
PEH A	Phosphonate monoester hydrolase A
PFU	Plaque forming units
PIPES	Piperazine-N,N`-bis(2-ethanesulfonic acid)
PVDF	Polyvinylidendifluoride
PVPP	Polyvinylpolypyrollidone
q.c.	quiescent centre
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per min.
RT	Room temperature

RT PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium dodecylsulfate
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethylethylendiamine
TIBA	2,3,5 Triiodbenzoic acid
Tris	Tris(hydroxymethyl)aminomethane
U	Unit of enzyme activity
V	Volt
WT	Wildtype
YAC	Yeast artificial chromosome
µg	Microgramm
µF	Microfaraday
µl	Microliter
Ω	Ohm
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
X-Gal	5-bromo-4-chloro-3-indolyl-phenylphosphonate
XPP	5-bromo-4-chloro-3-indoxyl phosphonate

Amino acids

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

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

dATP	2’-Deoxyadenosine-5’-triphosphat
dCTP	2’-Deoxycytosine-5’-triphosphat
dGTP	2’-Deoxyguanosine-5’-triphosphat
dTTP	2’-Deoxythymidine-5’-triphosphat
dNTP	2’-Deoxyribonucleosidtriphosphate

1 Introduction

On the margin of Chriby hills sits the mediaeval fortress “Buchlov”. From its terrace visitors have a beautiful view across the wide valley of the South Moravian river “Morava”. This is the place where, centuries ago, the tribunal of the hunter's court used to sit, and where the last farewells with the convicts were held. At this place you can admire even today an old peculiar tree with its strange root like branches – the linden tree of innocence. If you mention this noteworthy tree to residents sitting around at the traditional glass of Moravian wine, you will not only get the possibility to enjoy a taste of this marvellous vintage, but most probably will also have to listen to an old local legend describing the fate of lord Jindrich of Zástrizly who in the 16th century lived in this fortress.

"*Once lord Jindrich of Zástrizly went with his young valet for hunting. Several hours later he was found deceitfully slain with a dagger. The young valet was accused for this murder and imprisoned. After long days of unavailing torture he was condemned to death on the castle terrace. At this time the young man rose and pulled out a young linden tree growing nearby. With the words „I did not do this murder and if after a year this small tree will grow green, you will see you killed an innocent“ he set the linden tree inverted back into the soil. Impressed by these words the lords decided to wait for one year. A year later small green leaves indeed flourished from the previous roots and the young valet was set free*."

This old and rather romantic legend is a beautiful example that can be used to illustrate some important features of plants to laymen: the fascinating ability to adapt their development to cues continuously posed by the environment. Like animals, plants use the same principles to control their genetics and metabolism, but they had to develop their own strategies to accommodate the demands of their sessile and autotrophic life-style. In contrast to animals, which often respond to environmental stimuli by behavioural responses, plants respond by adapting both their development and their metabolism. Plants perceive different aspects of their environment like temperature, light intensity and quality, humidity, soil quality, gravity, parasite attack as well as many other cues. In contrast to most animals, they have very limited possibilities to change and accommodate their sites during their sessile life. As a consequence, they acquired unique mechanisms to continuously adjust their development throughout their lifespan. This is achieved by preventing some plant cells from irreversible commitment to a particular developmental pathway, by reactivation of cell division within existing tissues, and by regulation of differential growth. As primordia develop into organs the different parts grow at different rates to finally resume the specific form of a particular organ. How are these differential growth rates controlled? What are the genetic and molecular control mechanisms, which ensure that single cells within tissues or organs resume their ability to elongate and grow? Despite decades of intense research we are still at the beginning to understand the details of these complex processes. It has, however, become clear that several plant specific signalling molecules, termed phytohormones, play an important role in the regulation of these processes.

1.1 **Phytohormones**

The existence of phytohormones has already been proposed by the German botanist Julius von Sachs and his British colleagues Charles and Francis Darwin in the 19th century (for review see Thimann, 1977; Palme *et al*., 1997). The concept of phytohormones was developed a little bit later around the beginning of the 20th century when mammalian hormones were intensively studied (for overview see Kendrew, 1994). According to these concepts hormones refer to signalling molecules, which are thought to be synthesised in localised places, often in specialised tissues, and transported from there to target tissues where they elicit a response. The magnitude of each response is concentration related. Typically, the sensitivity of a tissue to a given hormone concentration is governed by the number of receptors on the cell surface or, in the case of, for example, steroid receptors in the cytosol (Muldoon *et al*., 1988). Many hormones with widely varying structures have been described and numerous hormone receptors including receptor kinases or G-protein coupled receptors have been uncovered (Smith *et al*., 1999). Most interestingly, recent research suggested that some membrane localised carrier proteins not only act as solute transporters but also as sensors or receptors (Ozcan *et al*., 1996; Lalonde *et al*., 1999).

Although most animal hormones fit this conceptual framework, phytohormones do not seem to strictly adhere to this concept. While some phytohormones act at places distant from the site of synthesis many others seem to act directly at their place of synthesis. In addition, their effects are often pleiotropic, suggesting the involvement of target cells with different sensitivities to perceive and transduce the phytohormonal signals into the final physiological responses (Trewavas, 1981; Mok *et al*., 1994; Palme and Gälweiler, 1999).

Phytohormones describe a group of plant specific signal molecules and synthetic compounds of related structure that are believed to play important roles in the regulation of plant differentiation and development. For almost a century plant scientists have endeavoured to identify these substances and to understand their nature and action. As a result, an enormous quantity of published information is available describing these substances and their effects. Today we know five classes of compounds, the "classic phytohormones" auxin, gibberellins, cytokinins, abscisic acid and ethylene (Davies, 1995). These substances are extended by a growing list of additional plant signal molecules like brassinosteroids, jasmonic acids, lipo-chitooligosaccharides, nitrogen monoxide, and numerous peptides. Broadly speaking, auxins and gibberellins are important for regulation of cell elongation, cytokinins as regulators of cell division, abscisic acid as a substance involved as inhibitor of many processes like seed dormancy, and ethylene as an important regulator of various developmental events like apical hook opening and fruit ripening (for recent discussion see Palme *et al*., 1997; Somerville, 2000).

As auxin has been the phytohormone, which was first discovered and, hence, is physiologically best characterised and because its molecular analysis is within the centre of this thesis, I will first give a brief historical summary on its discovery and characterisation. I will then proceed and discuss the main objectives and aims of my work.

1.2 **Auxins**

The discovery of auxins dates back to Charles and Francis Darwin's experiments on the phototropism of canary grass coleoptiles, which indicated the existence of transported signals (Darwin and Darwin, 1881). From their experiments they concluded that unilateral irradiation of these coleoptiles stimulated bending throughout the length of unshaded coleoptiles indicating the transmission of some "influence" to the other tissues where differential growth (i.e. elongation) was induced. A few years later Boysen-Jensen (1911) was able to pass this "influence" through gelatine and thus demonstrated for the first time its chemical nature. Modification of these early experimental attempts by Frits Went (1928) confirmed the nature of this growth promoting substance, which was therefore termed auxin (gr. "auxein" – to grow). These early studies also resulted in the establishment of the first quantitative bioassay for auxins. Shortly later, the chemical nature of auxin was determined as indole-3-acetic acid (Kögl *et al*., 1934), but it took more than ten years to confirm that auxin was the major growth promoting substance of higher plant tissues (Haagen-Smit *et al*., 1946). Since then an intensive analysis of auxins has been performed throughout the 20th century.

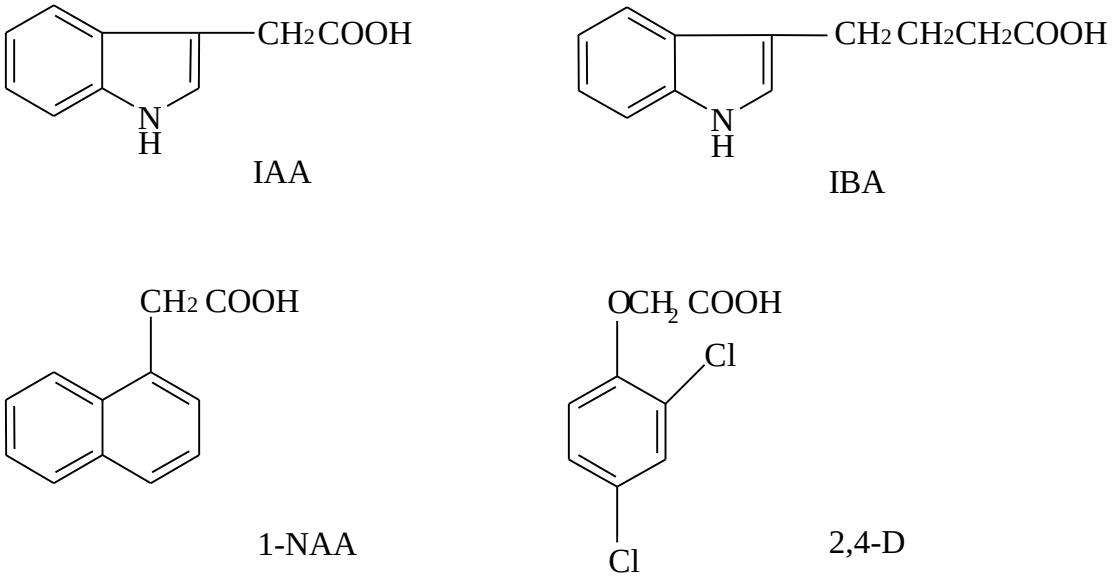
The most abundant naturally occurring auxin in plants is indole-3-acetic acid (IAA); other auxins are indole-3-butyric acid (IBA) and 4-chloroindole-3-acetic acid (4-Cl-IAA). There are also several synthetic auxins with higher metabolic stability, such as 1-naphthylacetic acid (1-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) which are commonly used in commercial agricultural applications. Some of the most important auxins are depicted in Figure 1.

Different forms of auxin have been identified – both free acids and conjugates. IAA can be reversibly conjugated either by an ester bond to sugars or myoinositol or by an amide bond to various amino acids or to small peptides and proteins (Cohen and Bandurski, 1982; J. Cohen, pers. communication). Several genes have been isolated which play a role in synthesis and/or cleavage of auxin conjugates (e.g. IAGLU, Szerszen 1994; ILR, Bartel and Fink, 1995). Little is known about the turnover of IAA, but several lines of evidence support the hypothesis of regulated degradation of free IAA (Tam *et al*., 1995).

Free auxins are usually regarded as physiologically active forms and have been shown to influence a surprisingly wide variety of processes (Davies 1995). Most prominent responses elicited by auxins are:

- cell enlargement and stem growth,
- cell divisions in the cambium and in tissue culture (in combination with cytokinins),
- phloem and xylem differentiation,
- root initiation and differentiation,
- tropic responses to light and gravity,
- apical dominance,
- leaf senescence.

Figure 1. Chemical structures of important auxins.
IAA: indole-3-acetic acid, IBA: indole-3-butyric acid, 1-NAA: 1-naphthylacetic acid, 2,4-D: 2,4-dichlorophenoxyacetic acid.



Nevertheless, the question remains, how does a single, chemically simple molecule of low information content mediate such a variety of apparently unrelated processes? We are still far from understanding this fascinating substance, but several possible ways to explain molecular mechanisms of auxin action can be envisioned:

- synergistic and antagonistic effects with other hormones and signals (e.g. cytokinins, ethylene or others),
- existence of several auxin receptors with different affinities for auxin,
- changes of auxin sensitivity in different tissues,
- local changes in auxin concentration and auxin gradients.

In particular, the last possibility has recently been supported by the discovery of local auxin gradients and visualisation of local auxin accumulations using endogenous (e.g. SAUR) or synthetic auxin responsive promotors (e.g. DR5) fused to GUS reporter genes (Li et al., 1994; Ulmasov *et al*., 1995; Ulmasov *et al*., 1997; Sabatini *et al*., 1999). These promotors were demonstrated to respond to endogenous auxin concentrations and therefore were used to indirectly monitor auxin levels within plants. Using transgenic *Arabidopsis* plants carrying the DR5::GUS construct Sabatini *et al*. (1999) were able to visualise responses to active auxins at cellular resolution. DR5::GUS plants showed increased GUS activity in the columella initial cells of the *Arabidopsis* root meristem suggesting higher auxin levels in these cells. Moreover, changes in the position of the GUS expression maximum in auxin responsive and transport mutants could be correlated with patterning defects in the distal root. Preliminary measurements of auxin concentrations are in good correlation with DR5::GUS activity suggesting the applicability

of this approach (Sandberg, pers. communication). How plants establish and maintain increased auxin levels in only a few cells within the root or in other tissues is still unclear. However, the fact that concentrations of free auxins are affected by a combination of *de novo* biosynthesis, reversible conjugation, oxidation and in addition by differential transport from auxin source to auxin sink tissues suggests that plants have a wide range of regulatory mechanisms to manipulate auxin concentrations at the cellular level (Bandurski *et al*. 1995).

Although a variety of mechanisms are possibly involved in the regulation of cellular auxin levels, auxin transport has been considered to be the prime process to deliver auxin from its sites of synthesis to its sites of action (Lomax *et al*., 1995). As to the relevance of this debate for my studies I will briefly summarise what is known on auxin transport and its role in plant development.

1.3 **Auxin transport**

Auxin is thought to be synthesised in young growing regions in the plant, predominantly in the shoot apex, young leaves and developing seeds (Normanly *et al*. 1991; Sandberg *et al*., 2000). From a large body of data it seems that almost any plant tissue can at certain times be a responsive target for auxin (Davies 1995). Therefore, auxin transport has been regarded as an essential process in plant development and probably is at the centre of molecular approaches to dissect the mechanisms of auxin action. Two main pathways describe the transport of auxin, a fast, non-directional transport in the phloem and a slower, directional polar transport pathway in different tissues.

1.3.1 The non-polar auxin transport in phloem

The evidence for the existence of non-polar auxin transport through the phloem was established through experiments with radioactively labelled auxin (Morris and Thomas, 1978). Labelled auxin was applied onto leaves and later analytically detected in differentiated phloem elements of leaves and in the vascular bundles of the stem. Transport occurred in both basipetal and acropetal directions. This transport pathway was also observed in the root vasculature (Lomax *et al*., 1995). The non-polar auxin transport system was found to be coupled with the transport of assimilates (e.g. sugars), and to proceed relatively fast (5-20 cm/h). This transport also seems to correlate well with the known transport pathways for the inactive auxin conjugates (Nowacki *et al*., 1980). Experiments with radioactively labelled auxin in pea established a connection between the non-polar and polar transport systems. The labelled auxin transported first within the phloem was later detected in the polar transport within parenchyma cells surrounding xylem indicating that both transport pathways may be directly or indirectly linked (Cambridge and Morris, 1996).

1.3.2 Lateral auxin transport

Already in the 1920s, Cholodny and Went independently were trying to hypothesise how auxin moves from the apex into the elongation zone, where it was thought to be asymmetrically distributed (Went 1974). In its simplest form the Cholodny-Went hypothesis explains shoot growth upon gravitropic and phototropic stimuli by elevated levels of auxin on the lower side of growing tissues thereby promoting elongation (Went 1974). This hypothesis assumes asymmetrical auxin redistribution in root or stem tissues upon gravitropic or phototropic stimulation. Subsequently, differential growth rates are acquired which ultimately result in bending of the stem or the root. (Hart, 1990). This concept was supported by experiments studying transgenic plants carrying an auxin responsive promoter fused to a GUS reporter gene. This construct was used as an indirect reporter for changes in cellular auxin levels. Transgenic tobacco plants expressing this construct showed indeed reversible asymmetrical redistribution of GUS activity in the stem upon gravitropic stimulation (Li *et al*., 1991). A correlation between asymmetric auxin distribution and growth rates has also been suggested to explain the root gravitropic responses (Feldman, 1985; Moctezuma and Feldman, 1999).

The molecular basis of the lateral auxin transport remains so far unknown, but auxin specific auxin efflux carrier proteins with a proposed localisation on the lateral side of plasma membranes of stem cells were postulated (Epel *et al*., 1992).

1.3.3 Polar auxin transport

The main feature of polar auxin transport is its uni-directional character. In contrast to non-polar auxin transport in phloem, which can be detected in both basipetal and acropetal directions, the polar auxin transport stream runs from the apex strictly basipetally with a velocity of 5-20 mm/h towards the root, and there probably acropetally towards the root tip as it is depicted in Figure 2 (Lomax *et al*. 1995). In the root tip another polar auxin transport stream was suggested. This occurs basipetally from the columella through the root epidermis (Ohwaki and Tsurumi, 1976)

Using radioactively labelled auxin this kind of transport was mainly detected in the cambium and adjacent, partially differentiated xylem elements (Morris and Thomas, 1978). Other reports claimed that parenchyma cells adjacent to the phloem were the place where polar auxin transport occurs (Goldsmith, 1977). Polar auxin transport requires energy, is specific for active free auxins and is considered as the main developmentally relevant auxin transport pathway (Lomax *et al*., 1995).

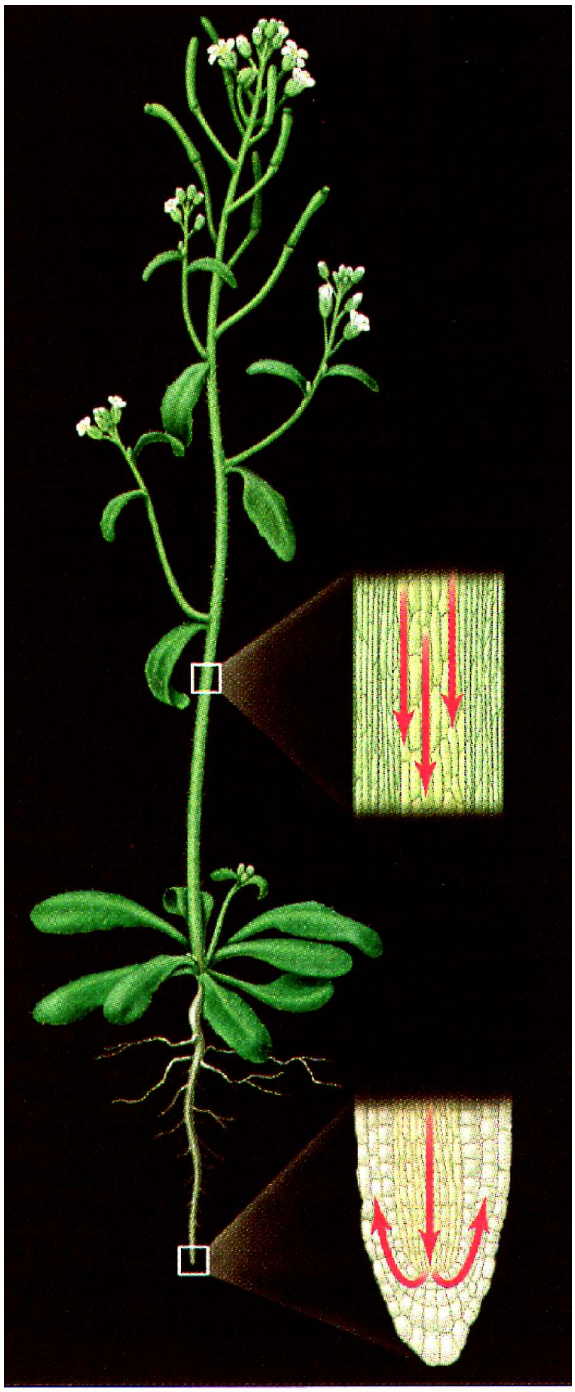


Figure 2. Polar auxin transport in an *Arabidopsis thaliana* plant.

Auxin is synthesised in young developing tissues and basipetally transported within the vascular tissue towards the root tip.
In the root meristem the transport stream is redirected and flows back through the epidermis to the elongation zone (adapted from Jones, 1998).

The energy requirements, saturability and effects of protein synthesis inhibitors suggest the existence of specific auxin transport proteins (i.e. auxin influx as well as auxin efflux carrier proteins). A coherent model for auxin transport, the chemiosmotic hypothesis, incorporating a large body of experimental data was formulated in the middle of the 1970s (Rubery and Sheldrake 1974; Raven 1975).

The chemiosmotic hypothesis

The chemiosmotic hypothesis describes a model for the polar transport of auxin through cell files and postulates the existence of auxin specific carrier proteins (Figure 3).

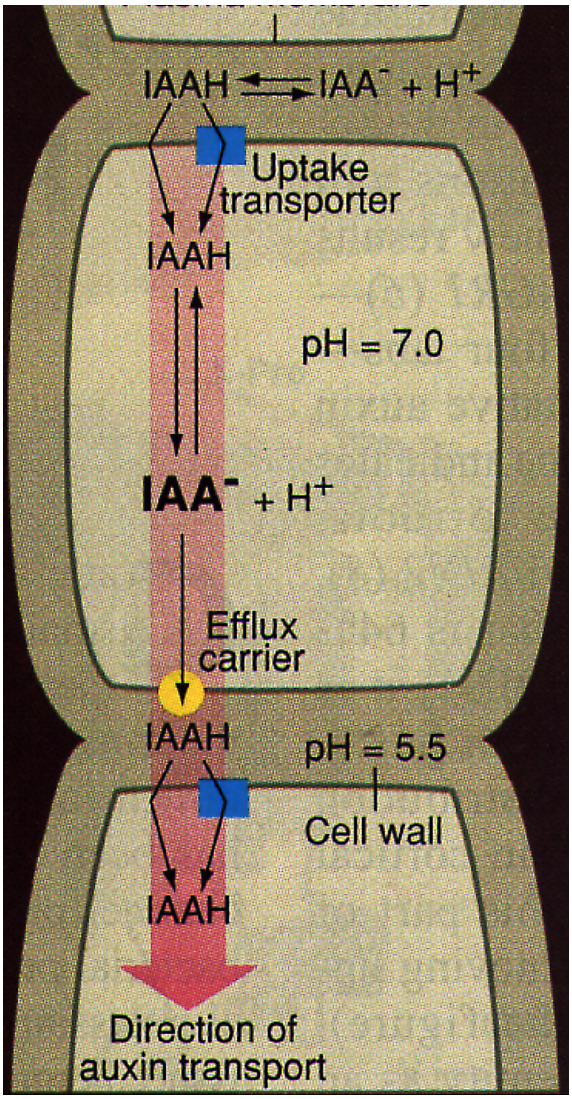


Figure 3. Chemiosmotic model of polar auxin transport.

The protonated IAA (IAAH) enters the cell from the more acidic apoplast through diffusion and specific uptake transporters. In the more basic environment of cytoplasm the IAAH is dissociated and becomes membrane impermeable IAA^- anion is "trapped" in the cell.
IAA^- is able to leave the cell only through specific efflux carriers, located at the basal side of cell (adapted from Jones, 1998).

In the relatively acidic environment of the cell wall (pH around 5,5) IAA exists predominantly in its protonated form (IAAH). This non-charged, lipophilic molecule passes easily through the plasma membrane by diffusion. In the more basic cytoplasm (pH around 7) the majority of IAAH dissociates and hence the resulting IAA^- anion is "trapped" in the cell due to its poor membrane permeability. For the efflux of IAA^- anions the existence of specific efflux carriers was postulated. The polarity of auxin transport was explained by postulating auxin efflux carrier proteins asymmetrically distributed within auxin transport competent cells.

In addition, auxin influx carriers are supposed to participate in polar auxin transport (Goldsmith, 1977; Morris *et al*., 1991). These carriers are believed to be saturable by high IAA concentrations and possibly works as an $IAA^-/2H^+$ cotransporter (Benning, 1986; Lomax *et al*., 1995).

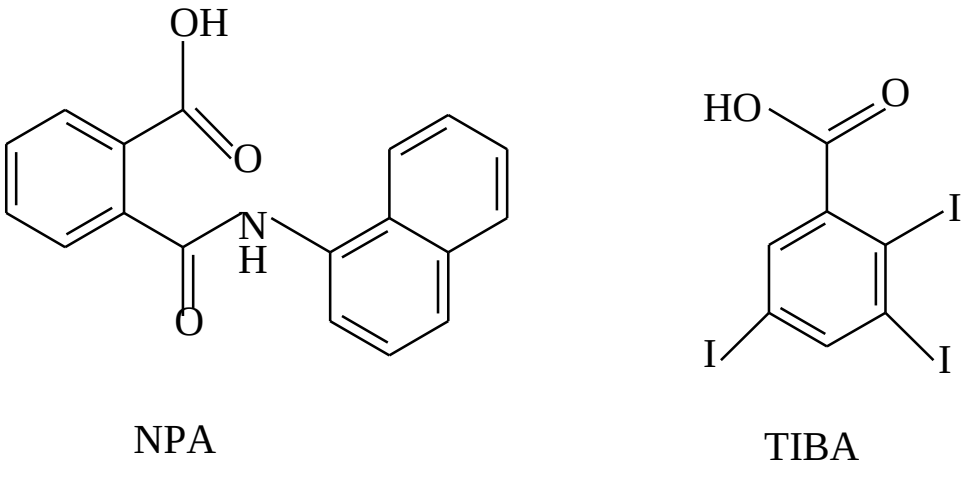
Auxin influx and efflux pathways can be physiologically distinguished using specific auxin efflux carrier inhibitors, coined phytotropins (e.g. 1-N-naphthylphthalamic acid (NPA); for overview see Rubery 1990). The existence of an NPA binding protein, forming part of the auxin efflux carrier complex was hypothesised. A first attempt to search for the NPA binding protein resulted in the isolation of monoclonal antibodies that inhibited NPA binding to pea membrane fractions (Jacobs and Gilbert, 1983). Interestingly, when used in immunolocalisation experiments these antibodies labelled some poorly defined however polarly localised structures within the pea vascular tissue. But as the antibody was lost soon after publication no conclusive information on the nature of these structures or the molecular identity of the target protein is yet available (Jacobs and Gilbert, 1983). Despite extensive studies on the NPA binding protein, little is known about its nature. NPA binding studies revealed different localisations for NPA binding proteins either at the periphery of the plasma membrane probably associated with the cytoskeleton (Cox and Mudday, 1994), or as integral membrane protein (Bernasconi *et al*., 1996). Photoaffinity labelling experiments of maize coleoptile proteins using azido-NPA revealed another group of proteins, named pm23 and pm24 representing a novel class of auxin-dependent microtubule associated proteins (Vente, 1996, Jakobs, 1998). Recently, an *Arabidopsis* mutant named *tir3* was isolated in a screen for NPA insensitive roots. This mutant was shown to have reduced NPA binding activity and auxin transport capacity (Ruegger *et al*., 1997). These results suggest the possibility that the *TIR3* gene may encode the NPA binding protein or some closely related protein (Hobbie, 1998). Additional experiments using auxin efflux and NPA binding studies in the presence of inhibitors of protein synthesis suggested the existence of a third unstable component of the auxin efflux carrier likely coupling the NPA binding protein to auxin transport protein (Morris *et al*., 1991).

1.3.4 The physiological effects of polar auxin transport inhibitors

Substances, which either act as antagonists or agonists of auxin action or auxin transport are valuable tools to dissect the molecular mechanisms of auxin action. So far, the best characterised and most effective substances inhibiting gravitropic and phototropic responses and the polar transport of auxin are phytotropins. These substances resulted from correlative exploration of structure-activity profiles of chemicals with auxin-like activity (Katekar and Geissler, 1977). Most widely recognised phytotropins such as 1-N-naphthylphthalamic acid (NPA) share as structural theme a benzoic acid ortho-linked with an aromatic ring system. However a number of very effective phytotropins such as 2,3,5-triiodobenzoic acid (TIBA) or the morphactins do not share this structural pattern (Figure 4). This could possibly suggest a diversity of auxin efflux carrier proteins, which however after molecular identification of these proteins remains to be determined. Interestingly, some naturally occurring compounds from the flavonoid family have similar effects to

NPA and have been considered to be natural regulators of polar auxin transport (Jacobs and Rubery, 1988).

Figure 4. Chemical structure of polar auxin transport inhibitors.
1-N-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA)



NPA TIBA

One of the most obvious effects of polar auxin transport inhibition is a complete abolishment of plant tropic responses. Already at low concentrations of NPA or TIBA both roots and shoots fail to react properly to stimulation by gravity or light and hence grow randomly in all directions. These experiments suggest that polar auxin transport is required for asymmetrical redistribution of auxin, which subsequently promotes or inhibits cell growth and elongation (Li *et al*., 1991).

The differential redistribution of auxin as a basis for differential cell elongation is also necessary for formation of the apical hook. In this process polar auxin transport is required since the treatment of seedlings with NPA or TIBA prevents hook formation (Lehman *et al*., 1996).

The question about the role of polar auxin transport in elongation growth has not been extensively addressed, despite the fact, that auxin is well known to play a role in cell elongation. NPA and TIBA are known to inhibit root and hypocotyl length in *Arabidopsis*. These observations were used to design screens for isolation of NPA insensitive mutants (Ruegger *et al*., 1997). Recent studies on the influence of NPA on root and hypocotyl elongation in *Arabidopsis* revealed, surprisingly, that NPA inhibits hypocotyl and root elongation only in light. In dark, no effects of NPA or TIBA on root and hypocotyl elongation were observed (Jensen *et al*., 1998). This suggests that polar auxin transport is involved in control of elongation only in light and that these processes seem to be uncoupled in the dark.

Other experiments with polar auxin transport inhibitors demonstrated that polar auxin transport is essential for the initiation and maintenance of polarised growth in developing embryos. Somatic embryos developing from hypocotyl-derived carrot callus cultures develop in the presence of phytotropins into globular embryos of enormous size (Schiavone *et al*.,

1987). Successive studies supported these observations. Zygotic embryos isolated from siliques of Indian mustard (*Brassica juncea* L.) were successfully cultured *in vitro*, thereby allowing the visualisation of developmental aberrations in the presence of various auxins, auxin antagonists and auxin transport inhibitors (Hadfi *et al*., 1998). Auxin transport inhibitors like NPA interfered with cotyledon separation and increased vascularisation in both hypocotyl and cotyledon tissues. These results highlight the important role of auxin and its transport in the progression from the radially symmetric globular embryo to the bilaterally symmetric heart stage embryo.

The manipulation of plants with auxin transport inhibitors also drew attention to the role of auxin in vascular development (Camus, 1949). Early grafting experiments demonstrated that buds induced the differentiation of new vascular tissues and the replacement of buds by auxin indicated that auxin was the major inductive signal (Wetmore *et al*., 1963). Several reports discussed auxin as a correlative and morphogenetic signal, which influences vascular maturation through a partially self-organising mechanism (Newcomb *et al*., 1970; Sachs, 1989). Recent experiments have shown that vascular development in leaves and cotyledons of *Arabidopsis* plants grown on different concentration of NPA was severely affected (Mattson *et al*., 1999): Secondary veins were not developed and, using higher NPA concentrations, the differentiation of the whole leaf vasculature was completely abolished. This has provided further evidence for a link between polar auxin transport and vascular development.

Polar auxin transport has been also shown to play a role in the initiation of lateral roots. *Arabidopsis* plants grown on NPA or TIBA fail to produce lateral roots (Muday *et al*., 1994).

Very recent experiments revealed a link between polar auxin transport and morphogenetic patterning. Growth of *Arabidopsis* roots on elevated concentrations of NPA caused a shift of auxin gradients resulting in changes of cell identity, division rate and cell polarity in the root meristem (Sabatini *et al*., 1999).

1.3.5 Molecular and genetic analysis of polar auxin transport

Various strategies have been used to identify proteins and genes important for auxin transport. For a long time biochemical approaches were predominant, but due to the rapidly progressing genome projects genetic approaches currently seem to result in significant progress.

Proteins involved in auxin transport were identified by labelling with radioactively labelled azido-IAA or -NPA, followed by subsequent purification of radiolabelled ("tagged") proteins on affinity columns or, alternatively, by using antibodies competing with IAA or NPA. The generation of antiidiotypic antibodies did not prove to be a useful strategy for isolation of auxin binding or transport proteins (Prasad and Jones, 1991). Using these methods several proteins have been identified and their *in vitro* binding to IAA or NPA demonstrated (Zettl *et al*., 1992; Palme *et al*., 1994; Vente, 1996).

Most progress on the characterisation of molecular components of polar auxin transport and auxin action has come from the analysis of *Arabidopsis* mutants. Many different screening strategies have been applied to find mutants affected in polar auxin transport and several mutants have been identified.

Some of these mutants have been selected on the basis of abnormal responses to auxin transport inhibitors. A mutant called *rcn1* was isolated, whose roots curl in presence of NPA in contrast to straight root growth in wild type (Garbers *et al*., 1996). This mutant showed a reduction in root and hypocotyl elongation and was also defective in apical hook formation. The *RCN1* gene was cloned and shown to encode a protein phosphatase II A subunit. *RCN1* may control the level of phosphorylation and thereby the activity of a component involved in polar auxin transport (Garbers *et al*., 1996).

The *tir* (transport inhibitor response) mutants were isolated on their ability to allow root elongation in the presence of NPA (Ruegger *et al*., 1997). These mutants define seven novel genetic loci. The mutant designed as *tir1* displays resistance to both auxin transport inhibitors and auxin (Ruegger *et al*., 1998). The *TIR1* gene was cloned and shown to encode a novel protein containing F-box and leucine-rich repeats (Ruegger *et al*., 1998). Related proteins are involved in control of the yeast cell cycle and processes mediating ubiquitin-associated degradation. Another mutant identified in the same screen called *tir3* displays a variety of morphological defects including reduced elongation of root and inflorescences, decreased apical dominance and reduced lateral root formation. Both auxin transport and NPA binding activity are dramatically reduced in *tir3* mutant (Ruegger *et al*., 1997).

Other mutants with altered polar auxin transport were selected on the basis of resistance or increased sensitivity to auxin or ethylene (e.g. *aux, axr, Dwf*; for overview see Hobbie and Estelle, 1994). The *axr* mutations define six novel loci that were isolated on their ability to allow elongation of roots on normally inhibitory concentrations of auxin (Hobbie and Estelle, 1994). Mutant plants containing the *axr1* mutation are dwarfs with impaired apical dominance and root gravitropism. The *AXR1* gene was cloned and shown to encode a novel protein with similarity to the ubiquitin activating enzyme E1 (Leyser *et al*., 1993). Thus both AXR1 and TIR1 proteins may participate in ubiquitin-mediated modification/degradation of regulatory proteins important for mediating auxin action.

Most mutants with defects in polar auxin transport were identified fortuitously in screens for developmental alterations and only later the connection to polar auxin transport was discovered (e.g. *pin1*, Okada *et al*., 1991*; mp*, Berleth and Jürgens, 1993); *gn*, Mayer *et al*., 1993). The *mp* mutant was isolated as an embryo mutant with defects in apical-basal pattern formation (Berleth and Jürgens, 1993). When grown on agar plates, adult *mp* plants were obtained, which showed a strong reduction in polar auxin transport (Przemeck *et al*., 1996). The *MP* gene was cloned and shown to encode a transcription factor from the ARF family. The MP protein was found to be expressed in developing vascular tissue (Hardtke *et al*., 1998). The defects in auxin transport in *mp* mutant suggest the possibility that the MP protein can regulate the expression of components of the polar auxin transport machinery. The *gn* mutant was isolated from the same screen as *mp*. Embryos of *gn* mutants display a variety of aberrations, including defects in apical-basal patterning, fused or improperly placed cotyledons. Some of these defects are reminiscent to defects observed when embryos are cultivated in the presence of polar auxin transport inhibitors (Berleth and Jürgens, 1993). The *GN* gene was cloned and the GN protein demonstrated to have guanine nucleotide

exchange activity for ARF GTPases (Steinman *et al.*, 1999). These small GTPases are known to play a role in control of vesicle trafficking between the Golgi apparatus and plasma membrane. Therefore it may be that the *GN* gene plays a role to properly localise components of the polar auxin transport machinery at the plasma membrane (Steinman *et al.*, 1999).

Most of the above-mentioned genes seem to be only indirectly involved in regulation of polar auxin transport, but there are also several other candidate genes likely encoding components of polar auxin transport machinery. These genes are the *AUX1* gene, the *AtPIN1* and the *AtPIN2* gene. They will be discussed in the next paragraph.

The AUX1 gene

As already mentioned, the existence of specific IAA influx carrier proteins was first postulated (Goldsmith, 1977) and later demonstrated by physiological studies (Benning, 1986). A disruption in the *AUX1* gene causes resistance to auxin and ethylene and affects root gravitropism in *Arabidopsis* seedlings (Bennett *et al.*, 1996). The *AUX1* gene encodes a 485 amino acid long protein sharing significant similarity with plant amino acid permeases, suggesting that this protein might be involved in the uptake of the tryptophan-like IAA. The *AUX1* gene was shown to be expressed in root tissues involved in mediating gravitropic responses (Friml, unpublished results). The recent demonstration that the membrane permeable 1-NAA but not the membrane impermeable IAA or 2,4-D was able to restore the gravitropic response in *aux1* mutant roots, provided another elegant hint for the hypothesis that AUX1 might act in the root as auxin influx carrier (Marchant *et al.*, 1999).

The AtPIN1 gene

According to the chemiosmotic hypothesis the IAA^- anion is trapped within the cell and is assumed to leave transport competent cells only through the activity of specific efflux carrier proteins (Lomax *et al.*, 1995). Recently, identification of the *AtPIN1* gene, supported this, up to now, hypothetical concept.

The *pin1* (also called *pin-formed*) mutant (Figure 5), with its characteristic needle like stem, was found in late 1950s. The dramatic morphological aberrations observed in this mutant can be easily phenocopied by growing wild-type *Arabidopsis* plants on media containing the auxin transport inhibitor NPA (Okada *et al.*, 1991). When the mutant was analysed in detail it was found that inflorescences of the *pin1* mutant showed a drastic reduction in basipetal auxin transport suggesting a defect in a gene encoding either a component of the auxin efflux machinery or an important regulatory component (Okada *et al.*, 1991). The *AtPIN1* gene was cloned by transposon tagging and found to encode a 622 amino acid protein with up to 12 putative transmembrane segments with similarity to a group of transporters from bacteria (Gälweiler *et al.*, 1998).

Using antibodies raised against a portion of AtPIN1, the protein was localised at the basal end of elongated parenchymatous xylem and cambial cells of *Arabidopsis* inflorescence axes (Gälweiler *et al.*, 1998), as well as on the acropetal side of stele cells in the root (Friml, unpublished results). Both AtPIN1 protein localisations were confirmed by *in situ* hybridisation and electronmicroscopy coupled to immunogold labelling (Gälweiler *et al.*,

1998). Recent analysis of transgenic plants carrying the *AtPIN1* promotor fused to the GUS reporter gene revealed the expression of *AtPIN1* throughout the vascular system (Teichmann, personal communication).

Figure 5. *pin1* (*pin-formed*) mutant from *Arabidopsis thaliana*

Also the role of *AtPIN1* during embryo development was studied. In early stages of embryogenesis the AtPIN1 protein was found localised in non-polar way in membranes of apical cell descendants with gradual polarity establishment during embryogenesis. This process was disrupted in *gn* mutants resulting in severe embryo defects, resembling those when embryos were treated with polar auxin transport inhibitors (Steinmann *et al*., 1999). Very recent experiments have shown that the AtPIN1 protein, when expressed in yeast, evokes an enhanced resistance to toxic fluoroindol compounds (Tietz, personal communication). These data therefore support the hypothesis, that AtPIN1 protein plays an important role in auxin efflux.

The AtPIN2 gene

AtPIN1 belongs to a family of related genes in *Arabidopsis*. The reverse genetic characterisation of another member of this family, *AtPIN2*, led to the identification of a mutant displaying severe defects in root gravitropism (Müller *et al*., 1998). This mutant was shown to be allelic to other known mutants, respectively *wav6-52*, *agr1*, *eir1*, affecting root gravitropism and auxin and ethylene sensitivity (Luschnig *et al*., 1998; Chen *et al*., 1998; Utsuno *et al*., 1998). The root specific AtPIN2 protein was localised in the cortex, epidermis and lateral root cap, predominantly at the basipetal side of cells (Müller *et al*., 1998; Friml, unpublished results). Here, it may facilitate auxin transport, as predicted by the Cholodny-Went hypothesis, to the elongation and differentiation zone and thereby regulate the gravitropic response of the root. The absence of the *AtPIN2* gene function not only caused an agravitropic phenotype consistent with the loss of an auxin efflux carrier in cells of the root cortex and epidermis, but also resulted in an auxin sensitive phenotype. The heterologous expression of the AtPIN2 protein in yeast caused enhanced resistance to toxic fluoroindol compounds, similarly to the heterologous expression of AtPIN1 (Luschnig *et al*., 1998; Tietz, personal communication). These data therefore support the hypothesis that *AtPIN2* gene plays a role in basipetal auxin transport in the *Arabidopsis* root.

1.4 **Aims of this work**

The major aim of this work was to identify, isolate and characterise new members of the *PIN* gene family in *Arabidopsis thaliana.* Two starting points inspired and promoted this work:

1) Several sequences homologous to *AtPIN1* and *AtPIN2* had been already identified in databases, suggesting the existence of a larger gene family (Gälweiler *et al*., 1998).

2) There are various plant developmental processes, known to be controlled by polar auxin transport. These processes are root and shoot phototropism, shoot gravitropism, shoot elongation and lateral root development. These processes are unlikely to be regulated by either the *AtPIN1* or the *AtPIN2* gene.

This raised the possibility that some up to now unknown homologues of *AtPIN1* and *AtPIN2* could participate in the regulation of these processes.

Based on this assumption the following strategy, summarised in Figure 6, was outlined:

1) To screen genomic and cDNA libraries from *Arabidopsis thaliana* for new *PIN* homologues using available *AtPIN1* and *AtPIN2* sequences.

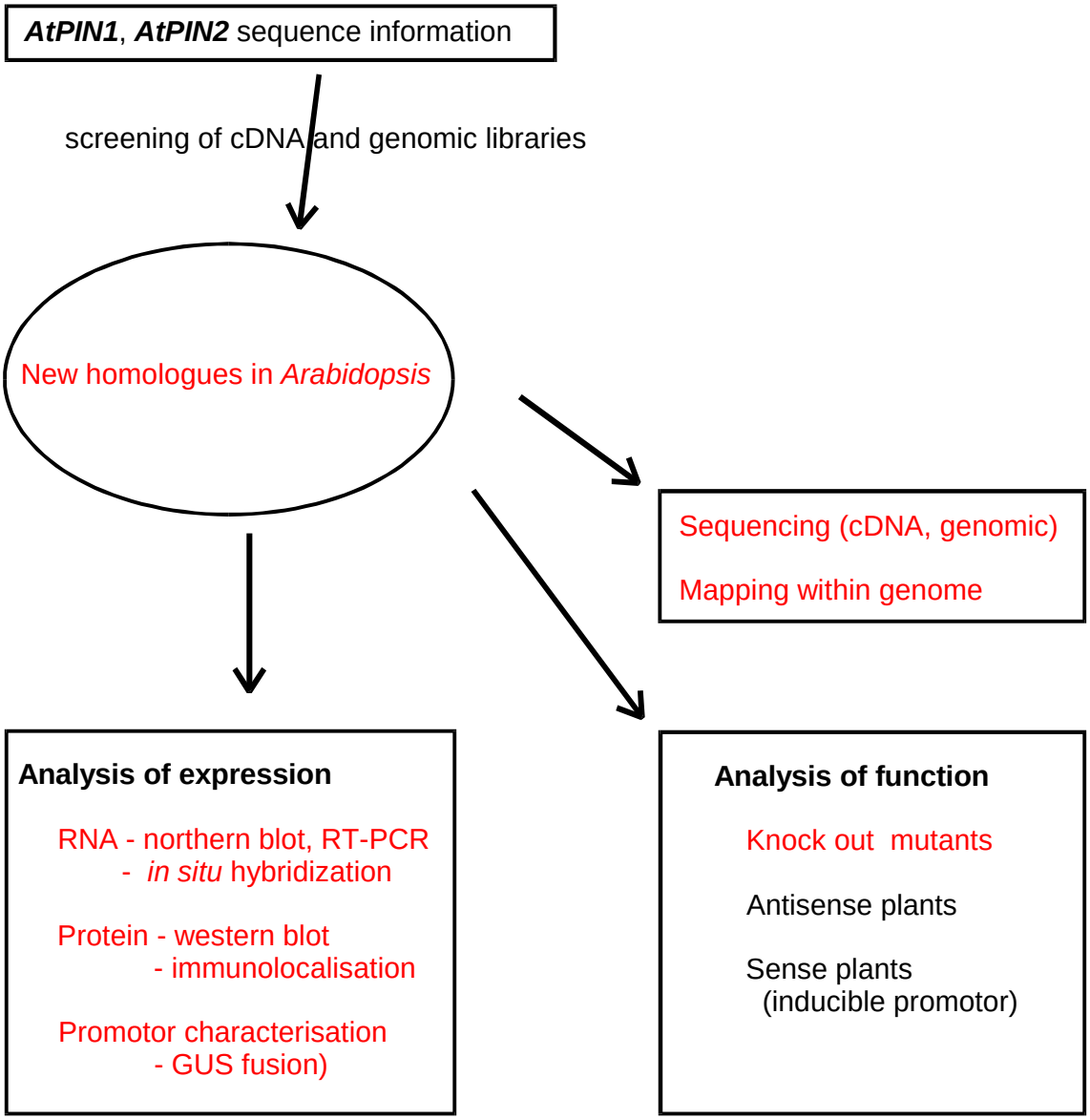
2) To study the expression of novel members of this family at the plant level by Northern blot analysis and, at cellular resolution, by *in situ* hybridisation techniques.

3) To generate specific antibodies and localise corresponding AtPIN proteins at the cellular and subcellular level.

4) To isolate mutant lines with disrupted *AtPIN* gene functions by reverse genetic approaches and to analyse these mutants.

Finally, a model integrating sequence, expression and functional data of new *AtPIN* genes, should be formulated, which allows their interpretation and thus hopefully uncovers some new, however still small pieces of the complex and puzzling mosaic of events occurring during polar auxin transport.

Figure 6. Research strategy for isolation and analysis of new *AtPIN* genes.
Accomplished analyses are marked.



2 Materials and methods

2.1 Materials

2.1.1 Plants

Arabidopsis thaliana L.	Cultivar Columbia Cultivar Landsberg erecta

2.1.2 Bacteria

Escherichia coli	
DH5α	F^-, ϕ80dlacZΔM15, Δ(lacZYA-argF), U169, endA1, recA1, hsdR17, (r_k^-- m_k^+), deoR, thi-1, supE44, λ-, gyrA96, relA1 (Hanahan, 1985).
XL1-Blue	F`::Tn10, pro A^+B^+, $lacI^q$Δ(lacZ), M15/recA1, endA1, gyrA96, thi, hsdR17, (r_k^-- m_k^+), SupE44, relA1 (Bullock et al., 1987)
Bl 21 DE3	*E. coli* B, F^-, ompT, dcm, hsdS
Agrobacterium tumefaciens	
GV3013 pMP90RK:	C58C1, Rif^r, Gm^r (Konz and Schell, 1986)

2.1.3 Phages

λ-GEM11	(*Promega*, Madison/USA)
R408	(Russel *et al*., 1986)
λ-Uni-ZAP^{TM} XR	(*Stratagene*, La Jolla/USA, 1991)

2.1.4 Plasmids

pBluescript II SK-	Amp^r (Short et al., 1988, *Stratagene*, USA)
pBluescript II KS-	Amp^r,(Short *et al.*, 1988, *Stratagene*, USA)
pUC 18	Amp^r, (Viera und Messing, 1982)
pET28a	Kan^r (*Novagen*)
all other vectors	(Friml, this thesis)

2.1.5 Nucleic acids libraries from *Arabidopsis thaliana*

IGF BAC:	p-beloBAC Kan-genomic library, ecotype Columbia (Mozo et al.,1999*)*
cDNA libraries:	λ-cDNA-libraries from Poly-A^+-mRNA from different tissues: root, guard cells, seedling and suspension culture, (host: phages). JG4-5 cDNA library from suspension culture (host: bacteria). pFL61 cDNA library from whole plants (host: bacteria). pXY112 cDNA library from aerial part of whole plant (host: bacteria). pSPORT cDNA-library from guard cells and epidermis stripes of *Arabidopsis thaliana* rosette leaves (Stracke, pers. com.) (host: bacteria).

2.1.6 Enzymes

DNA Kinase	*AGS*, Heidelberg
Random-Prime Kit	*Amersham Buchler*, Braunschweig
Alkaline Phosphatase	*Boehringer Mannheim*, Mannheim
Restriction enzymes	
Taq DNA polymerase	
T4 DNA ligase	
T4 DNA polymerase	
T7 RNA-polymerase	
T3 RNA-polymerase	*New England Biolabs*, Schwalbach
	Boehringer Mannheim, Mannheim
1 kb-ladder	

Gibco BRL, Eggenstein

Lysozyme
Proteinase K
Merck, Darmstadt

Deoxyribonuclease I
Driselase
Proteinase K
Ribonuclease A
DNase 1
Sigma , Munich

2.1.7 Chemicals

Dimethylformamide
Aldrich-Chemie, Steinheim

^{32}P-αdCTP
Amersham Buchler, Braunschweig

Ethanol
Methanol (HPLC grade)
J.T.Baker-Chemicals, Deventer/Holland

Urea ("ultra pure")
Bethesda Research Laboratories, Neu-Isenburg

Polyethyleneglycole 6000
BDH Biochemicals Ltd., Poole/England

Sodium dodecylsulfate (SDS)
Prestained SDS-PAGE Standard
Protein Assay, Dye Reagent Concentrate
Bio-Rad, Munich

Deoxyribonucleotide triphosphates
Dideoxyribonucleotide triphosphates
Boehringer, Mannheim

Agarose
Biozym, Hessisch Oldendorf

Agar-agar

Lugol
Merck, Darmstadt

Triton X-100
Calbiochem, Frankfurt

Tryptone
Agar
Difco Laboratories, Detroit/USA
Formamide
4-Methyl-Morpholine
Piperidine *Fluka*, Neu-Ulm

Seaplaque low melting agarose
FMC, Rockland/USA

Tris
Agarose, ultrapure
Gibco BRL, Gaithersburg
Gibco BRL, Eggenstein

Slowfade-Antifade Kit
Molecular Probes, Inc.

Trichlormethane (Chloroform)
Riedel-deHaen, Selze

Phenol
Rotiphorese 30 (30 % Acrylamid; 0,8 % Bisacrylamid)
Roth, Karlsruhe

Bromphenol Blue
2-Mercaptoethanol
Serva, Heidelberg

Adenosine triphosphate (ATP)
Ammoniumperoxodisulfate (APS)
Dalton Marker VII-L (SDS-PAGE molecular mass standard)
Dithiothreitol (DTT)
Diethyl pyrocarbonate (DEPC)
Ethidium bromide (EtBr)
Ficoll
Isopropyl β-D-thiogalactoside (IPTG)
Ampicillin
Rifampicin
Gentamicin
Carbenicillin

Hygromycin
Kanamycin
Indol-3-acetic acid (IAA)
1-Naphthylacetic acid (1-NAA)
2,4-Dichlorophenoxyacetic acid (2,4D)
Salmon sperm DNA
2-(N-Morpholino)ethansulfonic acid (MES)
3-(N-Morpholino)propanesulfonic acid (MOPS)
Murashige and Skoog Basal salt mixture medium (MS-Salt Base)
Polyvinylpyrrolidone (PVP)
Bovine serum albumin (BSA)
N,N,N',N'-Tetramethylethylendiamine (TEMED)
5-Bromo-4-Chloro-3-Indolyl-Phosphate (BCIP)
5-Bromo-4-chloro-3-indolyl -β-D-galactopyranoside (X-Gal)
5-Bromo-4-chloro-3-indolyl -β-D-glucuronide (X-Gluc)
5-Bromo-4-chloro-3-indolyl-phosphonate (XPP)
Sigma, Munich

2.1.8 Other Materials

Parafilm M
American National Can., Greenwich/USA

Hybond N
Amersham-Buchler, Braunschweig

Falcon tubes
Becton, Dickson Labware, Lincoln Park/USA

Miracloth
Calbiochem, La Jolla

Reaction tubes
0,5 - 2 ml
Eppendorf, Hamburg

Petri dishes
Jet Sorb Gel Extraction Kit
GENOMED GmbH, Bad Oyenhausen

Pipette tips
Greiner GmbH, Wupperthal

Intensifying Screen
Ektachrome 64T

Ektachrome 100Plus
X-ray film X-OMAT AR 5
Kodak, Rochester

3MM paper
Whatman, Maidstone

QIAquick column
QIAex column
QIAgen tip 20 / 100 (plus buffer P1, P2, P3, QB, QC, QF)
Qiagen, Hilden

2.1.9 Instruments

Photometer DU-40
Scintillation's-counter LS 7500
Centrifuge, L8-70
Beckman Instruments

Trio-Thermoblock (PCR)
Biometra

GenePulser™
BioRad, München

Sonifier
Branson

Centrifuge 5412
Eppendorf

Biofuge A
MegaFuge
MiniFuge GI
Hybridisation's oven
Heraeus Christ, Düsseldorf

HPLC HP-1090
Hewlett Packard

Microscope-Aristoplan
Microscope Leica DMRB
Microtom RM2065
Leitz, Wetzlar

Confocal laser scanning microscope Leica DMIRBE, TCS 4D
Leica

Phosphorimager
Molecular Dynamics, Sunnyvale

SpeedVac Concentrator
Savant Instr., Framingdale/USA

Analytical balance
Sartorius, Göttingen

Centrifuge RC-5B Superspeed
Sorvall Du-Pont Company

UV Crosslinker
Stratagene, La Jolla

Transilluminator TM-40, 254 nm
Ultra-Violett Products, Inc.

2.1.10 Media

2.1.10.1 Plant media

Ara medium (solid): per litre:
- 4,3 g MS (Basal Salt Med., *Sigma*)
- 0,5 g MES
- 0,1 g Myo-Inositol
- 15 g Sucrose
- pH 5,7 adjusted with KOH
- 8 g Agar
- 20 min autoclave
- add B_5 Vitamin

Ara medium (liquid): per litre:
- 4,3 g MS (Basal Salt Med., *Sigma*)
- 0,5 g MES
- 0,1 g Myo-Inositol
- 30 g Sucrose
- pH 5,7 adjusted with KOH
- 20 min autoclave
- add B_5 Vitamin

100 x B_5 vitamins: per litre:
- 0,1 g Glycine
- 0,1 g Nicotine acid
- 0,1 g Pyridoxine-HCl
- 1 g Thiamine-HCL

2.1.10.2 Phage media

NZCYM-Media (Sambrook et al., 1989):

per litre:
- 10 g NZ Amine
- 5 g NaCl
- 5 g Yeast extract
- 1 g Casaminoacid
- 2 g $MgSO_4$ x $7H_2O$
- 0,2 % Maltose
- pH 7,0 adjust with NaOH
- 20 min autoclave

Top-Agarose: per litre:
- 0.7 % Agarose
- 2 g $MgSO_4$

2.1.10.3 Bacterial media

dYT-Medium (*Stratagene*):

per litre:

10 g Yeast extract
16 g Trypton
10 g NaCl
pH 7,5

LB(Luria-Bertani)-Medium
(Maniatis *et al.*, 1982): per litre:

5 g Yeast extract
10 g Trypton
10 g NaCl

LB-Medium (Sambrook et al., 1989):

per litre:

10 g Bacto Tryptone
5 g Yeast extract
5 g NaCl
pH 7,5 adjust with NaOH

SOB-Medium: per litre:

20 g Tryptone
5 g Yeast extract
0,5 g NaCl

SOC-Medium: per litre:

20 g Tryptone
5 g Yeast extract
0,5 g NaCl
3,6g Glucose

YEP Medium: per litre:

10 g Peptone
10 g Yeast extract
5 g NaCl

YT-Medium: per litre:

5 g Yeast extract
8 g Tryptone
5 g NaCl

2.1.11 Other stock solutions

2.1.11.1 Antibiotics and hormones

Antibiotics	**Stock solutions**	**final concentration**
Ampicillin	100 mg/ml in H_2O	100 mg/l
Kanamycin	50 mg/ml in H_2O	50 mg/l
Hygromycin	50 mg/ml in PBS	15 mg/l
Rifampicin	100 mg/ml in DMSO	100 mg/l
Gentamycin	100 mg/ml in H_2O	100 mg/l
Carbenicillin	100 mg/ml in H_2O	400 mg/l

Hormones

IAA	1 mg/ml in 1 N NaOH	diverse
1-NAA	1 mg/ml in ethanol	diverse
2,4-D	1 mg/ml in DMSO	diverse
Kinetin	0,1 mg/ml in ethanol	diverse
pIP	20 mg/ml in DMSO	diverse
NPA	100 mM in DMSO	diverse

2.1.11.2 Other Substances

IPTG	0,1 M in H_2O	0,2 mM
X-Gal	20 mg/ml in DMF	0.004 %
X-Gluc	100 mg/ml in DMF	0,1 %
XPP	25 mg/ml in DMF	0,05 %
Ethidiumbromid	5 mg/ml in TE-Puffer	diverse

2.1.11.3 Antibodies

Anti-rabbit-IgG-FITC conjugate
Anti-rabbit-IgG coupled to alkaline phosphatase
Anti-rabbit-IgG coupled to horseradish peroxidase

2.1.12 Computer programmes

UNIX Version 8
GCG package (Genetics Computer Group, Sequence Analysis Software Package Version 7.0) (Devereux et al., 1984).
DNAstar-Software-package (*Lasergene*).
ImageQuant 4 (*Molecular Dynamics*)
Adobe Photoshop 4 (*Adobe*)
CorelDraw 7 (*Corel Corporation*)
Diskus (*Carl H. Hilgers*)

2.2 **Methods**

2.2.1 Methods for nucleic acids analysis

2.2.1.1 Isolation and purification of DNA

2.2.1.1.1 Small scale plasmid DNA isolation from *E. coli* by the "Boiling method"

Single bacterial colonies were transferred to 3-5 ml of LB medium and incubated o/n at 37° C with vigorous shaking. 1,5 ml of bacterial culture were poured into eppendorf tubes and shortly centrifuged at 13000 rpm. The supernatant was removed, the pellet resuspended in 100 µl of Tris-sucrose and 300 µl of MSTET solution and 4 µl of lysozyme solution (10 mg/ml in H_2O) were added. The samples were incubated for 60 s at 95 °C and centrifuged at 13000 rpm/ 15 min/ 4 °C. Pellet containing denatured proteins and chromosomal DNA was removed and 500 µl of cold isopropanol were added to the supernatant. The samples were centrifuged at 13000 rpm for 5 min at 4 °C. The pellet was washed with 500 µl of 70 % ethanol, dried in a speedvac, centrifuged and dissolved in 20 µl of sterile deionised water (Ausubeel, 1992).

LB medium:	1 % bacto-tryptone; 0,5 % bacto-yeast extract; 1 % NaCl
Tris-sacharose:	50 mM Tris/Cl (pH 8,0); 25 % sacharose
MSTET:	50 mM EDTA; 50 mM Tris/Cl (pH 8,0); 5 % sacharose; 5 % Triton X-100

2.2.1.1.2 Small scale plasmid DNA isolation from *E.coli* by the alkaline-lysis method

LB medium (5 ml) was inoculated with one single bacterial colony and incubated o/n at 37 °C. 3-4 ml of bacterial culture was shortly centrifuged, the supernatant removed and the pellet resuspended in 100 µl of solution I. 200 µl of solution II were added, gently mixed and kept on ice for 5 min. 150 µl of ice-cold solution III were subsequently added, samples were incubated on ice for 10 min and centrifuged (13000 rpm/ 15 min/ 4 °C). The supernatant was transferred to the new tube and DNA precipitated by addition of 500 µl of isopropanol. DNA was recovered by centrifugation at 13000 rpm for 10 min, washed with 70 % ethanol. The sediment was dried and dissolved in 250 µl of H_2O. After addition of 250 µl of 5 M LiCl the samples were incubated on ice for 15 min and centrifuged at 13000 rpm for 5 min. The supernatant was mixed to 1 ml of 100 % ethanol and kept at -20 °C for 20 min. DNA was recovered by centrifugation at 13000 rpm for 30 min, washed with 70 % ethanol, dried and dissolved in deionised water (Birnboim and Doly, 1979).

Solution I:	50 mM glucose; 25 mM Tris-HCl (pH 8,0); 10 mM EDTA (pH 8,0)
Solution II:	0,2 N NaOH; 1 % SDS
Solution III:	3 M KOAc (pH 4,5)

2.2.1.1.3 Large scale plasmid DNA isolation from *E. coli* and purification on Qiagen columns

Bacterial cells from 40 ml culture grown for 12-14 h were collected by centrifugation at 3500 rpm for 15 min at 4 °C. The pellet was resuspended in 4 ml of solution P1, followed by addition of an equal volume of solution P2. After mixing the tubes were left for 5 min at room temperature. 4 ml of solution P3 were added, the solution was gently mixed by inversion and centrifuged at 13000 rpm for 15 min at 4 °C. Qiagen-tip 100 columns were equilibrated with 4 ml of solution QBT and allowed to empty by gravity flow. The bacterial supernatants were loaded and the columns washed with 2 x 10 ml of solution QC. The DNA bound to the column was eluted with 5 ml of solution QF into 15 ml Corex tubes and precipitated with 0,7 volumes of isopropanol. The samples were centrifuged at 13000 rpm for 5 min at 4 °C. Pellet was washed once with 500 µl of 70 % ethanol, dried in a speedvac, centrifuged and dissolved in 50 µl of sterile deionised water.

Solution P1:	50 mM Tris/HCl (pH 8,0); 10 mM EDTA (pH 8,0); 100 µg/ml RNase
Solution P2:	0,2 N NaOH; 1 % SDS
Solution P3:	3.0 M KOAc (pH 5,5)
Solution QBT:	750 mM NaCl; 50 mM MOPS (pH 7,0); 15 % ethanol; 0,15 % TritonX-100
Solution QC:	1 M NaCl; 50mM MOPS (pH 7,0); 15 % ethanol
Solution QF:	1,25 M NaCl; 50mM MOPS (pH 8,5); 15 % ethanol

2.2.1.1.4 Isolation of genomic DNA from *A. thaliana*

Genomic DNA from *A. thaliana* leaf tissues, was extracted according to Saghai et al.(1984). In brief, about 0,5 g of leaf material were grind in liquid nitrogen, and the resulting powder transferred to 100 ml Erlenmeyer flask. 7 ml of extraction buffer were added and mixed thoroughly by shaking at 60 °C for 1 hour. 7 ml of chloroform-isoamylalcohol (10 : 1) were added and the resulting suspension was centrifuged (8000 rpm/ 15 min/ RT). The water phase was mixed with 2/3 volume of isopropanol and precipitated DNA was transferred into new eppendorf tube, washed with 1 ml of 75 % ethanol (30 min/ RT), dried and dissolved in 0,25 mM EDTA, 10 mM NH_4Ac. The genomic DNA was deproteinised with phenol-chloroform.

Extraction buffer:	1 M TrisCl (pH 8,0); 0,5 M EDTA; 5 M NaCl; 1 % CTAB

2.2.1.2 PCR amplification

PCR was performed by incubation of PCR reaction mixtures at three temperatures corresponding to the denaturing, annealing and extension steps in each cycle of amplification. In a typical reaction the DNA is denatured at 90 to 95 °C, the primers were allowed to anneal at 40 to 60 °C, and the extension was processed at 72 °C. 20-25 amplification cycles were used.

2.2.1.3 Separation of DNA by agarose gel electrophoresis

DNA fragments were analysed by electrophoresis in horizontal agarose gels. The agarose concentration depends on the size of the fragments to be resolved (Sambrook et al., 1989). Electrophoresis was performed at 5 V/cm using TBE buffer. DNA size markers 1kb ladder were used to estimate the size of unknown restriction fragments. After electrophoresis DNA was visualised on a transilluminator by fluorescence under UV light (254nm).

5 x Gel Loading Buffer:	40 % sucrose; 5 mM EDTA; 0,1 % SDS; 0,025 % Bromphenol Blue; 0,025 % Xylene Cyanol
TBE	90 mM Tris/Cl (pH 8,3); 90 mM boric acid; 2,5 mM EDTA; 100µg/l EtBr

2.2.1.4 Purification of DNA fragments from agarose gels using JETSORB DNA extraction kit

The DNA samples were separated by electrophoresis in an agarose gel and excision of the agarose gel slice with desired DNA fragment was performed under long wave UV illumination. The gel slice was transferred into a tube and its weight was determined. For each 100 mg gel slice the following kit components were added: 300 µl of buffer A1 and 10 µl of JETSORB suspension. The tube was vortexed and incubated at 50 °C for 15 min, mixed every 3 min during incubation. After short centrifugation the supernatant was removed, the pellet was washed with 300 µl of buffer A1 and two times with 300 µl of buffer A2. The Jetsorb pellet was air dried and the DNA fragment was eluted into 20 µl sterile H_2O (5 min at 50 °C). The pellet was recovered by centrifugation and the supernatant transferred into a new tube.

2.2.1.5 Enzymatic modification of DNA

2.2.1.5.1 Digestion with restriction enzymes

The digestion with restriction endonucleases was performed according to the supplier's recommendations. Digestion was carried out for 1 to 3 hours with 5-10 U of enzyme and stopped by inactivation at 75 °C for 10 min or by extraction with phenol chloroform followed by ethanol precipitation.

2.2.1.5.2 DNA ligation

To ligate DNA fragments with linearised vector, 1-2 U of T4 DNA ligase were used. To avoid recircularisation of the vector, the 5`- terminal phosphate group of the vector DNA was removed by alkaline phosphatase. Ligation was performed for at least 4 hours at 16 °C.

2.2.1.6 Isolation and purification of RNA

2.2.1.6.1 Isolation of *A. thaliana* total RNA

The total RNA was extracted according to Chomczynski at al. (1987). 200 mg of plant material were grind in liquid nitrogen and resuspended in 400 µl of GTC buffer. 40 µl of 2 M NaOAc (pH4) and 80 µl of chloroform were added and the suspension was extracted with 500 µl of phenol. After centrifugation (13000 rpm/ 20 min/ 4 °C), the aqueous phase was mixed with an equal volume of isopropanol. The nucleic acids were precipitated for 15 min at -20 °C and recovered by centrifugation (13000 rpm/ 15 min/ 4 °C). The pellet was resuspended in 300 µl GTC buffer and centrifuged (5 min/ 13000 rpm). The supernatant was mixed with an equal volume of isopropanol and centrifuged (13000 rpm/ 15 min/ 4 °C). The RNA pellet was washed with 80 % ethanol, dried for 30 min at room temperature and resuspended in 20 µl of deionised formamide.

GTC buffer:	4 M guanidiumthiocyanate; 25 mM Na citrate (pH7,0); 0,5 % N-Laurylsarcosin; 0,1 M 2-mercaptoethanol
Deionised formamide:	Bio-Rad Mixed resin (Anal Grade) A6 501-X8 2050 mesh was added to formamide mixed and filtered

2.2.1.6.2 Separation of RNA by formaldehyde agarose gel electrophoresis.

RNA was separated under denaturing conditions to prevent formation of secondary structures. This was achieved by inclusion of 6 % formaldehyde in the agarose gel. Before the separation, RNA aliquots were diluted with 1 volume of 2 x RNA loading buffer, denatured 5 min at 65 °C and cooled on ice. Electrophoresis was performed at 5V/cm for 4 to 6 hours.

RNA gel buffer (10 x):	0,2M MOPS; 50mM NaOAc; 10mM EDTA; pH 8,0
RNA run buffer (10 x):	0,2M MOPS; 50mM NaOAc; 10mM EDTA; pH7,0
RNA loading buffer (2 x):	66 % formamide; 2 x RNA gel buffer; 8 % formaldehyde; 0,1 % Xylene Cyanol; 0,1 % Bromphenol Blue; 0,1mg/ml ethidium bromide

2.2.1.7 Membrane transfer of nucleic acids

2.2.1.7.1 Southern blot

The DNA was separated under standard conditions on a 1 % agarose gel. The gel was equilibrated in 0,4 M NaOH for 15 minutes and DNA was transferred to a nylon membrane (Amersham, Hybond N+) using 0,4 M NaOH. After transfer, the membrane was washed with 1 x SSC and dried. The DNA fragments were crosslinked to the membrane by UV light (*Stratalinker, Stratagene*).

2.2.1.7.2 Northern blot

The RNA was separated under denaturing conditions by agarose gel electrophoresis. To remove the formaldehyde, the agarose gel was washed with water (5 min) and with 20 x SSC (20-30 min). The RNA was transferred to a nylon membrane (Amersham, Hybond N+) using 20 x SSC. After blotting, the membrane was washed with 2x SSC, dried and crosslinked to the membrane by UV light.

20 x SSC:	3M NaCl; 0,3M Na citrate; pH 7,0

2.2.1.8 Labelling of DNA probe with [α-^{32}P] dCTP

The synthesis of radioactive labelled DNA by the "random priming" method was done using the *redI* prime DNA labelling system (*Amersham*). The DNA fragment to be labelled was diluted to a concentration of 50 - 300 ng in 14,5 µl reaction mix. The DNA was denatured at 95 °C for 5 min, shortly centrifuged and 50 µCi of [α-^{32}P] dCTP, and 1 U of Klenow fragment were added. The reaction mixture was incubated for 30 min at 37 °C. Labelled DNA probe was purified by QIAquick nucleotide removal kit.

2.2.1.9 Hybridisation of ^{32}P - labelled probes

The membranes were pre-hybridised for 2 hours with pre-hybridisation solution at 68 °C. After probe addition, the filters were hybridised o/n at 68 °C. Unspecifically bound probe was removed by washing the membranes with 2 x SSC/ 0,1 % SDS at 68 °C (5 times/ 10 min) and with 0,5 x SSC/ 0,1 % SDS (15 min). ^{32}P radiolabel was quantified from freshly hybridised nylon membranes using a phosphor imager (Storm, *Molecular Dynamics*, Sunyvale,CA) and imaging plate (*Molecular Dynamics*) and analysed with ImageQuant software (version 4, *Molecular Dynamics*).

Pre-hybridisation buffer:	6 x SSPE; 6x Denhardt`s reagent; 0,5 % SDS
Hybridisation buffer:	4 x SSPE; 4x Denhardt`s reagent; 0,5 % SDS
20x SSPE:	NaCl; $NaH_2PO_4.H_2O$; 0,5 M EDTA
100x Denhardt`s reagent:	2 % BSA; 2 % Ficoll; 2 % PVP 360

2.2.2 Bacterial transformation

2.2.2.1 Preparation of competent *E. coli* cells

A colony of *E. coli* DH5α was inoculated into 5 ml of Bioth-X medium and incubated o/n at 37 °C. 0,2 ml of the overnight culture were diluted in 20 ml of fresh medium, and incubated at 37 °C to optical density A_{600} = 0,300. 5 ml of the culture were diluted in the 100 ml of fresh medium, and incubated under the same condition to optical density A_{600} = 0,500. Cells were harvested, kept on ice for 10 min and centrifuged (3000 rpm, 10 min at 4 °C). The supernatant was discarded, the cells gently resuspended in 7 ml of TFBI solution and incubated for 15 min on ice. After centrifugation, the cells were resuspended in 1ml TFBII and aliquoted in 100 µl samples, frozen in liquid nitrogen and stored at -70 °C.

TFB I:	15 % glycerol; 30mM KOAc; 50mM $MnCl_2$; 100mM RbCl; 10mM $CaCl_2$; pH 5,8 adjusted with acetic acid; sterilised by filtration through nitro-cellulose (0,22 µm)

TFB II:	15 % glycerol; 10mM MOPS (pH 7,0); 10mM RbCl; 75mM $CaCl_2$; sterilised by filtration through nitro-cellulose (0,22 µm)

2.2.2.2 Transformation of *E. coli*

For each transformation, a 50 µl aliquot of competent cells was allowed to thaw. DNA (10 to 100 ng) was added to competent cells, gently mixed and incubated on ice for 30 min. After heat shock (90 s at 42 °C), the cells were cooled for 5 min on ice and 1ml of LB medium was added. The transformation mixture was incubated for 1 hour at 37 °C. After centrifugation (300 rpm/ 5 min), 850 µl of supernatant were discarded, the cells resuspended in the remaining medium and plated (adapted from Hanahan, 1985)

2.2.2.3 Preparation of electrocompetent *A. tumefaciens* cells

A single colony of *A. tumefaciens* was inoculated into 16 ml of YEB medium and grown o/n at 28 °C. The overnight culture was used to inoculate 400 ml of YEB medium and grown to A_{600} = 0,500. The cells were sedimented (1000 rpm / 10 min/ 4 °C) and resuspended successively in 200 ml of ice cold 1 mM Hepes (pH 7,5), in100 ml ice cold 1mM Hepes (pH 7,5) and 10 ml ice cold 1 mM Hepes / 10 % glycerol (pH 7,5). Finally, the cells were resuspended in 800 µl 1 mM Hepes/ 10 % glycerol, aliquoted and frozen at -70 °C.

YEB medium:	0,5 % beef extract; 0,1 % yeast extract; 0,5 % peptone; 0,5 % sucrose; 2 ml/l 1 M $MgSO_4 .7H_2O$

2.2.2.4 Transformation of *A. tumefaciens*

An aliquot of frozen electrocompetent *A. tumefaciens* was thawed on ice and mixed with 1 µl of ligation mix. The electroporator was set to 25 µF, 2,5 kV and 200 Ω.The cell suspension was transferred into a 0,2 cm pre-cold electroporation cuvette. A single electroporation pulse was given and 1 ml of LB medium immediately added. After incubation at 28 °C for 2 h, the cells were plated (adapted from Dower et al., 1988).

2.2.3 Isolation of *En* insertion mutants by reverse genetic screening

To isolate transposon insertions in *AtPIN* genes a collection of *Arabidopsis* plants carrying *En-1* insertions was screened (Baumann et al., 1998). The three-step PCR based screening strategy allowed the identification of lines carrying individual insertions in *AtPIN* genes. In the first step, 28 different 3-Tray-Pools were tested with two, *AtPIN* gene specific primers in tandem with the two *En-1* specific primers. In total 112 PCR reactions were performed for each screened gene, loaded on the gel, blotted and hybridised with gene specific probes. Several positive pools for particular *AtPIN* genes were identified. In the second screening round, the DNA of single Tray pools as well as pools specific for Rows and Columns were tested to identify a single plant carrying mutations of interest. In total 15 PCR reactions for each positive 3-Tray-Pool were performed. The specificity of the PCR signal was confirmed by Southern blot analysis using hybridisation with *AtPIN* gene specific probe.

2.2.4 Methods for protein analysis

2.2.4.1 Determination of protein concentration

200 µl of 5x Bradford solution (*BioRad*) were mixed with the protein sample to a final volume of 1 ml. After 5 min incubation at room temperature, the absorption of the colour complex was measured at 595 nm in a spectrophotometer. The protein concentration was estimated with the help of a standard curve made with BSA (Bradford, 1976).

2.2.4.2 Separation of proteins in SDS polyacrylamide gels

The electrophoresis of protein extracts was performed in 10 % polyacrylamide gels. In brief, the separation gel mix was prepared, degassed and the polymerisation started by the addition of TEMED. The stacking gel was prepared similarly and loaded on top of the separation gel. Samples were mixed with loading buffer, heated (95 °C for 10 min) and loaded onto the gel. The electrophoresis was run at 80 V (Laemmli, 1970).

Running gel (10 %):	3 ml 30 % acrylamide/0,8 %bisacrylamide; 2,5 ml 4x buffer (1,5M Tris/Cl, pH8,8); 100 ml 10 % SDS; 50 ml ammonium persulphate; 5 ml TEMED; 4 ml H_2O
Stacking gel:	440 µl 30 % acrylamide/0,8 % bisacrylamide; 0,8 ml 4x buffer (0,5 M Tris/Cl; pH 6,8; 0,08 % SDS); 33 ml 10 % SDS; 16 ml ammonium persulphate; 2 ml TEMED; 2,03 ml H_20

2 x loading buffer:	50 mM Tris/Cl (pH 6,8); 100 mM Dithiothreitol; 2 % Bromphenol Blue; 10 % glycerol
Running buffer:	3 % Tris; 14,4 % Glycine; 1 % SDS

2.2.4.3 Coomasie blue staining

The polyacrylamide gel was incubated for 30 min at 69 °C with Coomassie. After staining, excess stain was removed by repeated washings in destaining solution (Neuhoff et al., 1985).

Coomassie staining solution:	50 % methanol; 10 % acetic acid; 0,1 % Brilliant Blue R250
Destaining solution:	10 % methanol; 10 % acetic acid;

2.2.4.4 Transfer of proteins (Western blot)

After SDS polyacrylamide electrophoresis proteins were transferred to polyvinylidene difluoride membrane (PVDF *Millipore*). The PVDF membrane was wetted in methanol, washed with water and equilibrated together with 3MM papers in Towbin buffer. The gel was placed adjacent to the PVDF membrane and pressed between sheets of 3MM paper. The semidry transfer of proteins was performed for 1h at 10-15V.

Towbin buffer:	25 mM Tris; 192 mM Glycine; 20 % methanol.

2.2.4.5 Immunostaining of Western blots

The membrane with the transferred proteins was washed in TBS (0,1 % Tween) and incubated for 1 hour in TBS (0,1 % Tween/ 3 % BSA). Incubation with the first antibody diluted 1:10000 in TBS (0,1 % Tween/ 3 % BSA) was performed for 1 hour. Unbound antibodies were removed by washing with TBS (0,1 % Tween). The secondary antibody (anti-rabbit antibodies coupled to alkaline phosphatase, raised in goat) were diluted to 1 : 15 000 in TBS (0,1 % Tween) and incubated with the PVDF membrane for 1 hour. After washing 3 x 10 min with TBS (0,1 % Tween), the PVDF membrane was equilibrated in reaction solution and subsequently incubated in substrate solution until the desired signal density was observed. The reaction was stopped by washing the membrane in water.

TBS:	50 mM Tris/Cl (pH 8); 150 mM NaCl
Reaction solution:	100 mM Tris/Cl pH(9,5); 5 mM $MgCl_2$; 100 mM NaCl
Substrate solution:	50 µl NBT; 37,5 µl BCIP in 10 ml of reaction solution

2.2.5 Tissue culture methods

2.2.5.1 Transformation of *A. thaliana*

Agrobacterium clones (GV3013) carrying the construct of interest were grown in 2 ml of YEB medium with gentamycin (10 mg/l), kanamycin (100 mg/l) and rifampicin (100 mg/l) overnight at 28 °C. *A. tumefaciens* cultures were 20 times diluted and further cultivated for 12-14 hours. Before transformation Silwet L-77 (500 µl/l) and 5 % sucrose were added into the *A. tumefaciens* culture. *A. thaliana* plants were grown under greenhouse conditions, at a density of 8 plants per pot (9cm ϕ). When the first emerged floral bolts were cut off to encourage growth of multiple secondary bolts. Transformation was done 5-10 days after clipping. The plants were dipped for 30 s into *A. tumefaciens* culture and covered with a plastic dome for 24h to maintain high humidity.

YEB medium:	0,5 % beef extract; 0,1 % yeast extract; 0,5 % peptone; 0,5 % sucrose; 2 ml/l; 1M $MgSO_4$

2.2.5.2 Selection and growth of transformed *Arabidopsis* plants under *in vitro* conditions

A. thaliana seeds were sterilised for 15 min in sterilisation solution. After washing three times in sterile H_2O seeds were dried. All steps were performed under sterile conditions in a flow bench. Sterile seeds were plated on MS medium with hygromycin (15 mg/l) as a selective substance.

Sterilisation solution:	5 % calcium hypochloride; 0,1 % Triton X-100
MS medium:	(see materials)

2.2.6 Histochemical detection of reporter enzyme activities

Activities of two reporter enzymes β-glucuronidase (GUS) or phosphonate monoester hydrolase (PEH A) were analysed in transgenic plants. Activities were detected using modified indigogenic methods according to Jefferson et al. (1987) or Dotson et al. (1996).

5-bromo-4-chloro-3-indoxyl β-D glucuronide or 5-bromo-4-chloro-3-indoxyl phosphonate were used as a substrates for enzyme activity localisation. Staining of non-fixed seedlings was performed in 0,1M phosphate buffer (pH 7) or bis-tris buffer (pH 9). Analyses were performed at a substrate concentration of 0,05 % either with or without addition of 1 mM potassium ferrocyanid and potassium ferricyanide. Seedlings were incubated in the substrate solution at 37 or 30 °C for diverse time periods. After staining green tissues were bleached by several washings in 80 % ethanol.

2.2.7 Detection of mRNA by *in situ* hybridisation

2.2.7.1 Whole mount *in situ* hybridisation

An *AtPIN3* specific probe was generated by subcloning a 620 bp fragment (nucleotides 841-1461) in pBSK. Digoxigenin-labelled sense and antisense probes were prepared by *in vitro* transcription according to the manufactures instructions (*Roche*). Three days old seedlings were fixed in fixation solution for 30 min. Chlorophyll was removed by incubation in MeOH (2 x 5 min) and EtOH (3 x 5 min). Seedlings were permeabilised in EtOH/xylene mixture (1:1) for 30 min, washed with EtOH (2 x 5 min) and rehydrated in EtOH/PBS (75, 50, 25 % series) for 10 min each. Postfixation was performed in fixation solution (without heptane) for 20 min followed by washes with PBS/Tween (2 x 10 min). The plant mRNA was released from proteins by proteinase K digestion (20 µg/ml in PBS for 15 min). The reaction was stopped with glycine (2 mg/ml) in PBS for 5 min. Seedlings were washed in PBS/Tween (2 x 10 min).

Hybridisation was performed as described (Ludevid et al., 1992). The samples were prehybridised in hybridisation mix (1h, 45 °C) and hybridised in hybridisation mix with 20-100 ng/ml probe (16h, 45 °C). Three washing steps were performed at 45 °C: (1. wash – 3 x (10, 60, 20 min); 2. wash – 1 x 20 min; 3. wash – 2 x 20 min) followed by wash at room temperature 3 x 10 min with PBS/Tween. The samples were further preincubated in 1 % BSA in PBS/Tween for 90 min/ RT and incubated with antibody for 4 hours (anti-DIG-AP-conjugated antibody, 1:2000 diluted in 1 % BSA in PBS/Tween). The samples were washed 8 x 20 min in PBS/Tween. Treatment of samples in AP buffer was performed for 10 min. Incubation with substrate was carried out in 1 ml of AP buffer supplemented with 5 µl NBT, 3,5 µl BCIP, 2 mM Levamisole. The staining was occasionally inspected under a binocular microscope. The reaction was stopped after 0,5 – 16 hours by changing of substrate solution against deionised water.

fixation solution:	5 % formaldehyde; 15 % DMSO; 0,08 M EGTA; 0,1 % Tween in PBS (pH 7,4); heptane (1:1) emulsion was shaken for 15 min to mix phases before use
hybridisation mix:	50 % formamide; 5 x SSC; 1 mg/ml denatured salmon sperm DNA; 0,1 mg/ml heparin; 0,1 % Tween 20
wash 1:	50 % formamide; 2 x SSC; 0,1 % Tween
wash 2:	2 x SSC; 0,1 % Tween
wash 3:	0,2 x SSC; 0,1 % Tween
PBS/Tween:	0,14 M NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 1,8 mM KH_2PO_4; pH 7,3 - 7,5; 0,1 % Tween
AP buffer:	0,1 M Tris pH 9,5; 0,1 M NaCl; 50 mM MgCl2; 0,1 % Tween
NBT:	50 mg/ml in 70 % DMF (store at 4 °C)
BCIP:	50 mg/ml 5-bromo-4-chloroindolylphosphate sodiumsalt in water (store at -20 °C)

2.2.7.2 *In situ* hybridisation on tissue sections

A. thaliana tissue pieces were fixed with 4 % paraformaldehyde in PBS for 1 hour. The embedding, sectioning, removal of paraffin and rehydration were performed as described for immunolocalisation (Chapter 2.2.9.2). Special care was taken to avoid RNase contamination. After rehydration the sections were treated with Proteinase K (30 min, 37 °C). The positive charging of tissue surface, which can cause background problems, was reduced by acetylation (10 min, RT). After washing (2 x 5 min with 2 x SSC) the samples were dehydrated by incubation in ethanol (30 %, 50 %, 70 %, 90 %, 100 %) and dried under vacuum for 1 hour. The slides containing tissue sections were incubated at 42 °C for 12 hours. Unbound probe was removed by successive washing (4 x 10 min, at RT with 4 x SSC/5 mM DTT; 1 x 30 min at RT with 2 x SSC/0,1 mM DDT; 1 x 5 min at RT with TBS-1). The unspecific binding was blocked with TBS-1/0,5 % Blocking Reagent (*Roche*) and the samples were incubated with Anti-DIG-antibody conjugate (1 : 5000 in TBS-1) in AP buffer (60 min). Afterwards, the slides were washed 3 x 5 min with TBS-1. After short equilibration in AP buffer, the substrate reaction was performed in dark. The staining was controlled using binocular microscope. After 0,5 – 16 hours the reaction was stopped by washing of slides with deionised water. Samples were briefly dried, embedded in 50 % glycerol and inspected with at the Leica DMRB microscope equipped with differential interference contrast (Nomarski optics).

Acetylation solution:	0,1 M TEA pH 7,0; 10 min 0,1 M TEA/0,25 % (v:v) Acetate anhydride pH 7,0
Hybridisation mix:	50 % Formamide (twice crystallised); 0,3 M NaCl; 10 mM Tris/HCl pH 7,5; 1 mM EDTA; 0,02 % Ficoll; 0,02 % PVP; 0,02 % BSA; 10 % Dextransulfate; 60 mM DTT; 500 mg/ml poly(A)RNA; 150 mg/ml yeast tRNA; DIG-labelled RNA-probe
20 x SSC:	0,3 M Na-citrate pH 7,5; 3 M NaCl
TBS-1:	0,1 M Tris/HCl pH 7,5; 150 mM NaCl
AP buffer:	0,1 M Tris/HCl pH 9,5; 0,1 M NaCl; 50 mM $MgCl_2$. For substrate reaction supplied with: 6,6 µl/ml NBT (50 mg/ml 70 % DMF); 3,3µl/ml BCIP (50 mg/ml 100 % DMF)

2.2.8 Production and purification of AtPIN3 specific antibodies

2.2.8.1 Small scale expression of His-AtPIN3 in *E. coli*

To express the hydrophilic region of the AtPIN3 protein the part of *AtPIN3* cDNA corresponding to nucleotides 990 - 1323 was amplified by PCR and ligated into pET-28a(+) (*Novagen*). The cloning was performed such that a terminal translation fusion with six N-terminal His codons was generated. *E. coli* (strain BL21 DE3) was transformed with this construct and several clones containing the plasmid were selected for small-scale expression. 5 ml LB medium were inoculated with 200 µl of an overnight bacterial culture and incubated for 2 h. Expression of polypeptide was induced by adding IPTG (final concentration: 1 mM) and the culture was incubated for 3 h. Crude protein extract was prepared from 800 µl of the bacterial culture and the level of protein expression was inspected by SDS-PAGE gel electrophoresis.

2.2.8.2 Large scale expression and affinity purification of His-AtPIN3

For antigen production, a large scale culture of *E. coli* (pET-His-AtPIN3(990-1323)) was grown. 100 ml of inoculated media were cultivated for 12 h at 37 °C. 40 ml of the culture were used for inoculation of 2 litres LB-Kan/ 10 mM $MgSO_4$-Medium. The culture was grown until an A_{595} = 0,8. Recombinant protein synthesis was induced by addition of IPTG (final concentration: 1 mM). After 2-3 hours vigorous shaking at 37 °C, the bacteria were harvested by centrifugation (10 min/ 4000 rpm). The cells were resuspended in 40 ml of buffer A. After extraction of proteins by 40 ml of phenol (pH 8) the mixture was sonicated for 5 min. The phases were separated by centrifugation at 5000 rpm at 4 °C. Proteins in the phenol phase were precipitated with 100 ml of EtOH. After centrifugation (6000 rpm) the protein pellet was resuspended in 100 ml of buffer B. The protein solution was applied onto a G 25 column equilibrated with buffer C. The DTE free protein solution was subsequently loaded onto a Ni^+-NTA column. This column was washed with 2 volumes of buffer C, one volume of buffer D and again 2 volumes of buffer C. Bound proteins were eluted with buffer C supplemented with 20 mM EDTA. EDTA from eluted proteins were removed by dialysis against buffer C. The proteins were concentrated by ultrafiltration using an Amicon chamber equipped with ultrafiltration membrane (Amicon, 206811).

Buffer A:	50 mM Tris; pH 8; 1 M NaCl; 10 mM b-ME
Buffer B:	50 mM Tris; pH 8; 6 M guanidium-hydrochloride; 0,1 M DTE
Buffer C:	50 mM Tris; pH 8; 0,5 M NaCl; 8 M Urea; 10 mM b-ME
Buffer D:	50 mM Tris; pH 8; 6 M guanidium-hydrochloride; 10 mM b-ME

2.2.8.3 Immunisation of rabbits

For antibody production, rabbits were injected with 50-100 µg of purified AtPIN3 protein added to an equal volume of Freund's complete adjuvant. After 14, 28 and 56 days, booster injections containing the same amount of antigen, but Freund's incomplete adjuvant, were done. Blood samples were taken before immunisation and on days 38 and 66 afterwards. Final bleeding was done on day 80. The immunisation of rabbits was performed by *Eurogentec*, Seraing / Belgium.

2.2.8.4 Affinity purification of antibodies

The anti AtPIN3 antibodies were affinity purified against AtPIN3 polypeptide purified by SDS PAGE and blotted to a PVDF membrane. About 50 µg of purified AtPIN3 polypeptide were separated by electrophoresis on a 10 % polyacrylamide gel and transferred to a PVDF membrane. After Ponceau staining, the membrane area containing the AtPIN3 polypeptide was isolated. The membrane strip was destained with TBS and incubated for 2 hours in buffer 1 to block the non-specific binding sites. An aliquot of the immune serum was diluted with four volumes of TBS and incubated with the membrane stripe for 2 h at 4 °C. The unbound antibodies were washed with TBS for 4 x 5 min, 4 °C. Bound specific antibodies were eluted with 2 x 1 ml buffer 2 for 1,5 min at 4 °C. Immediately after elution, the pH of both fractions was adjusted with 100 µl Tris/HCl (pH 8). For stabilisation, the antibodies solution was supplemented with 0,5 % BSA.

TBS:	8 g/l NaCl; 0,2 g/l KCl; 3 g/l Tris/HCl pH 7,2
Buffer 1:	1 % BSA; 0,05 % Tween 20 in TBS
Buffer 2:	0,1 M Glycine; 0,5 M NaCl; 0,05 % Tween 20; pH 2,6

2.2.9 Immunohistological detection of proteins

2.2.9.1 Whole mount immunolocalisation in *A. thaliana* roots

Arabidopsis seedlings (3-6 days old) were used for immunolocalisation of AtPIN3 protein using affinity purified antibody. Seedlings were fixed in a fixative (4 % paraformaldehyde in MTSB) for 1 hour under vacuum. After fixation seedlings were transferred into the columns of the In-situ Pro robot (*Abimed*). All subsequent steps of protocol were performed using the robot. The seedlings were washed with MTSB/0,1 % Triton. Cell walls were digested with 2 % Driselase in MTSB for 30 min and samples were 6 x washed with MTSB/0,1 % Triton. Incubation with 10 % DMSO/3 % NP-40 in

MTSB for 1 hour followed. After 6 x washing in MTSB/0,1 % Triton the seedlings were preincubated in 3 % BSA/MTSB for 1 hour. Samples were treated with the affinity purified primary antibody, diluted (1:100) in 3 % BSA/MTSB, for 5 hours at 37 °C. After extensive washing with MTSB/0,1 % Triton (8 x 10 min), the seedlings were incubated with secondary antibody (Anti-rabbit-IgG-FITC conjugate 1:200 in 3 % BSA/MTSB). Finally, the samples were washed 6 x with MTSB/0,1 % Triton and 6 x with deionised water. The seedlings were transferred into Slowfade Antifade mounting medium and inspected by confocal microscopy.

MTSB: 50 mM PIPES; 5 mM EGTA; 5 mM Mg SO_4; pH 7 adjusted with KOH

2.2.9.2 Detection of proteins on tissue sections

(I) Fixation

The *Arabidopsis* inflorescence axis was cut in 3-5 mm pieces and incubated in ice cold MeOH/HAC (3:1) fixative overnight at -20 °C. The fixed material was subsequently washed 3 times for 15 minutes in PBS.

(II) Embedding

Fixed and washed material was dehydrated in EtOH series: 10/30/50/70/90 % EtOH (each 15 min), 2 x 96 % EtOH (each 45 min), 2 x 100 % EtOH (each 45 min) and subsequently infiltrated with xylene:

- EtOH/xylene 3 : 1, 60 min
- EtOH/xylene 1 : 1, 60 min
- EtOH/xylene 1 : 3, 60 min
- 100 % xylene, 60 min
- 100 % xylene, overnight

Plant material in xylene was overlayered with melted paraffin (60 °C) and incubated at RT for 12 hrs. This layer of paraffin was replaced by freshly melted paraffin (60 °C) two times. The first incubation was performed at 42 °C for 12 hours and the second at 58 °C for 4 hours. Afterwards half of the xylene/paraffin volume was replaced by pure melted paraffin and incubated for 4 hours at 58 °C (step repeated 2 times) and for 12 hours at 58 °C (step repeated 4 times). Finally, stem pieces were mounted in heated (60 °C) moulds with melted paraffin, tissue was oriented and material was cooled rapidly down to solidify paraffin.

(III) Sectioning and antibody incubations

8 µm sections from fixed and paraffin embedded material were cut and transferred onto polyL-Lysine coated slides in a droplet of water. The sections were dried on the slides by incubation on the 42 °C plate (2h, o/n)

Deparaffinisation and rehydration followed as described:

- incubation of slides 20 min in 100 % xylene
- incubation of slides 10 min in 50 % xylene/50 % EtOH
- incubation of slides 10 min in 100 % EtOH
- drying of slides in fume hood 0,5 to several hours (slides can be frozen at -20 °C)
- selection of best slides (best structure conservation) by eye and microscope
- rehydration of sections by an ethanol series: 100/90/70/50/30/10 % ethanol (at RT for 5-8 min)
- incubation for ca 10 min in 1 x PBS

Afterwards the sections on slides were incubated in blocking solution at RT for 1 hour. Incubation with first antibody (1:10 to 1:100 diluted in blocking solution) was carried out in moisture chamber over night at 4 °C. Next day the material was washed 3 times in 1 x PBS/0,2 % Tween-20 at RT for 5-10 minutes and blocked once more for 1 hour at RT. Incubation with the secondary antibody (Anti-rabbit-IgG-FITC conjugate, 1 : 200) was performed for 4 hors at RT. Three washes in 1 x PBS/0,2 % Tween-20 at RT for 5-10 minutes and two washes in 1 x PBS without Tween-20 for 10 min closed the procedure. The sections were mounted in antifade reagent and inspected by confocal microscopy.

Blocking solution: 1 x PBS; 3 % non fat, filtered milk powder; pH 7,4

2.2.10 Phenotypic analysis of mutant lines

2.2.10.1 Hypocotyl and root length

The sterilised seeds of mutant and control plants were plated on Ara medium, vernalised for two days at 4 °C and grown in vertical position in continuous light for 6 days at 20 °C. The plates were photographed and the seedlings were in detail inspected using a binocular microscope or a microscope equipped with a videocamera (*Hitachi*, HV-C20A). The hypocotyl and root lengths as well as cell lengths of hypocotyl epidermis cells were measured with help of computer software DISKUS (*Carl H. Hilgers*).

2.2.10.2 Hypocotyl tropism responses

The sterilised seeds of mutant and control plants were plated on Ara medium, vernalised for two days at 4 °C. After vernalisation, a pulse of white light for 2 hours at 26 °C was applied followed by a 4 day incubation in dark (26 °C). Gravitropic response was induced

by turning plates for 90 degree. Plates were incubated at turned position for 24 hours. Phototropic response was induced by unilateral white light. Seedlings were exposed to the light treatment for 12 hours. The gravitropic and phototropic responses of seedlings were evaluated and documented.

2.2.10.3 Apical hook opening

The sterilised seeds of mutant and control plants were plated on Ara medium, vernalised for two days at 4 °C and grown in dark at 26 °C. In regular time intervals the plates were inspected and seedlings with opened apical hook counted. The experiment was terminated when all seedlings had opened apical hooks.

2.2.10.4 Root meristem pattern phenotype

Five days old *in vitro* grown seedlings were destained in 80 % ethanol for 24 hours and incubated in 0,24 M HCl/20 % methanol on 57 °C heat block for 15 min. This solution was replaced with 7 % NaOH in 60 % ethanol for 15 min at room temperature. Plants were rehydrated for 5 minutes each in 40 %, 20 % and 10 % ethanol, and infiltrated for 15 minutes in 5 % ethanol, 25 % glycerol. Seedlings were mounted in 50 % glycerol on glass microscope slides.

The starch of columella cells was stained in 1 % lugol solution (*Merck*) for 1 minute and rinsed in 50 % glycerol for 10 seconds. The samples were inspected using Leica DMRB microscope equipped with Nomarski optics.

3 Results

To contribute to the molecular analysis of polar auxin transport this thesis aims to study novel genes from *Arabidopsis thaliana* encoding important components of the auxin transport machinery. The major aim was to identify, isolate and characterise at the molecular and genetic level homologues of the putative auxin efflux carriers AtPIN1 and AtPIN2.

3.1 Identification of *AtPIN* homologues in genomic library

Since sequencing of the entire *A. thaliana* genome is close to completion and a large number of expressed sequenced tags (ESTs) are available in public databases, they were screened for novel homologues of *AtPIN1* and related sequences. The screen was performed using *AtPIN1* and *AtPIN2* cDNA sequences and predicted AtPIN1 and AtPIN2 amino acid sequences. Several partial EST sequences and one genomic sequence with more than 60% identity were identified indicating the existence of a larger *AtPIN* gene family in *Arabidopsis*.

After the successful identification of several members of the *AtPIN* family in databases, it was necessary to isolate *AtPIN* related genomic sequences and to search for corresponding full-length cDNAs. The most highly conserved sequences were selected for hybridisation to the IGF BAC library under low stringency conditions.

3.1.1 Identification of novel putative *AtPIN* loci in the IGF BAC library

For identification and isolation of new *AtPIN* genes the bacterial artificial chromosome library IGF BAC from *Arabidopsis thaliana* was used (Mozo *et al.,* 1998). The IGF BAC library consists of 10.752 clones with an average insert size of about 100 kb. Each clone is spotted twice onto a nylon membrane in a specific order, which allows after hybridisation rapid evaluation of signals and identification of positive clones. Analysis of the mean insert size of *Arabidopsis* genomic DNA inserted into the BAC clones and the number of BAC clones identified by hybridisation with probes corresponding to single *Arabidopsis* genes indicated an about 7-fold genome coverage. A database containing hybridisation results has been established by Mozo *et al.* (1998) which allows to rapidly map most novel genes within the *Arabidopsis* genome (http://www.mpimp-golm.mpg.de/101/mpi_mp_map/bac.html).

Seven different *AtPIN* specific probes were used for hybridisation. These probes were derived from *AtPIN1* and *AtPIN2* cDNA sequences, two homologous EST sequences (T04468, T43636) and one homologous genomic sequence (AB005242). Comparison between *AtPIN1*, *AtPIN2* cDNA sequences revealed that two regions, placed at the 5' and 3' terminus of the coding sequence, were highly conserved within the *AtPIN* family. These conserved regions were used to design probes specific for the *AtPIN* gene family. The following probes were used:

Probe pin1: full size *AtPIN1* cDNA,
Probe pin2: full size *AtPIN2* cDNA,
Probe t0: insert of EST clone T04468,
Probe t4: insert of EST clone T43636,
Probe 5'pin1: 5' conserved region of *AtPIN1* cDNA,
Probe 3'pin1: 3' conserved region of *AtPIN1* cDNA,
Probe pin5: part of *AtPIN5* genomic sequence amplified by PCR from *Arabidopsis* genomic DNA.

The probes were radioactively labelled and hybridised under low stringency conditions to the IGF BAC library spotted onto nylon filters. 153 positive signals were identified after hybridisation, which corresponded to 77 different IGF BAC clones. A summary of the data obtained is given in Table 1.

The position of identified IGF BAC clones in already mapped IGF BAC contigs was determined. Thereby it was possible to map most of the identified clones within the *Arabidopsis* genome. Positions in the genome, which were identified by at least two positive clones from the hybridisation screen were assumed to define a novel *AtPIN* gene related locus. Using this approach, it was possible to identify 10 new *AtPIN* loci in the *Arabidopsis* genome. These new putative *AtPIN* genes were designated *AtPIN3* through *AtPIN12*. The novel *AtPIN* loci together with the clones identifying them are listed in Table 2. The map position of the novel *AtPIN* related loci within *Arabidopsis* genome is depicted in Figure 7.

As new *AtPIN* loci were not considered those: (*i*) which were identified by a single hybridisation signal and (*ii*) which did correspond to a IGF BAC contig, where already *AtPIN* signals had been identified. It was not possible to map 12 additional "positive" IGF BAC clones within the *Arabidopsis* genome, due to the as yet incomplete characterisation of the IGF BAC library. These apparently "*AtPIN* unrelated" loci are listed together with unmapped clones in Table 3.

Table 1. Clones identified with different *AtPIN* probes in the *A. thaliana* IGF BAC library.
Probes are listed in first row (bold). Names of positive BACs are listed for each probe.

pin1	pin2	t0	t4	5'pin1		3'pin1		pin5
6D5	4D22	2B19	2I9	2I9	20H21	2I9	25K20	5E1
6G20	9A11	2I9	6D5	2L2	20L21	6D5	25M23	6C19
8B23	20L21	2M16	6F24	4A9	21A22	6F24	25O7	6E22
9A11	23D3	6F24	6G20	4D22	21M14	6G20	25P10	7C23
9D3	26O1	6G20	8M17	5L19	21N8	7M12	25P9	9A3
9O10	28C19	7F19	9A11	6D5	23D3	7O12	28C19	13O8
10A8	28M16	8M17	9D3	6F24	24B14	7P3	28D9	19E4
11H9	28N6	9A11	9O10	6G20	24K5	8M17	28I12	
12L8		9D3	10A8	7O1	24O19	8P22		
12O6		9K19	11H9	7O12	25F8	9D3		
16G14		9O10	12L8	8B23	25O7	9H24		
17D7		10A8	12O6	8M17	28C19	9O10		
20H21		12L8	15B23	9D3	28D9	10A8		
21A22		12O6	16G14	9F1	28I12	11H9		
21M14		14K22	17D7	9O10		12L8		
25F8		15B23	20H21	10A8		12O6		
25O7		16G14	22J4	11H9		16G14		
25P10		16O1	22L14	11P1		17D7		
28I12		20H21	23M3	12L8		20H21		
		22J4	25B18	12O6		20L21		
		22L14	25K17	16G14		21A22		
		25B18	28H13	17D7		21M14		
		25K17		19E14		21N8		
		26L18		1B15		25F8		
		28H13		1H20		25K19		

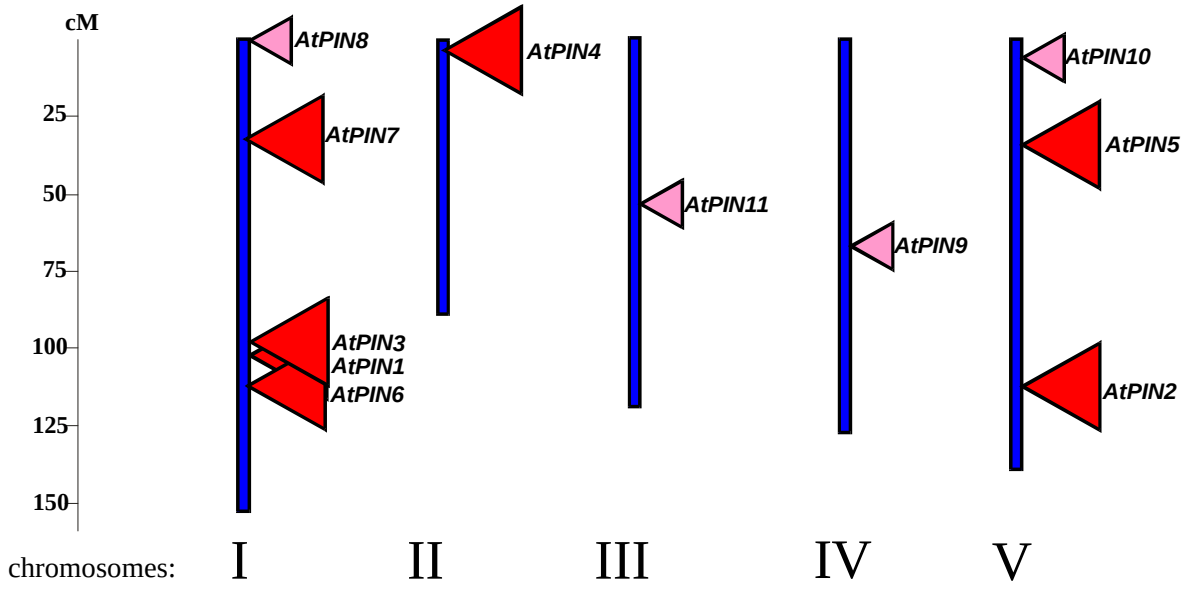
Table 2. Members of the *AtPIN* gene family identified in the IGF BAC library.
Map position (chromosome, close by marker, position in cM) and positive IGF BAC clones, which identified the corresponding *AtPIN* genes are listed.

gene	*PIN1*	*PIN2*	*PIN3*	*PIN4*	*PIN5*	*PIN6*	*PIN7*	*PIN8*	*PIN9*	*PIN10*	*PIN11*	*PIN12*
chrom.	**I**	**V**	**I**	**II**	**V**	**I**	**I**	**I**	**IV**	**V**	**III**	**?**
marker	**nga11**	**mi69**	**mi103a**	**rga**	**rnapol1**	**ADH**	**m235**	**cer1**	**mi475**	**mi121**	**m249**	**?**
position	**110 cM**	**115 cM**	**109 cM**	**1,8 cM**	**33 cM**	**114 cM**	**32 cM**	**0-4 cM**	**70 cM**	**12 cM**	**60 cM**	**?**
IGF BAC clones	6D5 25P10 8P22	4D22 23D3 26O1 28C19 28M16 28N6	6G20 8M17 9O10 12O6 20H21 22J4 22L14	2I9 6F24 9D3 10A8 11H9 16G14 17D7 23M3	5E1 6C19 6E22 7C23 9A3 13O8 19E4	1B15 1H20 4A9 7O12 8B23	25B18 25K17 28H13 12L8	9F1 11P1 19E14 24O19 25O7 28I12	2L2 5L19 8B2 24K5	7O1 28D9	21N8 7P3	21A22 25P9

Table 3. Loci identified by single positive clone and unmapped clones.

chrom.	contig	marker	position	clone	probe
I	1	m488	5,6 cM	25M23	3'pin1
III	2	fad7	14 cM	21M14	3'pin1, 5'pin1
III	5	a8a13	77 cM	7M12	3'pin1
IV	1	mi306	19,5 cM	15B23	t0, t4
iV	2	mi87	21cM	24B14	5'pin1
V	1	mi121	12 cM	28D9	3'pin1
V	5	m421	82,5 cM	25K19	3'pin1
V	6	CIC6C5	112 cM	25K20	3'pin1
V	7	mi335	133 cM	28C19	3'pin1, 5'pin1
Unmapped clones					
	2B19	2K19	2M16	7F19	9A11
	14K22	16O1	20L21	21A22	25F8
	9H24	26L18			

Figure 7. Mapping of *AtPIN* genes from *Arabidopsis thaliana.*
Large triangles mark the position of cloned and characterised genes, small triangles mark the position of putative new *AtPIN* homologues.



3.1.2 Isolation of AtPIN3 – *AtPIN7* genomic sequences

To start the molecular characterisation of several novel members of the *AtPIN* gene family, four IGF BAC clones were chosen for further analysis. It was necessary first to verify the results of the hybridisation screen and second to sequence at least portions of the novel putative *AtPIN* genes. Therefore, positive BAC clones were digested into smaller pieces. To determine which restriction fragments contained part of the *AtPIN* gene sequence, fragments were hybridised after Southern blot transfer to nylon membranes with an *AtPIN* gene family specific probe. The positive fragments were identified, subcloned and sequenced.

In order to achieve this, the DNA from IGF BAC clones 6G20, 2I9, 4A9, 28H13 was isolated and digested with BamHI restriction enzyme. Resulting fragments were inserted into pBS vector and cloned in *E. coli*. Positive transformants were plated with an appropriate density (5-10 colonies/cm^2) and allowed to grow overnight. The colonies were transferred onto nylon membrane and hybridised with the 3'pin1 probe. The clones corresponding to positive hybridisation signals were sequenced. This approach revealed partial sequences for *AtPIN3*, *AtPIN4*, *AtPIN6* and *AtPIN7*, respectively. Since the *AtPIN5* sequence was present in the *Arabidopsis* database, the genomic sequence was amplified directly by PCR using *AtPIN5* gene specific primers. The partial gene fragments for *AtPIN3 – AtPIN7* were further used for isolation of corresponding full-length cDNAs.

3.2 **Isolation and analysis of *AtPIN3-AtPIN7* cDNAs**

An important tool for the molecular and functional characterisation of novel *AtPIN* genes is the availability of full-length cDNAs. Therefore the *AtPIN* gene specific fragments isolated from IGF BAC clones were used to design probes for screening of various cDNA libraries. The following probes were generated: t0 (part of the *AtPIN3* specific EST), t4 (part of the *AtPIN4* specific EST), pin5 (PCR amplified fragment), pin6 (subcloned piece of the 4A9 IGF BAC clone), pin7 (subcloned fragment of the 28H13 IGF BAC clone). These probes were used to screen eight different *Arabidopsis* cDNA libraries. A summary of the screens that were performed is given in Table 4.

To screen for *AtPIN* cDNAs, bacteria and phages hosting the libraries were grown for 24 hours on solid medium, the DNA was transferred onto nylon membranes, denatured and crosslinked. Membranes were subsequently hybridised with radioactively labelled *AtPIN3 – AtPIN7* specific probes. Positive signals were identified by autoradiography. Pools containing positive clones were extracted and rescreened. After three rounds of screening, single, specific clones containing corresponding cDNA were isolated. All isolated cDNAs were sequenced. 11 different clones containing full-length cDNAs for 5 new *AtPIN* genes were identified. The full length cDNAs were characterised to identify the signals for initiation of translation, start and stop codons, polyadenylation signals and poly(A)$^+$ tail sequences. The cDNA derived sequences were also used to obtain the respective full length genomic sequences and promoters. All the characterised cDNAs were published in *A. thaliana* public database. The information for each new *AtPIN* gene/cDNA will be provided in the next chapter.

Table 4. Libraries used for screening of new *AtPIN* genes.

Origin of library	Vector	Host	Identified genes
Suspension culture I	JG4-5	bacteria	*AtPIN7*
Suspension culture II	Lambda ZapII	phages	none
Seedling	Lambda Yes	phages	*AtPIN6, AtPIN7*
Whole plant	pFL61	bacteria	*AtPIN3, AtPIN4, AtPIN5*
Aerial part	pXY112	bacteria	none
Guard cells	Lambda ZapII	phages	*AtPIN4*
Root	Lambda ZapII	phages	*AtPIN4*
Leaves	PSPORT	bacteria	*AtPIN3*

3.3 **Molecular analysis of new *AtPIN* genes**

In this chapter each new *AtPIN* gene will be described. The following information will be presented:

(1) from which cDNA libraries the gene was isolated,
(2) position of transcription and translation start,
(3) position of translation stop and polyadenylation signal,
(4) genomic and coding sequence information,
(5) the deduced exon/intron organisation,
(6) mapping within *Arabidopsis* genome
(7) accession number in *A. thaliana* database.

The genomic sequence was in all cases (*AtPIN3 – AtPIN7*) amplified by PCR from the corresponding IGF BAC clones using *AtPIN* gene specific primers and sequenced. Comparison of both genomic and cDNA sequence revealed the exon/intron organisation. The *AtPIN* genes were mapped using results from hybridisation analysis of the IGF BAC library. The following chapters give a brief summary on the newly identified *AtPIN* cDNAs and genes.

3.3.1 The *AtPIN3* gene

(1) The *AtPIN*3 full-length cDNA was isolated from 2 different cDNA libraries prepared from leaves and whole plant tissues, respectively.
(2) The transcription start was predicted at position -60 as CTCACTT sequence and translation start sequence contains a purine nucleotide (adenine) at position -3 and ATG start codon.
(3) The stop codon TAA is placed at position 1921 from ATG and a polyadenylation signal AATTAAA was identified at position 2201.
(4) The genomic sequence was amplified from IGF BAC clone 6G20 using *AtPIN3* cDNA specific primers corresponding to nucleotides 39 – 64 upstream and 2189 – 2213 downstream from ATG. The size of *AtPIN3* coding sequence comprises 1920 base pairs. The length of *AtPIN3* gene including introns is around 2.7 kb.
(5) The *AtPIN3* coding sequence consists of 6 exons disrupted by 5 introns.
(6) The *AtPIN3* gene maps on chromosome I at 108 - 111 cM from the top (marker mi103a).
(7) Accession number: AF087818.

The genomic structure of the *AtPIN3* gene and the *AtPIN3* coding sequence are presented in Figure 8 (page 55).

3.3.2 **The *AtPIN4* gene**

(1) The *AtPIN*4 full-length cDNA was isolated from 3 different cDNA libraries prepared from seedlings, leaves and whole plant tissues, respectively.
(2) The transcription start was predicted at position -30 as CTCATCA sequence and translation start sequence contains a purine nucleotide (adenine) at position -3 and ATG start codon.
(3) The stop codon TGA is placed at position 1837 from ATG and polyadenylation signal AATTAAA was identified at position 1912.
(4) The genomic sequence was amplified from IGF BAC clone 2I9 using *AtPIN4* cDNA specific primers corresponding to nucleotides 24 – 48 upstream and 1955 – 1978 downstream from ATG. The size of *AtPIN4* coding sequence comprises 1848 base pairs and the length of *AtPIN4* gene including introns comprises around 2.7 kb.
(5) The *AtPIN4* coding sequence consists of 6 exons disrupted by 5 introns.
(6) The *AtPIN4* gene maps on chromosome II at 0 - 4 cM from the top (marker rga).
(7) Accession number: AF087016.

The genomic structure of the *AtPIN4* gene and the *AtPIN4* coding sequence are presented in Figure 9 (page 56).

3.3.3 The *AtPIN5* gene

(1) The *AtPIN*5 full-length cDNA was isolated from a cDNA library prepared from whole plant tissue.
(2) The transcription start was predicted at position -160 as CCTATC sequence and translation start sequence contains a purine nucleotide (adenine) at position -3 and ATG start codon.
(3) The stop codon TGA is placed at position 1054 from ATG. A polyadenylation signal could not clearly be identified within the *AtPIN5* cDNA sequence.
(4) The genomic sequence was obtained as part of the P1 clone AB005242 from the *Arabidopsis thaliana* database. The size of *AtPIN5* coding sequence comprises 1053 base pairs. The length of *AtPIN5* gene including introns is around 1.9 kb.
(5) The *AtPIN5* coding sequence consists of 5 exons disrupted by 4 introns.
(6) The *AtPIN5* gene maps on chromosome V at 28 - 38 cM from the top (marker nga 106).

The genomic structure of the *AtPIN5* gene and the *AtPIN5* coding sequence are presented in Figure 10 (page 57).

3.3.4 The *AtPIN6* gene

(1) The *AtPIN6* full-length cDNA was isolated from a cDNA library prepared from seedlings.
(2) The transcription start was predicted at position -80 as CTCATA sequence and translation start sequence contains a purine nucleotide (adenine) at position -3 and ATG start codon.
(3) The stop codon TAA is placed at the position 1813 from ATG and a polyadenylation signal AATTAAA was identified at position 1853.
(4) The genomic sequence was amplified from IGF BAC clone 4A9 using *AtPIN6* cDNA specific primers corresponding to nucleotides 27 – 50 upstream and 1708 – 1729 downstream from ATG. The size of *AtPIN6* coding sequence comprises 1710 base pairs and the length of *AtPIN6* gene including introns is 3.6 kb.
(5) The *AtPIN6* coding sequence consists of 7 exons disrupted by 6 introns.
(6) The *AtPIN6* gene maps on chromosome I at 109 - 119 cM from the top (marker ADH).
(7) Accession number: AF087819.

The genomic structure of the *AtPIN6* gene and the *AtPIN6* coding sequence are presented in Figure 11 (page 58).

3.3.5 The *AtPIN7* gene

(1) The *AtPIN7* full-length cDNA was isolated from cDNA libraries prepared from seedlings and suspension culture, respectively.
(2) The transcription start was predicted at position -80 as GTCATC sequence and translation start sequence contains a purine nucleotide (adenine) at position -3 and ATG start codon.
(3) The stop codon TAA is placed at position 1846 from ATG and a polyadenylation signal AATTAAA was identified at position 1881.
(4) The genomic sequence was amplified from IGF BAC clone 28H13using *AtPIN7* cDNA specific primers corresponding to nucleotides 34 – 54 upstream and 1870 - 1892 downstream from ATG. The size of *AtPIN7* coding sequence comprises 1857 base pairs and the length of *AtPIN7* gene including introns is 2.6 kb.
(5) The *AtPIN7* coding sequence consists of 6 exons disrupted by 5 introns.
(6) The *AtPIN7* gene maps on chromosome I at 27 - 37 cM from the top (marker m235).
(7) Accession number: AF087820.

The genomic structure of the *AtPIN7* gene and the *AtPIN7* coding sequence are presented in Figure 12 (page 59).

3.3.6 Molecular analysis of new *AtPIN* genes - Summary

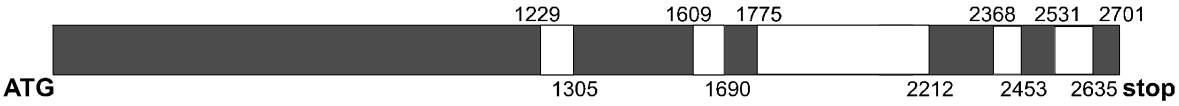
Five novel cDNA sequences with significant homology to the *AtPIN1* and *AtPIN2* genes have been isolated and sequenced. The identification of the transcription start sites, ATG start codons, polyadenylation signals and poly(A)$^+$ tail sequences suggest that the cDNAs are full length and represent the complete open reading frames. The genes were designated as *AtPIN3 – AtPIN7*. The analysis of the exon/intron organisation revealed that *AtPIN* coding sequences are interrupted by 4 to 8 introns. The *AtPIN* genes are placed on chromosomes I, II and V. *AtPIN1*, *AtPIN3* and *AtPIN6* were mapped in close proximity to each other between 110-115 cM from the top of the chromosome I. An overview of molecular properties of isolated *AtPIN* genes is listed in Table 5.

Table 5. Molecular properties of *AtPIN1 – AtPIN7* genes.

designation	length (Amino Ac.)	identity vs. AtPIN1	number of introns	position in genome
AtPIN1	622	100%	4	ch.I 110cM
AtPIN2	647	64%	8	ch.V 115cM
AtPIN3	640	73%	5	ch.I 109cM
AtPIN4	616	72%	5	ch.II 1.8cM
AtPIN5	352	61%	4	ch.V 33cM
AtPIN6	570	59%	6	ch.I 114cM
AtPIN7	570	72%	5	ch.I 32cM

Figure 8. The *AtPIN3* gene.
Exon/intron organisation of the *AtPIN3* gene (A) and its translated coding sequence (B) are depicted. Exons are displayed as black squares and introns as white squares with the positions of exon/intron borders indicated.

A

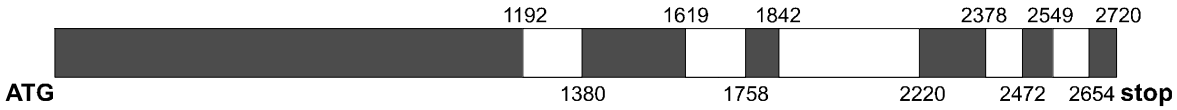


B

```
ATGATCTCATGGCACGACCTCTACACGGTCCTCACCGCCCGTGATTCCTCTCTACGTGGCCATGATCCTCGCTTACGGCTCTGTCCGGTGGTGGAAAATCT                100
 M  I  S  W  H  D  L  Y  T  V  L  T  A  V  I  P  L  Y  V  A  M  I  L  A  Y  G  S  V  R  W  W  K  I

TCTCACCCGACCAATGCTCCGGAATCAACCGTTTTGTCGCCATCTTCGCCGTCCCTCTCCTCTCTTTTCACTTCATCTCCACCAACAATCCTTACGCAAT           200
F  S  P  D  Q  C  S  G  I  N  R  F  V  A  I  F  A  V  P  L  L  S  F  H  F  I  S  T  N  N  P  Y  A  M

GAATCTCCGGTTCATCGCCGCCGACACTCTCCAAAAAATCATCATGTTGTCACTTTTAGTCCTATGGGCTAATTTCACTCGCTCCGGTAGCCTCGAGTGG           300
 N  L  R  F  I  A  A  D  T  L  Q  K  I  I  M  L  S  L  L  V  L  W  A  N  F  T  R  S  G  S  L  E  W

AGCATCACAATCTTTTCCCTTTCCACACTTCCCAACACTCTTGTTATGGGGATTCCTCTCTTGATCGCCATGTATGGCGAATACTCTGGTTCCCTCATGG           400
S  I  T  I  F  S  L  S  T  L  P  N  T  L  V  M  G  I  P  L  L  I  A  M  Y  G  E  Y  S  G  S  L  M

TCCAAATCGTCGTCCTCCAGTGTATCATCTGGTACACGCTTCTCCTTTTTCTCTTCGAGTTTCGTGGCGCCAAGATGCTCATCATGGAGCAGTTCCCTGA           500
V  Q  I  V  V  L  Q  C  I  I  W  Y  T  L  L  L  F  L  F  E  F  R  G  A  K  M  L  I  M  E  Q  F  P  E

GACGGCTGCTTCCATTGTTTCTTTCAAAGTCGAATCCGACGTCGTTTCGCTCGACGGCCATGATTTTCTTGAGACCGATGCAGAGATAGGTGACGACGGG           600
 T  A  A  S  I  V  S  F  K  V  E  S  D  V  V  S  L  D  G  H  D  F  L  E  T  D  A  E  I  G  D  D  G

AAGCTTCACGTCACCGTGAGAAAATCCAACGCTTCACGTCGTTCGTTCTGCGGCCCGAACATGACTCCACGGCCGTCAAATCTCACCGGAGCTGAGATTT           700
K  L  H  V  T  V  R  K  S  N  A  S  R  R  S  F  C  G  P  N  M  T  P  R  P  S  N  L  T  G  A  E  I

ATAGTCTCAGCACCACTCCTAGAGGCTCTAATTTCAACCACTCTGATTTTTACAACATGATGGGTTTCCCCGGTGGTCGTCTCTCCAATTTCGGTCCGGC           800
Y  S  L  S  T  T  P  R  G  S  N  F  N  H  S  D  F  Y  N  M  M  G  F  P  G  G  R  L  S  N  F  G  P  A

GGATATGTACTCCGTTCAATCATCTAGAGGTCCAACTCCTCGACCTTCAAACTTCGAGGAGAATTGCGCCATGGCATCCTCCCCGAGATTCGGGTATTAC           900
 D  M  Y  S  V  Q  S  S  R  G  P  T  P  R  P  S  N  F  E  E  N  C  A  M  A  S  S  P  R  F  G  Y  Y

CCTGGAGGAGGAGCCGGGTCTTATCCGGCTCCGAATCCAGAGTTCTCTTCAACCACCACATCTACCGCCAATAAAAGCGTCAATAAAAACCCGAAAGACG          1000
P  G  G  G  A  G  S  Y  P  A  P  N  P  E  F  S  S  T  T  T  S  T  A  N  K  S  V  N  K  N  P  K  D

TTAATACGAATCAGCAGACGACTCTTCCAACGGGCGGCAAGTCAAACAGCCATGACGCCAAGGAGCTTCACATGTTCGTCTGGAGCTCAAACGGGTCACC          1100
V  N  T  N  Q  Q  T  T  L  P  T  G  G  K  S  N  S  H  D  A  K  E  L  H  M  F  V  W  S  S  N  G  S  P

CGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGACAACGATCAAGGCGGAAGATCTGACCAAGGTGCTAAAGAGATCCGTATGTTAGTC          1200
 V  S  D  R  A  G  L  N  V  F  G  G  A  P  D  N  D  Q  G  G  R  S  D  Q  G  A  K  E  I  R  M  L  V

CCAGATCAATCTCACAACGGCGAGACCAAAGCTGTAGCTCATCCAGCAAGTGGAGATTTCGGAGGAGAACAACAATTTAGTTTCGCCGGAAAAGAAGAAG          1300
P  D  Q  S  H  N  G  E  T  K  A  V  A  H  P  A  S  G  D  F  G  G  E  Q  Q  F  S  F  A  G  K  E  E

AAGCAGAGAGACCAAAAGACGCCCGAGAATGGTCTAAACAAACTTGCTCCAAATTCCACGGCGGCGCTACAATCCAAGACAGGTCTAGGAGGAGCCCGAAGC          1400
E  A  E  R  P  K  D  A  E  N  G  L  N  K  L  A  P  N  S  T  A  A  L  Q  S  K  T  G  L  G  G  A  E  A

AAGTCAACGAAAAAATATGCCTCCGGCGAGTGTGATGACAAGGCTGATACTGATAATGGTTTGGAGGAAACTCATCAGAAACCCAAACACTTACTCTAGT          1500
 S  Q  R  K  N  M  P  P  A  S  V  M  T  R  L  I  L  I  M  V  W  R  K  L  I  R  N  P  N  T  Y  S  S

CTCATTGGACTTATTTGGGCTCTCGTCGCTTTCCGGTGGCACGTGGCAATGCCCAAAATCATTCAACAATCTATCTCCATTCTATCTGATGCTGGTCTTG          1600
L  I  G  L  I  W  A  L  V  A  F  R  W  H  V  A  M  P  K  I  I  Q  Q  S  I  S  I  L  S  D  A  G  L

GAATGGCAATGTTTAGTTTGGGCTTGTTCATGGCGTTGCAACCCAAATTAATCGCTTGTGGGAATTCACTGGCAACGTTTGCCATGGCGGTTAGGTTCCT          1700
G  M  A  M  F  S  L  G  L  F  M  A  L  Q  P  K  L  I  A  C  G  N  S  V  A  T  F  A  M  A  V  R  F  L

TACGGGTCCGGCCGTGATGGCGGTTGCTGCTATAGCCATCGGATTACGTGGTGATTTACTGCGTGTCGCTATAGTTCAGGCCGCATTACCTCAAGGAATT          1800
 T  G  P  A  V  M  A  V  A  A  I  A  I  G  L  R  G  D  L  L  R  V  A  I  V  Q  A  A  L  P  Q  G  I

GTGCCCTTTGTGTTTGCGAAGGAGTACAATGTTCATCCTGCTATTTTAAGTACAGGGGTAATATTTGGAATGCTTATAGCGCTTCCGATCACGCTGGTTT          1900
V  P  F  V  F  A  K  E  Y  N  V  H  P  A  I  L  S  T  G  V  I  F  G  M  L  I  A  L  P  I  T  L  V

ACTACATTCTACTCGGGTTATAA          1923
Y  Y  I  L  L  G  L  .
```

Figure 9. The *AtPIN4* gene.
Exon/intron organisation of the *AtPIN4* gene (A) and its translated coding sequence (B) are depicted. Exons are displayed as black squares and introns as white squares with the positions of exon/intron borders indicated.

A

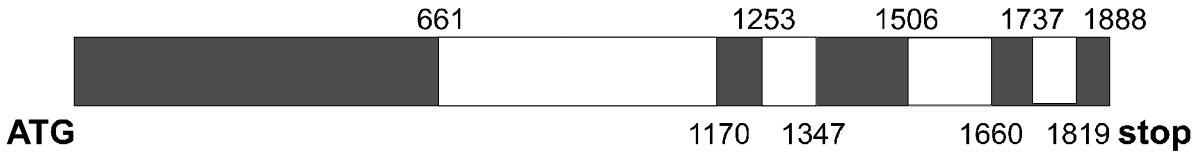


B

```
ATGATTACGTGGCACGACTTGTACACCGTCCTCACCGCCGTGGTACCACTTTACGTAGCTATGATTCTCGCCTACGGATCCGTACAGTGGTGGAAGATAT   100
 M  I  T  W  H  D  L  Y  T  V  L  T  A  V  V  P  L  Y  V  A  M  I  L  A  Y  G  S  V  Q  W  W  K  I

TCTCACCAGACCAGTGCTCCGGCATCAACCGCTTCGTCGCTATCTTCGCCGTCCCTCTCCTCTCCTTCCACTTCATCTCCACCAACGATCCTTACGCCAT   200
F  S  P  D  Q  C  S  G  I  N  R  F  V  A  I  F  A  V  P  L  L  S  F  H  F  I  S  T  N  D  P  Y  A  M

GAATTTCCGCTTCGTCGCCGCCGACACGCTTCAAAAAATCATCATGCTCGTCTTACTTGCTCTATGGGCTAACCTAACCAAGAACGGTAGCTTGGAGTGG   300
 N  F  R  F  V  A  A  D  T  L  Q  K  I  I  M  L  V  L  L  A  L  W  A  N  L  T  K  N  G  S  L  E  W

ATGATCACAATCTTCTCTCTCAGCACTCTCCCAAACACTCTTGTCATGGGGATCCCTCTGTTGATCGCCATGTACGGAACCTACGCAGGTTCTCTAATGG   400
 M  I  T  I  F  S  L  S  T  L  P  N  T  L  V  M  G  I  P  L  L  I  A  M  Y  G  T  Y  A  G  S  L  M

TCCAAGTCGTTGTTCTTCAGTGTATCATTTGGTACACTCTCCTTCTCTTCCTCTTCGAGTACCGTGGCGCTAAGCTTCTTATTATGGAGCAGTTCCCGGA   500
V  Q  V  V  V  L  Q  C  I  I  W  Y  T  L  L  L  F  L  F  E  Y  R  G  A  K  L  L  I  M  E  Q  F  P  E

GACTGGTGCGTCTATTGTTTCGTTTAAAGTTGAATCCGACGTCGTTTCTCTCGACGGTCATGATTTTCTTGAGACGGATGCTGAGATAGGAAACGACGGG   600
 T  G  A  S  I  V  S  F  K  V  E  S  D  V  V  S  L  D  G  H  D  F  L  E  T  D  A  E  I  G  N  D  G

AAGCTTCATGTTACCGTGAGGAAATCAAACGCATCGAGACGGTCACTGATGATGACTCCACGGCCTTCGAATCTTACCGGAGCTGAGATCTATAGTCTTA   700
 K  L  H  V  T  V  R  K  S  N  A  S  R  R  S  L  M  M  T  P  R  P  S  N  L  T  G  A  E  I  Y  S  L

GTTCGACTCCGAGAGGTTCTAACTTTAACCATTCTGATTTTTACTCTGTTATGGGGTTTCCCGGCGGGAGGCTTTCGAATTTTGGTCCGGCGGATTTGTA   800
S  S  T  P  R  G  S  N  F  N  H  S  D  F  Y  S  V  M  G  F  P  G  G  R  L  S  N  F  G  P  A  D  L  Y

CTCCGTTCAATCTTCTCGTGGTCCGACTCCACGGCCTTCGAACTTCGAAGAGAACAACGCCGTTAAATATGGATTTTACAATAACACTAACAGTTCTGTT   900
 S  V  Q  S  S  R  G  P  T  P  R  P  S  N  F  E  E  N  N  A  V  K  Y  G  F  Y  N  N  T  N  S  S  V

CCGGCGGCCGGTTCGTACCCGGCTCCGAACCCGGAGTTTTCAACCGGTACGGGTGTTTCAACTAAACCGAATAAAATTCCTAAAGAAAACCAACAGCAAC  1000
 P  A  A  G  S  Y  P  A  P  N  P  E  F  S  T  G  T  G  V  S  T  K  P  N  K  I  P  K  E  N  Q  Q  Q

TGCAAGAGAAAGATAGCAAAGCGAGCCATGACGCTAAGGAGCTTCACATGTTTGTTTGGAGCTCAAGCGCTTCTCCGGTCTCCGACGTGTTTGGCGGAGG  1100
L  Q  E  K  D  S  K  A  S  H  D  A  K  E  L  H  M  F  V  W  S  S  S  A  S  P  V  S  D  V  F  G  G  G

TGCAGGCGACAACGTGGCAACGGAACAATCTGAACAAGGTGCTAAGGAGATTCGGATGGTTGTCTCTGATCAACCTCGAAAGAGTGGTGGTGATGATATC  1200
 A  G  D  N  V  A  T  E  Q  S  E  Q  G  A  K  E  I  R  M  V  V  S  D  Q  P  R  K  S  G  G  D  D  I

GGCGGTCTTGATAGTGGAGAGGGAGAAAGAGAGATAGAGAAAGCTACAGCAGGGCTGAATAAAATGGGGTCTAATTCCACGGCGGAGCTAGAGGCGGCTG  1300
G  G  L  D  S  G  E  G  E  R  E  I  E  K  A  T  A  G  L  N  K  M  G  S  N  S  T  A  E  L  E  A  A

GTGGAGATGGCGGCGGCAACAACGGAACACATATGCCGCCGACAAGTGTGATGACACGACTGATATTGATAATGGTGTGGAGAAAGCTGATCAGAAACCC  1400
G  G  D  G  G  G  N  N  G  T  H  M  P  P  T  S  V  M  T  R  L  I  L  I  M  V  W  R  K  L  I  R  N  P

AAACACGTACTCCAGTCTAATCGGTCTCATATGGGCTCTTGTTGCTTACCGGTGGCATGTGGCTATGCCCAAAATATTACAACAATCCATCTCCATACTC  1500
 N  T  Y  S  S  L  I  G  L  I  W  A  L  V  A  Y  R  W  H  V  A  M  P  K  I  L  Q  Q  S  I  S  I  L

TCAGATGCTGGTCTTGGAATGGCTATGTTCAGCTTAGGTTTATTCATGGCACTTCAACCCAAAATCATTGCTTGTGGGAACTCTGTCGCCACGTTTGCCA  1600
S  D  A  G  L  G  M  A  M  F  S  L  G  L  F  M  A  L  Q  P  K  I  I  A  C  G  N  S  V  A  T  F  A

TGGCGGTCAGATTTATAACCGGTCCGGCCATCATGGCTGTTGCTGGGATTGCCATTGGCTTACACGGCGACCTTCTCCGTATAGCCATCGTTCAGGCTGC  1700
M  A  V  R  F  I  T  G  P  A  I  M  A  V  A  G  I  A  I  G  L  H  G  D  L  L  R  I  A  I  V  Q  A  A

GTTGCCTCAAGGAATAGTTCCGTTTGTGTTTGCAAAAGAGTACAATGTGCATCCCACGATTCTAAGCACTGGGGTCATATTTGGAATGTTAATAGCCTTA  1800
 L  P  Q  G  I  V  P  F  V  F  A  K  E  Y  N  V  H  P  T  I  L  S  T  G  V  I  F  G  M  L  I  A  L

CCTATAACTCTGGTCTACTATATTCTTCTTGGCCTTTGA                1839
P  I  T  L  V  Y  Y  I  L  L  G  L  .
```

Figure 10. The *AtPIN5* gene.
Exon/intron organisation of the *AtPIN5* gene (A) and its translated coding sequence (B) are depicted. Exons are displayed as black squares and introns as white squares with the positions of exon/intron borders indicated.

A

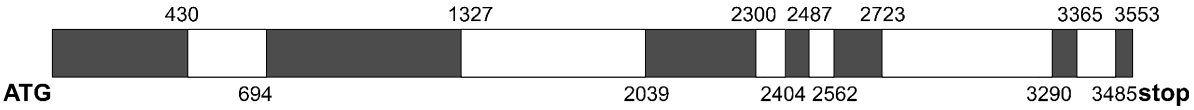


B

```
ATGATAAATTGTGGAGATGTTTACAAGGTAATCGAAGCAATGGTTCCACTCTATGTAGCCTTAATCTTAGGCTATGGCTCTGTGAAATGGTGGCACATCT      100
 M  I  N  C  G  D  V  Y  K  V  I  E  A  M  V  P  L  Y  V  A  L  I  L  G  Y  G  S  V  K  W  W  H  I

TCACGCGTGATCAATGTGATGCTATAAACCGTCTCGTTTGCTATTTCACCCTGCCGCTCTTCACCATTGAGTTCACGGCCCATGTCGACCCGTTCAACAT      200
F  T  R  D  Q  C  D  A  I  N  R  L  V  C  Y  F  T  L  P  L  F  T  I  E  F  T  A  H  V  D  P  F  N  M

GAATTACCGGTTCATCGCCGGCCGATGTCCTCTCTAAGGTCATCATAGTCACGGTCTTAGCGTTGTGGGCCAAATATAGCAACAAAGGAAGCTATTGTTGG      300
 N  Y  R  F  I  A  A  D  V  L  S  K  V  I  I  V  T  V  L  A  L  W  A  K  Y  S  N  K  G  S  Y  C  W

TCCATTACTAGTTTCTCTCTATGCACTCTTACTAACTCTCTTGTCGTTGGTGTGCCATTGGCTAAGGCAATGTACGGACAACAGGCTGTTGATCTGGTGG      400
 S  I  T  S  F  S  L  C  T  L  T  N  S  L  V  V  G  V  P  L  A  K  A  M  Y  G  Q  Q  A  V  D  L  V

TTCAATCCTCGGTATTTCAAGCCATCGTGTGGCTCACACTCTTGTTGTTTGTCTTAGAGTTTAGAAAAGCCGGATTTAGTAGTAATAATATCAGCGACGT      500
V  Q  S  S  V  F  Q  A  I  V  W  L  T  L  L  L  F  V  L  E  F  R  K  A  G  F  S  S  N  N  I  S  D  V

ACAAGTAGACAACATCAATATCGAAAGTGGAAAAAGAGAAACTGTGGTTGTGGGAGAGAAGTCGTTCCTTGAGGTCATGTCCCTCGTTTGGTTGAAGCTT      600
 Q  V  D  N  I  N  I  E  S  G  K  R  E  T  V  V  V  G  E  K  S  F  L  E  V  M  S  L  V  W  L  K  L

GCAACGAATCCAAATTGCTATTCTTGCATCCTTGGGATCGCATGGGCTTTTATTTCTAACAGGTGGCATCTGGAGCTGCCGGGCATATTAGAGGGTTCGA      700
 A  T  N  P  N  C  Y  S  C  I  L  G  I  A  W  A  F  I  S  N  R  W  H  L  E  L  P  G  I  L  E  G  S

TTCTTATAATGTCAAAAGCAGGAACAGGAACGGCCATGTTCAATATGGGAATATTCATGGCACTTCAAGAAAAGTTGATAGTATGTGGAACAAGCTTGAC      800
I  L  I  M  S  K  A  G  T  G  T  A  M  F  N  M  G  I  F  M  A  L  Q  E  K  L  I  V  C  G  T  S  L  T

TGTGATGGGAATGGTATTGAAGTTTATTGCCCGACCGGCGGCCATGGCCATCGGCTCTATTGTCCTTGGCCTCCACGGTGATGTCCTCCGCGTAGCCATC      900
 V  M  G  M  V  L  K  F  I  A  G  P  A  A  M  A  I  G  S  I  V  L  G  L  H  G  D  V  L  R  V  A  I

ATTCAGGCTGCTTTGCCACAATCGATTACTTCGTTCATTTTTGCTAAGGAATATGGATTACATGCAGATGTTCTAAGCACAGCGGTGATATTTGGGATGT     1000
 I  Q  A  A  L  P  Q  S  I  T  S  F  I  F  A  K  E  Y  G  L  H  A  D  V  L  S  T  A  V  I  F  G  M

TGGTCTCTCTACCTGTGCTCGTCGCCTACTACGCAGCTCTGGAGTTTATTCATTGA                                              1056
L  V  S  L  P  V  L  V  A  Y  Y  A  A  L  E  F  I  H  .
```

Figure 11. The *AtPIN6* gene.
Exon/intron organisation of the *AtPIN6* gene (A) and its translated coding sequence (B) are depicted. Exons are displayed as black squares and introns as white squares with the positions of exon/intron borders indicated.

A

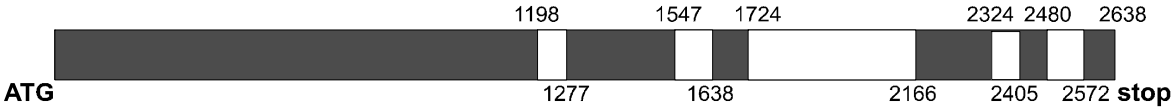


B

```
ATGATAACGGGAAACGAATTCTACACGGTGATGTGTGCCATGGCACCACTCTACTTCGCCATGTTCGTGGCTTATGGATCGGTAAAGTGGTGCAAGATCT   100
 M  I  T  G  N  E  F  Y  T  V  M  C  A  M  A  P  L  Y  F  A  M  F  V  A  Y  G  S  V  K  W  C  K  I

TCACGCCGGCGCAATGTTCCGGAATCAACCGTTTTGTCTCCGTTTTCGCCGTCCCAGTCCTCTCTTTCCACTTCATCTCACAAAACAACCCTTACAAAAT   200
F  T  P  A  Q  C  S  G  I  N  R  F  V  S  V  F  A  V  P  V  L  S  F  H  F  I  S  Q  N  N  P  Y  K  M

GGACACAATGTTCATCCTCGCCGACACTTTGTCCAAAATCTTCGTCTTCGTTTTGCTCTCTCTATGGGCCGTTTTCTTCAAAGCCGGTGGTCTCGATTGG   300
 D  T  M  F  I  L  A  D  T  L  S  K  I  F  V  F  V  L  L  S  L  W  A  V  F  F  K  A  G  G  L  D  W

CTCATCACTCTATTCTCAATCGCTACTCTCCCTAACACTCTCGTTATGGGCATACCTTTGCTTCAGGCCATGTATGGAGATTACACTCAAACCCTCATGG   400
 L  I  T  L  F  S  I  A  T  L  P  N  T  L  V  M  G  I  P  L  L  Q  A  M  Y  G  D  Y  T  Q  T  L  M

TCCAACTCGTTGTTCTTCAATGCATCATTTGGTATACTCTGCTACTATTCCTCTTTGAACTCCGAGCAGCGAGGTTGCTTATCCGAGCAGAGTTTCCGGG   500
V  Q  L  V  V  L  Q  C  I  I  W  Y  T  L  L  L  F  L  F  E  L  R  A  A  R  L  L  I  R  A  E  F  P  G

TCAAGCAGCCGGATCAATCGCCAAGATCCAAGTCGACGATGACGTCATTTCCTTAGACGGCATGGATCCGCTCCGTACAGAAACTGAAACCGATGTTAAC   600
 Q  A  A  G  S  I  A  K  I  Q  V  D  D  D  V  I  S  L  D  G  M  D  P  L  R  T  E  T  E  T  D  V  N

GGTCGGATCAGGCTCAGGATCCGACCGGTCCGTATCATCCGTACCTGATTCTGTTATGTCCTCCTCCTTGTGCTTAACCCCTAGAGCCTCTAATCTTTCTA   700
 G  R  I  R  L  R  I  R  R  S  V  S  S  V  P  D  S  V  M  S  S  S  L  C  L  T  P  R  A  S  N  L  S

ACGCCGAGATTTTCTCCGTCAACACTCCTAATAACCGTTTCTTCCACGGCGGAGGAGGAAGCGGTACCCTTCAGTTTTATAACGGTAGTAACGAGATCAT   800
N  A  E  I  F  S  V  N  T  P  N  N  R  F  F  H  G  G  G  G  S  G  T  L  Q  F  Y  N  G  S  N  E  I  M

GTTTTGTAACGGAGATTTAGGCGGGTTCGGATTTACTCGACCCGGATTAGGTGCAAGTCCTAGAAGGCTCTCGGGTTATGCTTCCTCCGATGCTTACTCG   900
 F  C  N  G  D  L  G  G  F  G  F  T  R  P  G  L  G  A  S  P  R  R  L  S  G  Y  A  S  S  D  A  Y  S

TTGCAGCCAACACCACGTGCCTCGAACTTTAACGAGCTTGACGTTAACGGAAACGGTACGCCGGTTTGGATGAAATCTCCTGCCGCCGGGAGAATATACC  1000
L  Q  P  T  P  R  A  S  N  F  N  E  L  D  V  N  G  N  G  T  P  V  W  M  K  S  P  A  A  G  R  I  Y

GGCAATCTTCGCCGAAGATGATGTGGGAGTCTGGGCAAAGACATGCAGCCAAAGATATCAATGGTACGTTTTCTTTGAAAGATGTGTCGTTTTCGTTTTT  1100
R  Q  S  S  P  K  M  M  W  E  S  G  Q  R  H  A  A  K  D  I  N  G  T  F  S  L  K  D  V  S  F  S  F  F

CGTTAGCACTGACACGACGGCGTTTAGTCAATTTTCTTCTCTTTTCGTGTTTTTCTTTATCATTGGATCAGTGCCAGAGAAGGAGATTAGCTTCAGAGAC  1200
 V  S  T  D  T  T  A  F  S  Q  F  S  S  L  F  V  F  F  F  I  I  G  S  V  P  E  K  E  I  S  F  R  D

GCACTTAAGGCTGCACCACAGGCTACGGCAGCCGGTGGTGGTGCTTCCATGGAGGAAGGAGCCGCCGGAAAAGACACAACTCCGGTGGCTGCGATTGGCA  1300
A  L  K  A  A  P  Q  A  T  A  A  G  G  G  A  S  M  E  E  G  A  A  G  K  D  T  T  P  V  A  A  I  G

AGCAAGAAATGCCAAGTGCAATTGTAATGATGCGCCTCATACTAACAGTCGTGGGCCGCAAGCTTTCTCGAAACCCTAACACTTATTCCAGCCTCTTAGG  1400
K  Q  E  M  P  S  A  I  V  M  M  R  L  I  L  T  V  V  G  R  K  L  S  R  N  P  N  T  Y  S  S  L  L  G

TCTTGTCTGGTCACTTATATCCTTCAAGTGGAATATACCGATGCCAAATATAGTGGATTTCTCGATAAAGATCATTTCAGATGCAGGTCTTGGGATGGCT  1500
 L  V  W  S  L  I  S  F  K  W  N  I  P  M  P  N  I  V  D  F  S  I  K  I  I  S  D  A  G  L  G  M  A

ATGTTTAGTTTAGGCCTGTTTATGGCGCTGCAGCCAAAGATGATCCCTTGTGGGGCGAAAAAGGCAACAATGGGGATGTTGATCAGGTTCATCTCAGGTC  1600
M  F  S  L  G  L  F  M  A  L  Q  P  K  M  I  P  C  G  A  K  K  A  T  M  G  M  L  I  R  F  I  S  G

CTCTTTTCATGGCTGGTGCTTCCCTTCTTGTCGGATTAAGAGGTTCTAGGTTACATGCTGCTATCGTACAGGCTGCTCTACCGCAAGGGATAGTACCATT  1700
P  L  F  M  A  G  A  S  L  L  V  G  L  R  G  S  R  L  H  A  A  I  V  Q  A  A  L  P  Q  G  I  V  P  F

TGTGTTTGCGAGGGAGTACAATTTGCATCCAGACCTACTCAGTACATTGGTTATCTTTGGGATGATAGTATCTTTACCAGTAACTATTTTGTACTACGTC  1800
 V  F  A  R  E  Y  N  L  H  P  D  L  L  S  T  L  V  I  F  G  M  I  V  S  L  P  V  T  I  L  Y  Y  V

CTCTTGGGCCTATGA          1815
L  L  G  L  .
```

Figure 12. The *AtPIN7* gene.
Exon/intron organisation of the *AtPIN7* gene (A) and its translated coding sequence (B) are depicted. Exons are displayed as black squares and introns as white squares with the positions of exon/intron borders indicated.

A



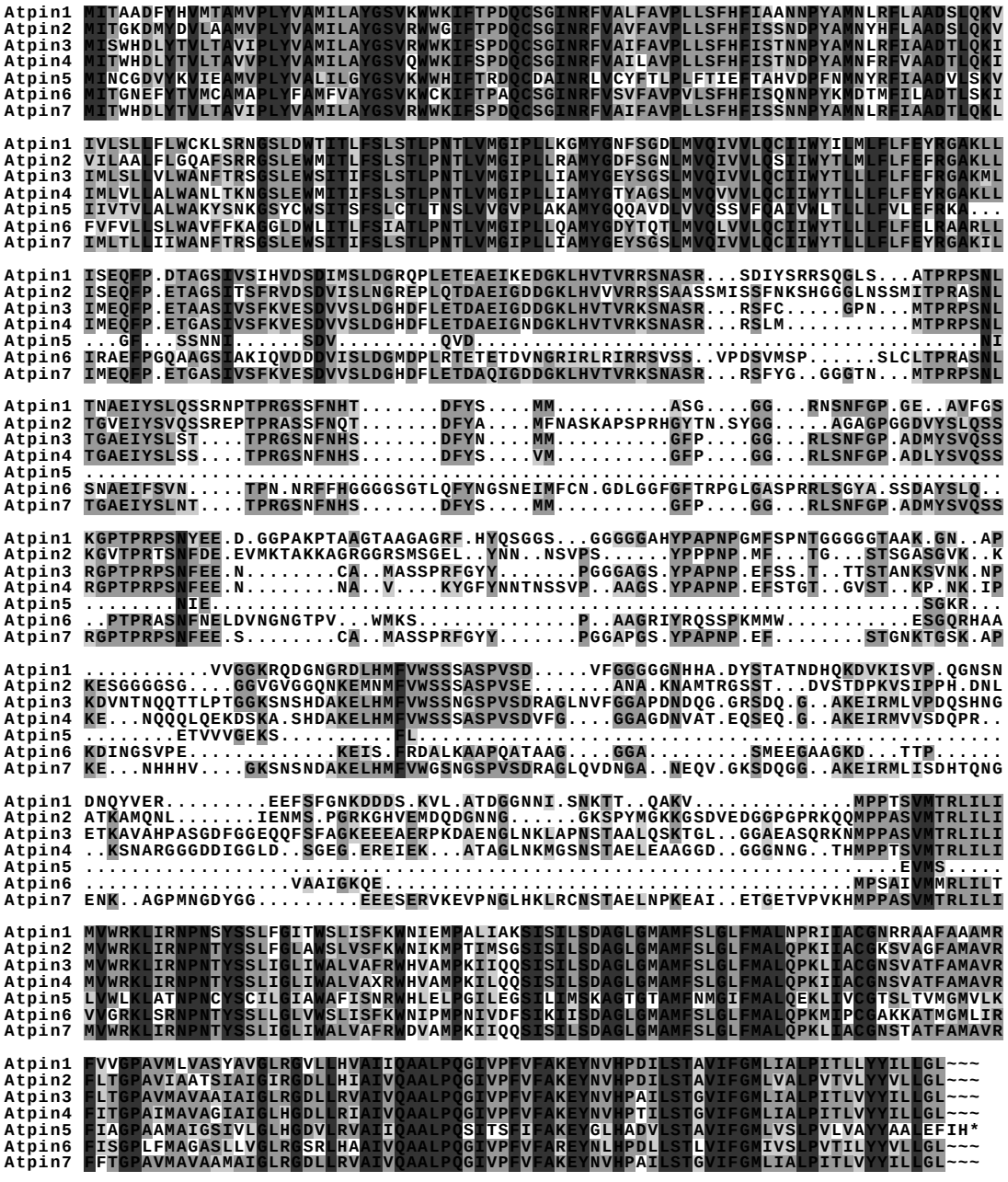
B

```
ATGATCACATGGCACGACCTCTACACCGTCCTCACGGCGGTGATACCACTCTACGTAGCCATGATCCTCGCTTACGGCTCAGTCCGGTGGTGGAAAATCT                100
 M  I  T  W  H  D  L  Y  T  V  L  T  A  V  I  P  L  Y  V  A  M  I  L  A  Y  G  S  V  R  W  W  K  I

TCTCACCAGACCAATGTTCCGGCATAAACCGCTTCGTCGCTATTTTCGCCGTCCCTCTTCTCTCTTTCCACTTCATCTCCTCAAACAATCCTTACGCAAT          200
F  S  P  D  Q  C  S  G  I  N  R  F  V  A  I  F  A  V  P  L  L  S  F  H  F  I  S  S  N  N  P  Y  A  M

GAATCTCCGATTCATCGCAGCCGATACACTCCAAAAACTAATCATGCTCACTCTCCTAATCATCTGGGCTAACTTCACTCGCTCCGGTAGTCTTGAATGG           300
 N  L  R  F  I  A  A  D  T  L  Q  K  L  I  M  L  T  L  L  I  I  W  A  N  F  T  R  S  G  S  L  E  W

AGCATCACAATCTTCTCTCTTTCAACACTTCCCAACACTCTCGTTATGGGAATACCTCTCTTGATCGCCATGTACGGAGAATATTCCGGCTCACTCATGG           400
 S  I  T  I  F  S  L  S  T  L  P  N  T  L  V  M  G  I  P  L  L  I  A  M  Y  G  E  Y  S  G  S  L  M

TTCAGATCGTTGTTCTTCAGTGTATAATCTGGTACACGCTTCTTCTCTTTCTTTTCGAATACAGAGGAGCTAAGATCTTGATCATGGAACAGTTTCCAGA           500
V  Q  I  V  V  L  Q  C  I  I  W  Y  T  L  L  L  F  L  F  E  Y  R  G  A  K  I  L  I  M  E  Q  F  P  E

GACTGGTGCTTCGATTGTATCGTTCAAAGTTGAGTCCGATGTTGTTTCTTTAGATGGACATGATTTTCTTGAAACTGATGCTCAAATCGGTGACGATGGT           600
 T  G  A  S  I  V  S  F  K  V  E  S  D  V  V  S  L  D  G  H  D  F  L  E  T  D  A  Q  I  G  D  D  G

AAGCTTCATGTTACGGTGAGAAAATCAAACGCTTCTCGGAGATCTTTTTACGGTGGTGGTGGTACTAATATGACTCCTCGTCCGTCTAATCTCACCGGAG           700
 K  L  H  V  T  V  R  K  S  N  A  S  R  R  S  F  Y  G  G  G  G  T  N  M  T  P  R  P  S  N  L  T  G

CTGAGATTTATAGTCTTAACACTACTCCAAGAGGATCTAACTTCAATCATTCTGATTTTTACTCTATGATGGGGTTTCCCGGTGGCCGGCTTTCGAACTT           800
A  E  I  Y  S  L  N  T  T  P  R  G  S  N  F  N  H  S  D  F  Y  S  M  M  G  F  P  G  G  R  L  S  N  F

TGGTCCGGCGGATATGTACTCTGTTCAGTCCTCGAGAGGACCTACTCCTAGACCTTCGAATTTTGAGGAGAGTTGTGCTATGGCTTCTTCGCCAAGATTC           900
 G  P  A  D  M  Y  S  V  Q  S  S  R  G  P  T  P  R  P  S  N  F  E  E  S  C  A  M  A  S  S  P  R  F

GGGTATTACCCGGGAGGAGCTCCCGGGTCGTACCCAGCTCCTAATCCGGAGTTTTCTACCGGTAATAAAACCGGTAGTAAAGCTCCAAAAGAGAATCATC          1000
 G  Y  Y  P  G  G  A  P  G  S  Y  P  A  P  N  P  E  F  S  T  G  N  K  T  G  S  K  A  P  K  E  N  H

ATCATGTAGGAAAATCAAATAGTAATGATGCTAAGGAGCTTCATATGTTTGTTTGGGGATCCAACCGGATCACCCGTTTCCGGACCGAGCTGGTCTTCAAGT          1100
H  H  V  G  K  S  N  S  N  D  A  K  E  L  H  M  F  V  W  G  S  N  G  S  P  V  S  D  R  A  G  L  Q  V

TGATAATGGAGCCAATGAACAAGTCGGAAAATCCGATCAAGGCGGTGCAAAAGAGATTCGAATGTTGATCTCTGATCATACTCAAAATGCTGGTCCGATG          1200
 D  N  G  A  N  E  Q  V  G  K  S  D  Q  G  G  A  K  E  I  R  M  L  I  S  D  H  T  Q  N  A  G  P  M

AACGGGGACTATGGCGGGGAAGAAGAGTCGGAGAGGGTAAAGGAAGTGCCTAACGGACTACACAAGCTTCGGTGTAACTCCACAGCAGAGCTAAACCCTA          1300
N  G  D  Y  G  G  E  E  E  S  E  R  V  K  E  V  P  N  G  L  H  K  L  R  C  N  S  T  A  E  L  N  P

AAGAAGCTATAGAAACGGGTGAAACTGTACCGGTAAAACATATGCCACCAGCGAGTGTGATGACTCGGCTGATATTGATAATGGTGTGGAGGAAACTCAT          1400
K  E  A  I  E  T  G  E  T  V  P  V  K  H  M  P  P  A  S  V  M  T  R  L  I  L  I  M  V  W  R  K  L  I

AAGAAACCCAAACACTTACTCTAGTCTCATTGGTCTCATTTGGGCTCTTGTTGCTTTCAGGTGGGATGTGGCAATGCCTAAAATTATTCAACAATCAATC          1500
 R  N  P  N  T  Y  S  S  L  I  G  L  I  W  A  L  V  A  F  R  W  D  V  A  M  P  K  I  I  Q  Q  S  I

TCAATTCTTTCTGATGCTGGTCTTGGTATGGCAATGTTCAGTTTGGGGTTGTTCATGGCATTGCAACCGAAATTAATTGCTTGCGGAAATTCGACGGCGA          1600
S  I  L  S  D  A  G  L  G  M  A  M  F  S  L  G  L  F  M  A  L  Q  P  K  L  I  A  C  G  N  S  T  A

CTTTTGCCATGGCGGTGAGATTCTTTACTGGACCAGCGGTAATGGCCGTGGCAGCAATGGCTATTGGATTACGTGGAGACCTATTGCCGTGTGGCCATTGT          1700
T  F  A  M  A  V  R  F  F  T  G  P  A  V  M  A  V  A  A  M  A  I  G  L  R  G  D  L  L  R  V  A  I  V

TCAAGCTGCATTGCCTCAAGGGATCGTGCCGTTTGTGTTTGCAAAAGAGTATAATGTTCATCCCGCAATCTTGAGTACAGGGGTAATATTTGGAATGCTT          1800
 Q  A  A  L  P  Q  G  I  V  P  F  V  F  A  K  E  Y  N  V  H  P  A  I  L  S  T  G  V  I  F  G  M  L

ATCGCACTACCGATTACACTTGTTTACTACATTTTACTCGGGCTATAA                                                      1848
I  A  L  P  I  T  L  V  Y  Y  I  L  L  G  L  .
```

3.4 **Analysis of AtPIN1-AtPIN7 protein sequences**

The cDNA sequences of *AtPIN* genes were used to predict the amino acid sequences, topology as well as other properties of the corresponding AtPIN proteins. Except for the AtPIN5 protein, all predicted protein sequences are approximately 600 amino acid long with molecular masses around 70 kDa. The predicted AtPIN5 protein is 352 amino acids long with a molecular mass of 55 kDa.

Figure 13. Comparison of predicted AtPIN protein sequences.



All AtPIN proteins share significant homology. Levels of identity and similarity are summarised in Table 6. They share around 60% identity. The AtPIN3, AtPIN4 and AtPIN7 display higher homology among themselves than the rest of the family (around 85% identity, Table 6). In Figure 13, where alignment of all AtPIN proteins is depicted, three domains are distinguishable: highly conserved N- and C- termini (80% identity) and a less conserved middle part (55% identity). In AtPIN5, the middle less conserved part is missing. A direct sequence comparison of AtPIN1 to AtPIN7 is depicted in Figure 13.

Table 6. Homology of AtPIN proteins.
In the upper half percentage of identity between different AtPIN proteins and in the lower part percentage of similarity are presented. More homologous members are marked.

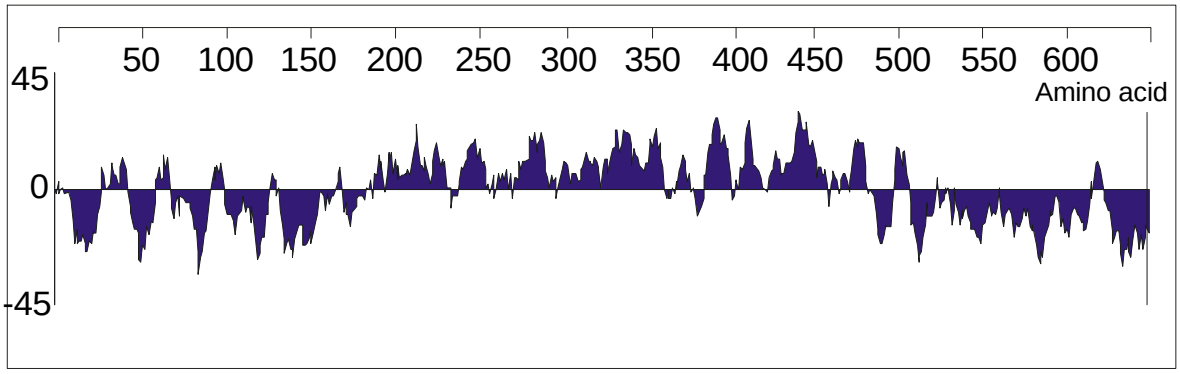
	AtPIN1	AtPIN2	**AtPIN3**	**AtPIN4**	AtPIN5	AtPIN6	**AtPIN7**
AtPIN1	x	64.9	66.7	65.4	56.1	52.6	65.1
AtPIN2	70.9	x	63.3	63.7	54.5	53.1	63.0
AtPIN3	73.7	69.4	x	**82.3**	53.9	53.5	**88.5**
AtPIN4	72.4	69.2	**85.6**	x	54.5	50.9	**80.7**
AtPIN5	61.2	68.6	68.0	68.6	x	53.2	52.6
AtPIN6	59.0	60.6	62.1	58.2	65.4	x	53.2
AtPIN7	71.7	69.1	**90.4**	**84.5**	66.0	62.3	x

%identity

%similarity

To chart the hydrophilic properties of AtPIN proteins, the computer software (Protean, *Lasergene*) was used to calculate the hydrophilic and hydrophobic plot (Kyte and Doolittle, 1982). The hydrophilic plot for the AtPIN3 protein is depicted in Figure 14 as an example to demonstrate some of the general features of AtPIN proteins. Hydrophobic amino acids are placed predominantly at the N- and C- termini of AtPIN proteins, the middle part consists mainly of hydrophilic amino acids.

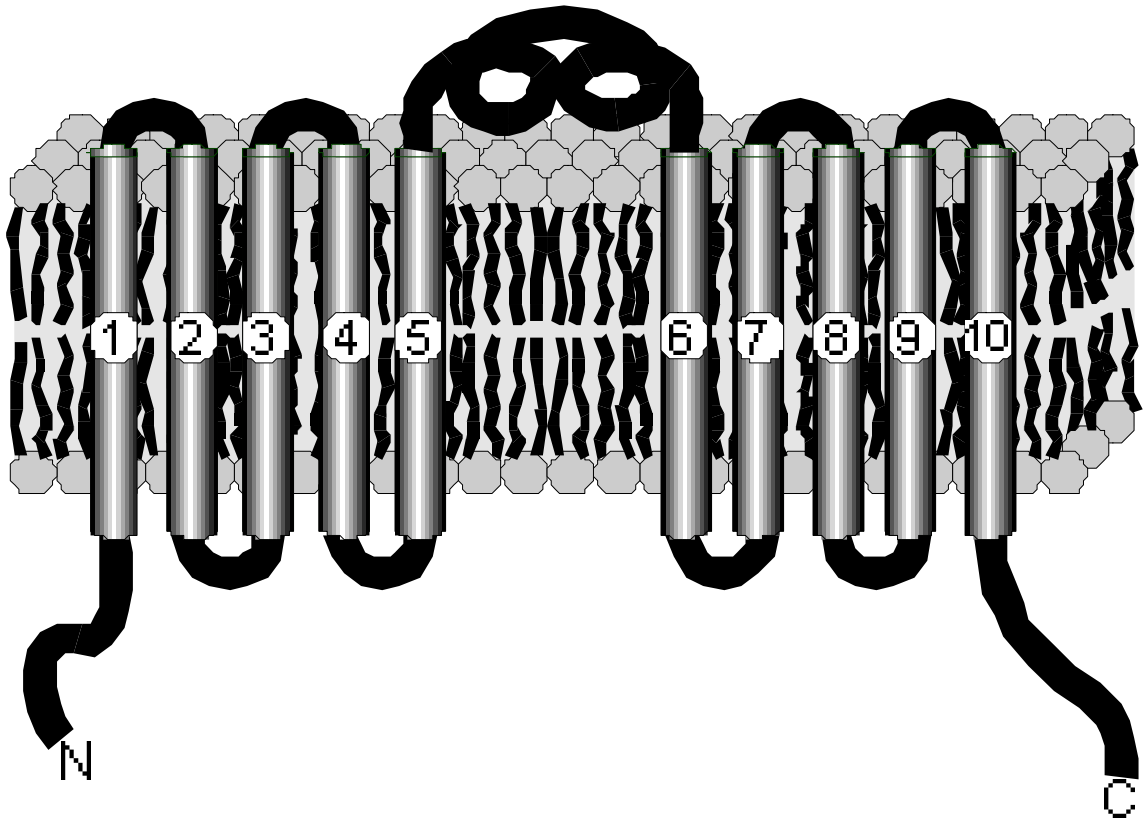
Figure 14. Hydrophilic plot of AtPIN3 protein.



The amino acid sequence of AtPIN proteins was used to deduce the topology of the proteins using protein prediction software: e.g. Psort (Nakai and Kanehisa, 1992, http://psort.nibb.ac.jp), PredictProtein (PHDtopology, Rost *et al.*, 1996, http://www.embl-Heidelberg.de/predictprotein/ predictprotein.html), Tmpred (Hofmann and Stoffel, 1993, http://ulrec3.unil.ch/software/ TMPRED_form.html) and Peptidestructure (GCG-Programm-Packet, Devereux *et al.*, 1984). The prediction software deduced from *AtPIN* sequences that except of AtPIN5 all other AtPIN proteins are transmembrane proteins with 8-10 transmembrane domains and large hydrophilic loops in the middle. The highly hydrophobic, putative transmembrane domains at the N- and C-terminal protein termini roughly correspond to conserved regions as the comparison of predicted AtPIN protein sequences in Figure 13 demonstrates. The middle, hydrophilic loop is less conserved.

The orientation of AtPIN proteins in the membrane and the spatial orientation of transmembrane domains could not be predicted with sufficient confidence and thus requires additional biochemical studies. The possible model for the AtPIN protein topology is depicted in Figure 15.

Figure 15. Model of putative topology of AtPIN proteins.

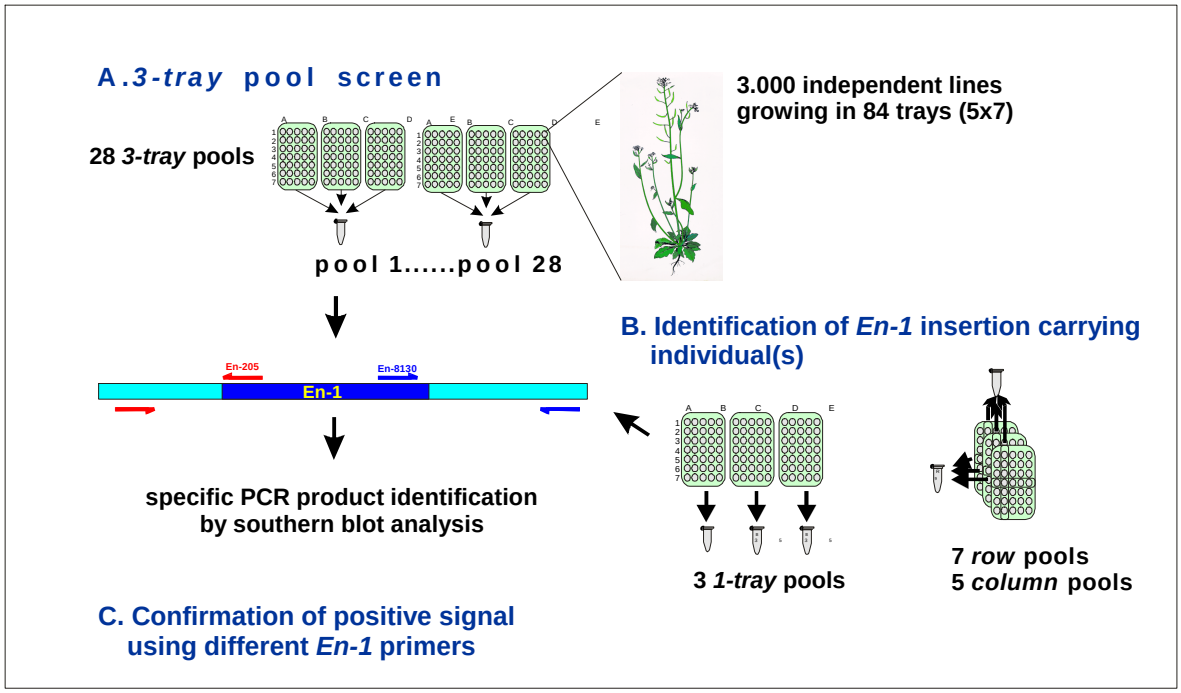


3.5 **Isolation of insertion mutants for *AtPIN*3 – *AtPIN*7 genes.**

Mutants are powerful tools to study the function of genes and proteins. Large collections of mutant plants have been generated by insertion of tags such as T-DNA or transposons. These plants are contributing significantly to our understanding of the function of diverse plant genes. These mutant libraries can be used in two different ways: (*i*) In forward screens, mutant plants with interesting phenotypes are selected and used further for identification of the disrupted genes. (*ii*) In reverse genetic screens, the sequence of a known gene is used for identification of mutant lines, carrying a mutation in the gene of interest.

For the functional analysis of members of the *AtPIN* gene family, the second reverse genetic strategy was adopted in order to isolate mutants in the *AtPIN3 – AtPIN7* genes. For this purpose the collection of *Arabidopsis* plants mutagenised by the maize transposon *En-1* was screened (Wisman *et al.*, 1998). This collection contains 8.000 independent lines with about 48.000 different transposon insertions. A PCR and hybridisation based screening procedure was used to isolate the mutant lines carrying *En-1* insertions within the *AtPIN3 – AtPIN7* genes (Baumann *et al.*, 1998). The schematic description of the screening strategy is depicted in Figure 16.

Figure 16. PCR based strategy of *En-1* mutant library screening.
In the first round, 28 *3-tray* pools were screened (A), in the second round the individual line was identified (B) and the positive results were confirmed (C).



3.5.1 *AtPIN3 – AtPIN7* gene specific oligonucleotides

For the PCR based reverse genetic screen of the *En-1* mutagenised *Arabidopsis* plant collection, *AtPIN* gene specific oligonucleotides (primers) compatible to *En-1* specific oligonucleotides were generated. Two pairs of specific primers for *AtPIN3*, *AtPIN4*, *AtPIN6* and *AtPIN7* genes were designed on the basis of known genomic DNA sequences of these genes. For the shorter *AtPIN5* gene, one pair of specific primers was sufficient for analysis. All *AtPIN* gene specific oligonucleotides, which were later used in PCR based screens of the *En-1* mutagenised *Arabidopsis* plant collection are listed in Table 7.

Table 7. *AtPIN3-AtPIN7* specific primers designed for screening the *En-1* mutant library (f - forward primer, r-reverse primer).

Gene	primer	Sequence (5'-3')
AtPIN3	p3f1	TCCTCTCACTTCTTCTTCTTCCTC
	p3f2	TGAGACGGCTGCTTCCATTGTTTC
	p3r1	TTTATTTATCTTTTCTTTGTCTCG
	p3r2	CTCTTTCTCGGGGCTTTCATAACC
AtPIN4	p4f1	CTCAAATGCCACTGTCTTCTCACT
	p4f2	TTTTTACTCTGTTATGGGGTTTCC
	p4r1	ATCTTCTTCTTCACCTTCCACTCT
	p4r2	TCTCTTTCTCCCTCTCCACTATCA
AtPIN5	p5f1	TTTCCTTTCGTTTGCTACTACTGC
	p5r1	TTATGACCTGAATGATGGCTACGC
AtPIN6	p6f1	AACTTAACCTACATAGTACATAC
	p6f2	TCCGTCAACACTCCTAATAAC
	p6r1	CAGTGTGTTGTGTGAATCATA
	p6r2	GAAGCTAATCTCCTTCTCTGG
AtPIN7	p7f1	CCTAACGGTTTCCACACTCA
	p7f2	ATTTTGAGGAGAGTTGTGCTAT
	p7r1	ACTTCTCCATTGAAGAGCTTA
	p7r2	TAGCTTCTTTAGGGTTTAGCTC

Prior to the mutant collection screening, the quality and specificity of both gene specific and *En-1* transposon specific primers were tested by PCR reactions in all combinations of forward and reverse primers and also in combinations with *En-1* transposon specific primers. As a template for all PCR reactions, wild type genomic DNA was used. In all

cases, specific products of correct size were amplified and no unspecific PCR products were detected (data not shown). From these test experiments it was concluded that all primers were suitable for screening of *En-1* mutagenised *Arabidopsis* plant collection.

3.5.2 Identification of *AtPIN3 – AtPIN7* insertion mutant lines

The three-step PCR based screening strategy, depicted in Figure 16, allowed the identification of mutant lines with *En-1* insertions in *AtPIN3 – AtPIN7* genes:

(1) In the first step, 28 different *3-tray* pools were tested with two *AtPIN* gene specific primers in tandem with two *En-1* specific primers. In total, 112 PCR reactions for each screened gene were performed. The PCR products were separated by agarose gel electrophoresis, blotted and hybridised with gene specific probes. Several positive pools for *AtPIN*3, *AtPIN*4, *AtPIN*5, *AtPIN*6 and *AtPIN*7 genes were identified, as presented in Table 8.

(2) In the second round of the screen, the single Tray-, Row- and Column-Pools from previously identified *3-tray* pools were tested in order to identify the single plants carrying the mutation of interest. In total 15 PCR reactions for each positive *3-tray* pool were performed and the specificity of the reactions was again confirmed by Southern blot and hybridisation with gene specific probes. Several independent mutant lines with insertions in *AtPIN*3, *AtPIN*4, *AtPIN*5, *AtPIN*6 and *AtPIN*7 genes were identified. A summary of the data is listed in Table 8.

(3) In the third round, all identified mutant lines were rescreened with another pair of gene specific and *En-1* specific primers to confirm the insertions in *AtPIN3 – AtPIN7*.

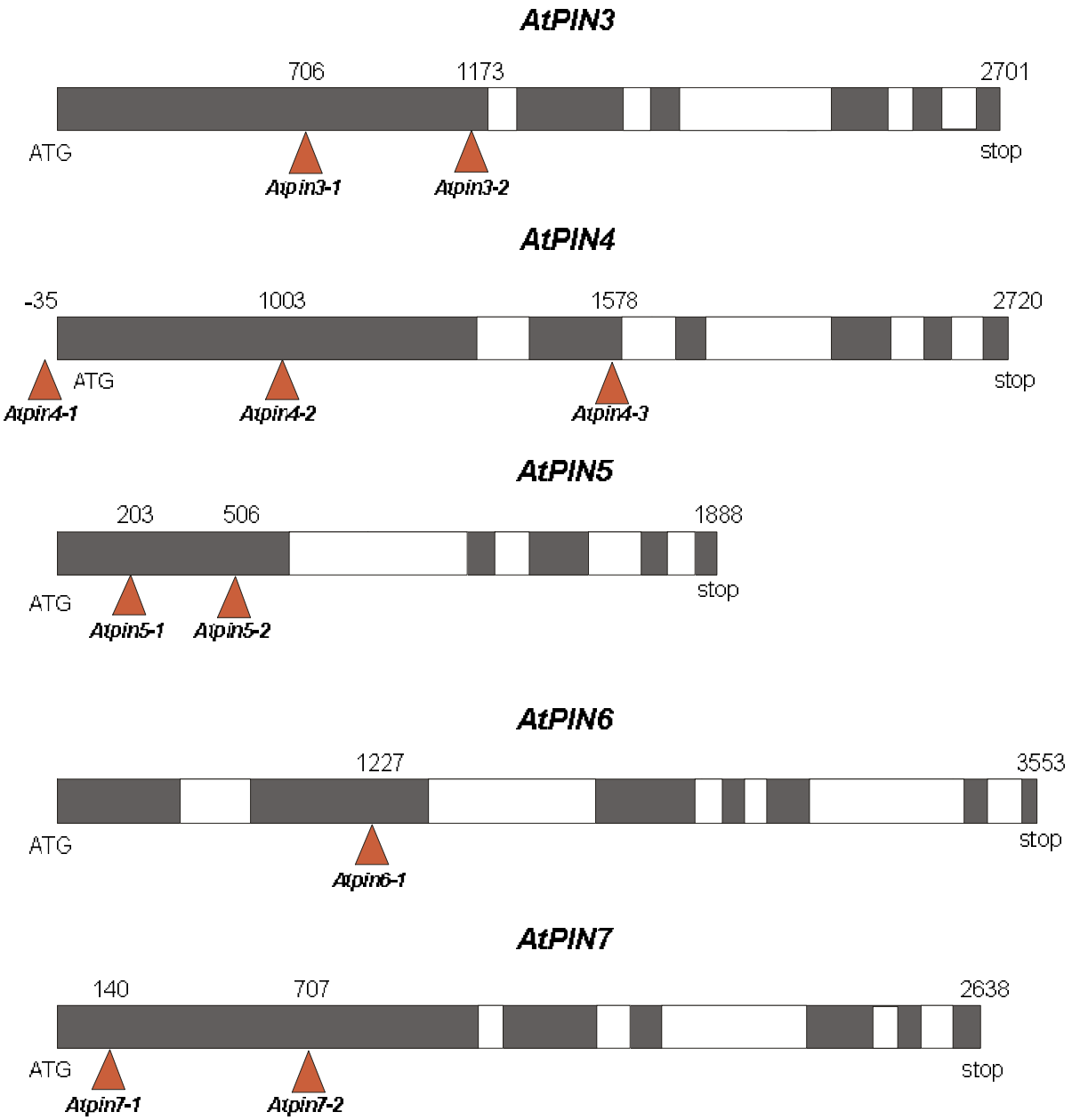
3.5.3 Localisation of *En-1* insertions within *AtPIN3 – AtPIN7* genes

To specify the precise position of inserted *En-1* transposons within the *AtPIN* genes, the flanking regions were amplified by PCR and sequenced. The comparison of flanking region sequences with gene sequences and *En-1* transposon sequence revealed the precise position of *En-1* transposon within the corresponding gene. The positions of *En-1* transposon are depicted in Figure 17. For the lines in which the open reading frame was disrupted by the mutation (*En-1* insertion in exon), homozygous lines within progeny were identified by Southern blot analysis. The identified homozygous plants were further used for phenotypic analysis.

Table 8. Mutant alleles identified for *AtPIN1-AtPIN7* genes in *En-1* mutant library.

Gene	3-Tray-Pool	Single line	*En* position	Allele
AtPIN3	T65-67	T65F1	Exon 1	*Atpin3-1*
	T6-8	T6I5	Exon 1	*Atpin3-2*
	T34-36			
	T48-49			
	T74-76			
	T24-26			
	T 96-98			
AtPIN4	T34-36	T36E3	Intron	
	T107-110	T108F6	Intron	
	T119-122	T122C4	Exon 3	*Atpin4-3*
	T99-101	T100D5	Intron	
	T12-14	T13E2	Exon 1	*Atpin4-2*
	T27-28	T28G1	Promotor	*Atpin4-1*
AtPIN5	T91-94	T91I4	Exon 1	*Atpin5-1*
	T123-126	T125D6	Exon 1	*Atpin5-2*
	T19-21	T21I6	Exon 1	*Atpin5-3*
	T143-146	T144A3	Exon 1	*Atpin5-4*
	T7-9	T8E1	Intron	
AtPIN6	T111-114	T113E3	Intron	
	T46-48			
	T16-18			
	T49-51	T50B4	Exon 1	*Atpin6-1*
	T95-98			
AtPIN7	T79-81			
	T37-39	T38B1	Intron	
	T115-118	T118D5	Promoter	*Atpin7-1*
	T13-15			
	T19-21			
	T73-76			
	T95-98			
	T59-61	T60D3	Exon 1	*Atpin7-2*
	T87-89	T88F6	Exon 1	*Atpin7-3*

Figure 17. Positions of *En-1* transposons in different *AtPIN* mutant alleles.
The positions of *En-1* transposons are depicted by triangles with precise positions marked. The exons are displayed in black and introns in white.



3.6 **Analysis of the *AtPIN3* gene**

The *AtPIN3* gene was first chosen for functional analysis. This included detailed expression studies, an analysis of the AtPIN3 protein localisation and characterisation of the *Atpin3* mutant phenotype.

3.6.1 Tools for analysis of *AtPIN3* expression and function

The isolation of the *AtPIN3* full length cDNA and mutant lines carrying *En-1* transposon insertions within the *AtPIN3* coding sequence were the basis for a detailed characterisation of the *AtPIN3* gene. It was planned to study *AtPIN3* expression by Northern blot analysis and *in situ* hybridisation, to analyse the localisation of the AtPIN3 protein within *Arabidopsis* tissues and cells using immunolocalisation techniques, and finally to analyse genetically the role of *AtPIN3* in *Arabidopsis* development using the *Atpin3* mutant.

To perform these analyses the following tools were needed:

(1) *AtPIN3* mRNA specific probe for Northern blot and *in situ* hybridisation,
(2) AtPIN3 protein specific antibodies for immunolocalisation,
(3) genetically characterised homozygous *Atpin3* mutant lines.

In the next sections the development of these tools will be described.

3.6.1.1 Isolation and characterisation of homozygous *Atpin3* mutant lines

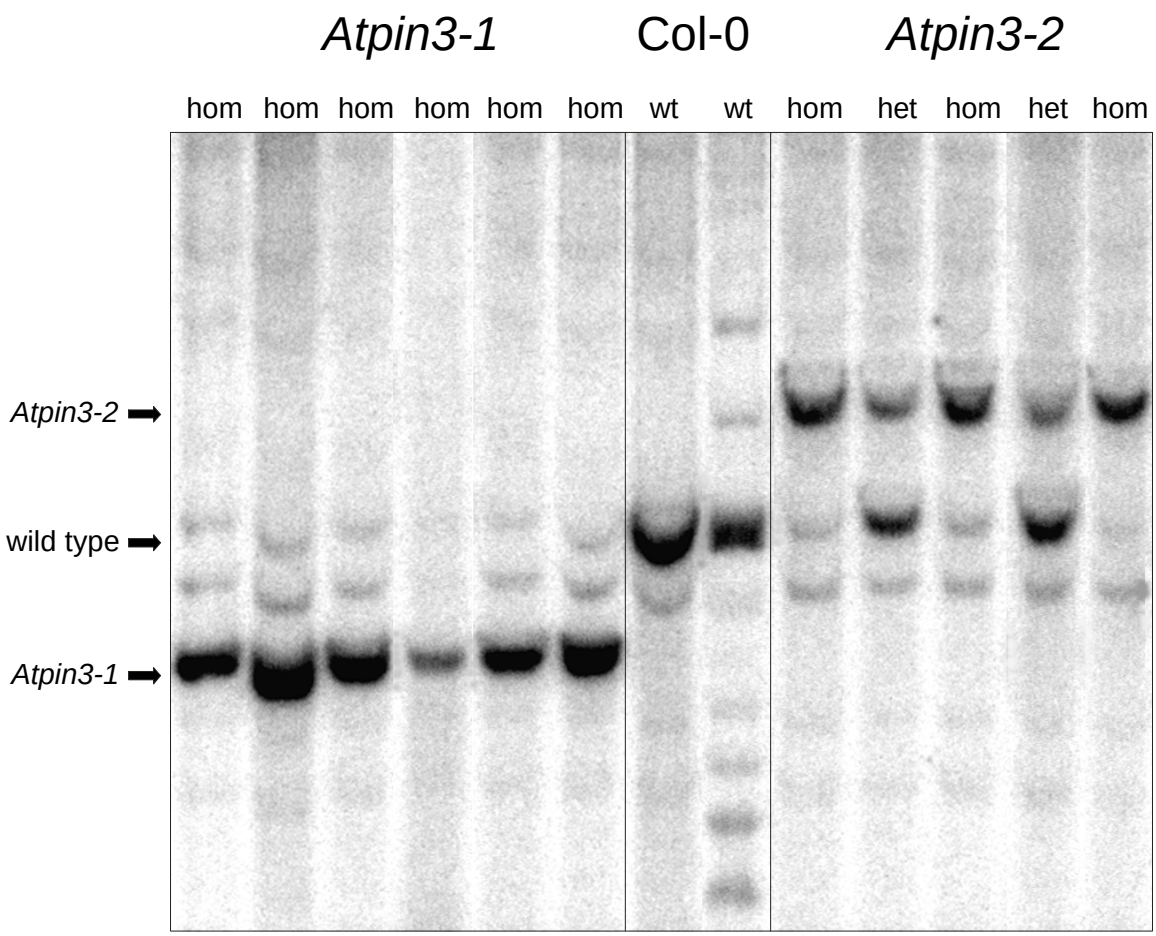
Screening of the *En-1* transposon mutagenised *Arabidopsis* collection revealed two independent heterozygous lines with insertions in the *AtPIN3* gene. The mutations were designated as *Atpin3-1* and *Atpin3-2* (Table 8, Figure 17). In order to be able to perform a phenotypic analysis of mutant plants, it was necessary to obtain homozygous plants, since the majority of knock-out mutations represent recessive mutations.

3.6.1.1.1 Isolation of homozygous *Atpin3* mutants

For identification of homozygous *Atpin3* mutants Southern blot analysis was used, since it allowed to distinguish the transposon tagged mutant allele from the wild type allele. Digestion of genomic DNA with appropriate enzymes resulted in restriction fragments of different lengths for tagged and wild type alleles. Therefore, *Atpin3-1* and *Atpin3-2*

progeny from previously isolated heterozygous plants were used as starting material to screen for homozygous plants. Genomic DNA was isolated from these plants and digested with the restriction enzyme Hind III. After electrophoretic separation, the DNA was blotted onto nylon membrane and hybridised with the *AtPIN3* specific probe. The predicted sizes of the diagnostic *AtPIN3* fragments were 2000 bp for the wild type allele, 1400 bp for the *Atpin3-1* mutant allele and 2800 bp for the *Atpin3-2* allele. As shown in Figure 18 several homozygous mutant plants were identified for both *Atpin3-1* and *Atpin3-2* alleles.

Figure 18. Isolation of *Atpin3-1* and *Atpin3-2* homozygous lines by Southern blot analysis.
Genomic DNA of Col-0 and different *Atpin3-1*, *Atpin3-2* progeny plants was digested with Hind III, separated by gel electrophoresis, and hybridised after Southern blotting to an *AtPIN3* specific probe corresponding to nucleotides 999 to 1449. Hybridisation signals specific for wild type, *Atpin3-1* and *Atpin3-2* (indicated by arrows) were distinguished and individual wild type (wt), heterozygous (het) and homozygous (hom) plants identified.



3.6.1.1.2 Northern blot analysis of *Atpin3* mutants

To verify whether *Atpin3* mutants were null alleles, total RNA was isolated from wild type, heterozygous and homozygous *Atpin3* seedlings, blotted onto nylon membrane and hybridised to an *AtPIN3* specific probe. The results of the Northern blot analysis are depicted in Figure 24 C. No *AtPIN3* specific mRNA could be identified in *Atpin3-1* and *Atpin3-2* homozygous plants. Therefore it was concluded that both *Atpin3-1* and *Atpin3-2* mutant alleles represent null mutations.

3.6.1.1.3 Identification of an *Atpin3-3* foot print mutation

A general feature of the *En-1* transposon is its mobility within the host genome. The *En-1* transposon excises with variable, probably condition dependent, frequency and inserts into new, often nearby positions. Occasionally, the excision of the *En-1* transposon causes a foot print mutation within the targeted gene by deletion or insertion of new bases during the excision process (Cardon *et al*., 1993; Wisman *et al*., 1998). This property of the *En-1* transposon was used to isolate a stable *Atpin3* mutant allele.

The progeny of homozygous *Atpin3-1* mutant plant was screened by Southern blot analysis. A single plant with *En-1* reversion but without restoration of wild type phenotype was identified. The *AtPIN3* gene in this plant was sequenced. A deletion of two base pairs was identified at the position of the previous *En-1* insertion (Figure 19, A). The deletion caused a shift in the open reading frame resulting in a stop codon (Figure 19, B). The foot print mutation was designated *Atpin3-3*. No AtPIN3 protein could be identified in *Atpin3-3* homozygous seedlings suggesting that the truncated protein was degraded (Chapter 3.6.3.2).

3.6.1.1.4 Identification of *Atpin3* mutant reversion

Besides causing foot print mutations the excision of the *En-1* transposon can also result in reversion of the mutant phenotype to the wild type phenotype. In such cases the *En-1* transposon excises and restores the original open reading frame (Cardon *et al*., 1993; Wisman *et al*., 1998). This provides evidence for a direct link between the mutation in a specific gene and the observed mutant phenotype.

To isolate reverted mutant alleles, the progeny of homozygous *Atpin3-2* mutant plants was examined with regard to hypocotyl gravitropism, which in *Atpin3* mutant plants was found to be severely impaired (see Chapter 3.6.4.3.4). Two seedlings displaying wild type hypocotyl gravitropic reaction were identified among 120 *Atpin3-2* mutant seedlings. These plants were analysed on the RNA and protein level. Both *AtPIN3* mRNA and AtPIN3 protein were identified in the putative revertants. The sequencing of *AtPIN3* locus in both plants confirmed the excision of *En-1* transposon and restoration of the wild type *AtPIN3* coding sequence.

Figure 19. The *Atpin3-3* foot print mutation.
The foot print mutation in *AtPIN3* coding sequence (A). Comparison of wild type and truncated AtPIN3 protein (B).

A

```
Atpin3-3  1055 TCACCCGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGA 1104
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type 1055 TCACCCGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGA 1104

Atpin3-3  1105 CAACGATCAAGGCGGAAGATCTGA..AAGGTGCTAAAGAGATCCGTATGT 1152
               |||||||||||||||||||||||  ||||||||||||||||||||||||
wild type 1105 CAACGATCAAGGCGGAAGATCTGAccAAGGTGCTAAAGAGATCCGTATGT 1154

Atpin3-3  1153 TAGTCCCAGATCAATCTCACAACGGCGAGACCAAAGCTGTAGCTCATCCA 1202
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type 1155 TAGTCCCAGATCAATCTCACAACGGCGAGACCAAAGCTGTAGCTCATCCA 1204
```

B

```
Atpin3-3     1 MISWHDLYTVLTAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFA 50
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type    1 MISWHDLYTVLTAVIPLYVAMILAYGSVRWWKIFSPDQCSGINRFVAIFA 50
                         .         .         .         .         .
Atpin3-3    51 VPLLSFHFISTNNPYAMNLRFIAADTLQKIIMLSLLVLWANFTRSGSLEW 100
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type   51 VPLLSFHFISTNNPYAMNLRFIAADTLQKIIMLSLLVLWANFTRSGSLEW 100
                         .         .         .         .         .
Atpin3-3   101 SITIFSLSTLPNTLVMGIPLLIAMYGEYSGSLMVQIVVLQCIIWYTLLLF 150
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type  101 SITIFSLSTLPNTLVMGIPLLIAMYGEYSGSLMVQIVVLQCIIWYTLLLF 150
                         .         .         .         .         .
Atpin3-3   151 LFEFRGAKMLIMEQFPETAASIVSFKVESDVVSLDGHDFLETDAEIGDDG 200
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type  151 LFEFRGAKMLIMEQFPETAASIVSFKVESDVVSLDGHDFLETDAEIGDDG 200
                         .         .         .         .         .
Atpin3-3   201 KLHVTVRKSNASRRSFCGPNMTPRPSNLTGAEIYSLSTTPRGSNFNHSDF 250
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type  201 KLHVTVRKSNASRRSFCGPNMTPRPSNLTGAEIYSLSTTPRGSNFNHSDF 250
                         .         .         .         .         .
Atpin3-3   251 YNMMGFPGGRLSNFGPADMYSVQSSRGPTPRPSNFEENCAMASSPRFGYY 300
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type  251 YNMMGFPGGRLSNFGPADMYSVQSSRGPTPRPSNFEENCAMASSPRFGYY 300
                         .         .         .         .         .
Atpin3-3   301 PGGGAGSYPAPNPEFSSTTTSTANKSVNKNPKDVNTNQQTTLPTGGKSNS 350
               ||||||||||||||||||||||||||||||||||||||||||||||||||
wild type  301 PGGGAGSYPAPNPEFSSTTTSTANKSVNKNPKDVNTNQQTTLPTGGKSNS 350
                         .         .         .         .
Atpin3-3   351 HDAKELHMFVWSSNGSPVSDRAGLNVFGGAPDNDQGGRSDQ*         391
               |||||||||||||||||||||||||||||||||||||||:.
wild type  351 HDAKELHMFVWSSNGSPVSDRAGLNVFGGAPDNDQGGRSERGAKEIRMLV 400
```

3.6.1.2 Preparation of *AtPIN3* specific *in situ* hybridisation probe

To study cell specific expression of the *AtPIN3* gene, probes for *in situ* analysis had to be prepared. To generate the *AtPIN3* specific probe a region was selected encompassing part of the *AtPIN3* cDNA from the hydrophilic region corresponding to nucleotides 999 to 1449. This part was selected since it shows a very low homology to other members of the *AtPIN* gene family. The chosen part was amplified by PCR, using primers with BamHI and EcoRI restriction site adapters, as depicted in Figure 20. The purified fragment was cloned into the pBS vector between T7 and T3 promoters and used as a template for the synthesis of digoxigenin labelled sense and antisense RNA probes by *in vitro* transcription. The antisense probe was used to specifically localise the *AtPIN3* mRNA by *in situ* hybridisation experiments and the sense probe was used as a negative control.

3.6.1.3 Heterologous expression of AtPIN3 and generation of specific antibodies

Northern blot analysis revealed that *AtPIN3* was expressed in various *Arabidopsis* organs (Chapter 3.6.2). To localise AtPIN3 protein more precisely, specific antibodies were raised against recombinantly expressed AtPIN3 protein sequence. These antibodies were used to detect the AtPIN3 protein at the tissue and cell level.

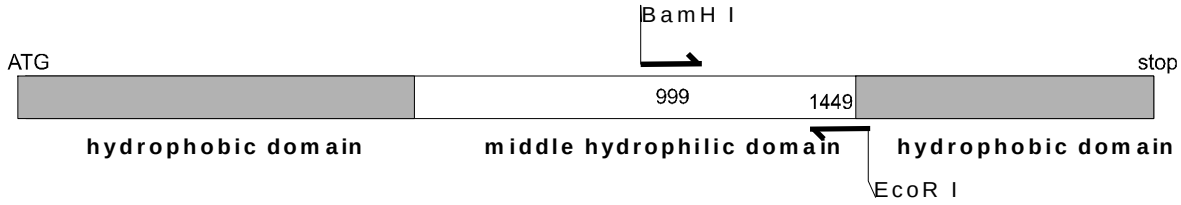
3.6.1.3.1 Production of His-AtPIN3 recombinant protein in *E. coli*

To allow production of an AtPIN3 specific antiserum, *AtPIN3* was expressed in *E. coli*. To achieve this, a translational fusion was generated fusing part of the hydrophilic AtPIN3 middle region with six N-terminally placed histidines. The part of the protein between amino acid residues 334 to 483 is the least conserved region within the *AtPIN* family, displays high antigenicity (Jameson and Wolf, 1988) and therefore appears to be best suited for production of AtPIN3 specific antibodies. The region of the *AtPIN3* cDNA between nucleotides 999 and 1449 was amplified by PCR using primers with BamHI and EcoRI restriction site adapters, as shown in Figure 20. The purified fragment was ligated into the pET28a expression vector allowing a translational fusion with six histidines. Such histidine tags enable easy and efficient purification of fusion proteins on Ni-NTA affinity columns.

The fusion construct was introduced into *E. coli*. Protein expression was induced by addition of IPTG. Clones expressing the recombinant protein (His-AtPIN3) after induction were selected by SDS-PAGE and used for large scale production. His-AtPIN3 was released from bacterial extracts, bound to a Ni-NTA column and eluted with EDTA containing buffer after extensive washing of the column. The purity of His-AtPIN3 was checked by SDS-PAGE and found to be greater than 60% (Figure 21). To obtain pure protein the His-AtPIN3 specific SDS-PAGE gel band (marked by an arrow in Figure 21) was cut out. Afterwards the protein was lyophilised and used for immunisation of rabbits.

Figure 20. Cartoon showing the part of the *AtPIN3* cDNA chosen for expression in *E. coli* (A) and the protein sequence of His-AtPIN3 fusion protein (B).

A

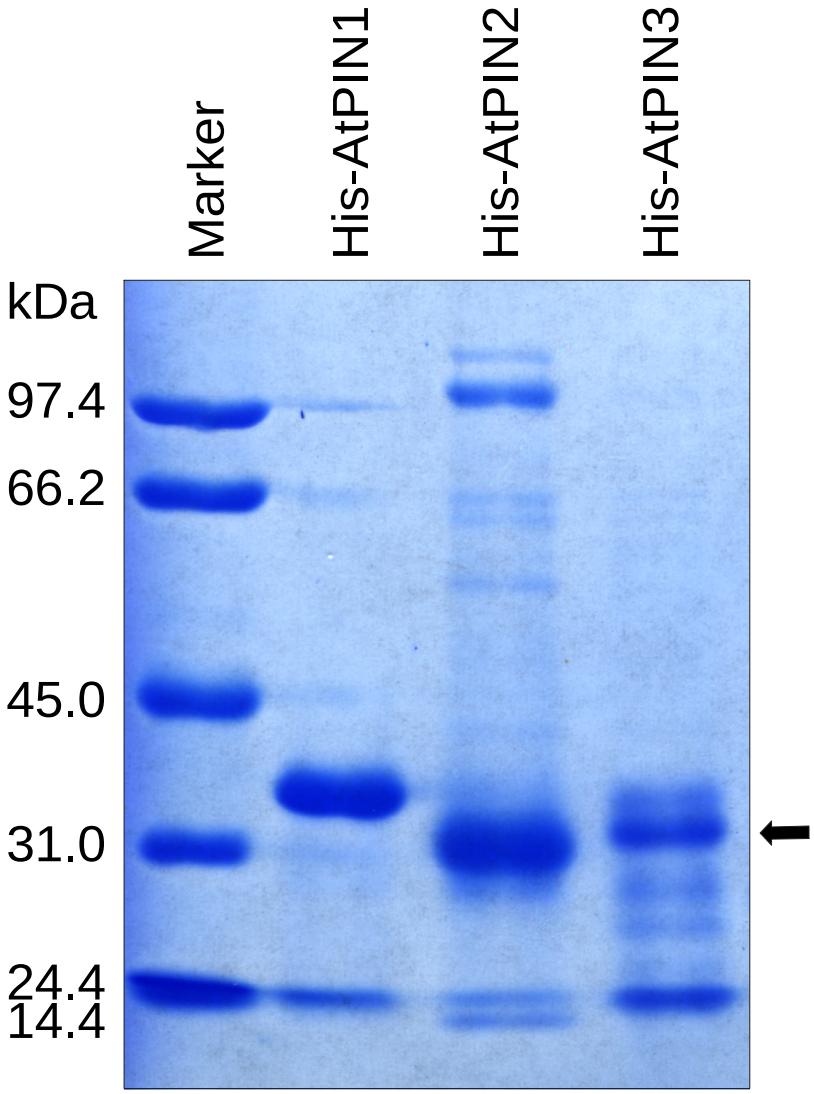


B

Histidine tag

```
  1  MGSSHHHHHH SSGLVPRGSH MASMTGGQQM GRGSGSVNTN QQTTLPTGGK
 51  SNSHDAKELH MFVWSSNGSP VSDRAGLNVF GGAPDNDQGG RSDQGAKEIR
101  MLVPDQSHNG ETKAVAHPAS GDFGGEQQFS FAGKEEEAER PKDAENGLNK
151  LAPNSTAALQ SKTGLGGAEA SQRKNMPPAS VMTRLIEFEL RRQ ACGRTRA
```

Figure 21. Coomasie stained SDS-polyacrylamide gel with AtPIN1, AtPIN2 and AtPIN3 Ni^{+}-NTA affinity purified fusion proteins.
In each lane 20 µg of protein were loaded. The His-AtPIN1 and His-AtPIN2 fusion proteins were used as controls.



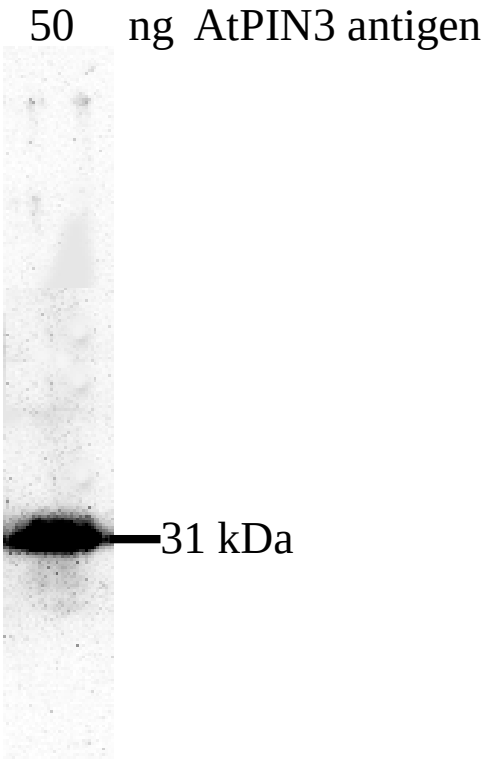
3.6.1.3.2 Characterisation and purification of AtPIN3 specific antiserum.

Immunisation of rabbits was performed by *Eurogentec* (Gent, Belgium). Immunisation consisted of three boosts in 14 days intervals with a final boost after another 30 days. Preimmunisation serum was collected prior to the first immunisation. Two bleedings were performed at days 38 and 66 after the first boost. The final bleeding was performed 80 days after the first immunisation.

The antiserum obtained from the final bleeding was characterised by Western blot analysis. Different antiserum dilutions (1:500 - 1:5000) were used to detect recombinant AtPIN3 antigen on a Western blot. As little as 50 ng of the recombinant antigen were detected by the antiserum in a dilution of 1:1000. No cross-reactions with bacterial proteins were observed. The Western blot for characterisation of the AtPIN3 specific antiserum is shown in Figure 22.

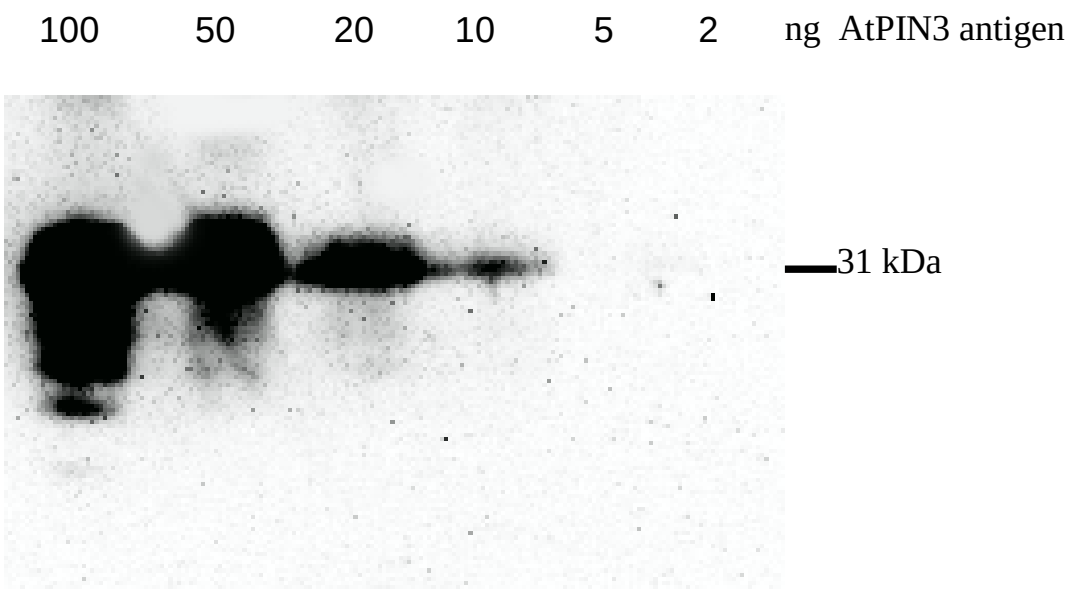
Figure 22. Characterisation of crude unpurified AtPIN3 specific antiserum by Western blot analysis.

50 ng of purified AtPIN3 recombinant protein was detected by AtPIN3 antiserum in a dilution of 1:1000.



The serum was also tested on Western blots with total *Arabidopsis* leaf protein extracts and proteins extracted from microsomal fractions. No AtPIN3 specific signal could be detected. This may be due to low AtPIN3 levels in leaf tissue. Based on this observation a final evaluation of the specificity of the antiserum in plants was at this time not yet possible. To increase the sensitivity and specificity of the AtPIN3 antiserum, this was affinity purified. One hundred microgram of recombinant His-AtPIN3 were blotted after SDS-PAGE onto PVDF. A stripe of PVDF containing His-AtPIN3 was cut out and incubated with AtPIN3 specific antiserum to bind AtPIN3 specific antibodies. After intensive washing bound antibodies were eluted and again used for characterisation in Western blot analysis. The purified antiserum detected 10 ng of recombinant AtPIN3 antigen in a dilution of 1:200, without any cross-reaction to other bacterial proteins, and therefore appeared to be suited for immunolocalisation experiments. The Western blot used for characterisation of affinity purified AtPIN3-specific antibodies is shown in Figure 23.

Figure 23. Detection limit of affinity purified AtPIN3 antibodies.
AtPIN3 antigen was separated by SDS-PAGE and Western blotted onto PVDF.



3.6.2 Northern blot analysis of *AtPIN3* expression

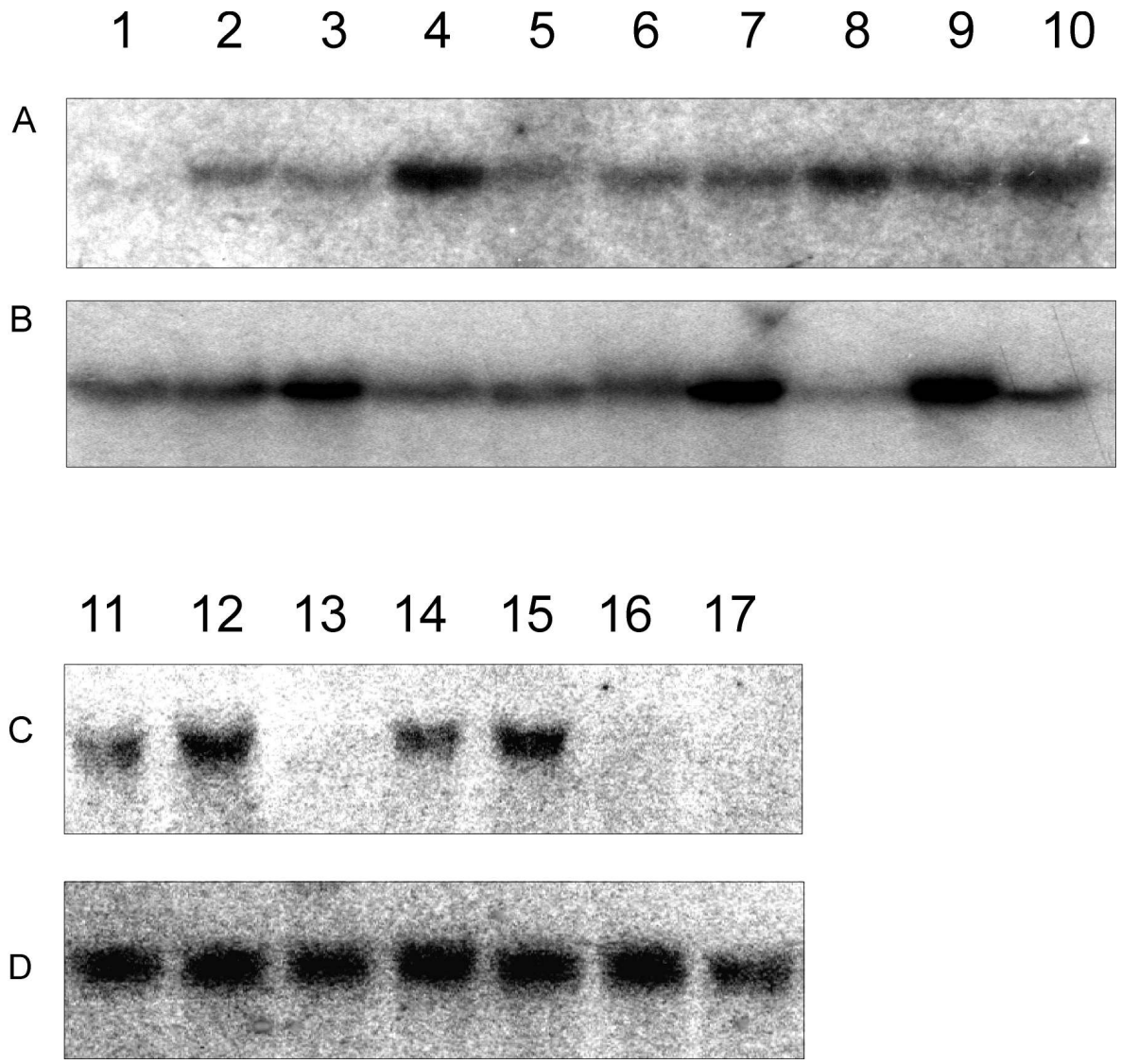
Northern blot analysis was performed with RNAs extracted from various *Arabidopsis* organs to study steady state expression levels of *AtPIN3* mRNA.

Total RNA was isolated from various *Arabidopsis* (Col-0) organs: root, inflorescence, leaves, seedlings, cotyledons, siliques. In addition, the total RNA from suspension cultures was extracted. After electrophoretic separation, the RNA was blotted onto nylon membrane and hybridised to an *AtPIN3* specific probe. The radiolabelled probe was prepared from the middle part of *AtPIN3* cDNA (nucleotides 999 - 1449). To control RNA loading, the blot was hybridised to an *ACTIN* probe.

The results of the Northern blot analysis are shown in Figure 24. The *AtPIN3* specific transcripts were detected in inflorescence axes, roots, leaves, seedling, flowers and siliques. The highest amount of *AtPIN3* mRNA was detected in roots and inflorescence axes. No *AtPIN3* expression was detected in suspension culture. The *AtPIN3* expression in the inflorescence axis was inspected in more detail using different parts from apex to base. *AtPIN3* mRNA was detected in young elongating parts located at the upper part of the main inflorescence axes and in side branches. No *AtPIN3* specific signal was detected in older basal parts.

The phenotypic analysis of *Atpin3* mutants has suggested that *AtPIN3* expression might be regulated by light (Chapter 3.6.4). Therefore the influence of light on *AtPIN3* expression was examined in light and dark grown seedlings. No differences in *AtPIN3* expression levels between light and dark grown seedlings were found.

Figure 24. Northern blot analysis of *AtPIN3*.
Total RNA from different *Arabidopsis* organs was isolated, subjected to Northern blot analysis and hybridised to an *AtPIN3* specific probe (A, C) or later to an *ACTIN* probe (B, D). Lanes: (1) suspension culture, (2) roots from liquid culture, (3) cauline leaves, (4) inflorescences, (5) siliques, (6) seedlings, (7) rosette leaves, (8) roots, (9) flowers, (10) cotyledons, (11) upper stem, (12) side stem, (13) lower stem, (14) seedling dark, (15) seedling light, (16) seedling *Atpin3-1*, (17) seedling *Atpin3-2.*



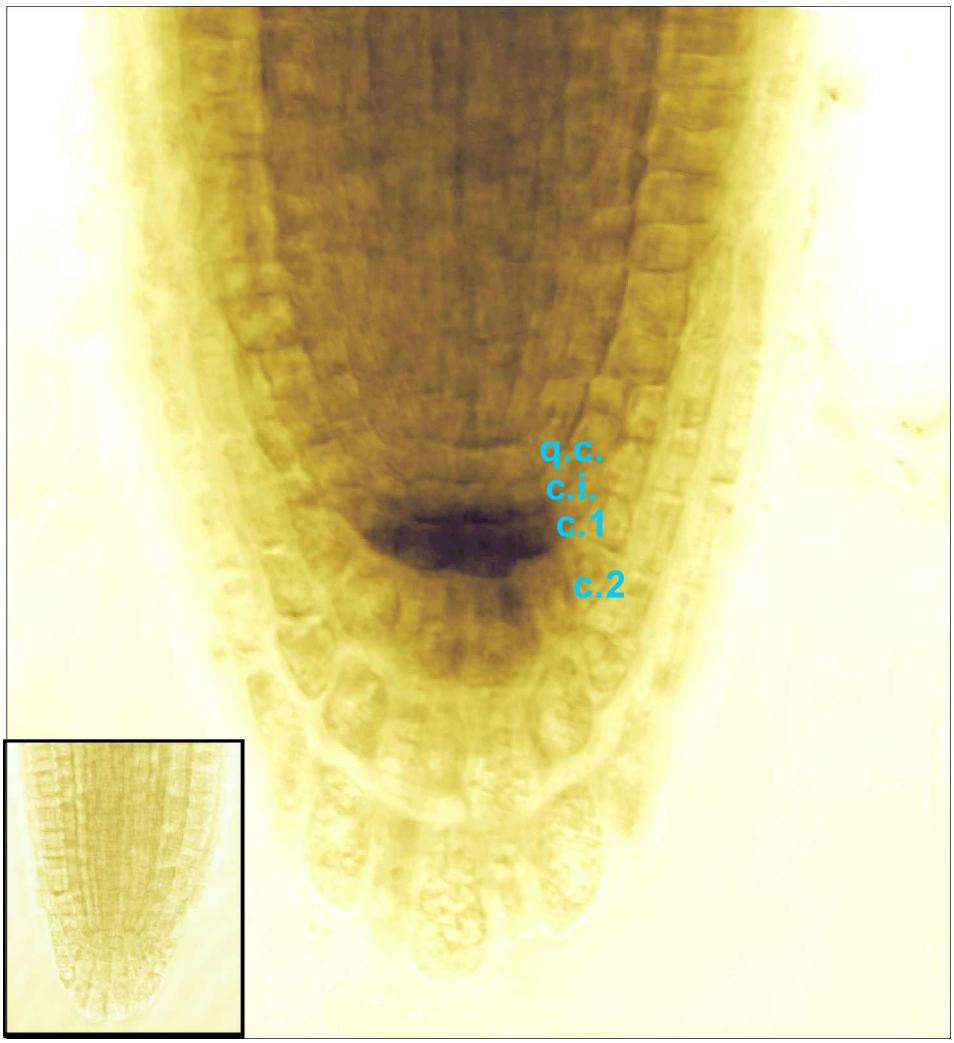
3.6.3 *AtPIN3* in the *Arabidopsis* root

Northern blot analysis revealed that *AtPIN3* is expressed in *Arabidopsis* roots. To detect *AtPIN3* mRNA and AtPIN3 protein expression at cellular level, *in situ* hybridisation and immunolocalisation experiments were performed. Also a role of the AtPIN3 protein was examined by phenotypic analysis of *Atpin3* mutant roots.

3.6.3.1 Localisation of *AtPIN3* mRNA in roots by *in situ* hybridisation

Whole mount *in situ* hybridisation was used to analyse the spatial distribution of *AtPIN3* mRNA in *Arabidopsis* root.

Figure 25. *AtPIN3* specific whole mount *in situ* hybridisation with *Arabidopsis* roots.
Digoxigenin labelled *AtPIN3* antisense transcripts were used as probes (for details see Materials and methods, Chapter 2.2.7.1). *AtPIN3* mRNA was detected in the first columella tier (c.1) of the distal root meristem, accompanied by a weaker signal in the second columella tier (c.2). Quiescent centre (q.c.), columella initials (c.i.), first (c.1) and second (c.2) columella tiers are marked. No *AtPIN3* specific signal was detected using the sense probe (inset).

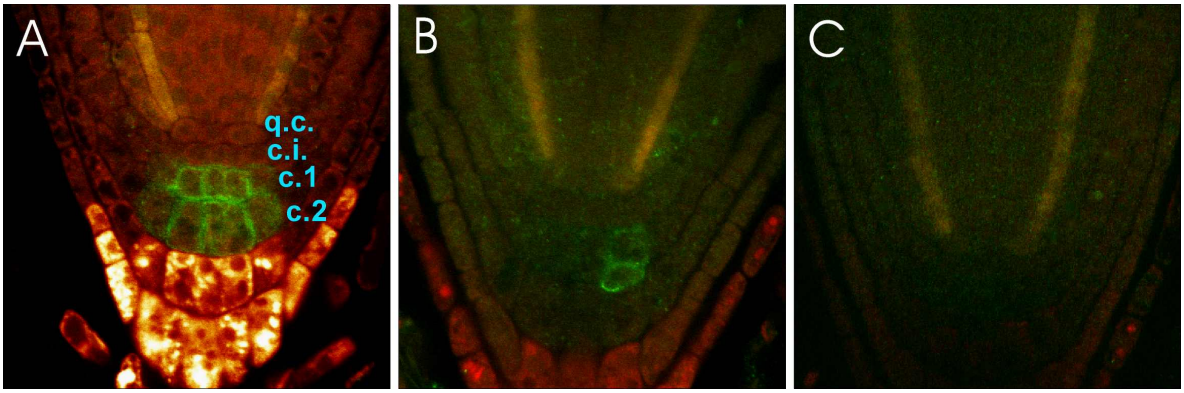


Five days old *Arabidopsis* seedlings were fixed, permeabilised and subjected to *in situ* hybridisation with an *AtPIN3* specific probe as described in Chapter 2.2.7.1. Hybridisation signals were visualised using anti-DIG antibodies. The *AtPIN3* mRNA specific signal was detected in the root tip in the cells of the first columella tier accompanied by a weaker signal in second columella tier cells (Figure 25). No *AtPIN3* specific signal was detected in the root tip using the sense control probe (Figure 25 inset).

3.6.3.2 AtPIN3 protein localisation in root

Whole mount immunolocalisation experiments were performed on 4 day old wild type *Arabidopsis* seedlings and *Atpin3-1, Atpin3-2, Atpin3-3* mutant seedlings. Seedlings were fixed, permeabilised and incubated with affinity purified AtPIN3 specific antibodies (for details see Chapter 2.2.9.1). For visualisation of signal, secondary antibodies conjugated to FITC were used and samples were subsequently inspected by laser scanning confocal microscopy. The AtPIN3 protein signal was detected at the plasma membrane of the first columella daughter cells and in several cases also in second columella daughter cells (Figure 26 A). AtPIN3 immunolocalisation in homozygous *Atpin3* mutant roots revealed no AtPIN3 specific signal (Figure 26 C). The absence of a signal in *Atpin3* null mutants indicates that the antibodies indeed bind specifically only to AtPIN3. In a few cases (less than 5%, n=87), somatic reversion of *En-1* transposon was observed and *AtPIN3* expression was restored in few columella cells further confirming the specificity of antibodies (Figure 26 B).

Figure 26. Localisation of AtPIN3 in the *Arabidopsis* root meristem.
AtPIN3 was detected in wild type root meristem in first (c.1) and second (c.2) tier of columella cells (A). No AtPIN3 protein was localised in the quiescent centre (q.c.) and in columella initials (c.i.). No AtPIN3 protein was detected in homozygous *Atpin3* mutant plants (C). In rare cases somatic reversion was observed in *Atpin3* homozygous plants restoring AtPIN3 expression (B).



3.6.3.3 Analysis of *Atpin3* mutant roots

Expression analysis and protein localisation experiments revealed the presence of *AtPIN3* mRNA and AtPIN3 protein in first and second columella tiers in the distal root meristem. The role of AtPIN3 in root development was studied by comparing wild type with *Atpin3* mutant roots.

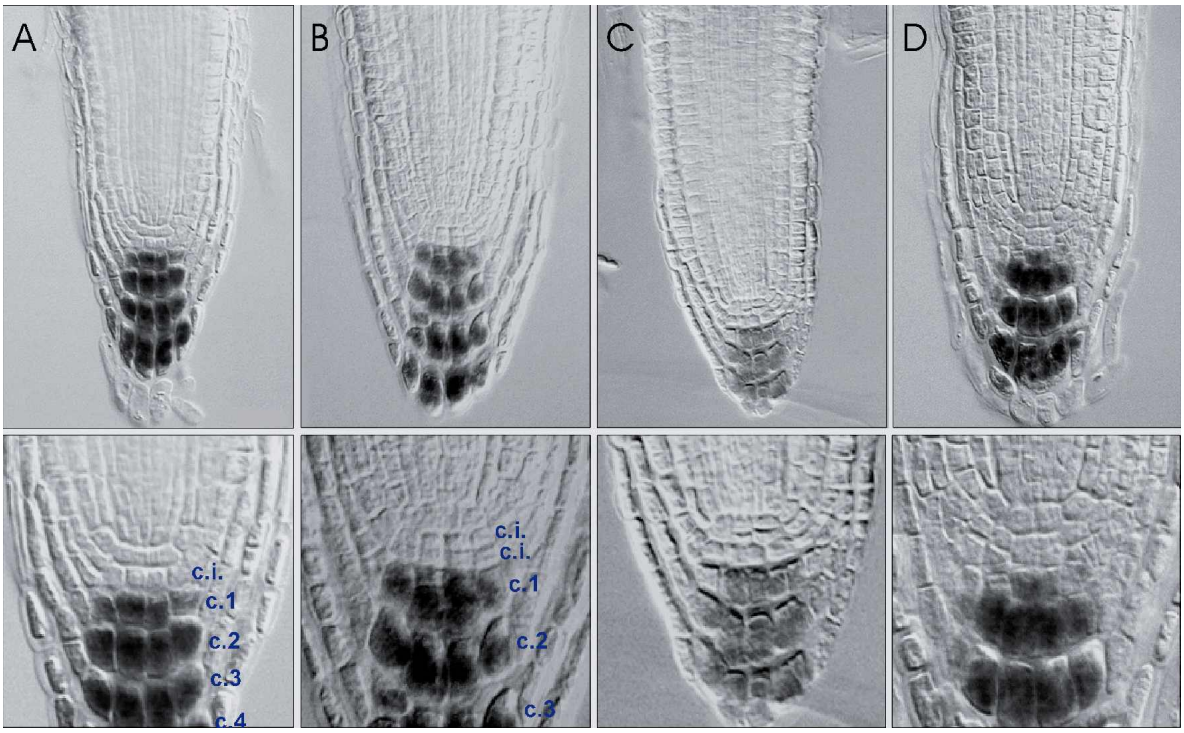
The length of six days old seedling roots grown in light and dark were examined. Significant differences in root length between wild type (Col-0) and *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutant plants were observed. Homozygous *Atpin3* seedlings grown in continuous light displayed 30 % shorter roots than wild type seedlings (30±8 %, Col-0: n=58; *Atpin3*: n=66) (Figure 30). In dark grown seedlings no differences between Col-0 wild type and *Atpin3* mutant plants were found (Col-0: n=54; *Atpin3*: n=62) (Figure 32).

The *Atpin3* root length phenotype was phenocopied by growing wild type plants on 0.1 μM NPA. NPA treated seedlings grown in light displayed a 33% shorter roots than untreated seedlings (33±8 %, Col-0: n=58; Col-0 on NPA: n=56). No differences in root length between untreated and NPA treated dark grown seedlings were found (Col-0: n=54; Col-0 on NPA n=62).

The cell pattern of the root meristem in Col-0 wild type plants and in *Atpin3-1*, *Atpin3-2* and *Atpin3-3* mutant plants was examined. Starch grains were stained with lugol solution and thus the identity of differentiated columella cells determined. In wild type roots, one tier of columella initials without starch and four tiers of differentiated columella cells containing starch were present in more than 98% of the seedlings (n=117). In contrast, significant changes in the pattern of the *Atpin3* root meristem were observed. In 84 % (n=127) of the *Atpin3* mutant plants one additional layer of columella initials was found and in 88 % (n=127) of *Atpin3* mutants, only 3 tiers of columella cells were found (Figure 27).

The phenotype of the *Atpin3* mutant in root meristem can be described in terms of change in cell identity in comparison to wild type. In wild type roots, cells of first columella tier, where *AtPIN3* gene is expressed, are differentiated and contain starch. In *Atpin3* mutant plants, these cells fail to differentiate and maintain their columella initial-like undifferentiated status.

Figure 27. Root meristem phenotype of wild type and *Atpin3* mutant plants.
Starch grains in the root meristem of *Arabidopsis* wild type (Col-0) and *Atpin3* mutant plants were stained with lugol to determine the identity of columella cells. In wild type plants, one tier of columella initials (c.i.) and four tiers of columella cells (c.1, c.2, c.3, c.4) were detected (A). More than 80 % *Atpin3* mutant plants contained two tiers of columella initials (c.i.) (B) and only 3 tiers of columella cells (C). Intermediate stages were also found in which part of the columella initial cells differentiated to the columella cells while others maintained its columella initial identity (D). The lower panel shows a magnification of lugol stained columella cells from (A)-(D).



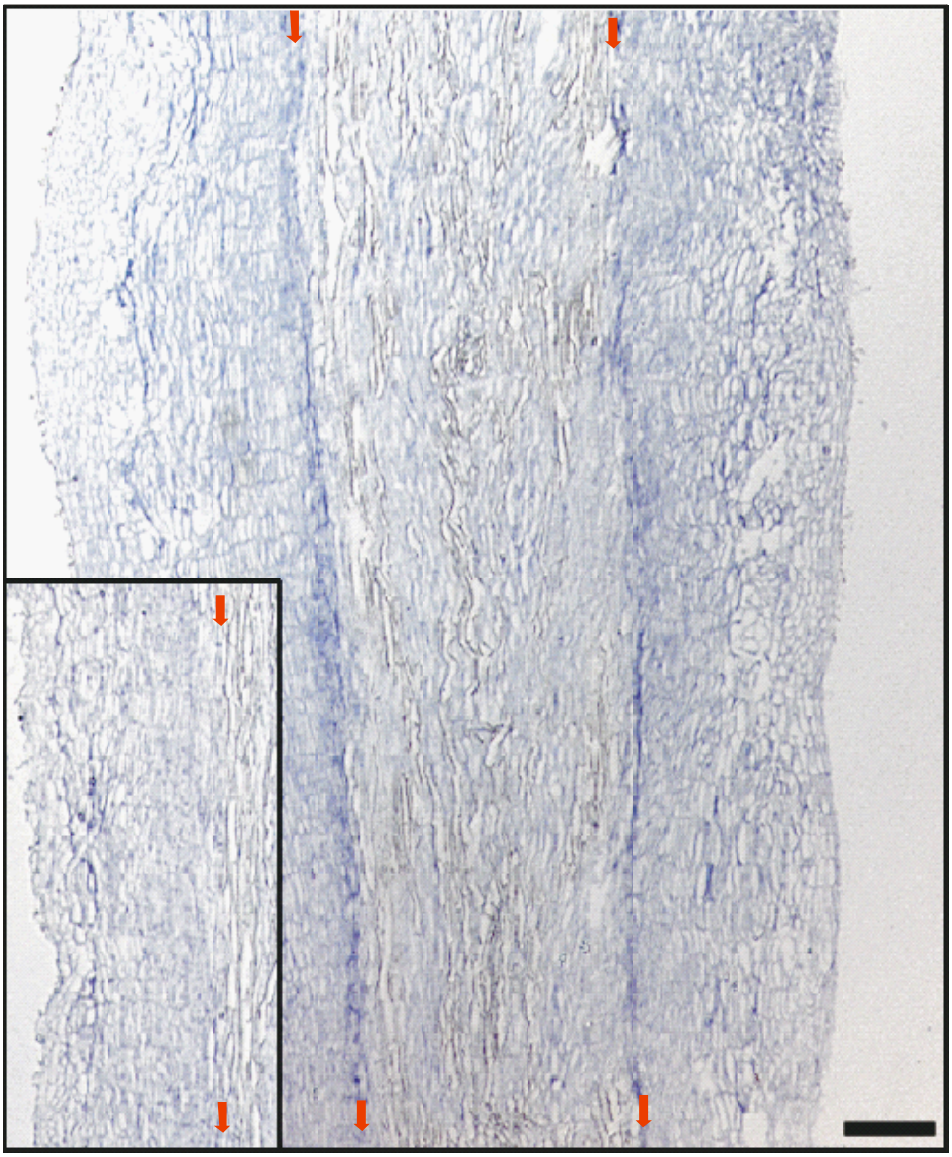
3.6.4 AtPIN3 in the *Arabidopsis* hypocotyl

Northern blot analysis revealed that *AtPIN3* was expressed in shoots and seedlings, suggesting that *AtPIN3* might be expressed in hypocotyls. Therefore *AtPIN3* expression and AtPIN3 protein localisation were studied in detail in the hypocotyl sections of wild type and *Atpin3* mutant plants.

3.6.4.1 *AtPIN3* mRNA localisation in hypocotyl by *in situ* hybridisation

The spatial distribution of *AtPIN3* expression was analysed by *in situ* hybridisation in longitudinal sections of hypocotyls.

Figure 28. *AtPIN3* specific *in situ* hybridisation in sections of *Arabidopsis* hypocotyls.
AtPIN3 mRNA was detected in hypocotyl endodermis (indicated by arrows). No *AtPIN3* specific signal was detected using sense probe (inset).

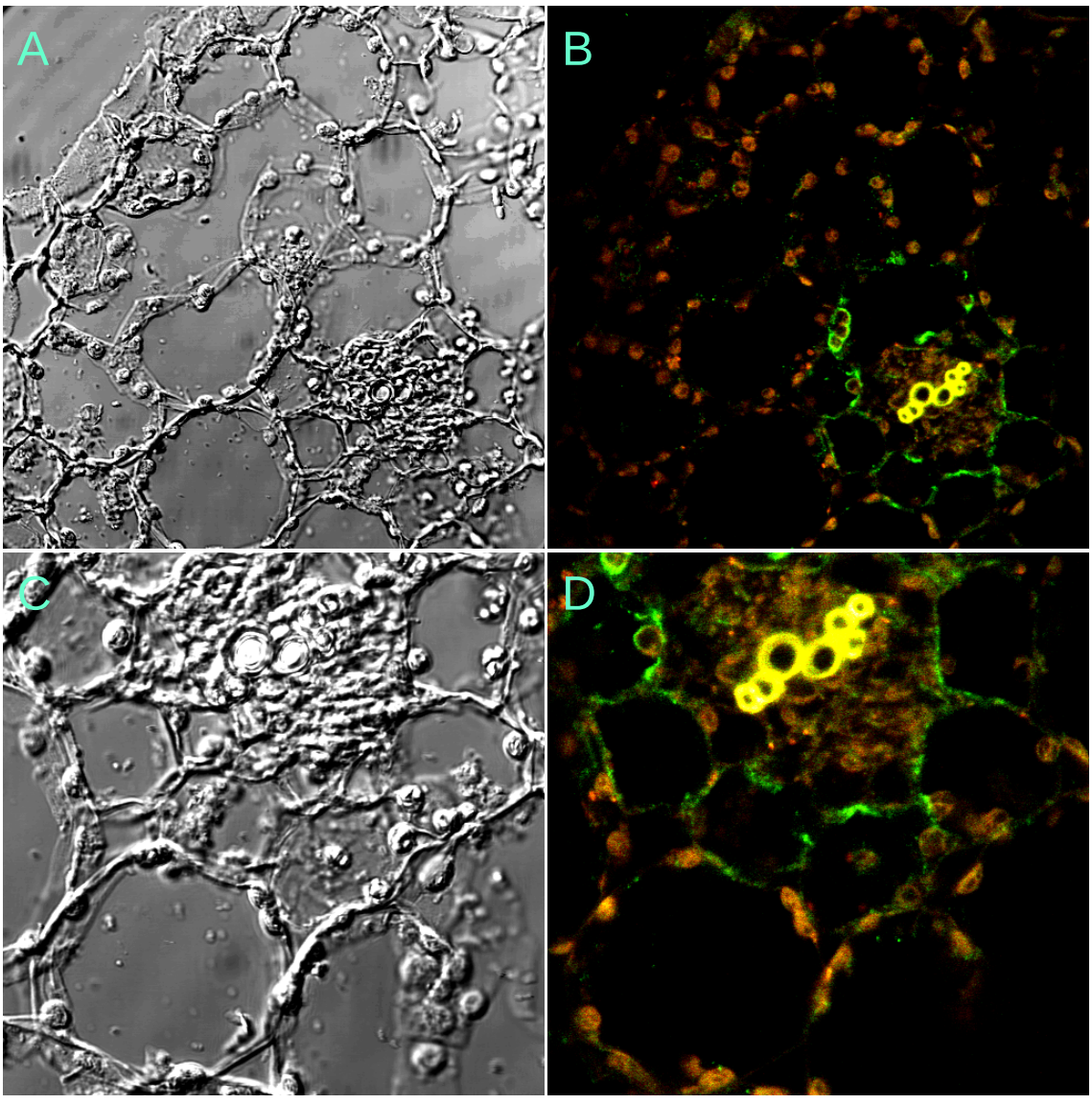


Fourteen days old *Arabidopsis* plants were fixed, embedded in paraffin, sectioned and hybridised to *AtPIN3* specific digoxigenin labelled sense and antisense probes (for details see Material and Methods, Chapter 2.2.7.2). *AtPIN3* mRNA specific signal was detected in the endodermis cells (Figure 28). No signal was detected using the sense probe (Figure 28, inset)

3.6.4.2 AtPIN3 protein localisation in hypocotyl

In order to localise the AtPIN3 protein in *Arabidopsis* hypocotyls, immunolocalisation experiments with AtPIN3 specific antibodies were performed on transversal hypocotyl sections.

Figure 29. Localisation of AtPIN3 protein in *Arabidopsis* hypocotyls.
AtPIN3 protein (green signal) was detected at the plasma membrane of endodermis cells (B, D). Corresponding differential interference contrast images of the transversal hypocotyl sections (A, C).



Six days old *Arabidopsis* seedlings were fixed, embedded in PEG and sectioned. Ten micrometers thick sections were first incubated with AtPIN3 specific antibodies and than with FITC conjugated anti-rabbit IgG secondary antibodies. Laser scanning confocal microscopy revealed that the AtPIN3 protein was localised in endodermis cells of hypocotyls (Figure 29), confirming *in situ* hybridisation data (see 3.6.4.1). Interestingly, higher AtPIN3 levels were detected at the plasma membrane on the inner side of endodermis cells (Figure 29 D). In control experiments with *Atpin3* mutant seedlings, no AtPIN3 specific signal was detected (data not shown).

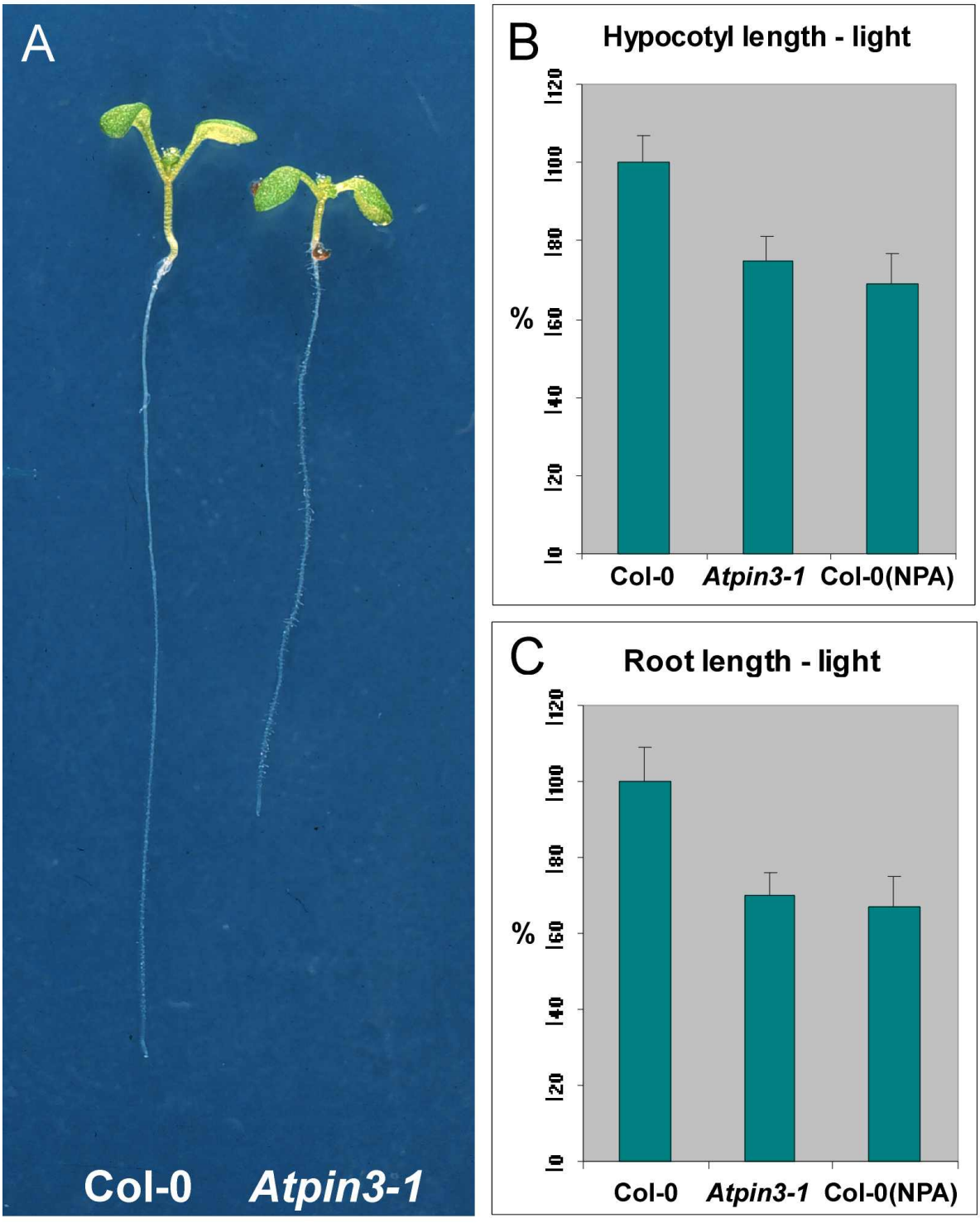
3.6.4.3 Hypocotyl phenotype of *Atpin3* mutants

The localisation of *AtPIN3* expression products in endodermis cells of *Arabidopsis* hypocotyls suggested a function of AtPIN3 in hypocotyl growth and development. In order to study this function, hypocotyl growth and development in *Atpin3* mutants were analysed.

3.6.4.3.1 Hypocotyl elongation in light

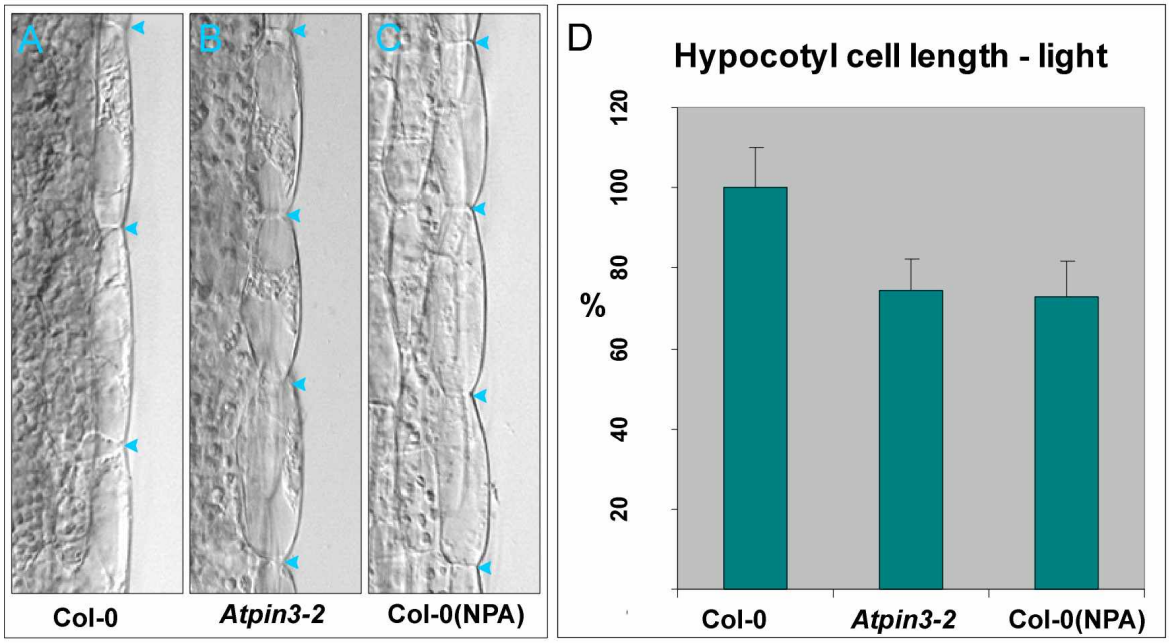
The hypocotyl length of light grown seedlings was studied. Col-0 wild type and *Atpin3* mutant seedlings were grown on agar plates in vertical position under continuous illumination. After 5 days of cultivation hypocotyl lengths (hypocotyl/root junction to apical meristem) were measured. Significant differences between wild type and *Atpin3*-1, *Atpin3*-2, *Atpin3*-3 mutants were observed. *Atpin3* seedlings grown in continuous light displayed 30 % (29 ±7 %, *Atpin3*: n=66; Col-0: n=58) shorter hypocotyls than Col-0 wild type plants (Figure 30). This phenotype was phenocopied by growing wild type plants on NPA. Light grown wild type seedlings treated with 0.1 µm NPA displayed beside defects in hypocotyl and root gravitropism also around 30 % (31 ±7 %, Col-0: n=54; Col-0 on NPA: n=56) shorter hypocotyls in comparison to untreated wild type plants (Figure 30 B).

Figure 30. Hypocotyl and root elongation phenotype of *Atpin3* light grown mutants.
Wild type (Col-0) and *Atpin3* mutant plants grown in continuous light for 5 days (A). Comparison of the average hypocotyl lengths (B) and average root lengths (C) of Col-0, *Atpin3* mutant plants and Col-0 plants grown on 0.1 μM NPA.



To determine if the shorter hypocotyls in *Atpin3* plants and NPA treated wild type plants are caused by a defect in cell elongation or by a change in cell division activity, the length of hypocotyl epidermis cells was determined for wild type, *Atpin3* mutant and NPA treated wild type plants. It was found that the length of epidermis cells of *Atpin3* mutant hypocotyls as well as those of wild type plants grown on 0.1 µM NPA were by 30 % shorter than hypocotyl cells of untreated Col-0 wild type plants, as it is shown in Figure 31 (28±6 % for *Atpin3* mutant, *Atpin3*: n=54; Col-0: n=63; 30±7 % for Col-0 on NPA, Col-0 on NPA: n=61; Col-0: n=63). This result suggests that shorter hypocotyls in *Atpin3* mutant plants are caused by a defect in cell elongation.

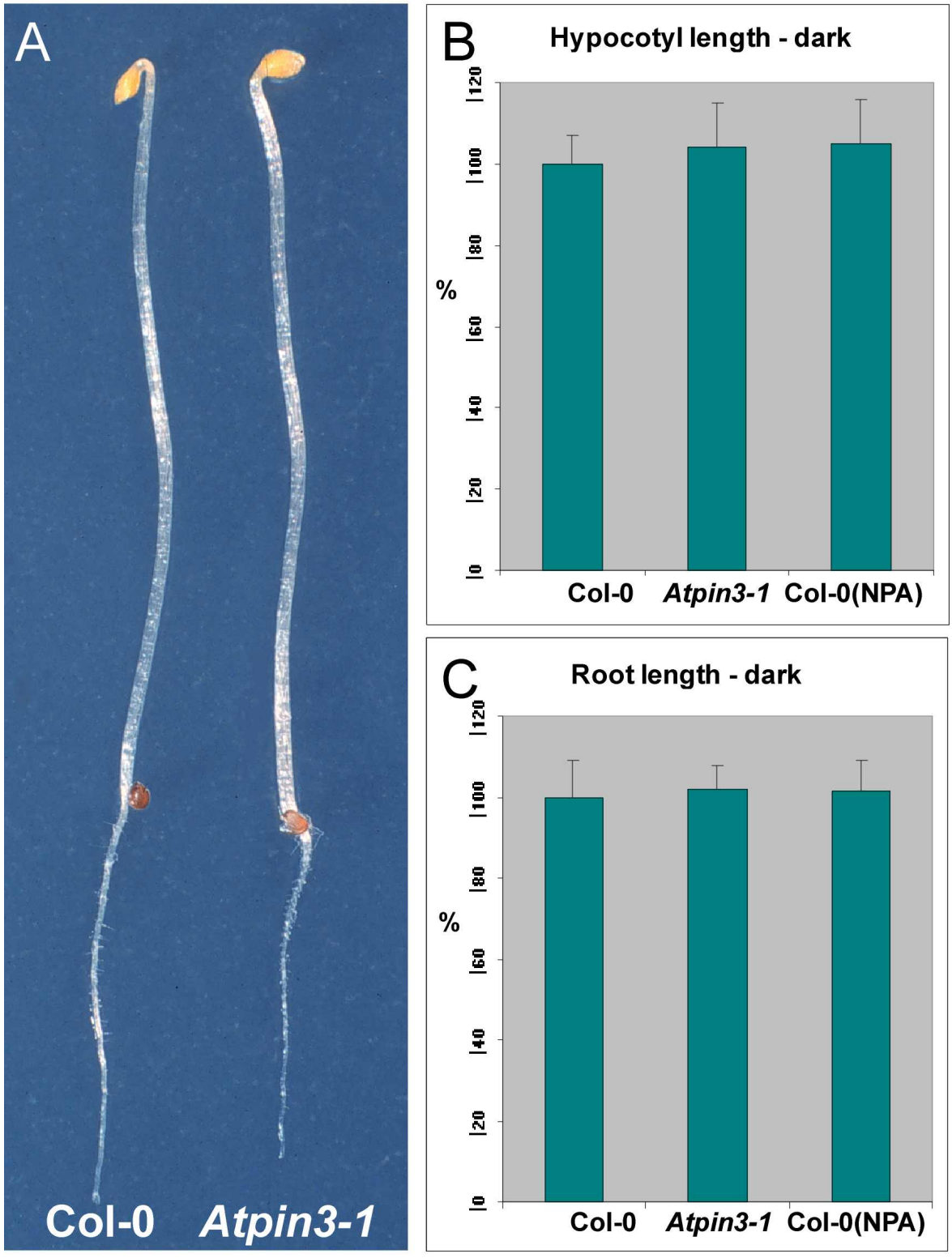
Figure 31. Comparison of hypocotyl cell lengths of Col-0 and *Atpin3* mutant seedlings. Seedlings were grown in permanent light. Col-0 wild type (A), *Atpin3* mutant (B) Col-0 plants grown on 0.1 µM NPA. Comparison of the average epidermal cell lengths in hypocotyls (D).



3.6.4.3.2 Hypocotyl elongation in dark

The hypocotyl length in etiolated wild type, *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutant and NPA treated wild type seedlings was examined. The Col-0 wild type and *Atpin3* mutant seedlings were grown on agar plates with or without 0.1 µM NPA in vertical position in dark. After 5 days of cultivation the lengths of hypocotyls ranging from hypocotyl/root junction to apex were measured. As shown in Figure 32, no significant differences in hypocotyl lengths between wild type, *Atpin3* mutants and seedlings grown on NPA were found (*Atpin3*: n=62; Col-0: n=54; Col-0 on NPA: n=62).

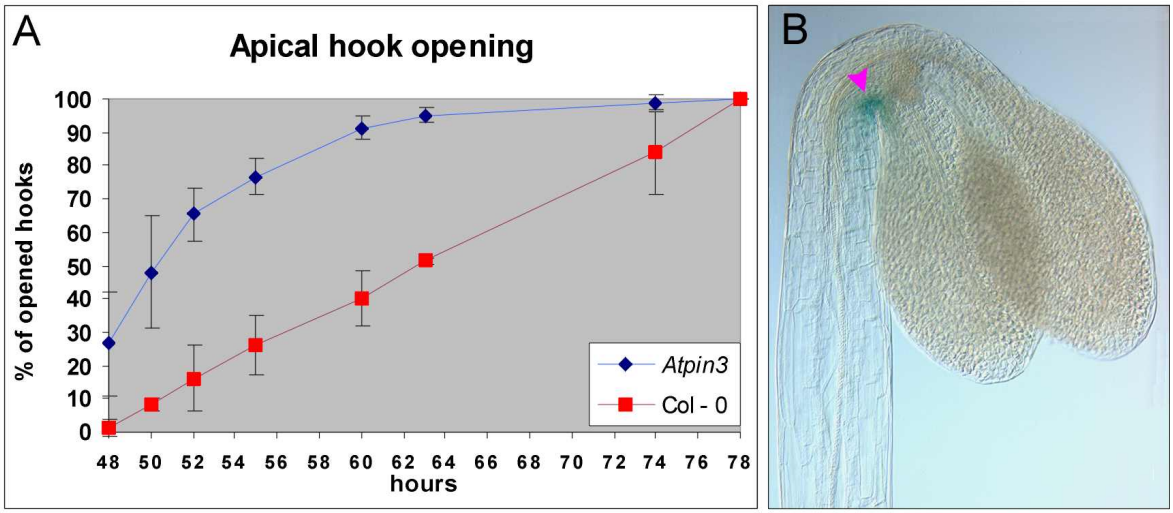
Figure 32. Hypocotyl and root elongation phenotype of dark grown *Atpin3* mutants.
Wild type (Col-0) and *Atpin3* mutant plants were grown in the dark for 5 days (A). Comparison of the average hypocotyl lengths (B) and the average root lengths (C) between Col-0, *Atpin3* mutant plants and Col-0 plants grown on 0.1 μM NPA.



3.6.4.3.3 Apical hook opening

Polar auxin transport inhibitors like NPA are known to disturb the process of apical hook opening (Lehman *et al.*, 1996). The underlying molecular mechanisms controlling this process are far from being understood. To monitor changes in auxin levels during apical hook opening, transgenic *Arabidopsis* plants were used harbouring a hydrolase reporter gene (PEH A, Dotson *et al.*, 1996) under the control of synthetic auxin responsive promotor (DR5rev; Benkova, pers. communication; Ulmasov *et al.*, 1997). This reporter is supposed to be active in cells with elevated auxin concentration. As shown in Figure 33 B, DR5rev reporter activity was detected only at the inner edge of the apical hook. These data suggest that (*i*) differential auxin distribution occurs during apical hook opening and that (*ii*) this distribution is probably mediated by polar auxin transport. This interesting question was examined in Col-0 wild type and *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutant plants. More than 300 seeds of *Atpin3* mutant and control plants were plated on agar plates and grown in dark. In regular time intervals, the plates were inspected and seedlings with opened apical hook were counted. The experiment was terminated when all seedlings had opened apical hooks. Cumulative results for *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutant seedlings and Col-0 seedlings were evaluated and compared. As shown in Figure 33, significant differences between wild type and *Atpin3* mutant seedlings were found. *Atpin3* mutant seedlings opened their apical hooks about 12 hours earlier than wild type suggesting a role of AtPIN3 in apical hook opening.

Figure 33. Apical hook opening phenotype of *Atpin3* mutant.
The ratio of dark grown seedlings with already opened apical hook for Col-0 and *Atpin3* mutants was counted in regular time intervals after germination (A). Differential expression of auxin responsive promotor (marked by red arrow) during apical hook opening in Col-0 suggests that lateral auxin redistribution is involved in this process (B).

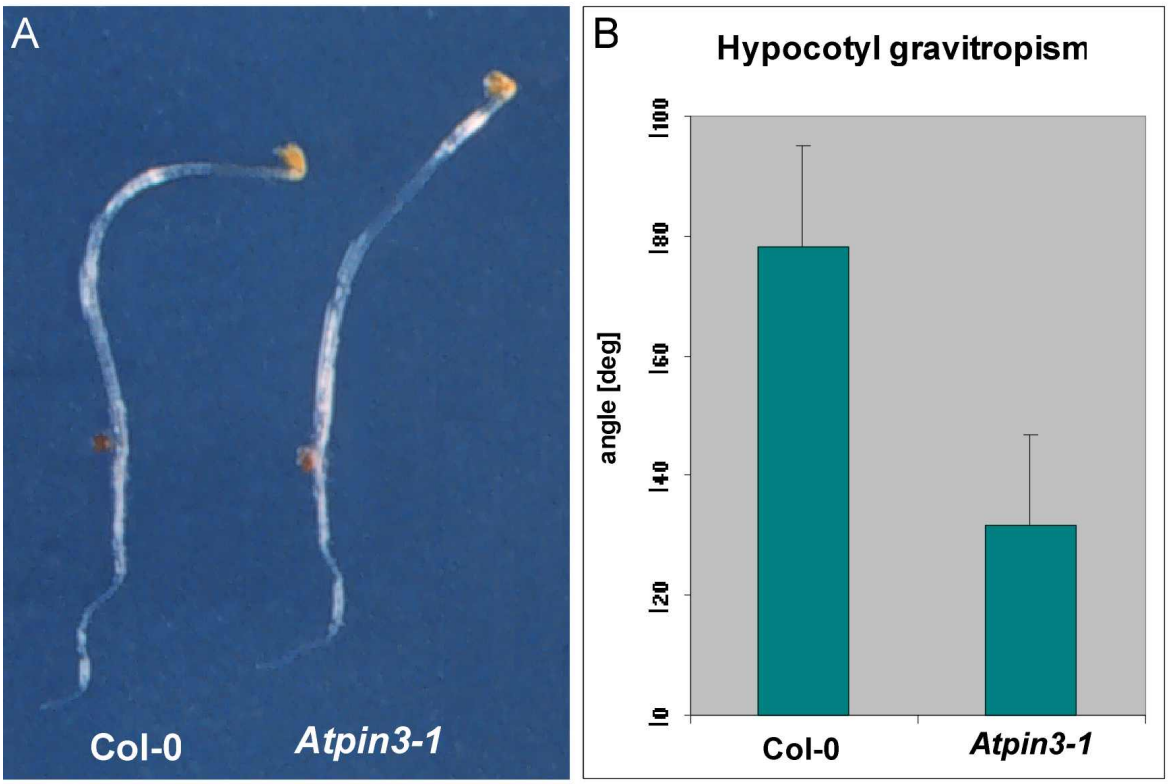


3.6.4.3.4 Hypocotyl tropism phenotype

One of the most pronounced effects of polar auxin transport inhibition on plants is a complete abolishment of tropic responses (Li *et al*., 1991). Control of lateral auxin distribution may be essential for this process and also an important role of endodermis in shoot tropic responses was reported (Li *et al*., 1991, Watahiki *et al*., 1999). Therefore gravitropic as well as phototropic responses of hypocotyls were analysed in *Atpin3* mutant seedlings. The angle of hypocotyl bending after gravity or light stimulation in Col-0 wild type and *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutant plants was measured.

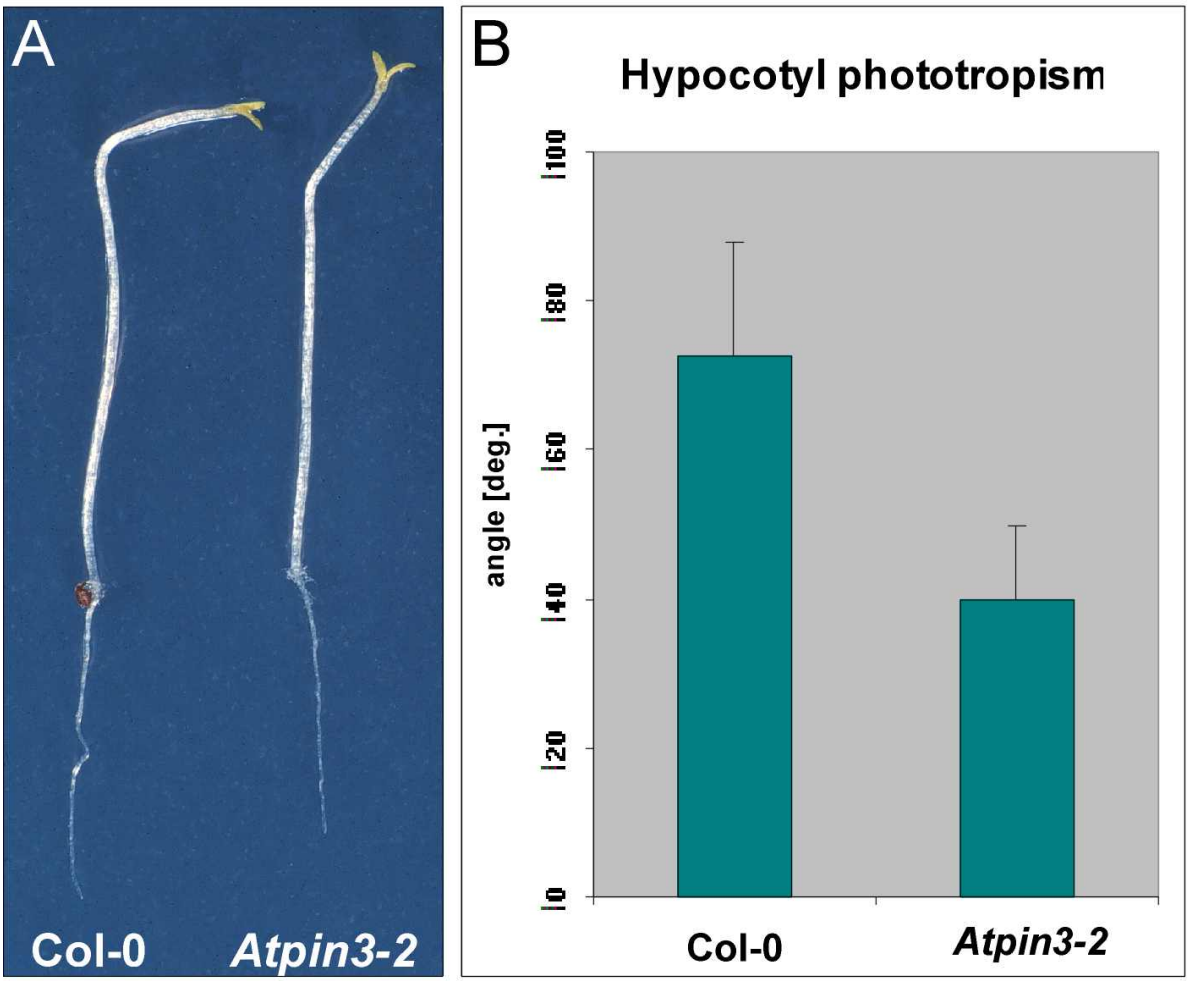
Col-0 and *Atpin3* mutant plants were grown on agar plates in vertical position in dark. Tropic responses were induced 4 days after germination either (*i*) by gravitropic stimulation induced by turning the plates by 90 degrees for 24 hours or (*ii*) by phototropic stimulation using unilateral white light for 10 hours. The angle of hypocotyl bending to previous vertical direction was determined and evaluated. Significant differences in both gravitropic and phototropic responses between Col-0 and *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutant seedlings were found. As shown in Figure 34, the hypocotyl gravitropic response in *Atpin3* mutants was reduced almost by 60 % compared to wild type seedlings (59±19 %, *Atpin3*: n=72; Col-0: n=65).

Figure 34. Hypocotyl gravitropism phenotype of *Atpin3* mutants.
Wild type (Col-0) and *Atpin3* mutant plants after 24 hours of gravistimulation (A). Average of hypocotyl bending angle for Col-0 and *Atpin3* mutant seedlings (B).



The phototropic response in *Atpin3* mutants was around 50 % less pronounced than in wild type seedlings (45±13 %, Col-0: n=78; *Atpin3*: n=81) as it is shown in Figure 35. These results suggest a role of AtPIN3 in the hypocotyl tropic responses, most probably in lateral redistribution of auxin.

Figure 35. Hypocotyl phototropism phenotype of *Atpin3* mutants.
Wild type (Col-0) and *Atpin3* mutant plants after 10 hours of unilateral white light stimulation (A). Comparison of hypocotyl bending between Col-0 and *Atpin3* mutant seedlings (B).



3.6.5 *AtPIN3* in the *Arabidopsis* inflorescence axis

Northern blot analysis revealed the presence of *AtPIN3* transcripts predominantly in the upper, elongating part of inflorescence axis. Therefore the *AtPIN3* mRNA and the AtPIN3 protein localisation in the inflorescence was examined by *in situ* hybridisation and immunolocalisation experiments. Moreover the phenotype of *Atpin3* knock out mutant plants was analysed to elucidate the function of the AtPIN3 protein in *Arabidopsis* inflorescence axis.

3.6.5.1 *AtPIN3* mRNA localisation in inflorescence axes by *in situ* hybridisation

The spatial distribution of *AtPIN3* mRNA was analysed on longitudinal sections of inflorescence axis by *in situ* hybridisation. Inflorescences of 18 day old *A. thaliana* plants were fixed, embedded in paraffin and hybridised with *AtPIN3* specific digoxigenin labelled probe. *AtPIN3* specific signals were detected in stem endodermis cells, as it is depicted in Figure 36. This result nicely correlates with the observations made for hypocotyls. No *AtPIN3* specific signal was detected using the sense control probe (Figure 36, inset).

3.6.5.2 AtPIN3 protein immunolocalisation in inflorescences

In situ hybridisation experiments showed the presence of *AtPIN3* mRNA in inflorescence endodermis cells. In order to localise the protein, immunolocalisation experiments were performed in the same tissue. For this purpose, the affinity purified AtPIN3 specific antibodies were used. Immunolocalisation experiments were performed on transversal and longitudinal inflorescence sections of three weeks old plants. Inflorescence axis segments were fixed, embedded in PEG, sectioned and incubated with AtPIN3 specific antibodies. AtPIN3 protein signal was detected only in younger, upper parts of inflorescence axes and was exclusively localised in endodermis cells (Figure 37). This result is consistent with the Northern blot and *in situ* hybridisation data (Chapter 3.5.2 and 3.5.6.1). The AtPIN3 protein was predominantly localised polarly at the lateral side of endodermis cells (Figure 37 F). In rare cases also basal or non-polar localisation of AtPIN3 protein was observed (Figure 37 D).

Figure 36. *AtPIN3 in situ* hybridisation in *Arabidopsis* inflorescence axes.
AtPIN3 specific signal was detected in longitudinal inflorescence sections in endodermis cells (indicated by arrows). No *AtPIN3* specific signal was found using sense control probe (inset).

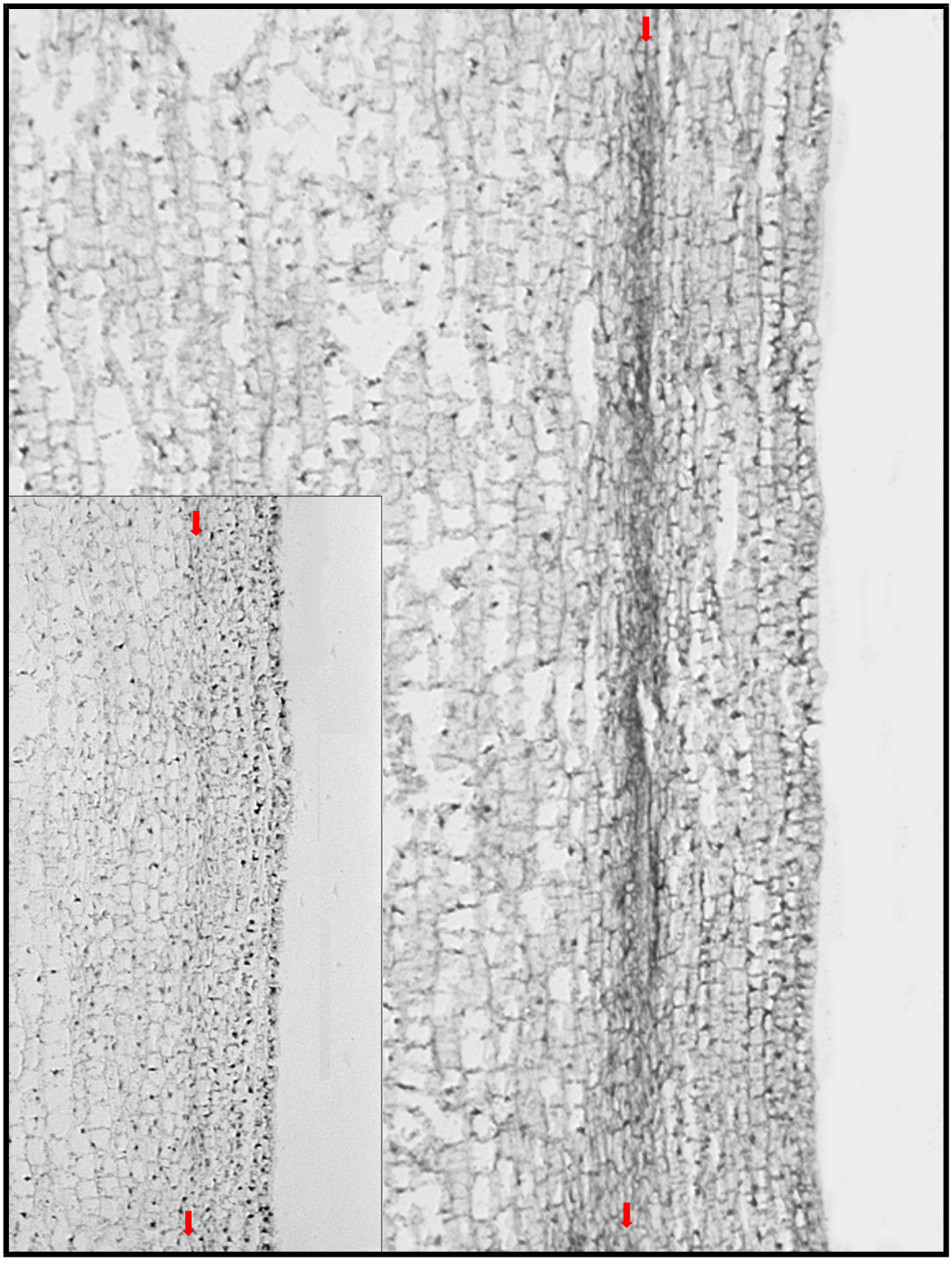
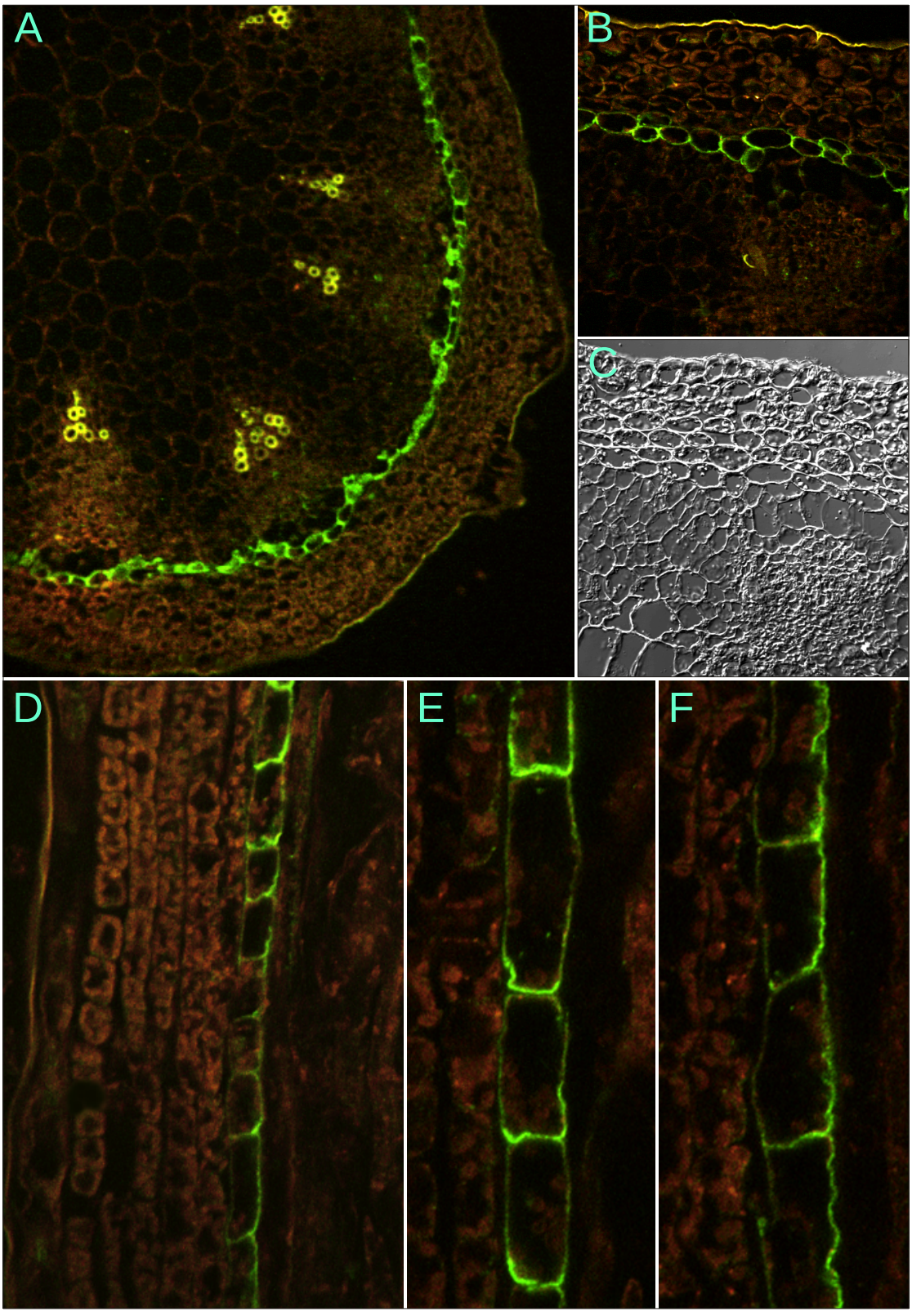


Figure 37. Localisation of the AtPIN3 protein in *Arabidopsis* inflorescence axes.
Transversal sections (A, B, C) and longitudinal sections (D, E, F) were incubated with AtPIN3 specific antibodies and FITC coupled secondary antibodies. AtPIN3 specific signals (shown by green colour) were detected at the membrane of endodermis cells, predominantly at their lateral side (F) in few cases also at their basal side (E).

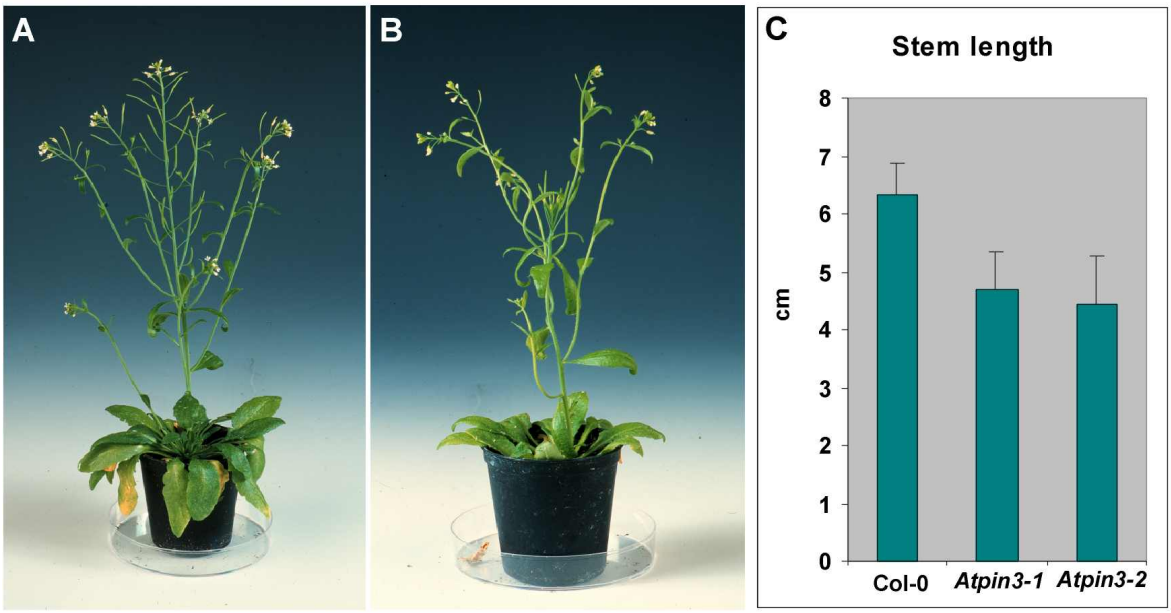


3.6.5.3 Stem length of *Atpin3* mutants

Both, the *AtPIN3* mRNA and protein were detected in endodermis cells of young inflorescence axes. To study the function of AtPIN3 in this tissue, the *Atpin3* mutants were analysed. *Atpin3* mutants and Col-0 wild type plants were grown under controlled conditions in a growth chamber. The stem lengths of three weeks old plants were measured. Significant differences between wild type and *Atpin3-1*, *Atpin3-2*, *Atpin3-3* mutants were observed. *Atpin3* plants grown in long days displayed 30 % (29±11 %, Col-0: n=51; *Atpin3*: n=54) shorter inflorescence axes than wild type plants (Figure 38). As shown in Figure 38B the inflorescence in some cases completely failed to elongate.

These data suggest that AtPIN3 plays a similar role in stems like in hypocotyls, probably regulating auxin exchange between the pith and epidermis. This hypothesis is supported by AtPIN3 protein localisation at the lateral side of endodermis cells.

Figure 38. Inflorescence axis phenotype of *Atpin3* mutants.
Three weeks old Col-0 (A) and *Atpin3* (B) plant. Comparison of the average stem lengths of Col-0 and *Atpin3* plants (C).



3.7 **Analysis of the *AtPIN4* gene**

After analysis of the *AtPIN3* gene, a detailed characterisation of the *AtPIN4* gene was performed. Similar to the *AtPIN3* gene analysis, the analysis of *AtPIN4* included expression and protein localisation studies as well as a characterisation of the *Atpin4* mutant phenotype. These analyses were performed along the same strategic guidelines as applied for *AtPIN3* analysis. Therefore, to avoid redundancy in the presentation, the description of tools will not be presented to the same detail as already done for *AtPIN3*.

3.7.1 Tools for analysis of *AtPIN4* expression and function

The starting points for analysis of the *AtPIN4* gene were the availability of a full-length cDNA (Chapter 3.2) and several mutant lines with transposon insertions in the *AtPIN4* coding sequence (Chapter 3.5.2). For detailed analysis of the *AtPIN4* expression and function, additional tools were necessary:

(1) homozygous mutant lines carrying *En-1* transposon insertions within the *AtPIN4* coding region,
(2) *AtPIN4* specific probes for Northern blot analysis and *in situ* hybridisation,
(3) antibodies to localise the AtPIN4 protein within *Arabidopsis* tissues and cells.

In the next sections, the generation and use of these tools will be briefly described.

3.7.1.1 Isolation and characterisation of homozygous *Atpin4* mutant lines

Screening of the *En-1* transposon mutagenised *Arabidopsis* plant collection revealed three independent heterozygous lines with insertions in the *AtPIN4* gene. The mutations were designated as *Atpin4-1*, *Atpin4-2*, and *Atpin4-3* (Table 8, Figure 17). In order to be able to perform a phenotypic analysis of mutant plants, it was necessary to obtain homozygous plants.

Homozygous *Atpin4* mutants were identified by Southern blot analysis in a similar way as already described in Chapter 3.6.1.1. Digestion of genomic DNA with Xba I resulted in diagnostic restriction fragments for tagged and wild type alleles with predicted sizes for the wild type allele of 2800 bp, for the *Atpin4-1* mutant allele of 3800 bp and for the *Atpin4-2* of 1800 bp. In order to visualise the diagnostic fragments after Southern blotting the nylon membrane was hybridised with a *AtPIN4* specific radiolabelled probe (corresponding to *AtPIN4* cDNA between nucleotides 1429 – 2012). To identify the *Atpin4-3* allele, the genomic DNA was digested with BamH I and the nylon membrane obtained after Southern blotting was hybridised to another *AtPIN4* specific probe (corresponding to *AtPIN4* cDNA between nucleotides 679 - 1149). This resulted in

diagnostic fragments with predicted sizes for the wild type allele of 7000 bp and for the *Atpin4-3* mutant allele of 9000 bp. We were able to distinguish wild type, heterozygous and homozygous plants in progeny of isolated heterozygous plants (data not shown). Several homozygous plants were found for each respective allele (*Atpin4-1, Atpin4-2* and *Atpin4-3*).

The homozygous *Atpin4* mutant lines were further characterised with respect to *AtPIN4* expression. In order to verify the absence of *AtPIN4* mRNA and protein in *Atpin4* mutant lines, Northern blot analysis and immunolocalisation experiments were performed. Total RNA was isolated from homozygous *Atpin4* mutant seedlings, blotted onto nylon membrane and hybridised to *AtPIN4* specific probe (nucleotides 679 - 1149). The *AtPIN4* mRNA signal was seen in heterozygous plants (Figure 39, C lane1). No *AtPIN4* mRNA was detected in *Atpin4-1, Atpin4-2* and *Atpin4-3* homozygous plants (Figure 39, C lanes 2-4). From this result it was concluded that all isolated *Atpin4* mutant lines represent null alleles. These results were confirmed by immunolocalisation experiments in wild type and mutant plants. No AtPIN4 protein was dectable in roots and embryos of homozygous *Atpin4* mutants (Figure 40, D; 42, F).

3.7.1.2 Preparation of *AtPIN4* specific *in situ* hybridisation probe

To study cell specific expression of the *AtPIN4* gene, probes for *in situ* hybridisation analysis were prepared. To generate the *AtPIN4* specific probe, a region was selected encompassing part of the *AtPIN4* cDNA from the middle, diverged region corresponding to nucleotides 679 to 1149. This region was amplified by PCR, using primers with BamH I and EcoR I restriction site adapters, similar as it was depicted for *AtPIN3* in Figure 20. The purified PCR fragment was ligated into the pBS vector between T7 and T3 promoters and used as a template for the synthesis of digoxigenin labelled sense and antisense RNA probes by *in vitro* transcription. The antisense probe was used to specifically localise the *AtPIN4* mRNA by *in situ* hybridisation experiments and the sense probe was used as a negative control.

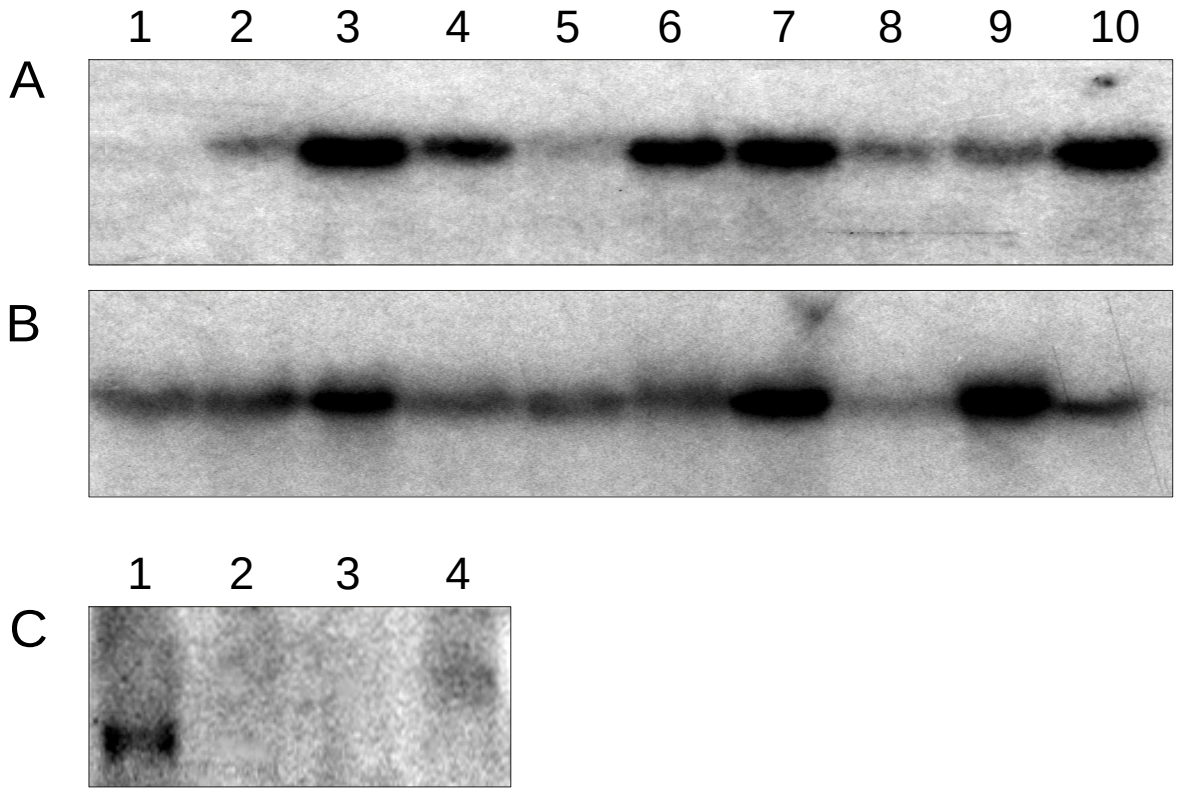
3.7.1.3 Preparation of AtPIN4 specific antibodies

Immunolocalisation of AtPIN4 protein was performed using polyclonal antibodies raised against a portion of the recombinantly expressed linker region connecting the N- and C-terminal hydrophobic segments. AtPIN4 specific antibodies were generated exactly as described for AtPIN3 antibody (Chapter 3.6.1.3). To obtain AtPIN4 antigen the part of the AtPIN4 protein between amino acids 243 to 400 was expressed as a translational fusion with 6 N-terminally placed His residues in *E. coli*. The His-AtPIN4 was produced in *E. coli,* affinity purified by Ni^{+}-NTA chromatography and used for immunisation of rabbits. Antibodies were affinity purified using His-AtPIN4 protein as affinity matrix. Both crude and affinity purified anti-AtPIN4 antibodies were characterised by Western blot analysis. The detection limit of affinity purified AtPIN4 specific antibody was around 2 ng for His-AtPIN4 antigen (data not shown).

3.7.2 Northern blot analysis of *AtPIN4* expression

Northern blot analysis of AtPIN4 expression was performed with total RNAs extracted from various *Arabidopsis* (Col-0) organs (e.g. roots, inflorescence axes, leaves, seedlings, cotyledons, siliques) and from suspension cultures harvested 4 days after subculturing. After electrophoretic separation the RNA was blotted onto nylon membrane and hybridised with an *AtPIN4* specific, radiolabelled probe corresponding to nucleotides 679-1149. The membrane was subsequently hybridised with an *ACTIN* probe to monitor sample loading. The results of the Northern blot analysis are shown in Figure 39. High levels of *AtPIN4* specific transcript were detected in cauline and rosette leaves, cotyledons and young, 6 days old *in vitro* grown seedlings. No *AtPIN4* expression was detected in suspension cultured cells. No specific signal was detected in homozygous mutant seedlings.

Figure 39. Northern blot analysis of *AtPIN4* mRNA in different *Arabidopsis* organs.
Northern blot analysis of total mRNA extracted from different *Arabidopsis thaliana* organs. Hybridisation was performed with a radiolabelled *AtPIN4* cDNA probe corresponding to nucleotides 679 to 1149. (1) Wild type suspension culture cells; (2) roots from *Arabidopsis* plants grown in liquid culture; (3) cauline leaves; (4) inflorescence; (5) siliques; (6) seedlings grown on MS agar for 6 days; (7) rosette leaves; (8) roots (9) flowers; (10) cotyledons (A). All blots were re-hybridised to a constitutively expressed *ACTIN* gene as loading control (B). Northern blot of total RNA extracted from ten days old heterozygous and homozygous *Atpin4* mutant seedlings (1) *AtPIN4::Atpin4-1*; (2) *Atpin4-1::Atpin4-1*; (3) *Atpin4-2::Atpin4-2*; (4) *Atpin4-3::Atpin4-3* (C).

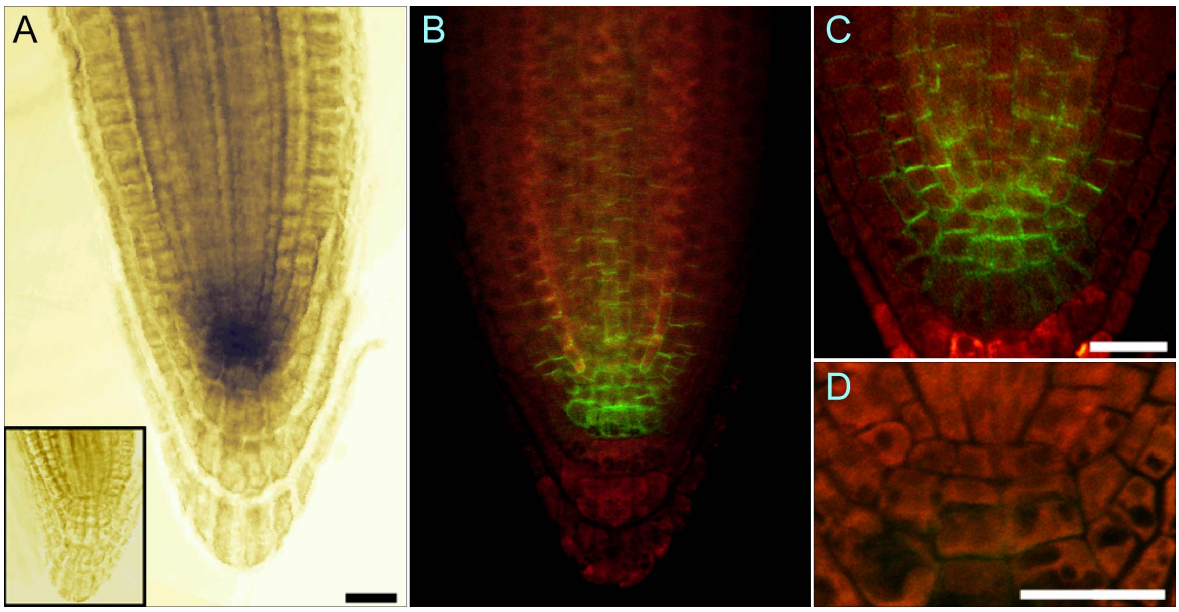


3.7.3 Localisation of *AtPIN4* mRNA and protein in roots

To localise *AtPIN4* mRNA in roots whole mount *in situ* hybridisation was performed using *AtPIN4* specific probe. The experiments were performed as described in Materials and Methods, Chapter 2.2.7.1. After visualisation of hybridisation signals with AP-conjugated anti-DIG antibodies, the *AtPIN4* mRNA was detected in the quiescent centre and surrounding cells (Figure 40, A). Hybridisation signals were observed only when antisense, but not when sense probes were used for hybridisation (Figure 40 A, inset).

Localisation of AtPIN4 protein was determined by immunolocalisation experiments using affinity purified AtPIN4 specific antibodies. The AtPIN4 signal was visualised with the FITC-conjugated secondary antibodies. Greenish signals as a result of immunolocalisation experiments were observed in the quiescent centre and surrounding cells, which is in agreement with *in situ* hybridisation results (Figure 40, B). The magnification of the region shown in Figure 40, C, shows a distinct staining of quiescent centre cells as well as of all initial cells and cells surrounding the quiescent centre. Both polar and non-polar membrane localisation of AtPIN4 protein was observed depending on cell types expressing *AtPIN4*. Cells of the columella and columella initials did not show a polar localisation of AtPIN4. Quiescent centre cells displayed not very pronounced polarity with the slight majority of AtPIN4 protein at the acropetal side of cells. In cells of proximal meristem (endodermis, cortex and vascular initials and their daughters) AtPIN4 is localised strictly polarly, at the acropetal side of cells. As expected for null mutants we did not find signals in roots from *Atpin4 -1*::*Atpin4 -1*, *Atpin4 -2*::*Atpin4 –2* and *Atpin4 -3*::*Atpin4 -3* homozygous lines indicating the specificity of AtPIN4 antibodies.

Figure 40. Whole mount *in situ* hybridisation and immunolocalisation of *AtPIN4* specific mRNA and protein in *Arabidopsis* roots.
Whole mount *in situ* hybridisation of a 4 day old seedling roots using an *AtPIN4* specific antisense probe (A). Inset shows control hybridisation with sense probe (A, inset). Localisation of the AtPIN4 protein at the plasma membrane of quiescent centre and surrounding cells (B). Enlargement showing the quiescent centre area, polar localisation of AtPIN4 in proximal meristem cells at the acropetal side and non-polar localisation in columella cells (C). Same region as in C showing roots from homozygous *Atpin4 -1::Atpin4 –1* plants (D). The *AtPIN4* mRNA is visualised by dark brown colours, whereas the AtPIN4 protein is visualised by green colour. Autofluorescence of the root tissue is indicated by red colour. Scale bars, 20 µm.



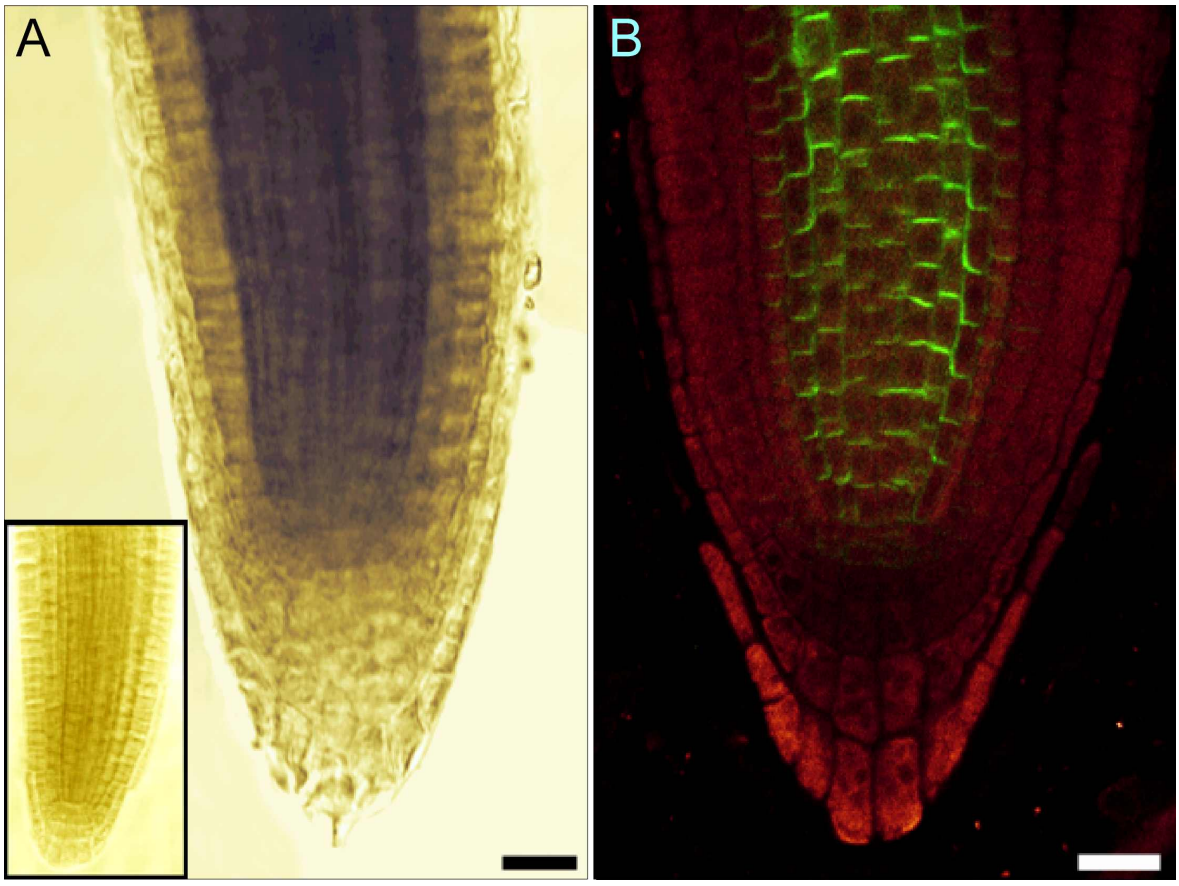
3.7.4 Localisation of *AtPIN1* mRNA and protein in roots

To address possible functional redundancy between AtPIN1 and AtPIN4 proteins in the root tip, a detailed expression analysis of *AtPIN1* in roots was performed. To localise *AtPIN1* mRNA in roots whole mount *in situ* hybridisation was used (for details see Material and Methods, Chapter 2.2.7.1). The *AtPIN1* specific probe was generated as described previously (Gälweiler *et al.*, 1998) and used for whole mount *in situ* hybridisation analysis. After visualisation of hybridisation signals with AP-conjugated anti-DIG antibodies a distinct hybridisation pattern different from *AtPIN4* was found (Figure 41, A). The antisense probe, however not the sense probe (Figure 41, A, inset), labelled the central stele of the root indicating the *AtPIN1* gene expression in this region.

For localisation of the AtPIN1 protein, antibodies raised against part of the middle hydrophilic region of the AtPIN1 protein were used. The AtPIN1 specific antibodies were described (Gälweiler, 1998; Gälweiler et al., 1998) and kindly made available by Dr. L. Gälweiler. Antibodies were affinity purified and used to infiltrate the root tissues prepared

for whole mount analysis (for details see Materials and Methods, Chapter 2.2.9.1). The AtPIN1 specific signal was detected in cells of central stele (Figure 41, B). Polar localisation of the AtPIN1 protein predominantly at the acropetal side of stele cells was observed.

Figure 41. Whole mount *in situ* hybridisation and immunolocalisation of *AtPIN1* specific mRNA and protein in *Arabidopsis* roots.
Whole mount *in situ* hybridisation of a 4 day old seedling root using an *AtPIN1* specific antisense probe (A). Inset shows control hybridisation with sense probe (A, inset). Localisation of the AtPIN1 protein. AtPIN1specific signal was detected using a FITC-coupled secondary antibodies in cells of central root stele (B). The *AtPIN1* mRNA was visualised by dark brown colours, whereas the AtPIN1 protein was visualised by green colour. Autofluorescence of the root tissue is indicated by red colour. Scale bars, 20 μm.

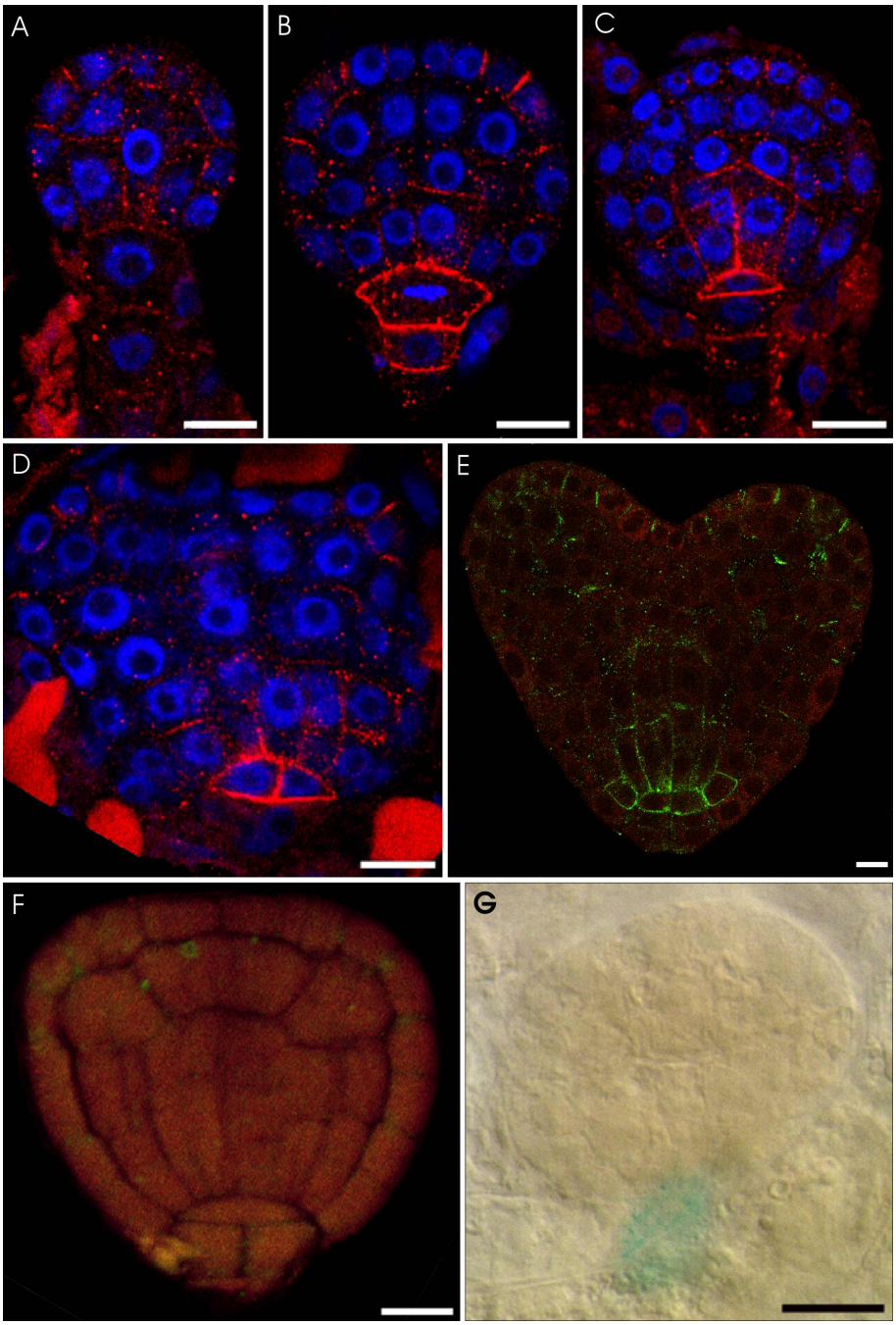


3.7.5 AtPIN4 protein localisation in embryos

To study the *AtPIN4* expression during embryogenesis, we conducted whole mount immunolocalisation studies in fixed globular and heart stage embryos using AtPIN4 specific antibodies. The results demonstrate that in early globular embryos (Figure 42, A) no specific staining was detected, whereas in a later globular stage a strong staining of the hypophysis cell, precursor of the quiescent centre, was becoming visible (Figure 42, B). The AtPIN4 was also found in the upper suspensor cell at the basal membrane. During this stage and the subsequent developmental stages additional signals became visible in the surrounding initials and daughter cells, particularly within the basal provascular cells (Figure 42, B, C, D). In the heart stage, not only the cells derived from the lenticular cell and destined to form the future quiescent centre were stained. Additionally, cells of the endodermis initials were strongly stained (Figure 42, E). No signals were observed in embryos isolated from the three different null mutant lines (*Atpin4-1*, *Atpin4-2* and *Atpin4-3*) thereby confirming the specificity of our antibodies (Figure 42, F).

Also transgenic plants were generated carrying a construct with the *AtPIN4* promoter (nucleotides - 1659 to -1) fused to the *uidA* (GUS) gene as a reporter. These plants were used to systematically study *AtPIN4* expression pattern (data not shown). Embryos were isolated from these plants and stained. The blue indigo colour in the region where the lenticular cell and upper suspensor cells are situated confirmed the specificity of the signals visualised by AtPIN4 antibodies (Figure 42, G).

Figure 42. AtPIN4 protein localisation in *Arabidopsis* developing embryos.
Embryos were isolated, fixed and whole mount immunostaining was performed according to published procedures (Steinmann *et al*., 1999). Specific signals were visualised after binding of Cy3-or FITC-labelled secondary antibodies and viewed with a Leica confocal laser scanning microscope (A-F). Staining in transgenic *AtPIN4*promotor::GUS embryos (G) was inspected with a Leica DMRB microscope equipped with differential interference contrast. AtPIN4 in early globular stage (A). Globular stage shortly before hypophysis division (B). Late globular stage (C). Triangular stage (D). Heart stage (E). Triangular stage of *Atpin4 -3::Atpin4 -3* homozygous embryo (F). Globular stage: GUS staining of transgenic embryos carrying an *AtPIN4*promotor::GUS construct (G). AtPIN4 localisation is indicated by red colour (Cy3-conjugated secondary antibody; A-D) or by green colour (FITC-conjugated secondary antibody; E, F). The DNA of nuclei was stained with DAPI (A-D) indicated by blue colour. Autofluorescence of tissue (D, E) is indicated by brown colours. Scale bars, 10 µm.



3.7.6 Disruption of *AtPIN4* affects DR5 expression and patterning in *Arabidopsis* root

To test the hypothesis, whether the *AtPIN4* gene plays a role in auxin transport, the possible alterations in the pattern of auxin accumulation in the root tip after loss of AtPIN4 function were studied. Transgenic plants carrying a DR5::GUS construct have already been successfully used to visualise auxin accumulation in transgenic *Arabidopsis* plants (Sabatini *et al*., 1999). Possible changes in auxin accumulation resulting from *AtPIN4* activity were examined here using a similar strategy. For practical reasons another reporter construct was used consisting of a reverted auxin responsive element TGTCTC ninemer (Ulmasov *et al*., 1997) fused to the CaMV35S minimal promotor (DR5rev) and the PEH A reporter gene (Dotson *et al*., 1996), which was kindly provided by Dr. E. Benkova. This construct was introduced in both wild type and *Atpin4* mutant plants and DR5rev activity in the presence and absence of IAA was monitored. In addition the staining of columella cells with lugol was studied to demonstrate possible changes in cell patterning.

In 7 days old *Arabidopsis* wild type roots the Dr5rev::PEH A reporter was found to be active in columella initial cells and the subsequent cell layers (Figure 43, C). In *Atpin4* mutant roots, however, DR5rev::PEH A reporter activity was shifted to a zone surrounding the quiescent centre and even up into the vascular initial cells (Figure 43, D). Treatment of wild type roots with 1 µM NPA, a substance supposed to block auxin transport, had a similar consequence resulting in a shift and spatially broader activation of DR5rev::PEH A (Figure 43, E). These results suggest that both auxin transport inhibition and *Atpin4* mutation change the auxin gradients and maximal DRrev::PEH A reporter activity in the *Arabidopsis* root meristem.

Even more dramatic changes in DR5rev reporter activity were observed when wild type or *Atpin4* plants carrying the DR5rev::PEH A construct were incubated with 10 µM IAA for 5 hours. For wild type roots a maximum of DR5rev::PEH A activity was observed around the quiescent centre and the columella initial cells (Figure 43, F). This qualitatively did not differ from IAA untreated roots suggesting that the cellular machinery controlling cellular auxin levels was able to cope with the exogenously applied IAA levels and to maintain apparently normal endogenous auxin gradients. In contrast, in *Atpin4* mutants treated with exogenous IAA, the DR5rev::PEH A maximum shifted above the quiescent centre and the vascular initials with a concomitant dramatic increase in staining across the whole root tip (Figure 43, G). This means that *Atpin4* mutant plants are not able to cope with exogenously applied IAA like wild type plants. This suggests that disruption of the *AtPIN4* gene results in a strong defect in control of auxin levels. This finding was further supported by experiments, where IAA was applied simultaneously with NPA. In this combination the *Atpin4* mutant phenotype was phenocopied in wild type plants (data not shown).

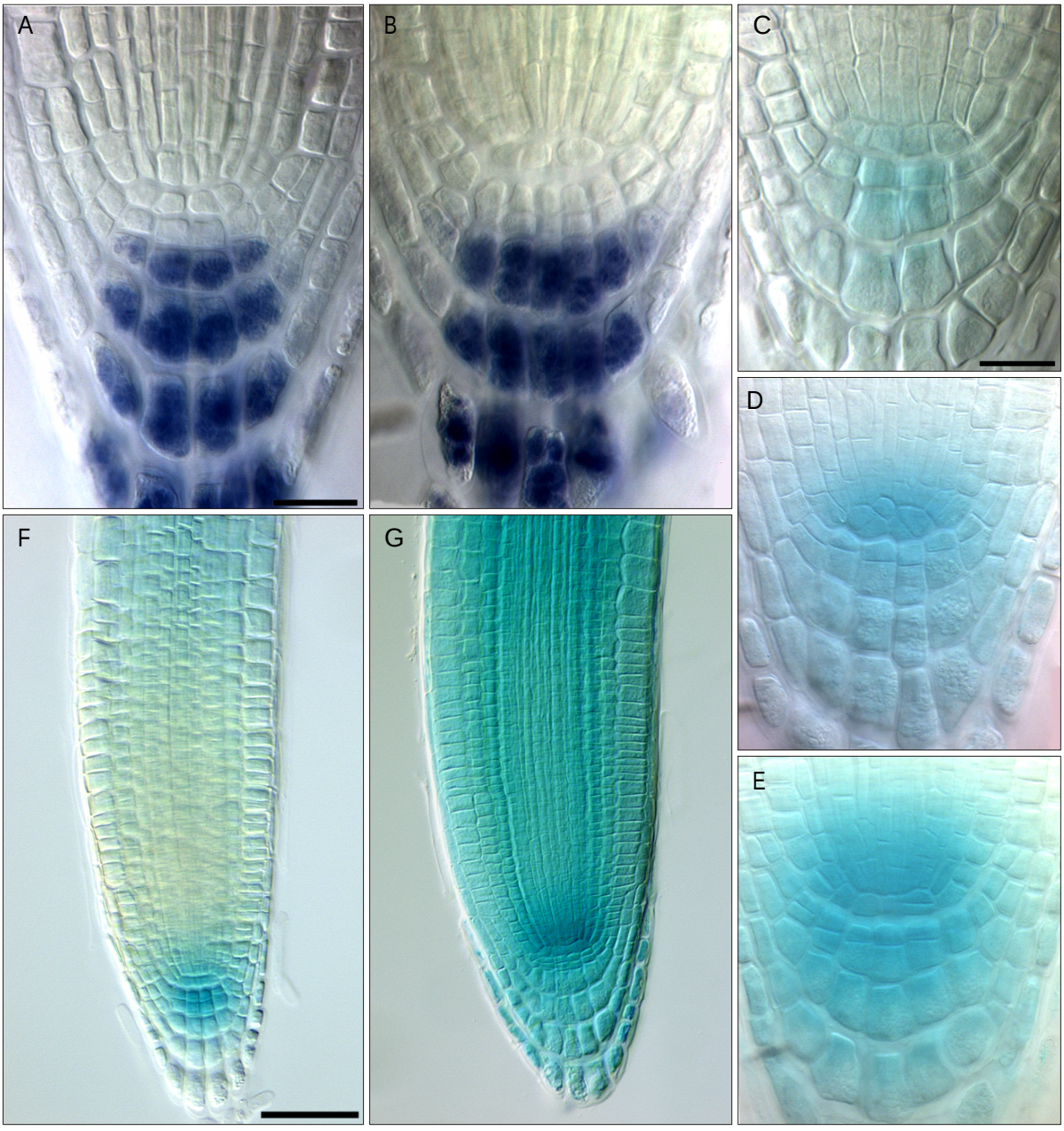
These interesting observations suggest different cellular auxin levels in the various tissues. In collaboration with Prof. G. Sandberg auxin and auxin conjugate levels are currently directly measured by liquid chromatography coupled to mass spectroscopy.

The changes in auxin distribution and gradients are known to be connected to morphogenetic defects (Sabatini *et al*., 1999). Therefore changes in cell patterns within the *Atpin4* mutant root meristem were examined. The cell identity of differentiated columella cells was determined by staining of 6 days old wild type and *Atpin4* mutant roots with lugol solution and the pattern of root meristem was inspected using a microscope equipped with differential interference contrast (Figure 43, A, B). The following aberrations in *Atpin4* mutant comparing to wild type roots were found:

(1) the normally well defined quiescent centre is missing, cells in positions of the wild type quiescent centre divided in irregular planes,
(2) cells of the distal root meristem have aberrant shapes,
(3) more tiers of columella initials were observed,
(4) more cell files of differentiated columella cells were found.

This cell patterning phenotype of *Atpin4* mutant can be phenocopied by growing wild type plants on 1 μM of the polar auxin transport inhibitor NPA. These results provide another functional link between AtPIN4 function, polar auxin transport and cell patterning in *Arabidopsis* root.

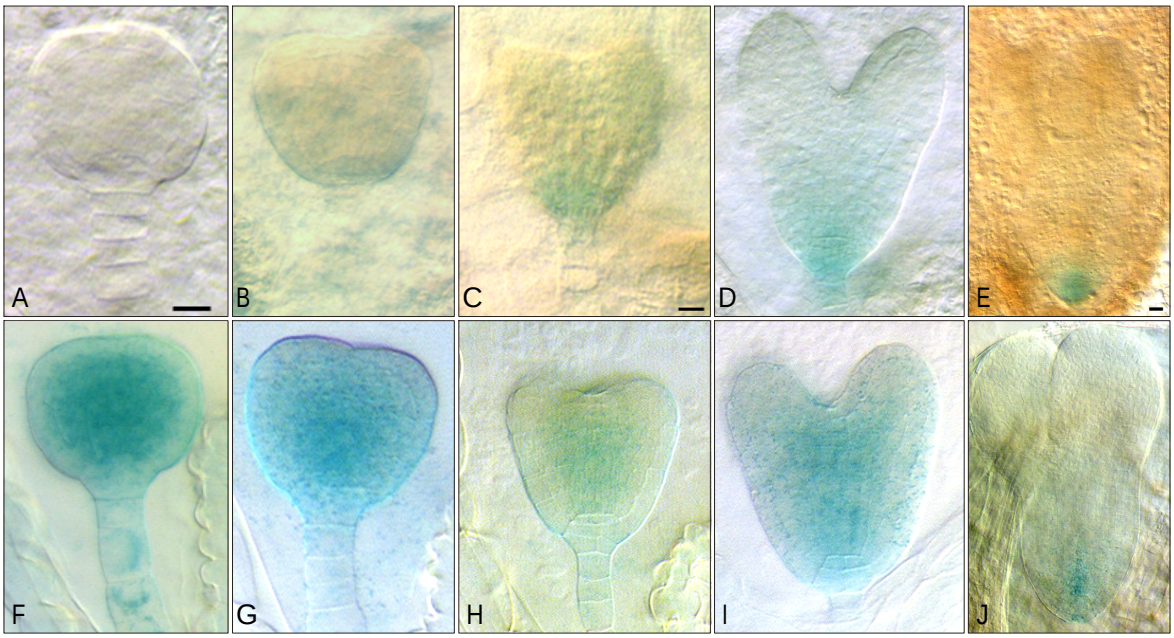
Figure 43. Lugol staining and analysis of DR5rev::PEH A expression in wild type and *Atpin4* roots.
The cell pattern of wild type and *Atpin4* mutant roots was studied. Root tips from wild type and *Atpin4-1* plants were treated with lugol to stain starch containing columella cells (A, B). DR5rev::PEH A activity was analysed in root tips of transgenic wild type (*Arabidopsis* Col-0) plants (C), *Atpin4-1* plants (D), and in wild type plants treated with the polar auxin transport inhibitor NPA (E). DR5rev::PEH A reporter activity was analysed in root tips of transgenic wild type (*Arabidopsis* Col-0) plants (F) *Atpin4-1* plants (G) after incubation with10 µM IAA for 5 hours. Lugol stained starch is dark violet coloured (A, B), while PEH A activity is marked by blue colour (C-G). Scale bars 20 µM (A-E), 50 µM (F, G).



3.7.7 Disruption of *AtPIN4* affects DR5 expression in *Arabidopsis* embryo

DR5rev::PEH A has proven to be a useful reporter of auxin levels in *Arabidopsis* seedlings (Dr. Benkova, pers. com.). This reporter allowed visualisation of differential auxin levels in wild type and *Atpin4* roots (Figure 43). It was important to test whether embryos of wild type and *Atpin4* mutants express different PEH A patterns. Figure 44, A-E demonstrates that wild type embryos express PEH A only very weakly at heart stage at the basal part of the embryo. Staining intensity was increased in later heart and torpedo stage embryos (Figure 44, D, E). In contrast, *Atpin4* embryos showed a strikingly different PEH A pattern with a very strong staining already in the globular embryo. Staining of the central inner, probably provascular regions, was reduced during progression to torpedo stage. These staining patterns demonstrate that probably due to lack of *AtPIN4* function auxin levels were higher resulting in elevated levels of auxin upregulated PEH A reporter activity. Moreover in wild type embryos basaly localised auxin maximum was in *Atpin4* embryos mislocalised. These data strongly indicate that AtPIN4 is involved in auxin transport process(es) important for early *Arabidopsis* development. More detailed studies aiming to dissect its influence on other, morphogenetic processes are currently underway.

Figure 44. Auxin distribution in wild type and *Atpin4* mutant embryos.
Embryos of transgenic wild type and *Atpin4-1* plants carrying the auxin sensitive promoter DR5rev::PEH A construct were analysed for PEH A reporter gene activity as described in Materials and methods. PEH A reporter activity was monitored in wild type (A-E) and *Atpin4* null mutants (F-J). (A, F) globular embryos; (B, G) triangular embryo; (C, H) early heart stage; (D, I) heart stage; (E, J) torpedo stage. Scale bars, 10 μM.



4 Discussion

"Auxins regulate plant growth and development" is a sentence that has been repeatedly written in numerous articles and reviews dealing with this pleiotropic plant growth regulator (e.g. Reinecke and Bandursky, 1987). Despite enormous progress in the dissection of hormonally regulated signal transduction networks of yeast and higher animals we still know only very little on similar networks in plants. In particular, how auxin molecularly mediates its effects is still a "black box". This apparent lack of knowledge on the molecular mechanisms of auxin signalling is puzzling, as there exists a hiatus in knowledge in this field, particularly concerning the responses of plants to external indole-3-acetic acid applications. There is less knowledge available on how auxin is synthesised and even less on how it is perceived and transduced in its final physiological reactions. Surprisingly, much more is known about its transport from assumed biosynthetically active sites to its sites of action (Lomax *et al*., 1995). Overall this is a curious notion probably reflecting the fact that most of these data are a result of "spray and pray" experiments in which auxins or specific chemical inhibitors were externally applied and physiological effects monitored. Most of these experiments were technically simple, cheap and unpretentious. Nevertheless, when some of the observations gained by these approaches were re-evaluated by using genetically engineered plants in which phytohormone concentrations were changed from within by expression of bacterial hormone biosynthetic genes a surprisingly good correlation was found (for discussion see Palme *et al*., 1991; Spena, 1993).

It has also become clear from a wide range of studies that the profound effects of auxin on turgor, elongation, cell division, differentiation, programmed cell death and morphogenetic processes such as those on lateral roots and vascular tissues, formation of adventitious roots and trichomes, branching of shoots, or tropic responses are at least partially mediated by controlling the expression pattern of a number of important target genes (for recent review see Sitbon and Perrot-Rechenmann, 1997). Understanding the signal transduction cascade leading to altered gene expression holds the key in world wide attempts to elucidate the primary mechanism(s) of auxin action. An array of genetic and molecular experimental strategies is currently applied by a number of laboratories to address this important question. At a recent EMBO workshop (Estelle *et al*., 2000) the most advanced outcome of these experimental efforts with rather confusing than enlightening results was extensively discussed. It became clear that there is an ever increasing number of genes encoding auxin regulated transcription factors (i.e. the IAA and the ARF gene families; for review see Abel and Theologis, 1996; Sitbon ad Perrot-Rechenmann, 1997). Most of these genes are primary response genes acting independently of protein synthesis. This implies that the cellular machinery for their activation is already pre-existing and that the signal transduction pathways activated by auxin transduce their signals to the nucleus probably via post-translational processes. We know currently very little about these pathways. The best data yet available are those suggesting an important role for an auxin modulated proteolytic pathway responsible for negative regulation of the stability of nuclear repressors or transcription factors. But how is auxin linked to these

cellular signalling and proteolytic pathways? What are the receptors for auxin? The search for auxin receptors has not yet led to the discovery of proteins, which convincingly fit the general concepts of hormone receptors. On the other hand we do know that ethylene, another important phytohormone, does act through binding to receptors sharing significant homologies with the bacterial two-component signalling systems (Chang and Meyerowitz, 1994). Does this mean that auxin perception is different from other phytohormones? Rainer Hertel once speculated that it might not be unlikely that auxin acts not through "classic" receptors but instead the auxin efflux carriers act as primary receptors (Hertel, 1983). This was an interesting speculation far ahead even todays experimental evidence in the auxin field. Nevertheless, it is interesting to note that the concept of dual functions of solute carriers in both transport and sensing is currently intensively discussed for plant and yeast sugar carriers (Lalonde *et al*., 1999). Thus, with auxin efflux carrier proteins and their genes becoming molecularly available, Hertel's hypothesis will soon become testable.

Arguments to study auxin transport

Extensive research over several decades has revealed the importance of polar auxin transport in plant development. (Lomax *et al*., 1995; Palme and Gälweiler, 1999; Palme, 2000). In view of the past and present problems to formulate a convincing conceptual framework for auxin perception and action, it was recently predicted that from molecular dissection of auxin transport, important aspects of auxin action will become clear (Palme and Gälweiler, 1999). With the help of mutants impaired in auxin transport these experiments could be designed to address more specific questions on auxin action. Equally well one could envision that through mutants in the auxin delivery system some developmental patterning responses obscured by pleiotropic auxin effects may become genetically dissectable. In this light the recent cloning of genes encoding important polarly localised auxin transport proteins were considered as breakthroughs (Gaelweiler *et al*., 1998; Müller *et al*., 1998; Steinmann *et al*., 1999). An important consideration in designing future approaches to dissect auxin action and to address questions whether auxin efflux carriers indeed represent the elusive auxin receptors were questions regarding the family of genes sharing similarity with initially cloned genes and their functional uniqueness or redundancy. Cloning of new members of this family from *Arabidopsis* and their molecular genetic analysis could pave the way for a more detailed understanding of the molecular basis of several important aspects of auxin action.

Therefore this work deals with the characterisation of new members of the *AtPIN* gene family. The general strategy for this work is summarised in Figure 6 involving, besides identification and characterisation of *AtPIN* related genes, the isolation of mutants in some of these genes and their detailed molecular and functional characterisation.

4.1 **Identification of new members of the *AtPIN* gene family**

In the post-genomic era when complete genome sequences of many organisms stored in data bases are readily available for *in silico* analysis, it is easy to identify gene families and study their characteristics. Although sequencing of the *Arabidopsis* genome will come much earlier then originally expected to completion (probably by the end of this year) we are still facing large genome regions unsequenced. When we embarked on this project three years ago, we had to use rather conventional strategies to identify all members of the *AtPIN* gene family. Instead of using for example genomic lambda clone or cosmid libraries we chose a BAC library for our studies. BAC clones contain much larger (100 kb) regions of genomic DNA than cosmids, are relatively easily grown, amplified, or stored in microtitre plates. The IGF BAC library seemed best suited for our purpose because of its high coverage of the *Arabidopsis* genome (about 7-fold; Mozo *et al*., 1998). Using probes derived from *AtPIN1* and *AtPIN2* we identified a large number of BAC clones as described in Chapter 3.1.1. Are all of these clones related to *AtPIN* genes? As most of the IGF BAC clones are not sequenced yet, we had to develop criteria, which allowed a pre-selection among the many signals. In fact, by considering only those signals as positive signals which were found at least in two different BAC clones within a contig, we could reduce the number of potential candidate BACs to about twelve. Our mapping analysis using known BAC contigs suggests the presence of probably 12 *AtPIN* genes in *A. thaliana*, an estimation however solely resting on the analysis of hybridisation and mapping of the IGF BAC clones with AtPIN specific probes (http://www.mpimp-golm.mpg.de/101/mpimp_map/bac.html). Several possible pitfalls possibly causing misinterpretations have to be considered. It is well known that BAC libraries contain with a frequency of approximately 1 % chimeric clones which can cause incorrect clone mapping and thereby artificially increase the number of identified loci (Osoegawa *et al*., 2000). Another problem can be a lack of specificity of *AtPIN* probes resulting in some cross hybridisation with unrelated genomic sequences. Alternatively, due to the low hybridisation stringency applied for our BAC hybridisation with AtPIN, association of *AtPIN* with unrelated genomic sequences might even have been favoured. Moreover, some PIN homologues may show only very little similarity on the DNA level, but relatively high homology on the protein level. This fact became indeed evident when the *AtPIN5* gene was found in the data bases. This gene was not identified by cross hybridisation with conserved probes derived from *AtPIN1* and *AtPIN2* sequences because of DNA sequence divergence. Despite all these drawbacks our screening strategy successfully identified at least five new *AtPIN* genomic sequences (*AtPIN3 – AtPIN7*) from the IGF BAC clones. Whether the additionally identified signals represent also new *AtPIN* genes (*AtPIN8 – AtPIN12*) or whether the other less well characterised signals found in our hybridisation experiments represent less conserved *AtPIN* genes or no *AtPIN* genes at all was outside the scope of this work and remains to be determined later.

4.2 **Mapping of new *AtPIN* genes**

The *AtPIN3 – AtPIN7* genes were mapped within the *A. thaliana* genome using information present in the IGF BAC database (Mozo *et al*., 1998; http://www.mpimp-golm.mpg.de/101/mpi_mp_map/bac.html). The evaluation of our hybridisation results allowed to determine genomic positions for these genes as well as for the other putative homologues *AtPIN8-AtPIN12*.

An interesting question was whether some *AtPIN* genes were clustered in the *Arabidopsis* genome. Several reports have presented information about the existence of gene clusters in plants (Jia *et al*., 1997, Martin *et al*., 1993, Salmeron *et al*., 1996). These authors reported that genes with identical or similar functions were often placed in the genome close to each other on chromosomal stretches of up to 50 kb. This was observed for both tomato and *Arabidopsis*. Progress in sequencing of the *A. thaliana* genome allowed to address this question in more detail. It was found that between 10 to 20 % of all predicted ORFs apparently occur as clusters of related genes, indicating that local sequence duplication and subsequent divergence may generate the gene pool from which gene families evolve (Wambutt *et al*., 2000).

Our mapping data of members of the *AtPIN* gene family fully support these observations. Three *AtPIN* genes (*AtPIN1*, *AtPIN3* and *AtPIN6*) were in close proximity to each other between 110-115 cM from the top of chromosome I. High sequence and also possible functionally homology (demonstrated for *AtPIN1* and *AtPIN3* genes) suggests that these genes indeed could create a functional cluster.

Another interesting question was whether already characterised mutants related to phytohormone action map closely to the position of new *AtPIN* genes. Such mutants could be functionally related or even allelic to new *AtPIN* loci. This was already demonstrated for the *Atpin2* mutant, which was allelic to *agr1*, *waw6* and *eir1* mutants (Müller *et al*., 1998).

The *AtPIN3* gene was mapped 103 cM from the top of chromosome I, close to the *AtPIN1* and *AtPIN6* genes. Another gene called *TIR2* maps at the same position (Jensen *et al.*, 1998). The *tir2* mutant was isolated from a screen for auxin transport inhibitors resistant plants (Ruegger *et al.*, 1997). The phenotype of the *tir2* mutant displays very similar features to the *Atpin3* mutant phenotype (e.g. shorter hypocotyl in light grown seedlings and impaired hypocotyl gravitropism). The question, whether *AtPIN3* and *TIR2* are allelic, is currently tested but still awaits final analysis. The sequencing of the *AtPIN3* gene in *tir2* mutant background as well as allelic tests between *Atpin3* and *tir2* are underway.

The *AtPIN4* gene mapped around 1.8 cM from top of chromosome II. Two mutants called *emb91* and *emb34* map in the same region (Meinke *et al.*, 1979). Both mutants display an embryo lethal phenotype. However, they have not been cloned yet. A possible functional relationship is supported by the fact that the *AtPIN4* gene is strongly expressed

during embryogenesis but no firm conclusions can be drawn before a detailed genetic analysis is performed and completed.

The *AtPIN5* gene was mapped 33 cM down from top of chromosome V. Two mutants called *nph4* and *tir7* map at the same area (Stowe-Evans *et al.*, 1998, Ruegger *et al.*, 1997). The *nph4* mutant displays defects in auxin sensitivity and phototropism. *tir7* mutant roots are more resistant to auxin transport inhibitors. Both mutants have not been cloned yet and their possible functional connection to *AtPIN5* is still unclear. Close to the *AtPIN7* - *AtPIN12* loci no possible related mutants have been identified.

The analysis of novel *AtPIN* genes not only revealed gene clustering for some of these genes but also identified interesting mutants close by with defects resembling some of the properties observed for mutants in *AtPIN* genes. We have however to await the outcome of the ongoing allelism tests and sequence analysis before final conclusions can be drawn.

4.3 *AtPIN3-AtPIN7* cDNAs and predicted AtPIN proteins

For functional studies it was important to have full-length cDNAs for all *AtPIN* genes. To isolate these cDNAs from various cDNA libraries specific probes were derived from partial BAC clone sequences and used for hybridisation under low stringency conditions. Several cDNAs corresponding to each of the *AtPIN3* - *AtPIN7* genes were isolated and characterised at the molecular level.

All cDNAs except *AtPIN5* contained typical sites for transcription starts, translation starts, stop codons and polyadenylation signals. As consensus transcription start sequence CTCATCA was reported to occur upstream of the translation start (Joshi, 1987). The translation start sequence contains a conserved purine nucleotide at the -3 position and an ATG start codon for methionine fitting the consensus motifs identified earlier (Joshi, 1987). At the 3' -terminus the stop codon TAA or TGA terminates the coding region. In addition, polyadenylation signals like AATTAAA and poly(A)$^{+}$ tail sequence are placed downstream in the untranslated region (UTR).

The *AtPIN3* cDNA was isolated from leaf and whole plant cDNA libraries. This correlated well with the broad expression in various tissues found in Northern blotting experiments. The *AtPIN3* gene encodes a protein of 640 amino acids. The transcription start was predicted at position −60 as CTCACTT sequence. The translation start sequence contains an adenine at position −3 and an ATG start codon. The stop codon TAA is placed at position 1921 downstream of the ATG. A polyadenylation signal AATTAAA occurs at position 2201.

The *AtPIN4* cDNA was found in whole plant, guard cell and root cDNA libraries. The *AtPIN*4 gene encodes a protein of 616 amino acids. The transcription start sequence CTCATCA was predicted at position -30. The translation start sequence contains as an

adenine at position –3 and ATG coding for methionine. The stop codon TGA is at position 1837. A polyadenylation signal was identified at position 1912.

The *AtPIN5* cDNA was isolated from whole plant cDNA library. Interestingly, this gene appeared to be expressed at lower levels when compared to other *AtPIN* genes. The *AtPIN5* gene encodes a protein of 352 amino acids. A transcription start sequence CCTATC occurs at -160 position. The translation start sequence contains an adenine at position –3 and an ATG codon for methionine. The stop codon TGA was identified at position 1054. In contrast to other *AtPIN* cDNAs, the homology with other AtPIN sequences was particularly weak around the 3'-terminus (see Figure 13). No polyadenylation signal was found in the cDNA. Therefore the possibility, that the 3' prime end was not isolated, can not be excluded.

The *AtPIN6* cDNA was isolated from a seedling cDNA library. The *AtPIN6* gene encodes a protein of 570 amino acids. The transcription start sequence CTCATA was identified at position -80. The translation start sequence contains an adenine at position –3 and ATG codon for methionine. The stop codon TGA is at position 1813. A polyadenylation signal was identified at position 1853.

The AtPIN proteins share 65-80% identitical amino acids. The N- and C-termini are more conserved (85% of identity), in contrast to the less conserved middle part. Within the *AtPIN* gene family, a subfamily of more related members can be distinguished. This however does not correlate with the *AtPIN* gene clusters found. Protein topology prediction software identified 4-5 transmembrane domains at the N- and 4-5 transmembrane domains at the C-terminal region. It seems that the transmembrane domains and their spatial organisation are crucial for PIN proteins as these regions are highly conserved. Moreover, several point mutations affecting the function of the *AtPIN2* gene were detected only in sequences encoding transmembrane domains (Utsuno *et al.*, 1998). The role of the hydrophilic loop connecting both hydrophobic regions is still unclear. This region could be important for (*i*) the regulation of transport activity of the protein, (*ii*) determination of the affinity or specificity for substrates, (*iii*) for specific interactions with other proteins or (*iv*) specific regulation of PIN activity by cellular signalling networks.

4.4 Isolation of *Atpin3 – Atpin7* mutants

Mutants are powerful tools for the elucidation of gene function. Typical mutant collections used in plant research are plant collections mutagenised by either EMS, T-DNA or transposons. EMS mutagenesis causes mainly point mutations by single base pair exchange and enables to achieve large numbers of alleles with different single nucleotide changes. This allows to elucidate the role of individual amino acid residues in protein function (Hoffmann, 1980). The cloning of EMS mutagenised genes has been tedious in the past, but the sequencing of the *A. thaliana* genome and the development of SNP based technologies will in close future speed up this process significantly thereby opening

tremendous opportunities for gene-function studies (Cho *et al*., 1999; Mc Callum *et al*., 2000).

The main advantages of T-DNA or transposon induced mutations are (i) the possibility of rapid cloning of the targeted genes identified in forward screens, and (ii) the relatively easy application of the reverse genetic methodology for functional analysis of already cloned genes. The maize *En-1* transposon based *Arabidopsis* mutant collection was used for reverse genetic screening of mutations in *AtPIN3 – AtPIN7* genes (Wisman *et al*., 1998). The advantage of this collection is the relatively large number of insertions (about 10 to 15 per plant) in a relatively small number of lines (app. 8.000). This enables to perform efficiently and rapidly reverse genetic screens. A drawback of this technology is that individual lines usually carry several independent mutations. This frequently causes difficulties in the interpretation of phenotypes. On the other hand, the mobility of the *En-1* transposon can be used to identify gene functions because of reversion of mutant phenotypes to wild type due to *En-1* excision. Occasionally, the excision of *En-1* transposon causes foot print mutations within targeted genes by deletion or insertion during the excision process (Wisman *et al*., 1998). This property of the *En-1* transposon was successfully used in this work to isolate stable mutations (e.g. *Atpin3-3*).

The *En-1* transposon mutant collection was screened in order to find lines with disruptions in *AtPIN3 – AtPIN7* genes. In total, ten lines have been identified with insertions in *AtPIN3 – AtPIN7*. These lines were further characterised. The precise position of the *En-1* transposon within the gene was determined by sequencing of flanking regions. The absence of *AtPIN3 - AtPIN7* specific transcripts was confirmed by Northern blot analysis and, for AtPIN3 and AtPIN4, the absence of the respective proteins was further confirmed by immunolocalisation experiments.

AtPIN3. Two mutations within the *AtPIN3* gene were identified and designated *Atpin3-1* and *Atpin3-2*. The *En-1* transposons were found to be inserted at positions 706 and 1173, respectively. A stable foot print mutation was detected within the *Atpin3-1* progeny and designated as *Atpin3-3*. Two plants from the *Atpin3-2* progeny were identified, in which the excision of *En-1* transposon caused reversion to wild type. All *AtPIN3* mutant alleles were characterised by Northern blot analysis and immunolocalisation experiments and were found to be null mutations. Homozygous *Atpin3* plants were identified by Southern blot analysis and used for phenotypic characterisation. The availability of two independent null mutant lines and phenotypic revertants allowed a correlation between aberrant phenotypes and mutations in the *AtPIN3* gene. Moreover, the isolated stable foot print mutation will be useful for elucidation of genetic interactions with other genes in double or other combinatory mutants.

AtPIN4. Three mutations within the *AtPIN4* gene were identified and designated as *Atpin4-1, Atpin4-2* and *Atpin4-3*. The *En-1* transposons were inserted respectively at positions –35, 1003 and 1578, respectively. All *AtPIN4* mutant alleles were characterised by Northern blot analysis and immunolocalisation experiments and found to be null mutations. The *Atpin4* homozygous plants were identified by Southern blot experiments and used for phenotypic characterisation (Friml *et al*., 2000). For future research the isolation of stable *Atpin4* mutation will be needed. This can be achieved either by reverse

genetic screening in stable transposon mutant subpopulations or by identification of stable foot print mutations in the progeny of *Atpin4-1*, *Atpin4-2* or *Atpin4-3*.

AtPIN5. Two mutations within the *AtPIN5* gene were identified and designated as *Atpin5-1* and *Atpin5-2*. The *En-1* transposons were inserted at positions 203 and 506 respectively. The characterisation of *AtPIN5* mutant alleles by Northern blot analysis and immunolocalisation experiments are still in progress. Preliminary results show that both *Atpin5* mutations are very unstable. Therefore the screening for other stable mutations in this gene such as T−DNA insertions have been started.

AtPIN6. One mutation within the *AtPIN6* gene was identified and designated *Atpin6-1*. The *En-1* transposon was inserted at position 1227. The *Atpin6* homozygous plants were identified by Southern blot analysis and used for phenotypic characterisation (Friml, unpublished results). However, although the *Atpin6-1* mutant can be used for the analysis of the *AtPIN6* function in plant development, the isolation of additional, independent alleles would considerably facilitate this analysis.

AtPIN7. Two mutations within *AtPIN7* gene were identified and designated *Atpin7-1* and *Atpin7-2*. The *En-1* transposons were inserted at positions 140 and 707, respectively. Both *AtPIN7* mutant alleles were characterised by Northern blot analysis and were found to be null mutations. The *Atpin7* homozygous plants were identified by Southern blot analysis and used for phenotypic characterisation (Friml, unpublished results). However, as for the other *AtPIN* gene mutations we need stable *Atpin7* alleles for further analysis. These screenings are still ongoing.

Up to now, the phenotypic analysis for the *Atpin3* and the *Atpin4* mutant (Friml et. al., 2000) have been completed. The characterisation of the other *Atpin5*, *Atpin6* and *Atpin7* mutant phenotypes is still in progress.

4.5 Are all PIN proteins involved in polar auxin transport?

The AtPIN1 and AtPIN2 proteins were discussed to play a role in polar auxin transport especially in auxin efflux (Okada *et al*., 1991, Gälweiler *et al*., 1998, Steinmann *et al*., 1999, Müller *et al*., 1998; Luschnig *et al*., 1998; Chen *et al*., 1998; Utsuno *et al*., 1998, Palme and Gälweiler, 1999). Until now only indirect evidences have accumulated supporting this hypothesis. A role of these proteins in auxin transport is favoured by:

- phenotypes of *Atpin* mutants,
- polar localisation of AtPIN proteins in auxin transport competent cells,
- disruption of AtPIN protein functions causing changes in auxin responsive promotor activity,
- heterologous expression of AtPIN1 and AtPIN2 proteins in yeast,
- homology of AtPIN proteins with prokaryotic transport proteins.

Below, some more details are listed illustrating the putative role of the studied *AtPIN* genes/proteins in auxin transport processes.

The phenotypes of *Atpin* mutants.

The strongest evidence supporting a role of *AtPIN* genes in auxin efflux came from the analysis of *Atpin* mutants. In addition, most of the alterations in plant growth and development caused by disruption of AtPIN protein function can be phenocopied by treatment of wild type plants with polar auxin transport inhibitors suggesting their direct or indirect involvement in auxin transport.

Atpin1 mutant: The knitting needle like stem and the lack of lateral organs are the main characteristics of *Atpin1* mutant phenotype. The *Atpin1* mutant plants resemble strongly wild type plants growing on polar auxin transport inhibitors (Gälweiler *et al*., 1998). Most importantly, *Atpin1* mutant plants show a drastic reduction of basipetal auxin transport in stems (Okada *et al*., 1991).

Atpin2 mutant: It has been demonstrated that root gravitropism is abolished by inhibition of polar auxin transport (Lomax *et al*., 1995). Roots of *Atpin2* mutant failed to respond to gravity. Analysis of this mutant supports the Cholodny-Went theory indicating that polar, lateral and backward auxin transport is important for this response (Müller *et al*., 1998; Luschnig *et al*., 1998; Chen *et al*., 1998; Utsuno *et al*., 1998).

Atpin3 mutant: It has been demonstrated that auxin transport is involved in root and hypocotyl elongation processes in light grown, but not in dark grown *Arabidopsis* seedlings (Jensen *et al*., 1998). Hypocotyls and roots of *Atpin3* mutants are shorter in comparison to wild type plants. This phenotypic difference between *Atpin3* and Col-0 seedlings is prominent only in light grown plants. Also apical hook opening, hypocotyl gravitropism and hypocotyl phototropism are altered in *Atpin3* mutants in a similar way as when wild type plants are grown on inhibitors of auxin transport.

Atpin4 mutant: Recently auxin gradients and auxin transport have been reported to be crucial for maintaining correct root meristem patterns (Sabatini *et al*., 1999). Moreover, treatment of *A. thaliana* seedlings with polar auxin transport inhibitors caused severe changes in root meristem patterns. Interestingly, *Atpin4* mutant plants show drastic changes in expression pattern of an auxin sensitive synthetic promotor as well as patterning defects in embryos and root meristems (Friml *et al*., 2000).

Atpin7 mutant: *Atpin7* mutant roots show in the presence of exogenous NAA or IAA significantly higher inhibition of elongation than wild type roots. This suggests an increased sensitivity of *Atpin7* mutants to exogenous applied auxins (Friml, unpublished results). This mutant phenotype again seems to be best explained by a defect in auxin transport.

Polar localisation of AtPIN proteins in auxin transport competent cells.

Auxin efflux carrier proteins were hypothesised for a long time to have a polar localisation in auxin transport competent cells. Still it was considered to be a surprise when the AtPIN1 protein was localised at the basal side of elongated parenchymatous xylem and cambial cells of *Arabidopsis* inflorescence axes (Gälweiler *et al*., 1998; Jones, 1998), as well as on the acropetal side of stele cells in the root (Friml *et al*., 2000). These are the cells, where basipetal and acropetal auxin transport in stems and roots were proposed to take place (Lomax *et al*. 1995). In excellent agreement with previously formulated models, the AtPIN2 protein was found polarly at the basipetal side of lateral root cap and epidermis cells, where another backwards directed polar auxin transport stream was proposed to run basipetally from the columella through the root epidermis towards the distal elongation zone (Ohwaki and Tsurumi, 1976).

Disruption of AtPIN protein function causes changes in auxin responsive promotor activity.

The synthetic auxin responsive promotor containing auxin responsive promotor elements TGTCTC (DR5, Ulmasov *et al*., 1997) fused with the GUS reporter gene was introduced into the *Atpin1* and *Atpin2* mutant plants. Activity of DR5 construct was demonstrated to reflect high endogenous auxin levels. When compared to wild type roots characteristic changes in DR5 activity were observed in *Atpin1* and *Atpin2* root meristems (Sabatini *et al*., 1999). A similar construct with a DR5rev promotor (Ulmasov *et al*., 1995, 1997) fused to the PEH A reporter gene (Benkova *et al*., in preparation) was introduced into *Atpin4* mutant plants. Drastic changes in DR5rev promotor activity in *Atpin4* mutant roots and embryos were observed suggesting alterations of auxin concentrations in various cells due to a loss of *Atpin4* function (Figure 42; Friml *et al*., 2000).

Heterologous expression of AtPIN1 and AtPIN2 proteins in yeast.

The heterologous expression of the AtPIN2 in yeast caused enhanced resistance to the yeast toxin fluoroindole, a substance with some limited structural similarity to tryptophane and auxin (Luschnig *et al*., 1998). It has been recently demonstrated that heterologous expression of AtPIN1 protein in yeast also caused the resistance to toxic fluoroindole (Tietz, personal communication). These data can be interpreted to indicate a transport function for AtPIN1 and AtPIN2 proteins in yeast. More detailed studies, in particularly the use of homologous plant expression systems, need to be performed before an auxin specific transport function can be confirmed.

Homology of AtPIN proteins to prokaryotic transport proteins.

The AtPIN1 and AtPIN2 proteins were demonstrated to share similarity with prokaryotic and eukaryotic transporters (Palme and Gälweiler, 1999, Müller *et al*., 1998; Luschnig *et al*., 1998; Chen *et al*., 1998; Utsuno *et al*., 1998). Especially the N- and C-terminal hydrophobic parts display homology to malonate transporter mdcF (Hoenke *et al*., 1997). Also bacterial efflux carriers that remove toxic compounds from the interior of the cell (*arsB*, Diorio *et al*. 1995 and *sbmA*, Salomon and Farias, 1995) are related to AtPIN1 and AtPIN2 proteins. The same topology similarity and sequence homology to the

transport proteins was also found for the rest of identified AtPIN proteins suggesting that they all exhibit similar function.

The central question remains whether all PIN proteins represent catalytic subunits of the auxin efflux machinery or participate only indirectly in this process or even have, as Hertel (1983) speculated, auxin receptor activity. To answer these questions, direct auxin transport assays have to be developed to establish directly carrier functions and to determine their substrate specificity and kinetic properties. It will also be important to determine if different AtPIN proteins transport auxin with either low or high affinity as this may identify possible candidates with receptor function. Nevertheless the data accumulated so far, especially from studies of genetic and phenotypic defects caused by disruption of different members of the *AtPIN* family, provide already sufficient evidence to argue that apparently most of the isolated *AtPIN* genes seem to be involved in some important aspects of auxin transport.

4.6 **Why do plants need so many *PIN* genes?**

If we consider the number of identified *AtPIN* genes and if we assume that all of them exhibit similar functions in auxin efflux, the question arises why plants need such a diversity of *AtPIN* genes/proteins? One explanation could be that through specifically regulated promotors an efficient spatio-temporal cellular regulation could be imposed on the auxin transport system. In addition, the different AtPIN proteins could also differ in some of important catalytic properties even if involved in the same transport process thus changing substrate specificity (e.g. for other natural auxins or auxin conjugates) or affecting kinetic properties (e.g. rates).

Regulation of expression.

Several motifs for regulation of transcription by cellular or external cues were found in *AtPIN1 – AtPIN7* promotor sequences (e.g. for light, ABA, gibberellin, auxin and ethylene) (Gälweiler, 1999, Müller, 1997, Friml, unpublished results). Although these *in silico* identified sequence motifs need experimental confirmation, it is not unlikely that expression of different *AtPIN* genes might be specifically regulated by internal and external cues thus allowing the plant to adapt the polar auxin transport machinery to diverse developmental and environmental situations. However, the argument of differential temporal and spatial expression of the various *AtPIN* genes can not fully explain the diversity of the *AtPIN* gene family. In several cases we observed the simultaneous expression of several *AtPIN* genes within the same cells. This has been demonstrated, for example, for *AtPIN3* and *AtPIN4* genes in the first columella tier of root caps and for *AtPIN1* and *AtPIN4* genes in the region where vascular and endodermis initials are located (Friml *et al*., 2000). This may indicate the involvement of these proteins in some more distinct cellular functions.

Auxin affinity and transport rate.

It has been suggested that different cells within the plant contain different auxin levels (Eklöf *et al*., 1997; Sabatini et. al., 1999) and that different physiological processes are regulated by different amounts of auxin. To achieve such defined auxin levels, auxin transporters with different (low or high) affinities for auxin and different transport rates would be needed. Biochemical discrimination of high and low affinity auxin transporters would also be a prerequisite to experimentally address the question about a sensor function of some of these transporters.

Regulation and interactions.

The process of auxin transport is likely to be highly regulated. The possible relationship to ethylene and cytokinin signal transduction pathways has been discussed many times (Brzobohaty *et al*., 1994; Mok, 1994). The diversity of AtPIN proteins can represent the basis for specific regulation e.g. by interactions with specific regulatory proteins and cellular signalling networks. This may indeed be the context that allows in specific cells to either polarly or non-polarly localise specific AtPIN proteins in membranes. Moreover, the recent finding of downregulation of AtPIN1 expression in *Arabidopsis* plants expressing the cytokinin biosynthetic *ipt* gene from *Agrobacterium tumefaciens* suggests an even more intricate relationship between auxin transport, auxin action and cytokinins (Grosskopf-Kroiher *et al*., 2000). Using the available mutants as well as novel mutagenic approaches (e.g. suppressor mutant isolation in combination with, for example, GFP-tagged PIN proteins) may allow to molecularly dissect the pathways that decide at which membrane the particular AtPIN protein will be placed. Therefore, it is expected that future experiments using non-destructive *in vivo* labelling or other innovative experimental approaches, will not only show the dynamic movements of AtPIN proteins between different cellular compartments, but will also allow to genetically dissect their regulatory pathways.

4.7 **What have we learned from analysis of *AtPIN3* and *AtPIN4*?**

AtPIN3 and *AtPIN4* are two of the newly identified *PIN* genes that were identified and molecularly characterised during the course of this work. Both genes encode proteins of about 60 kDa sharing the well known "PIN-like" protein topology.

Localisation

The "PIN-like" protein topology includes two highly conserved hydrophobic, membrane segments, which are interconnected by a diverged more hydrophilic residues containing linker region. This latter feature allowed, similar to AtPIN1 and AtPIN2, to generate antibodies of sufficient specificity to discriminate between both proteins in immunocytochemical studies. The specificity of both antibodies was strikingly demonstrated by (*i*) the presence of the immunolocalisation signals in those root cells in which by *in situ* hybridisation the corresponding mRNA was found, (*ii*) the absence of signals in null mutants, and the (*iii*) reappearance of signals in cells that had reverted to wild type after somatic excision of the *En-1* transposon (as example see Figure 26). Whole mount analysis allowed detection of strong AtPIN3 specific signals in the first tier of differentiated columella cells. A weaker signal was detected in more than 50 % of the analysed roots in the second tier of columella cells. No polarity in signal distribution was observed. In transversal sections of hypocotyls and stems AtPIN3 was clearly identified in the endodermis. Longitudinal sectioning of these tissues detected a primarily laterally positioned signal towards the inner cortex. Sometimes this signal was found to be shifted more down to a basal orientation. The reasons for this differential location are not yet understood and remain to be studied using real time image analysis. Non-polar AtPIN4 signals were mostly found in cells of the quiescent centre. Signals were also found in all initial cells surrounding the quiescent centre. Interestingly, in these provascular cells AtPIN4 polarity reappeared with distance from the quiescent centre. Again no signals were found in the *Atpin4-1 Atpin4-2* and *Atpin4-3* null mutant lines (Figure 40). Based on topology prediction both proteins are predicted to be integral plasma membrane proteins. The subcellular localisation in plasma membranes was indeed shown by immunoelectronmicroscopy. This analysis was performed in collaboration with Prof. Mendgen clearly resolving signals at the plasma membrane (data not shown).

Possible cellular functions of *AtPIN3* and *AtPIN4* in root cell patterning.

Although *AtPIN3* and *AtPIN4* are expressed in a variety of different tissues and cells during *Arabidopsis* development, it is useful to consider their expression in the root meristem for functional interpretation. The root meristem consists of a simple and well-defined system of dividing and differentiating cells illustrated in different colours in Figure 45 (Dolan *et al*., 1993; http://www.bio.uu.nl/˜mcbroots). In the centre of the meristem the grey coloured quiescent centre cells (q.c.) are situated. These cells divide only rarely. AtPIN4 was non-polarly expressed in the lenticular cell of embryo, a cell considered to be the precursor for the q.c. of the primary root.

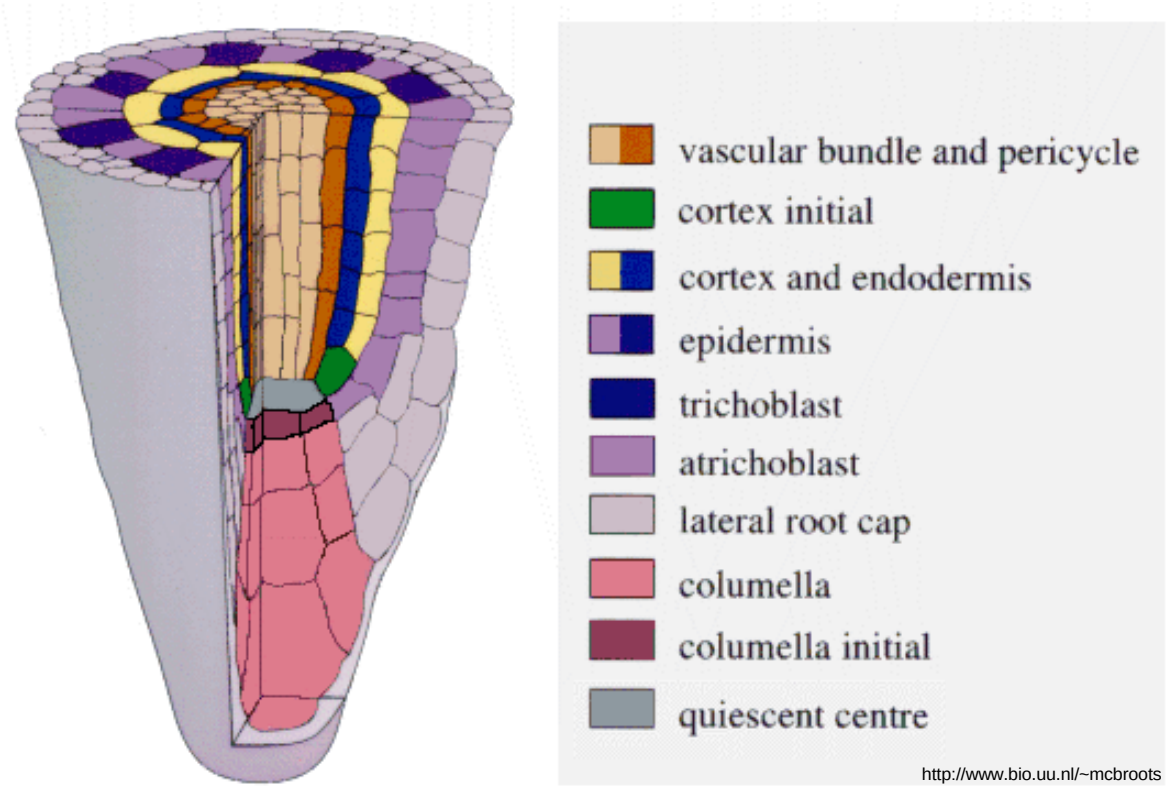
Consequently, non-polar AtPIN4 localisation was also found in the q.c. The non-polar localisation of AtPIN4 may be rationalised if we assume that AtPIN4 is involved in rapid export of auxin out from these cells thereby reducing auxin levels, cell division activity and helping to maintain the stem cell potential for this cell group. If this assumption is correct, we should find after establishment of effective biochemical assays, (*i*) faster kinetic auxin transport properties for AtPIN4 and (*ii*) null mutants with patterning defects in this region.

As to the lack of convenient assay systems, the first assumption was not yet testable, but preliminary inspection of mutant primary roots with disrupted *AtPIN4* function indeed revealed highly specific patterning defects. The additional drastically altered expression pattern of an auxin sensitive synthetic promotor in wild type and mutant seedlings further corroborates our hypothesis and confirms an important role for auxin in root meristem patterning.

Interestingly, we found very specific changes in root columella cell differentiation when *Atpin3* null mutants were inspected. In cells of wild type primary roots, *AtPIN3* mRNA and protein was found in the first tiers and, frequently, in the second tiers of columella cells. Interestingly, after staining of starch granules with lugol, mutant columella cells remained as undifferentiated initial cells. The finding of AtPIN3 expression in starch containing columella cells is also of wider importance for understanding the role of auxin in graviperception and transduction. Graviperception is thought to occur in these cells probably due to cytoskeletal or other subcellular rearrangements induced by changing the position of starch granules after gravitropic stimulation (MacCleery and Kiss, 1999).

If AtPIN3 would play a role in this process, graviperception or transduction should be affected in *Atpin3* mutants. However, only subtle defects in root gravitropism were observed in *Atpin3* mutants. Our preliminary results suggest that at least two another *AtPIN* genes are expressed in root columella exhibiting probably partially redundant function.

Figure 45. Anatomy of *Arabidopsis* root meristem according to Dolan *et al.*, 1993



Possible role of AtPIN3 in lateral auxin transport

Lateral auxin transport is supposed to play a role in lateral auxin redistribution in shoot and thus regulated shoot elongation and tropic responses. The lateral AtPIN3 localisation found in shoot hypocotyls and stems suggested a role in lateral auxin distribution and mediation of tropic responses. As shown in Figures 34 and 35 *Atpin3* mutants are indeed impaired in both hypocotyl phototropism and gravitropism confirming some of the concepts formulated by the Cholodny-Went hypothesis pointing to a reduction in lateral auxin distribution and hence lack of appropriate elongation response of respective tissues. Another pleasing observation confirming a role of AtPIN3 in lateral auxin distribution was made during analysis of apical hook opening. Apical hooks are structures at the apical part, which are developed by *Arabidopsis* seedlings when grown in the dark. Differential cell elongation growth results in opening when seedlings break through the soil where illumination with white light occurs. When we compared wild type and mutant seedlings (*Atpin3-1*, *Atpin3-2*, and *Atpin3-3*) we found a significantly earlier apical hook opening by mutant seedlings. These data strongly indicate that AtPIN3 plays an important role in differential elongation responses in shoot. Since we did not observe a full loss of the hypocotyl tropic response, we hypothesise that another, probably redundant, but not yet functionally characterised *AtPIN* gene is involved in control of this process

Are AtPIN1, AtPIN3 and ATPIN4 involved in partially redundant functions?

In two cases, partial functional redundancy among AtPIN proteins was indicated. The *AtPIN1* and *AtPIN4* genes were found to be expressed in the proximal meristem above the quiescent centre. If we assume partial functional redundancy for both genes we should expect that reduction of auxin transport to similar degrees results in similar phenotypic defects. This was indeed found with aberrant cell divisions in the quiescent centre for both *Atpin1* and *Atpin4* mutants (data not included; Friml *et al*., 2000). In the region where columella initials (c.i.) and first tier columella cells (c1) are located, a similar colocalisation was found for AtPIN3 and AtPIN4 proteins. As expected, disruption of *Atpin3* and *Atpin4* mutants displayed similar cell identity changes (first columella tier cells to columella initial cells) (Friml *et al*., 2000).

5 Perspectives

The work presented in this thesis allowed the identification of five novel members of *AtPIN* gene family. At present time (May 2000), a detailed analysis of the *AtPIN3* and *AtPIN4* genes has been almost fully accomplished while the analysis of *AtPIN5*, *AtPIN6* and *AtPIN7* is still in progress. Several interesting experiments for future research emerge from this work. The results from these experiments possibly will not only contribute to the understanding of the role of *PIN* genes in polar auxin transport but are also likely to be of wider relevance for our understanding of some important aspects of plant development. Of specific interest will be, for example, to perform cell ablation experiments and to study the consequences of individual cell disruptions in wild type and mutant backgrounds and the effects of loss of auxin transport function in individual cells on root cell division and differentiation. Alternatively, stable mutants can be used to isolate new (e.g. suppressor) mutants or to generate mutants regulating the membrane trafficking of AtPIN proteins. This will hopefully help to unravel some of the secrets of auxin transport and auxin action in patterning and establishment of cell and organ polarity.

6 Bibliography

Abel, S., Theologis, A. (1995): A polymorphic bipartite motif signals nuclear targeting of early auxin-inducible proteins related to PS-IAA4 from pea (Pisum sativum). *Plant J.* 8, 87-96.

Abel, S., Theologis, A. (1996): Early genes and auxin action. *Plant Physiol*. 111, 9-17.

Abel, S., Ballas, N., Wong, L.M., Theologis, A. (1996): DNA elements responsive to auxin. *Bioessays* 18, 647-54.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (1993): Current protocols in molecular biology. *Green Publishing Associates, John Wiley & Son, Inc.*, New York.

Bandurski, R.S., Cohen, J.D., Slowin, J., Reinecke, D.M. (1995): Auxin biosynthesis and metabolism. In: *Plant hormones, physiology and molecular biology* (Davies, P.J., ed.). *Martinus Nijhoff Publishers, Kluwer Academic Publishers Group*, Dordrecht, 39-65.

Bar-Peled, M., Bassham, D.C., Raikhel, N.V. (1996): Transport of proteins in eukaryotic cells: more questions ahead. *Plant Mol. Biol.* 32, 223-249.

Bartel, B. and Fink, G.R. (1995): ILR1, an aminohydrolase that releases active indole-3-acetic acid from conjugates. *Science* 268, 1745-1748.

Baumann, E., Lewald, J., Saedler, H., Schulz, B., Wisman,E. (1998): Successful PCR-based reverse genetic screen using an En-1-mutagenised *Arabidopsis thaliana* population generated via single-seed descent. *Theoretical & Applied Genetics* 97, 729-734.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., Feldmann, K.A. (1996): *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. *Science* 273, 948-950.

Benning, C. (1986): *Planta* 169, 228-237.

Berleth, T. and Jürgens, G. (1993): The role of the *Monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. *Development* 118, 575-587.

Bernasconi, P., Bhavesh, P.C., Reagan, J.D., Subramanian, M.V. (1996): The N-naphthylphthalamic acid-binding protein is an integral membran protein. *Plant Physiol.* 111, 427-432.

Birnboim, H.C. and Doly, J. (1994): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*. 7, 1510-1513.

Bleecker, A.B. (1991): Genetic analysis of ethylene responses in *Arabidopsis thaliana*. *Symp. Soc. Exp. Biol*. 45, 149-58.

Boysen-Jensen, P. (1913):"Uber die Leitung des phototropischen Reizes in der Avenakoleoptile". *Ber. Deut. Bot. Ges*. 31, 559-566.

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

Brzobohaty, B., Moore, I., Palme, K. (1994): Cytokinin metabolism: implications for regulation of plant growth and development. *Plant Mol. Biol.* 26, 1483-97.

Bullock, W. O., Fernandez, J. M. et al. (1987): Xl1-blue a high efficiency plasmid transforming rec-a *Escherichia-coli* strain with beta galactosidase selection. *Biotechniques* 5, 376-377.

Cambridge, A. P. and Morris, D. A. (1996): Transfer of exogenous auxin from the phloem to the polar auxin transport pathway in pea (*Pisum sativum* L.). *Planta* 199, 583-588.

Camus, G. (1949): Recherches sur le role de bourgeones dans les phenomenes de morphogenes. *Revue Cytologie et Biologie Vegetale* 11, 1-195.

Cardon, G. H., M. Frey, et al. (1993): Mobility of the maize transposable element En/Spm in *Arabidopsis thaliana*. *Plant Journal* 3 773-3784.

Chen, R., Hilson P., Sedbrook, J., Rosen, E., Caspar, T., Masson, P.H. (1998): The *Arabidopsis thaliana AGRAVITROPIC1* gene encodes a component of polar auxin-transport efflux carrier. *Proc. Natl. Acad. Sci. USA* 95, 15112-15117.

Cho, R.J., Mindrinos, M., Richards, D.R., Sapolsky, R.J., Anderson, M., Drenkard, E., Dewdney, J., Reuber, T.L., Stammers, M., Federspiel, N., Theologis, A., Yang, W.H., Hubbell, E., Au, M., Chung, E.Y., Lashkari, D., Lemieux, B., Dean, C., Lipshutz, R.J., Ausubel, F.M., Davis, R.W., Oefner, P.J. (1999): Genome-wide mapping with biallelic markers in *Arabidopsis thaliana*. *Nat. Genet*. 23, 203-207.

Chomczynski, P. and Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.

Cohen, J.D. and Bandurski, R.S. (1982): Chemistry and physiology of the bound auxins. *Annu. Rev. Plant Physiol.* 33, 403-430.

Cox, D.N. and Muday, G.K. (1994): NPA binding-activity is peripheral to the plasma membrane and is associated with the cytoskeleton. *Plant Cell* 6, 1941-1953.

Darwin, C. und Darwin, F. (1881): The power of movement in plants (Deutsche Übersetzung: Das Bewegungsvermögen der Planze). Darwins gesammelte Werke Bd. 13, *Schweizer-bart´sche Verlagsbuchhandlung*, Stuttgart.

Davies,P.J. (1995): Plant hormones, physiology, biochemistry and molecular biology. *Martinus Nijhoff Publishers Group*, Dordrecht.

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

Diorio, C., Cai, J., Marmor, J., Shinder, R., DuBow, M.S. (1995): An *Escherichia coli* chromosomal ars operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. *J. Bacteriol*. 177, 2050-2056.

Dotson, S.B., Lanahan M.B., Smith, A.G., Kishore, G. M. (1996): A phosphonate monoester hydrolase from *Burkholderia caryophilli* PG2982 is useful as a conditional lethal gene in plants. *Plant Journal* 10, 383-392.

Dower, W.J., Miller, J.F., Ragsdale, C.W. (1988): High efficiency transformation of *E.coli* by high voltage electroporation. *Nucleic Acids Res*. 16, 6127-6145.

Eklof, S., Astot, C., Blackwell, John, Moritz, T., Olsson, O., Sandberg, G. (1997): Auxin-cytokinin interactions in wild-type and transgenic tobacco. *Plant and Cell Physiology* 38, 225-235.

Epel, B.L., Warmbrodt, R.P. and Bandurski., R.S. (1992): The ethylene signal transduction pathway in plants. *Science* 268, 667-675.

Estelle, M., Jones, A., Perrot-Rechemann, C. (2000) EMBO Workshop on Auxin, Calcattagio, France

Feldman, L.J. (1985): Root gravitropism. *Physiol. Plant*. 65, 341-344.

Friml, J. (2000) EMBO Workshop on Auxin, Calcattagio, France

Friml, J., Wisniewska, J., Schelhass, M., Tänzler, P., Tretyn, A., Palme, K. (1999): Analysis of the *AtPIN3* gene from *Arabidopsos thaliana*. *Biologia plantarum* 42(suppl.), S20.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A., Palme, K. (1998): Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* 282, 2226-2230.

Gälweiler, L. (1999): Isolierung und Charakterisierung des *PIN1*-Gens von *Arabidopsis thaliana* L. Dissertation an der Biologischen Fakultät II der Universität Hohenheim.

Garbers, C., A. Delong, et al. (1996): A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in *Arabidopsis*. *EMBO J.* 15, 2115-2124.

Goldsmith, M.H.M. (1977): The polar transport of auxin. *Ann. Rev. Plant. Physiol*. 28, 439-478.

Haagen-Smit, A. J., Dandliker, W. B., Wittwer, S. H., and Murneek, A. E. (1946): Isolation of 3-indoleacetic acid from immature corn kernels. *Amer. J. Bot*. 33,118-120.

Hadfi, K., Speth, V., Neuhaus G. (1998): Auxin-induced developmental pattrens in *Brassica juncea* embryos. *Development* 125, 879-887.

Hanahan, D. (1985): Techniques for transformation of *Escherichia coli*. Glover, D.M. (ed), DNA cloning Vol1, a *IRL Press*, Oxford, Washington DC, 109.

Hardtke, C.S. and Berleth, T. (1998): The *Arabidopsis* gene *MONOPTEROS* encodes a transription factor mediating embryo axis formation and vascular development. *EMBO J.* 17, 1405-1411.

Hart, J.W. (1990): Plant tropism and other movements, *Unwyn Hyman*; London.

Hertel, R. (1983): The mechanism of auxin transport as a model for auxin action. *Z. Pflanzenphysiol*. 112, 53-67.

Hobbie, L., Timpote, C., Estelle, M. (1994): Molecular genetics of auxin and cytokinin. *Plant Mol. Biol.* 26, 1499-1519.

Hobbie, L.J. (1998): Auxin:molecular genetic approaches in *Arabidopsis*. *Plant Physiol. Biochem.* 36, 91-102.

Hoenke, S., Schmid, M., Dimroth, P. (1997): Sequence of a gene cluster from *Klebsiella pneumoniae* encoding malonate decarboxylase and expression of the enzyme in *Escherichia coli*. *Eur. J. Biochem*. 246, 530-538.

Hoffmann, G.R. (1980): Genetic effects of dimethyl sulfate, diethyl sulfate, and related compounds. *Mutat. Res.* 75, 63-129.

Hofmann, K., Stoffel, W. (1993): TMBASE- A database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler* 374, 166-170.

Jacobs, M., Gilbert, S.F. (1983): Basal localization of the presumptive auxin transport carrier in pea stem cells. *Science* 220, 1297-1300.

Jacobs, M. and Rubery, P. H. (1988): Naturally occuring auxin transport regulators. *Science* 241, 346-349.

Jakobs, S. (1998): Charakterisierung der Miasproterine ZmPM23 und ZmPM42. Dissertation an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln.

Jameson, B. A. and H. Wolf (1988): The antigenic index: a novel algorithm for predicting antigenic determinants. *Computer Applications in the Biosciences* 4, 181-186.

Jefferson, R.A. (1987): Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep*. 5, 387-405.

Jensen, P.J., Hangarter, R.P., Estelle, M. (1998): Auxin trasport is required for hypocotyl elongation in light-*grown Arabidopsis. Plant Physiol.* 16, 455-462.

Jia, Y., Loh, Y.T. (1997): Alleles of *Pto* and *Fen* occur in bacterial speck-susceptible and fenthion-insensitive tomato cultivars and encode active protein kinases. *Plant Cell* 9, 61-73.

Jones, A.M., Im ,K.H., Savka ,M.A., Wu ,M.J., Dewitt, N.G., Shillito, R., Binns, A.N., (1998): Auxin dependent cell expansion mediated by overexpressed auxin-binding protein. *Science* 282, 1114-1117.

Joshi, C.P. (1987a): An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nucl. Acids Res.* 15, 6643-6653.

Joshi, C.P. (1987b): Putative polyadenylation signals in nuclear genes of higher plants: A compilation and analysis. *Nucl. Acids Res.* 15, 9627-9640.

Katekar, G.F., Geisler, A.E. (1977): Auxin transport inhibitors. *Plant Physiol.* 60, 826-829.

Kendrew, J. (1994): The encyclopedia of molecular biology. *Blackwell Science*. Oxford.

Konz, C. and Schell, J. (1986): The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. and Gen.Genet*. 204, 383-396.

Kögl, F. und Kostermans, D.G.F.R. (1934): Hetero-Auxin als Stoffwechselprodukt niederer pflanzlicher Organismen. Isolierung aus Hefe. *Hoppe Seyler's Z. Physiol. Chem*. 228, 113-121.

Kyte, J. and Doolittle, R. F. (1982): A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157, 105-132.

Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lalonde, S., Boles, E., Hellmann, H., Barker, L., Patrick, J.W., Frommer, W.B., and Ward, J. (1999): The dual function of sugar carriers: transport and sugar sensing. *Plant Cell* 11, 707-726.

Lehman, A., Black, R. and Ecker, J.R. (1996): *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. *Cell* 85, 183-194.

Leyser, H.M.O., Lincoln, C., Timpte, C., Lammer, D., Turner, J., Estelle, M. (1993): The auxin-resistant gene *AXR1* of *Arabidopsis* encodes a protein related to ubiquitin-activating enzyme E1. *Nature* 364, 161-164.

Li, Y., Hagen, G., Guilfoyle, T.J. (1991): An auxin-responsive promoter is differentially induced by auxin gradients during tropisms. *Plant Cell* 3, 1167-1176.

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

Lomax, T. L., Muday, G. K., Rubery, P. H. (1995): Auxin transport. In: *Plant Hormones, Physiology, Biochemistry and Molecular Biology*. (Davies, P.J., ed.). *Martinus Nijhoff Publishers, Kluwer Academic Publishers Group*, Dordrecht, 509-530.

Ludevid, D. , Höfte, H. , Himelblau, E. and Chrispeels, M.J. (1992): The expression pattern of the tonoplast intrinsic protein-tip in *Arabidopsis thaliana* is correlated with cell enlargement. *Plant Physiol.* 100, 1633-1639.

Luschnig, C., Gaxiola, R., Grisafi, P., Fink, G. (1998): EIR1, a root specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana. Genes Dev.* 12, 2175-2187.

Maniatis, T., Fritsch, E.F. und Sambrook, J. (1982): *Molecular Cloning. A Laboratory Manual.* CSHL; *Cold Spring Harbor*, New York.

Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann C., Bennett M.J. (1999): AUX1 regulates root gravitropism in *Arabidopsis* by facilitating uptake within root apical tissue. *EMBO J.* 18, 2066-2073.

Martin, G.B., M.C., De Vicente, *et al*. (1993): High-resolution linkage analysis and physical characterization of the *Pto* bacterial resistance locus in tomato. *Mol. Plant Microbe Interaction* 6, 26-34.

Mattson, J., Sung, Z.R., Berleth, T. (1999): Responses of plants vascular system to auxin transport inhibition. *Development* 126, 2979-2991.

Mayer, U., Buettner, G. and Jürgens, G., (1993): Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. *Development* 117, 149-162.

Meinke, D.W., Sussex, I.M. (1979): Isolation and characterization of six embryo-lethal mutants of *Arabidopsis thaliana*. *Dev. Biol.* 72, 62-72.

Meuwly, P. and P. E. Pilet (1991): Local treatment with IAA induces differential growth responses In *Zea-mays* L. roots. *Planta* 185, 58-64.
McCallum, C.M., Comai, L., Greene, E.A., Henikoff, S. (2000): Targeted screening for induced mutations. *Nat. Biotechnol*. 18, 455-457.

Moctezuma, E., Feldman, L.J. (1999): Auxin redistributes upwards in graviresponding gynophores of the peanut plant. *Planta* 12, 180-186.

Mok, M.C. (1994): Cytokinins and plant development. In Mok, D.W.S. and Mok, M.C, (eds.) Cytokinins, activity and structure, pp. 155-166. *CRC Press*, Boca Raton.

Morris, D.A. and Thomas, (1978): A microautoradiographic study of auxin transport in the stem of intact pea seedlings (*Pisum sativum* L.). *J. Exp. Bot.* 29, 147-157.

Morris, D.A., Rubery, P.H., Jarman, J. und Sabater, M. (1991): Effects of inhibitors of protein synthesis on transmembrane auxin transport in *Curcurbita pepo* L hypocotyl segments. *J. Exp. Bot.* 42, 773-783.

Mozo, T., Dewar, K., Dunn, P., Ecker, J.R., Fischer, S., Kloska, S., Lehrach, H., Marra, M., Martienssen, R., Meier-Ewert, S., Altmann, T. (1999): A complete BAC-based physical map of the *Arabidopsis thaliana* genome. *Nat. Genet.* 22, 271-275.

Muday, G. K. and Haworth, P. (1994): Tomato root growth, gravitropism, and lateral development: Correlation with auxin transport. *Plant Physiology and Biochemistry* 32(2): 193-203.

Muldoon, T.G., Watson, G.H., Evans, A.C., Steinsapir, J. (1988): Microsomal receptor for steroid hormones: functional implications for nuclear activity. *J. Steroid. Biochem*. 30, 23-31.

Müller, A., Guan, C., Gälweiler, L., Tänzler, P., Huijser, P., Marchant, A., Pary, G., Bennet, M., Wisman, E., Palme, K. (1998): *AtPIN2* defines a locus of *Arabidopsis* for root gravitropism control. *EMBO J.* 17, 6903-6911.

Müller, A. (1997): Genetische und molekulare Analyse des *PIN2*-Gens aus *Arabidopsis thaliana* L. Dissertation an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln.

Nakai, K. and Kanehisa, M. (1992): A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* 14, 897-911.

Neuhoff, V., Stamm, R. and Eibl, H.J. (1985): Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: A systematic analysis. *Electrophoresis* 6, 427-448.

Newcomb,W.and Wetherell, D.F. (1970): The effects of 2,4,6-trichlorophenoxyacetic acid on embryogenesis in wild type carrot tissue cultures. *Bot. Gaz.* 131, 242-245.

Normanly, J., Cohen, J.D., Fink, G.R. (1991): *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. *Proc. Natl. Acad. Sci.USA* 90, 10355-10359.

Nowacki, J., Bandurski, R.S. (1980): Myo-inositol esters of indole-3-acetic acid as seed auxin precursors of *Zea mays* L. *Plant Physiol*. 65, 422-427.

Ohwaki, Y., Tsurumi, S. (1976): Auxin transport and growth in intact roots of *vicia-faba. Plant Cell Physiol.* 17, 1329-1342.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J. and Shimura, Y. (1991): Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* 3, 677-684.

Osoegawa, K., Tateno, M., Woon , P.Y., Frengen, E., Mammoser, A.G., Catanese, J.J., Hayashizaki, Y., de Jong, P.J. (2000): Bacterial artificial chromosome libraries for mouse sequencing and functional analysis. *Genome Res.* 10, 116-28.

Ozcan, S., Dover, J., Rosenwald, A.G., Wolfl, S., Johnston, M. (1996): Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proc. Natl. Acad. Sci. USA* 29, 93.

Palme, K., Hesse, T., Moore, I., Campos, N., Feldwisch, J., Garbers, C., Hesse, T., Schell, J. (1991): Hormonal modulation of plant growth: the role of auxin perception. *Mech. Dev.* 33, 97-106.

Palme, K., Hesse, T., Garbers, C., Simmons, C., Soll, D. (1994): The *ERabp* gene family: structural and physiological analyses. *Basic Life Sci*. 62, 155-61.

Palme, K., Redhead, C., Kristoffersen, P. (1996): Phytohormones and signal transduction in plants. Endocrenology: Basic and Clinical Principles (P. M. Conn and S. Melmed, eds), *Humana Press Inc*., Totowa, NJ.

Palme K, Bischoff F, Cvrckova F, Zarsky V. (1997): Small G-proteins in *Arabidopsis thaliana*. *Biochem. Soc. Trans*. 25, 1001-1005.

Palme, K., Galweiler, L. (1999): PIN-pointing the molecular basis of auxin transport. *Curr. Opin. Plant Biol.* 2, 375-381.

Palme, K. (2000) EMBO Workshop on Auxin, Calcattagio, France.

Prasad, P.V., Jones, A.M., (1991): Putative Receptor for the Plant Growth Hormone Auxin Identified and Characterized by Anti-Idiotypic Antibodies. *Proc. Natl. Acad. Sci. USA* 88, 5479-5483**.**

Przemeck, G. K. H., Mattsson, J. et al. (1996): Studies on the role of the A*rabidopsis* gene *Monopteros* in vascular development and plant cell axialization. *Planta* 200, 229-237.

Raven, J.A. (1975): Transport of indolacetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. *New Phytol*. 74, 163-172.

Reinecke, D.M., and Bandurski, R.S. (1985): Auxin biosynthesis and metablism. In P.J Davies: Plant hormones and their role in plant growth and development. pp. 24-42. *Martinus Nijhoff Publishers*. Dordrecht.

Rost, B., Fariselli, P. et al. (1996): Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Science* 5, 1704-1718.

Rubery, P.H. und Sheldrake, A.R. (1974): Carrier-mediated auxin transport. *Planta* 118, 101-121.

Rubery, P.H., (1990): Phytotropins: receptors and endogenous ligands. *Symp. Soc. Exp. Biol.* 44,119-46.

Ruegger, M., E. Dewey, et al. (1997): Reduced naphthylphthalamic acid binding in the *Tir3* mutant of A*rabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. *Plant Cell* 9, 745-757.

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J., Estelle, M. (1998): The TIR protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes and Development* 12, 198-207.

Russel, M., S. Kidd, et al. (1986): An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene* 45, 333-338.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett J. Guilfoyle ,T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., Scheres, B. (1999): An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. *Cell* 99, 463-472.

Sachs, T. (1989): The development of vascular networks during leaf development. *Curr. Top. Plant Biochem. Physiol*. 18, 168-183.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A., Allard, R.W., (1984): Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* 81, 8014-8018.

Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., Staskawicz, B.J. (1996): Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell* 12, 123-33.

Salomon, R.A., Farias, R.N. (1995): The peptide antibiotic microcin 25 is imported through the TonB pathway and the SbmA protein. *J. Bacteriol*. 177, 3323-5.

Sambrook, J., Fritsch, E.F., et al. (1989): Molecular Cloning a Laboratory Manual Second Edition Vols. 1 2 and 3. *Sambrook, J., E. F. Fritsch And T. Maniatis. Molecular Cloning: A Laboratory Manual, Second Edition.*

Sandberg, G., Ljung, K., Bellini, K., Bhalearo, R.P. (2000): EMBO Workshop on Auxin, Calcattagio, France.

Schiavone, F.M. and Cooke, T.J. (1987): Unusual patterns of somatic embrygenesis in the domesticated carrot developmental effects of exogenous auxins and auxin transport inhibitors. *Cell Differentiation* 21, 53-62.

Sitbon, F. and Perrot-Rechenmann, C. (1997): Expression of auxin-regulated genes. *Physiol. Plant.* 100, 443-455.

Smith, R.G., Palyha, O.C., Feighner, S.D., Tan, C.P., McKee, K.K., Hreniuk, D.L., Yang, L., Morriello, G., Nargund, R., Patchett, A.A., Howard, A.D. (1999): Growth hormone releasing substances: types and their receptors. *Horm. Res*. 51, Suppl 3, 1-8.

Somerville, C. (2000): The twentieth century trajectory of plant biology. *Cell* 7, 13-25.

Spena, A., Estruch, J.J., Schell, J. (1992): On microbes and plants: new insights in phytohormonal research. *Curr. Opin. Biotech*. 3, 159-163.

Spena, A. (1993): Transgenic plants altered in phytohormone metabolism. *Acta Botanica Gallica* 140, 693-700.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C.L., Paris, S., Gälweiler, L., Palme, K., Jürgens, G. (1999): Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* 286, 316-318.

Stowe-Evans, E.L., Harper, R.M., Motchoulski, A.V, Liscum, E. (1998): NPH4, a conditional modulator of auxin-dependent differential growth responses in *Arabidopsis*. *Plant Physiol.* 118, 1265-75.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D,, Dean, C., Ma, H., Martienssen, R. (1995): Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* 9, 1797-1810.

Szerszen, J.B., Sczyglowski, K., Bandurski, R.S. (1994): *IAGLU*, a gene from *Zea mays* involved in conjugation of growth hormone indole-3-acetic acid. *Science* 265, 1699-1701.

Tam, Y.Y., Slovin, J.P. and Cohen, J.D. (1995): Selection and characterization of a-methyltryptophan resistant lines of *Lemna gibba* showing a rapid rate of indole-3-acetic acid turnover. *Plant Physiol.* 107, 77-85.

Thimann, K.V. (1977): Hormone action in the whole Life of plants. Amherst: The Universtity of Massachusetts.
Trewavas, A. (1981): How do plant growth substances work? *Plant Cell Envir*. 4, 203-228.

Trewavas, A. (1982): Growth substances sensitivity : the limiting factor in plant development. *Physiol. Plant*. 55, 60-72.

Ulmasov, T., Liu, Z.B., Hagen, G., Guilfoyle, T.J. (1995): Composite structure of auxin response elements. *Plant Cell* 7,1611-23.

Ulmasov, T., Murfett, J., Hagen, G., Guilfoyle, T.J. (1997): Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 9, 1963-71.

Utsuno, K., Shikanai, T., Yamada, Y., Hashimoto, T. (1998): *AGR*, an Agravitropic locus of *Arabidopsis thaliana*, encodes a novel membrane protein family member. *Plant Cell Physiol.* 39, 1111-1118.

Vente, A. (1996): Untersuchungen zur Funktion der auxinbindenden Proteine pm23 und pm24 aus *Zea mays* L. Dissertation an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln.

Viera, J., Messing, J. (1982): The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19, 259-268.

Wambutt, R. *et al.* (2000): Progress in *Arabidopsis* genome sequencing and functional genomics, *J. Biotechnol*. 78, 281-292.

Watahiki, K., Tatematsu, K., Fujihira, K., Yamamoto, M., Yamamoto, K. (1999): The *MSG1* and *AXR1* genes of *Arabidopsis* are likely to act independently in growth-curvature responses of hypocotyls. *Planta* 207, 362-369.

Went, F.W. (1928): Wuchsstoff und Wachstum. *Rec. Trav. Bot. Neerl.* 25, 1-116.

Went, F.W. (1974): Reflections and speculations. *Annu. Rev. Plant Physiol*. 25, 1-26.

Wetmore, R.H. and Rier, J.P. (1963): Experimental induction of vascular tissues in callus of angiosperms. *American J. Botany* 50, 418-430.

Wisman, E., Hartmann, U., Sagasser, M., Baumann, E., Palme, K., Hahlbrock, K., Saedler, H., Weisshaar, B. (1998): Knock-out mutants from an En-1 mutagenized *Arabidopsis thaliana* population generate phenylpropanoid biosynthesis phenotypes. *Proc. Natl. Acad. Sci. U S A* 95, 12432-12437.

Zettl, R., Feldwisch, J., Boland, W., Schell, J., Palme, K. (1992) Azido-(3,6-$^{3}H_2$)-N-1-naphthylphtalamic acid, a noval photoactivatable probe for auxin efflux carrier proteins from higher plants: Identification of a 23kDa protein from maize coleoptile plasma membranes. *Proc. Natl. Acad. Sci.USA* 89, 480-484.

7 Zusammenfassung

Das Phytohormon Auxin ist für die Morphogenese, das axiale und tropische Wachstum, die Leitgewebedifferenzierung ebenso wie für viele andere pflanzliche Prozesse von Bedeutung. Der Transport dieses Phytohormons spielt eine wichtige Rolle bei der Vermittlung der physiologischen Wirkungen dieses Hormons. Auf auxintransportdefizienten Mutanten aus *Arabidopsis thaliana* aufbauend, gelang kürzlich in unserer Arbeitsgruppe die Isolierung der ersten zellpolar positionierten Auxineffluxcarrierproteine (AtPIN1, AtPIN2). Wegen des breiten Spektrums zellulärer und gewebespezifischer Auxinflüsse liegt die Vermutung nahe, daß eine Familie von Proteinen in *Arabidopsis thaliana* vorliegt. Die Isolierung neuer Mitglieder und Aufdeckung der Genvielfalt dieser Familie war das zentrale Thema der vorliegenden Arbeit.

Mit Gensonden, die von schon bekannten Mitgliedern der PIN Genfamilie abgeleitet wurden, wurde geprüft, wieviele Mitglieder dieser Familie wahrscheinlich in *A. thaliana* vorliegen. Die Überprüfung einer geordnet vorliegenden genomischen BAC Bank legte eine Zahl von wenigstens 12 Mitgliedern nahe. Weitere potentielle Kandidaten wurden identifiziert, aber nicht weiter charakterisiert. 5 Kandidaten (*AtPIN3* bis *AtPIN7*) wurden für weitergehende Untersuchungen ausgewählt. Die Charakterisierung beinhaltete (1) die Isolierung vollständiger cDNAs, (2) die Kartierung der genomischen *loci*, (3) die rekombinante Expression von Fragmenten und Herstellung von Antikörpern, (4) die gewebe- und zellspezifische Analyse der Expression ausgewählter *AtPIN* Gene (mRNA und Protein), und (5) die Isolierung und nachfolgende genetische und zellbiologische Charakterisierung von Insertionsmutanten. 2 dieser Gene (*AtPIN3* und *AtPIN4*) wurden für eine detailierte Analyse ausgewählt.

Die Kartierung der Gene *AtPIN3-AtPIN7* unter Verwendung einer BAC Bibliothek ergab, daß AtPIN3, AtPIN6 und AtPIN7 wahrscheinlich in einem Gencluster auf Chromosom 1 liegen. AtPIN4 wurde auf Chromosom 2 und AtPIN5 auf Chromosom 5 lokalisiert. Für alle Gene konnten Insertionsmutanten identifiziert werden. Diese wurden genetisch charakterisiert (Reversion, Footprintmutanten, homozygote Linien). Insertionsmutanten der Gene *AtPIN3* und *AtPIN4* wurden für die in dieser Arbeit beschriebenen funktionellen Untersuchungen herangezogen.

In silico Analysen ergaben, daß alle aus den Gensequenzen vorhergesagten Proteine eine zu AtPIN1 grundsätzlich ähnliche Topologie aufweisen. In den N- und C-terminalen Bereichen werden jeweils 2 aus wahrscheinlich 5-6 Transmembransegementen bestehende hydrophobe Bereiche vorhergesagt. Diese sind durch eine hydrophile Schleife miteinander verbunden. AtPIN5 scheint keine oder nur eine sehr kurze Schleifenregion zu haben.

Die hydrophile Schleifenregion wurde für heterologe Expressionstudien ausgewählt. Bereiche mit hoher Antigenizität und Sequenzdiversität wurden als translationale Fusionsproteine mit 6 N-terminalen Histidinen in *E. coli* hergestellt, aufgereinigt und zur Herstellung polyklonaler Antikörper in Kaninchen eingesetzt. Diese Antikörper wurden anschliessend affinitätsgereinigt und für zellbiologische Untersuchungen eingesetzt. In

dieser Arbeit wurde die Herstellung und Nutzung der Antikörper für AtPIN3 und AtPIN4 beschrieben.

Die Expression der Gene *AtPIN3* und *AtPIN4* wurde detailiert untersucht. Diese Untersuchung ergab, daß das AtPIN3 Gen in verschiedenen Geweben und Zelltypen exprimiert vorliegt. Eine spezifische Expression von *AtPIN3* wurde in Endodermiszellen im Hypokotyl und der Blütenstandsachse nachgewiesen. Eine detailierte Untersuchung durch whole mount *in situ* Hybridisierung zeigte ein erhöhtes Expressionsniveau in der Wurzelhaube. Diese Expression konnte durch Immunlokalisation für das AtPIN3 Protein bestätigt werden. Das AtPIN3 Protein lag in den Zellen der Wurzelhaube nicht polar verteilt vor. Eine vorzugsweise laterale Verteilung des AtPIN3 Proteins wurde in den Endodermiszellen des Hypokotyls und des Stengels beobachtet. Die Analyse der Mutanten zeigte, daß in diesen Mutanten das *AtPIN3* Leseraster zerstört worden war. Somit konnte immuncytochemisch keine AtPIN3 Expression mehr nachgewiesen werden. Die Zerstörung des *AtPIN4* Gens resultierte in einer Veränderung der Musterbildung. Diese Mutanten zeigten in Weisslicht eine charakteristische Reduzierung des Hypokotyl- und Stengelwachstums. Die Öffnung des Hypokotylhakens war gegenüber Wildtyp *Arabidopsis*pflanzen deutlich verzögert. Diese Pflanzen zeigten außerdem ein gestörtes phototropes und ein gestörtes gravitropes Wachstum. Zusammengenommen deuten alle bislang verfügbaren Daten auf eine Funktion des AtPIN3 Proteins in der lateralen Auxinverteilung hin.

Die Expression des *AtPIN4* Gens wurde durch whole mount *in situ* Hybridisierung in Wurzelspitzen nachgewiesen. Die immunzytochemische Analyse des AtPIN4 Proteins bestätigte dieses Expressionsmuster. In Embryonen konnte die Expression schon im globulären Stadium gezeigt werden. Im Verlauf der Embryonalentwicklung wurde eine dynamische Veränderung der Expression festgestellt. Besonders charakteristisch war die Expression in der Lenticularzelle und in den sich hieraus entwickelnden Nachfolgezellen des ruhenden Zentrums. Postembryonal wurde AtPIN4 im ruhenden Zentrum und in den diesen Bereich umgebenden Initialzellen nachgewiesen. Je nach Zelltyp wurde eine polare oder apolare Lokalisierung des AtPIN4 Proteins beobachtet. In Nullmutanten mit zerstörter *AtPIN4* Funktion wurden auffällige Veränderungen im Expressionsmuster eines auxinsensitiven synthetischen Promotor nachgewiesen. Diese Ergebnisse lassen auf eine Veränderung des Auxingradienten im Wurzelbereich schliessen. Ausserdem wurden im Meristem auffällige Veränderung in der Ausprägung der Musterbildung fest gestellt. Weiterführende genetische und biochemische, zum Zeitpunkt der Abgabe dieser Arbeit aber noch nicht vollständig abgeschlossene Arbeiten, deuten auf eine wichtige Rolle des AtPIN4 Proteins in der Auxinumverteilung hin, die in der *Arabidopsis* Wurzelspitze erfolgt.

In dieser Arbeit wurden zwei der fünf neu isolierten *AtPIN* Gene funktionell charakterisiert. Durch Einsatz eines breiten Spektrums genetischer und molekularer Techniken konnte zum erstenmal ein klarer Zusammenhang zwischen Auxintransport und Musterbildungsprozessen aufgezeigt werden.

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